Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editor: Victor R. Preedy

Vinood B. Patel
Victor R. Preedy *Editors*

Biomarkers in Kidney Disease

Biomarkers in Disease: Methods, Discoveries and Applications

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In the past decade there has been a sea change in the way disease is diagnosed and investigated due to the advent of high throughput technologies, such as microarrays, lab on a chip, proteomics, genomics, lipomics, metabolomics, etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. Furthermore, the discovery of many new markers have not been put into clinical practice, partly because of their cost and partly because some scientists are unaware of their existence or the evidence is still at the preclinical stage. In some cases the work needs further scientific scrutiny. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of *Biomarkers in Disease: Methods, Discoveries and Applications*. The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

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Biomarkers in Kidney Disease

With 208 Figures and 142 Tables

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Volume Preface

In the present volume, ***Biomarkers in Kidney Disease***, we have sections on

- **General Aspects**
- **Circulating and Body Fluid Biomarkers**
- **Specific Diseases and Conditions**
- **Molecular, Cellular, and Histological Variables**
- **Functional and Structural Variables**
- **Resources**

While the Editors recognize the difficulties in assigning particular chapters to particular sections, the book has enormously wide coverage and includes the following areas, analytes, and conditions: osmolal gap, metabolic acidosis, metabolomics, hypoxia, micro-RNAs, creatinine, spot urine markers, neutrophil gelatinase-associated lipocalin (NGAL), chemokines, angiotensinogen, flow cytometry, leucocytes, lymphocytes, exosomes, *N*-acetyl-beta-D-glucosaminidase (NAG), endothelin, methylated arginines, albuminuria, cystatin C, homocysteinemia, fetal beta2-microglobulin, proteinuric biomarkers, apelin, copeptin, BLyS and APRIL cytokines, glutathione transferase, growth arrest-specific protein 6 (Gas6), urokinase receptor, urea nitrogen, allograft damage index (CADI), antibody arrays, malondialdehyde, matrix metalloproteinase-2 (MMP-2), plasminogen activator inhibitor-1 (PAI-1), fibrosis, kidney biopsies, next generation sequencing (NGS), cell-cycle arrest biomarkers, integrin-linked kinase (ILK), molecular biomarkers, M-type phospholipase A2 receptor, ultrasound elastography, aortic pulse wave velocity, renal arterial resistance index, pulmonary pressure, glomerular filtration rates, and erythrocyte width. A wide spectrum of acute and chronic conditions are described including, nephritis, neoplastic disease, transplantation, allograft damage, cystic fibrosis, diabetes, IgA nephropathy, focal segmental glomerulosclerosis, renal microthrombosis, and dialysis.

There are also many other analytes and conditions described within this volume.

Finally, the last chapter is devoted to locating resource material for biomarker discovery and applications.

The chapters are written by national or international experts and specialist. This book is specifically designed for clinical biochemists, nephrologists, specialists working within the field of kidney disease and treatments, health scientists, epidemiologists, and doctors and nurses, from students to practioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and libraries as a reference guide.

April 2015 Victor R. Preedy
London Vinood B. Patel

Series Preface

In the past decade, there has been a sea change in the way disease is diagnosed and investigated due to the advent of high-throughput technologies and advances in chemistry and physics, leading to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology, immunoassays, or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is still at the preclinical stage. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the book series **Biomarkers in Disease: Methods, Discoveries and Applications**. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and treatments. It links conventional approaches with new platforms. The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Potential Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in **Potential Applications to Prognosis, Other Diseases, or Conditions** pertains to speculative or proposed areas of research, cross-transference to other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The Series is expected to prove useful for clinicians, scientists, epidemiologists, doctors, and nurses, and also academicians and students at an advanced level.

April 2015 Victor R. Preedy
London

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Dr. Vinood B. Patel B.Sc., Ph.D., FRSC is currently a Reader in Clinical Biochemistry at the University of Westminster and honorary fellow at King's College London. He presently directs studies on metabolic pathways involved in liver disease, particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol, and fatty acids in the pathophysiology of liver disease. Other areas of interest are identifying new biomarkers that can be used for diagnosis and prognosis of liver disease, understanding mitochondrial oxidative stress in Alzheimer's disease, and gastrointestinal dysfunction in autism. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his Ph.D. in Protein Metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr. Patel is a nationally and internationally recognized liver researcher and was involved in several NIH-funded biomedical grants related to alcoholic liver disease. Dr. Patel has edited biomedical books in the area of nutrition and health prevention, autism, and biomarkers and has published over 150 articles, and in 2014 he was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy B.Sc., Ph.D., D.Sc., FRSB, FRSH, FRIPHH, FRSPH, FRCPath, FRSC is a senior member of King's College London (Professor of Nutritional Biochemistry) and King's College Hospital (Professor of Clinical Biochemistry; Honorary). He is attached to both the Diabetes and Nutritional Sciences Division and the Department of Nutrition and Dietetics. He is also founding and

current Director of the Genomics Centre and a member of the School of Medicine. Professor Preedy graduated in 1974 with an Honours Degree in Biology and Physiology with Pharmacology. He gained his University of London Ph.D. in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993 he gained his second doctoral degree, for his contribution to the science of protein metabolism in health and disease. Professor Preedy was elected as a Fellow of the Institute of Biology (Society of Biology) in 1995 and to the Royal College of Pathologists in 2000. He was then elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health and Hygiene (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public Health, and in 2012 a Fellow of the Royal Society of Chemistry. In 2015, the Society of Biology received its Royal Charter, so Professor Preedy became an FRSB. In his career, Professor Preedy worked at the National Heart Hospital (part of Imperial College London) and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on biomedical sciences and has a long-standing interest in analytical methods and biomarkers, especially their applications to the study of health and disease. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 500 articles, which includes peer-reviewed manuscripts based on original research, reviews, abstracts, and numerous books and volumes.

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Part I

General Aspects

Biomarkers in Kidney Transplantation

1

Mohsen Nafar and Shiva Samavat

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Abstract

Kidney transplantation is the optimal renal replacement therapy. The progressions in immunosuppressive drugs improved the short-term survival, but 10-year graft survival is about 50 %, only. Acute or chronic rejection, drug nephrotoxicity, and transplant glomerulopathy all have adverse impacts on graft survival. Most of these events are the result of over- or under-immunosuppression.

On the other hand, tolerance as a state of no immunosuppression in the presence of functioning graft is an ultimate goal of transplantation.

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In order to individualize treatments and recognize the optimal level of immunosuppression, noninvasive methods for diagnosis of acute rejection and tolerance have been developed, and biomarkers in the shade of technological advances would help physician in this way. Peripheral blood cell, plasma, and urine are readily accessible and perfect specimens for identification of biomarkers. This review is focused on recently developed biomarkers in acute rejection and tolerance as the two most important processes in decision-making about immunosuppressive therapy. The clinical utilities and limitations of these markers are discussed in details.

Keywords

Kidney transplantation • Acute rejection • Tolerance • Biomarker • Genomics • Proteomics • miRNA

Abbreviations

AR	Acute rejection
ATI	Acute tubular injury
ATN	Acute tubular necrosis
AUC	Area under the curve
BPAR	Biopsy-proven acute rejection
CAD	Chronic allograft dysfunction
CAMR	Chronic antibody-mediated rejection
CE-MS	Capillary electrophoresis mass spectrometry
CMV	Cytomegalovirus
COT	Clinical operational tolerance
Cr	Creatinine
CXCL-10	C-X-C motif chemokine 10
DGF	Delayed graft function
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
Foxp3	Forkhead/winged helix transcription factor
IF/TA	Interstitial fibrosis/tubular atrophy
IRI	Ischemia-reperfusion injury
IS	Immunosuppression
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MMP-8	Matrix metalloproteinase-8
NPV	Negative predictive value
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PPV	Positive predictive value
qPCR	Quantitative polymerase chain reaction
RT-qPCR	Real-time quantitative polymerase chain reaction
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry

TCMR	T-cell-mediated rejection
TG	Transplant glomerulopathy
TOL	Tolerance
Treg	Regulatory T-cells
UMOD	Uromodulin
UTI	Urinary tract infection
VEGF	Vascular endothelial growth factor

Key Facts

Key Facts of Operational Tolerance

- Operational tolerance is a state of stable graft function despite cessation of immunosuppressive drug for more than a year without evidences of chronic rejection.
- Most cases were reported in liver transplantation.
- The majority of cases in renal transplantation are due to noncompliance or intentional withdrawal due to lymphoproliferative disorders.
- Lack of donor-specific antibodies and donors of young age are related to operational tolerance.

Key Facts of Costimulatory Signal

- T-cell activation requires two signals.
- Signal 1 is an antigen-specific pathway that involves T-cell receptor and major histocompatibility complex.
- Signal 2 is the result of other T-cell surface receptors and their ligands on antigen-presenting cell.
- Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and CD28 and their ligands B7-1 and B7-2 are the major receptors involved.
- CTLA-4 binding to B7-1 and B7-2 is an inhibitory signal and leads to anergy.
- Abatacept and belatacept are CTLA4Igs that block costimulatory signal.
- CTLA4Ig is a competitive inhibitor of CD28 binding.
- Targeting receptors and/or ligands in costimulatory pathway is a way to increase graft survival.

Definitions

ELISA The enzyme-linked immunosorbent assay is based on antigen and antibody interaction and enzyme-induced color changes in substrate. Antigens are attached into wells in a plate. Then an antibody that can bind to the antigen and is linked to an enzyme is added. The next step is the addition of substrate. The reaction causes color

change in the substrate, and the intensity of the color signal is indicative of the amount of antigen present.

Genomics Genomics is a combination of genome detection methods (polymerase chain reaction) and bioinformatics to detect the whole genome in a cell and to identify the function and pathways that are involved.

Microarray Microarray is one of the tools in genomics, which is consisted of a glass slide with DNA molecules attached to it in specific spots. It detects gene expression, and the data is processed and normalized and the results are expressed in a gene expression matrix. The information from microarray studies is presented either in absolute measures or expression ratio.

MicroRNAs MicroRNAs (miRNAs) are 21–23-nucleotide noncoding RNAs that regulate posttranscriptional gene expression by binding to target mRNA leading to either the degradation of mRNA or inhibition of their transcription.

Proteomics Proteomics is the analysis of the whole protein content of a biofluid. The changes in the proteomes are caused by changes in synthesis or modifications during the course of biologic or pathologic processes. These modifications can be used as specific markers of the process.

SELDI-TOF technique One of the proteomic techniques for profiling the proteome of different types of samples using mass spectrometer. This technique does not need sample preparation procedure and may serve as a diagnostic tool. Low resolution and lack of reproducibility are some of the limitations of this technique.

Introduction

Kidney transplantation is the most physiologic renal replacement therapy. Despite significant improvement in 1-year graft survival, long-term graft survival improvement was minimally increased (Hariharan et al. 2000).

During the early phase (first 2 weeks, mostly) of kidney transplantation, factors affecting the outcome are those related to the status of the donated kidney, ischemia-reperfusion injury, acute tubular necrosis (ATN), and the resulting delayed graft function (DGF). Acute rejections whether antibody-mediated or cell-mediated ones are other determinants of graft survival especially during the first posttransplant year.

During recent years, advances in immunosuppressive protocols lead to better short-term graft survival. On the contrary, the incidence of highly sensitized recipients, extended criteria donors, and marginal kidney quality are rising, and therefore detecting patients at higher risk of acute rejection and prompt intervention is critical to save the organ.

The early-phase insults might occur subclinically and consequently cause chronic allograft rejection, transplant glomerulopathy, and end in chronic allograft loss.

Detecting rejection based on currently available techniques (increased serum creatinine or allograft biopsy) is either inaccurate, late, or invasive.

There is an urgent need for markers of graft status from early to late phase of transplantation to ensure timely diagnosis of events before irreversible histologic damage occurred.

On the other hand, overzealous immunosuppression causes infection and malignancies in the long term. It would be wise to adjust immunosuppressive regimes according to the immunologic risk of each individual patient (Lodhi and Meier-Kriesche 2011).

In order to define a biomarker or a panel of biomarkers for a specific process, apart from accuracy, precision, and validity, one must describe the clinical utility of the marker, such as when to evaluate and the frequency of assessments. Additionally, these biomarkers must be clinically available and cost effective.

Biofluids such as blood and urine are readily available and relatively noninvasive samples with the ability of repeated sampling and follow-up monitoring.

Finding and proving the clinical use of biomarkers of ATN, DGF, acute rejection, transplant glomerulopathy (TG), chronic allograft dysfunction (CAD), and tolerance would help to prolong allograft survival. In the following sections, biomarkers of acute rejection and allograft tolerance will be discussed as a guide for immunosuppression therapy.

Biomarkers of Allograft Rejection

Diagnosis of acute rejection is currently based on histologic assessment of allograft sample, which is invasive and has a minor risk of bleeding complications. Additionally, current markers such as serum creatinine cannot detect subclinical rejections (Rush et al. 1994). To improve clinical outcome, there is a need to find markers that predict events before histopathologic and mostly irreversible evidences of rejection become evident and have the ability to differentiate rejection from other causes of allograft inflammation and dysfunction such as pyelonephritis, viral infection, and ATN.

Differentially expressed proteins in blood or urine sample of transplant patients might help to have early diagnosis, predict outcome, and response to therapy in a noninvasive way.

Urine Biomarkers

Urine is an easily accessible biofluid, which allows repeated sampling and reflects intrarenal processes.

Perforin, Granzyme B, and Fas-L mRNA

The major players in cell-mediated rejection are cytotoxic T-cells. $CD8^+$ T-cells are first cells that appear at the scene of rejection. Activated cytotoxic T-cells release granzyme B and perforin. Perforin allows granzyme B to enter the target cells and lead to cell death via mitochondrial apoptotic pathways. Additionally, a small portion of endothelial cell death is mediated by Fas-ligand (Fas-L) pathway (Choy 2010). Apart from $CD8^+$ T-cells, $CD30^+$ T-cells have been proven to be involved in

alloimmunity, and CD30 acts as a costimulatory molecule (Süsal et al. 2011). Thus, urinary cytotoxic markers might be helpful in diagnosis of acute rejection.

Urinary concentration of perforin and granzyme B mRNA was elevated in 24 patients with biopsy-proven acute rejection (BPAR) compared with 22 patients with other diagnoses (chronic allograft nephropathy, toxic tubulopathy, ATN, and nonspecific findings). The ROC curve for perforin mRNA at the cutoff of 0.9 fg per microgram of total RNA showed 83 % sensitivity and specificity for diagnosis of acute rejection. At the cutoff point of 0.4 fg per microgram of total RNA for granzyme B mRNA, granzyme B had 79 % sensitivity and 77 % specificity in identifying acute rejection (Li et al. 2001). These data demonstrate diagnostic value of cytotoxic markers; however, the question is whether they could distinguish acute rejection from other etiologies of inflammation. In a study, urinary mRNA levels of perforin, granzyme B, and Fas-L were followed longitudinally in 37 cadaveric transplant patients by the means of real-time PCR assay. Urine samples were collected during the episodes of BPAR, cytomegalovirus (CMV) infection and disease, urinary tract infection (UTI), DGF, and CAD. Perforin, granzyme B, and Fas-L mRNA levels were significantly higher in BPAR than controls with stable graft function. Interestingly, the urinary levels of markers were not significantly different among patients with BPAR, UTI, CMV infection or disease, and DGF (Yannaraki et al. 2006). Therefore these markers are not specific for acute rejection and are evidences of graft inflammation.

Granzyme A mRNA

Granzyme A along with granzyme B is the most abundant cytolytic molecules of the effector T-cells. It also triggers inflammation by induction of cytokines. Its role as a biomarker of subclinical and clinical T-cell-mediated rejection (TCMR) has been evaluated in a study on 60 patients in six different groups, including those with stable graft function, CMV infection, calcineurin inhibitor toxicity, subclinical rejection (SCR), TCMR-I (with prominent tubulitis), and TCMR-II (with moderate or severe intimal arteritis and tubulitis). High urinary granzyme A mRNA was able to differentiate patients with SCR and TCMR-I from those stable graft function and calcineurin inhibitor toxicity. However, this marker was also elevated in patients with CMV infection; thus, confronting an increased urinary granzyme A, one must rule out the presence of CMV infection by CMV-PCR (van Ham et al. 2010).

It seems that granzyme A could be a useful marker in diagnosis of subclinical rejection after exclusion of CMV infection and gives the clinician enough time to promptly treat the patients before occurrence of irreversible damage.

Foxp3 mRNA

Regulatory T-cells are known since 1975 and have regulatory role in immune response and are involved in tolerance. In the biopsy samples of acute rejection, increased infiltration of Tregs along with effector T-cells has been shown. The immunoregulatory role of Tregs was proven in acute rejections as they controlled further damage. Forkhead/winged helix transcription factor (Foxp3) is

expressed by Tregs and could be used as a marker of their presence and activity (Brown and Wong 2008).

Urinary expressions of Foxp3 mRNA along with CD3E, perforin, and CD25 were significantly higher in patients with biopsy-proven acute rejection compared with those with chronic allograft nephropathy and stable graft function. Foxp3 mRNA level was inversely correlated with severity of acute rejection. Interestingly, there was no correlation among other markers (perforin, CD3E, and CD25) and serum creatinine in patients with acute rejection. Urinary Foxp3 mRNA was predictive of acute rejection episode reversibility, and at the cutoff of 3.46, it had a sensitivity of 90 % and specificity of 73 % in prediction of reversal of graft function. Furthermore, the combination of serum creatinine and the Foxp3 mRNA level was more accurate in predicting the reversal of acute rejection with 96 % specificity. The results indicate that the higher the Foxp3 mRNA level, the greater the chance of reversal of acute rejection. These are all in line with damage controlling role of Tregs (Muthukumar et al. 2005).

Thus, increased urinary Foxp3 mRNA is useful in diagnosis as well as predicting the outcome of acute rejection.

Cytokine/Chemokine mRNA

Cytokines and chemokines (chemotactic cytokines) play a major role in the inflammatory cascade. Each cytokine represents activation of a specific pathway.

C-X-C motif chemokine 10 (CXCL-10) also known as interferon gamma-induced protein 10 (IP-10) is secreted by monocytes, endothelial cells, and renal tubular and mesangial cells in response to interferon-γ (IFNγ). CXCL-10 by binding to its receptor CXCR-3 on activated T-cells and natural killer cells leads to leukocyte recruitment during acute rejection (Ho et al. 2011).

Data suggested that urinary CXCL-10 elevation preceded serum creatinine rise. Urine CXCL-10 can be used as a marker of inflammation and can distinguish tubulitis (histologic characteristic of cellular rejection) from fibrosis. In a study of 91 patients with a wide range of histologic findings from normal to various degrees of tubulitis (borderline, subclinical, and clinical tubulitis) and those with interstitial fibrosis and tubular atrophy (IF/TA), urine CXCL-10-to-creatinine (CXCL-10/Cr) ratio at the cutoff of 2.87 ng/mmol had 81.8 % sensitivity and 86.4 % specificity in differentiating normal histology from subclinical and clinical tubulitis. At the lower cutoff of 1.97 ng CXCL-10/mmol Cr, the sensitivity and specificity for diagnosis of normal histology versus borderline or subclinical tubulitis were 73.3 % and 72.7 %, respectively (Ho et al. 2011).

Along with CXCL-10, the other CXCR-3 ligand, CXCL-9, was shown to be correlated with subclinical rejection. At the cutoff of 7.5 ng/mmol Cr, CXCL-9 had 86 % sensitivity and 64 % specificity in diagnosis of subclinical tubulitis from normal histology or borderline tubulitis. Urinary CXCL-10 and CXCL-9 were not elevated in those with IF/TA as a sole histologic finding (Schaub et al. 2009).

The advantage of these chemokines is earlier appearance in urine than CXCR-3, perforin, and granzyme B and therefore timely recognition of subclinical tubulitis.

These chemokines have same accuracy in pediatric as well as adult transplant patients (Jackson et al. 2011).

Unlike granzyme B and perforin, urine CXCL-10 level is not increased in other inflammatory processes such as UTI and CMV infection (Ho et al. 2011). Tubulointerstitial inflammation by BK virus and ischemia-reperfusion injury (IRI) might increase urinary levels of CXCL-9 and CXCL-10. Therefore, it is necessary to exclude BK virus infection by plasma PCR. The effects of IRI would not last more than 2 months, and thereafter urine chemokines could be reliable markers of tubulitis due to rejection (Schaub et al. 2009). The influence of UTI on urine chemokines is controversial; thus, to be on the safe side, it is better to rule out UTI by negative urine cultures.

A group evaluated the clinical utility of CXCL-9 in risk stratification, prediction of acute rejection in patients with acute graft dysfunction, and prediction of late graft loss. In the setting of acute graft dysfunction, urinary levels of CXCL-9 mRNA had a negative predictive value (NPV) of more than 92 % in putting acute rejection aside. As its positive predictive value (PPV) was about 61–67 %, this biomarker could not be used instead of the gold standard tissue biopsy, but the high NPV might help to avoid the unnecessary invasive kidney biopsy. The NPV was independent of recipient age, HLA mismatch, and de novo donor-specific antibodies. The elevated urine CXCL-9 mRNA level preceded the serum creatinine increment by almost 30 days, and thus it could be used as a predictor of intragraft inflammation days before the clinically evident increase in serum creatinine and as a guide for prompt treatment. Additionally, high urine CXCL-9 mRNA level at 6 months posttransplantation could predict >30 % decrement in estimated glomerular filtration rate (eGFR) at 24 months posttransplantation. In this study, urinary level of CXCL-9 was higher in patients with acute rejection than in those with BK virus infection (Hricik et al. 2013).

Briefly, the urinary mRNA of CXCL-9 is a promising marker to rule out acute rejection and graft inflammation based on its high NPV. As measurement of CXCL-9 protein by ELISA is easier and more reliable in clinical settings, according to the current data, its use to exclude acute rejection is suggested.

OX40/OX40-L mRNA

During T-cell activation along with T-cell receptor (TCR) and major histocompatibility complex (MHC) interaction on antigen-presenting cells (APC), there are second regulatory signals consisted of costimulatory and co-inhibitory pathways (Fig. 1). The major molecular players of these pathways are from either the immunoglobulin superfamily (CD28, CTLA-4, CD80 and CD86, PD-1, and PD-L) or the TNF family (CD40, CD40L, OX40, and OX40-L) (Ford et al. 2014).

OX40 interaction with its ligand causes memory T-cell generation and cytokine production and results in Th2 response and leads to acute rejection. On the contrary, PD-1 and PD-L ligation acts as an inhibitory signaling pathway on T-cells. In a study, the urinary mRNA expression of costimulatory pathway members was compared between patient with stable graft function and those with biopsy-proven acute rejection. The group reported significantly increased levels of OX40, OX40-L, and PD-1 mRNA in urinary cells of patients with acute rejection. PD-1L levels were not

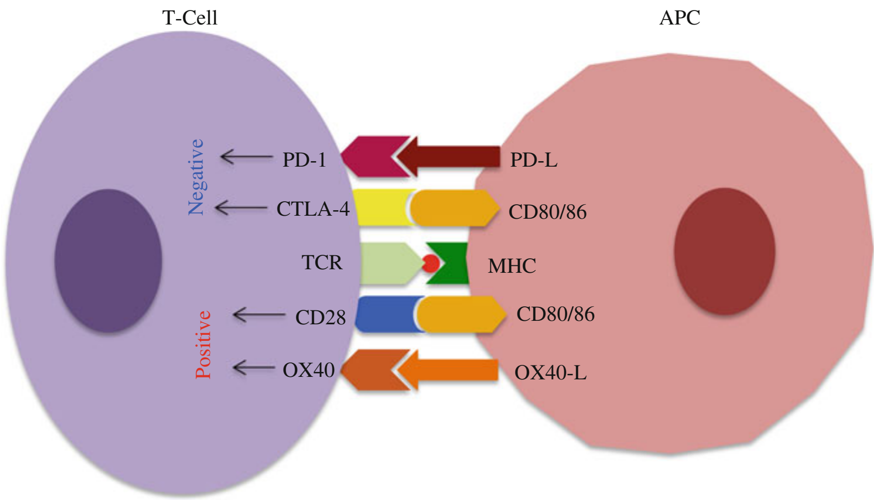

Fig. 1 The costimulatory pathway. Costimulatory signaling results from interaction of ligands on antigen-presenting cells (*APCs*) and the related protein on T-cells. Signals with positive effect lead to T-cell proliferation and cytokine production, and signals with negative effects cause anergy and apoptosis. *CTLA-4* cytotoxic T-lymphocyte-associated protein 4, *MHC* major histocompatibility complex, *PD-1* programmed cell death protein 1, *PD-L* programmed cell death protein 1 ligand, *TCR* T-cell receptor

different between the two groups. OX40 mRNA level alone at a cutoff of 5.98 had a sensitivity of 81 % and specificity of 88 % in diagnosis of acute rejection. When combined with urinary levels of mRNA for OX40-L, PD-1, and Foxp3, the sensitivity and specificity would rise to 95 % and 92 %, respectively. Also the higher OX40-L mRNA level (cutoff value of 3.79) predicted the higher probability of reversal of acute rejection (sensitivity of 69 % and specificity of 100 %) (Afaneh et al. 2010).

Thus, OX40 and its ligand might be used as diagnostic and also predictive biomarker of acute rejection.

mRNA Signature

In a recent study, investigators introduced a urinary mRNA profile instead of a single mRNA in approach to kidney transplant patient with acute graft dysfunction by the means of RT-qPCR. They suggested an mRNA signature with the ability to differentiate acute rejection (AR) from acute tubular injury (ATI).

Combination of urinary values of CD3E, CD105, TLR4, CD14, complement factor B, and vimentin mRNAs formed a diagnostic signature that differentiated AR from ATI. Data suggested that using this signature decreases the unnecessary allograft biopsies. Among patients with AR, a five-mRNA diagnostic model was developed that differentiated acute cellular rejection (ACR) from antibody-mediated rejection (AMR). This model was consisted of CD3E, CD105, CD14, CD46, and 18S rRNA with the area under the curve of 0.81 (95 % confidence interval,

0.68–0.93). Decision curve analysis to assess the clinical benefit was performed in this study (Matignon et al. 2014).

Briefly, using the signature model of mRNAs helps decreasing the number of biopsies in patients with acute graft dysfunction.

Urine miRNAs

MicroRNAs (miRNAs) are 21–23-nucleotide noncoding RNAs that regulate post-transcriptional gene expression by binding to target mRNA leading to either the degradation of mRNA or inhibition of their transcription. They play a role in almost every cellular pathway, and each cell type has its own miRNA pattern. The miRNA profile is representative of the ongoing biologic process and could be evaluated in different biofluids such as urine, blood, and other body fluids. Despite its high cost, RT-qPCR has the ability of detecting a wide range of miRNA when compared with microarray (Mas et al. 2013).

Lorenzen et al. were the first group evaluating the diagnostic role of urine miRNA in acute rejection. Using RT-qPCR, urine samples of 62 patients with biopsy-proven acute rejection were compared with those of patients with stable graft function. The initial data found 21 differentially expressed miRNAs among patients and controls. Among these miRNAs, miR-210 and miR-10b were downregulated, and miR-10a was upregulated in patients with acute rejection compared to the controls with stable graft function. Lower levels of miR-201 were correlated with faster eGFR decline and more severe rejection. Successful reversal of acute rejection normalized the miR-210 and miR-10b levels. The variations in urine levels of miR-210 were independent of the presence of leukocyturia and UTI and age (Lorenzen et al. 2011). If further validation studies confirm these findings, miR-210 could serve as a noninvasive biomarker in diagnosis of acute rejection. However, based on the results from samples collected before evolution of rejection, miR-210 could not predict the impending episodes of acute rejection.

Urine Proteomics

In search for biomarkers, urine proteome profile comes to the center of attention. It is the indicator of local processes in kidney and systemic events that might change urine proteins. In order to characterize urine proteome profile in acute rejection, several studies have been performed (Table 1). Some are discussed in more details.

In a study on 73 patients with graft dysfunction who underwent indication biopsy, by the means of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), two differentially expressed peptides were identified. In patients with acute rejection compared with other causes of graft dysfunction, urinary expression of human β-defensin-1 (HBD-1) was reduced, and urinary expression of α-1-antichymotrypsin (ACT) was elevated. Both of these markers are part of inflammatory and immune responses. When used in combination, the elevated ACT and decreased HBD-1 levels, the sensitivity and specificity for diagnosis of acute rejection would be 85.7 % and 80.2 %, respectively (O'Riordan et al. 2007).

Table 1 Selected urine biomarker for acute allograft rejection

Biomarker	Detection method	Cutoff	AUC	Sensitivity (%)	Specificity (%)	Reference
sHLA-DR	ELISA	15 U/mL	0.88	80	98	Ting et al. (2010)
sUPAR	ELISA	NA	NA	NA	NA	Roelofs et al. (2003)
VEGF	ELISA	3.64 pg/μmol Cr	0.871	85.1	78.4	Peng et al. (2008)
MASP2	LC-MS/MS	NA	NA	NA	NA	Loftheim et al. (2012)
CD103 mRNA	RT-qPCR	8.16 copies/μg Cr	0.73	59	75	Ding et al. (2003)
TIM-3 mRNA	RT-qPCR	1.2[a]	0.96	84	96	Manfro et al. (2008)
ChrY dd-cfDNA[b]	dPCR	≥3 copies of ChrY/K μg Cr	0.80	81	75	Sigdel et al. (2013)

AUC area under curve, *CE-MS* capillary electrophoresis mass spectrometry, *ChrY dd-cfDNA* chromosome Y donor-derived cell-free DNA, *Cr* creatinine, *dPCR* digital polymerase chain reaction, *MASP2* isoform 2 of mannan-binding lectin serine protease 2, *MMP-8* matrix metalloproteinase-8, *NA* not available, *RT-qPCR* real-time quantitative polymerase chain reaction, *sUPAR* soluble urokinase-type plasminogen activator receptor, *TIM-3* T-cell immunoglobulin mucin domain 3, *VEGF* vascular endothelial growth factor

[a]By the relative quantification method $2^{-\Delta\Delta CT}$

[b]It is a sensitive marker for diagnosis of acute allograft injury, but it is not that specific to distinguish acute rejection from BK virus nephropathy

Metzger et al. conducted a multicenter study on 103 transplant patients to identify biomarkers of acute subclinical and clinical rejection and the role of confounding conditions such CMV infection, BK virus infection, and UTI. Capillary electrophoresis mass spectrometry (CE-MS) analyses were used to evaluate urine peptide pattern. Not a single peptide was able to discriminate rejection from other clinical conditions with an acceptable specificity, but a panel of 14 differentially expressed peptides was extracted with the area under the curve (AUC) of 0.89. In order to further validate the panel, the group used it in a validation set and reached an AUC of 0.91 and 93 % sensitivity and 78 % specificity. The presence of UTI and CMV infection did not cause any misclassification. Most of the peptides in this panel were collagen α-1 fragments, which could be an indicator of extracellular matrix degradation and matrix metalloproteinase-8 (MMP-8) activity (Metzger et al. 2011).

Sigdel et al. conducted a shotgun proteomic study with capillary LC-MS/MS on 92 urine samples of patients, including those with biopsy-proven acute rejection, stable graft function, nephrotic syndrome, and healthy controls. The advantage of this study is that they further validated the identified markers by ELISA in an independent set of samples, which is more cost effective, and affordable assay for clinical use. Most of the discriminating proteins in the acute rejection group were MHC antigens, complement pathway proteins, and extracellular matrix proteins. Applying ELISA, they reported significantly decreased uromodulin (UMOD) (AUC = 84.6 %) and CD44 (AUC = 97.3 %) in those with acute rejection with a

Table 2 Urine biomarker panels in diagnosis of acute allograft rejection

Biomarker panel	Detection method	Reference
ANXA11 (↑), integrin α3 (↑), integrin β3 (↑), TNF-α (↑)	Antibody microarrays and reverse capture protein microarray	Srivastava et al. (2011)
IP-10 (↑), MIG (↑), I-TAC (↑)	Luminex assays	Huang et al. (2014)
UMOD (↓), SERPINF1 (↑), CD44 (↑)	LC-MS/MS	Sigdel et al. (2010)
COL1A2, COL3A1, UMOD, MMP-7, SERPING1, TIMP1[a]	LC-MS and multiple reaction monitoring (MRM)	Ling et al. (2010)
HLA-DRB1 (↑), fibrinogen beta (↑), fibrinogen gamma (↑)	iTRAQ	Sigdel et al. (2014)
ID-3796 peptide and 13 collagen α (I, III) fragments	CE-MS	Metzger et al. (2011)
CLCA1 (↑), PROS1 (↑), and KIAA0753 (↑)[b]	2D-LC-MS/MS	Sigdel et al. (2014b)

ANXA11 annexin A 11, *COL1A* collagen type 1 α, *CLCA1* calcium-activated chloride channel regulator-1, *IP-10* IFN-induced protein 10, *I-TAC* IFN-induced T-cell chemoattractant, *MIG* monokine induced by IFNγ, *MMP-7* matrix metalloproteinase-7, *PROS1* vitamin K-dependent protein S, *SERPINF1* pigment epithelium-derived factor (PEDF), *SERPING1* serpin peptidase inhibitor, *TIMP1* tissue inhibitor of metalloproteinase 1, *TNF-α* tumor necrosis factor-α, *UMOD* uromodulin

[a]Gene expression

[b]Exosomal proteins

correlation coefficient of 0.99 and 0.84, respectively, and significantly elevated pigment epithelium-derived factor (PEDF, SERPINF1) levels (AUC = 93.2 %) with a correlation coefficient of 0.78. Thus, this pattern of peptides could verify acute rejection in transplant patients with high sensitivity and specificity independent of age, proteinuria, and immunosuppression protocol (Sigdel et al. 2010) (Table 2).

As there are concerns about the confounding factors such as the amount of proteins in urine (the effect of highly abundant proteins on identification of proteins with lower abundance) and BK virus nephropathy (a pathologically challenging diagnosis), the group conducted a study based on urine peptidomic analysis by LC-MS and multiple reaction monitoring (MRM) on 70 urine samples from 50 transplant patients. Peptidomic analysis provides information about disease-related modification on proteins (proteolytic and antiproteolytic activities). The abundance of UMOD and collagen peptides (COL1A2 and COL3A1) in urine was lower in patients with acute rejection. Evaluating the transcriptome in kidney tissue of these patients demonstrated higher gene expression for matrix metalloproteinase-7 (MMP-7), tissue inhibitor of metalloproteinase 1 (TIMP1), and the serpin peptidase inhibitor (SERPING1) in patients with acute rejection. The abovementioned changes were independent of the presence of BK nephropathy. Apart from being a specific biomarker profile, this panel sheds light on the underlying mechanism of injury during acute rejection and subsequent chronic graft fibrosis: the collagen cascade (Ling et al. 2010).

Recently, the isobaric tags for relative and absolute quantitation (iTRAQ) proteomic technique was used to identify biomarkers of acute rejection. The proteins then were validated by ELISA. Of a total of 389 measured proteins, nine were highly specific for acute rejection. These were identified as: HLA class II protein HLA-DRB1, keratin-14 (KRT14), histone H4 (HIST1H4B), fibrinogen gamma (FGG), actin-beta (ACTB), fibrinogen beta (FGB), fibrinogen alpha (FGA), keratin-7 (KRT7), and dipeptidyl-peptidase-4 (DPP4). These markers could differentiate acute rejection from chronic allograft injury and BK virus nephropathy. Further validation, by ELISA in independent samples, showed increased urinary levels of HLA-DRB1, fibrinogen beta, and fibrinogen gamma (Sigdel et al. 2014a).

Overall, urine peptidomics and proteomics are raising horizon in the land of biomarker studies. The identified profile needs to be validated by a less time and cost-consuming technique such as ELISA for routine clinical utility.

Blood Biomarkers

Evaluating blood biomarkers is also a minimally invasive way to diagnose acute rejection. However, the diagnostic profile might be confounded by systemic milieu, and its sensitivity and specificity might decline. Numerous markers were introduced by different studies using various techniques, but clinical validation is needed before routine application (Table 3).

Table 3 Selected serum biomarker for acute allograft rejection

Biomarker	Method	Sample	Reference
Granzyme B, perforin, Fas-L	RT-PCR	PBL	Vasconcellos et al. (1998)
Foxp3	RT-PCR	PBL	Aquino-Dias et al. (2008)
IFNγ – producing memory T-cell	ELISPOT	Pretransplant PBML	Nickel et al. (2004)
Nitric oxide		Serum	Bellos et al. (2011) and Masin-Spasovska et al. (2013)
PECAM1	ELISA	Serum	Chen et al. (2010)
HLA class I (ABC)	Flow cytometry	Peripheral blood CD3+/CD8+ T lymphocytes	Tian et al. (2009)
Titin, kininogen-1, and LPS-BP	iTRAQ	Plasma	Freue et al. (2010)
IL-1R antagonist, IL-20, and sCD40 ligand	Luminex™ bead array analysis	Serum	Xu et al. (2013)

ELISA enzyme-linked immunosorbent assay, *ELISPOT* enzyme-linked immunosorbent spot, *IL-1R* interleukin-1 receptor, *iTRAQ* isobaric tagging for relative and absolute protein quantification, *LPS-BP* lipopolysaccharide-binding protein, *PBL* peripheral blood leukocytes, *PBML* peripheral blood mononuclear cells, *PECAM1* platelet endothelial cell adhesion molecule 1

CD30

CD30 as a marker of Th2-type immune response has been shown to be associated with allograft outcome (Pelzl et al. 2002). Soluble CD30 (sCD30) as a potential marker of an alloimmunity reaction was evaluated in 203 living kidney transplant patients before, on the fifth day posttransplantation, and at the time of acute increase in serum creatinine with ELISA kit. sCD30 levels among patients with BPAR were compared with those of patients with stable graft function and non-rejection cause of acute allograft dysfunction (including CMV infection, ATN, and calcineurin inhibitor toxicity). sCD30 level on the fifth day posttransplantation with the cutoff value of 41 U/ml predicted the occurrence of acute rejection in the first 6 months with a sensitivity and specificity of 70 % and 71.7 %, respectively. It could not predict the 2-year graft survival. Pretransplant sCD30 level could not predict acute rejection, and there was a significant elevation in sCD30 level during the episodes of BPAR. Thus, sCD30 level after transplantation and its changes could be used as a predictor of acute rejection (Nafar et al. 2009). In a multicenter study on 2,322 transplant patients, investigators demonstrated an association between day 30 posttransplant CD30 level and 3-year graft survival. CD30 levels $\geq$40 U/ml on day 30 were associated with high anti-HLA antibody activity and could be considered as a marker of alloimmunity (Süsal et al. 2011). Same results were obtained in an earlier study, of course with smaller sample size but longer follow-up of 5 years posttransplantation (Delgado et al. 2009). Thus, posttransplant CD30 level might be utilized as a marker of increased alloimmunity and if proved by clinical trials might be used as a guide to immunosuppressive dose adjustment.

Genomics

In order to enhance the sensitivity and specificity of peripheral blood diagnostic tests, transcriptional profile (genomics) was utilized by the means of microarray studies. Gene expression in peripheral blood samples was extensively evaluated in association with acute rejection. Since 1998 that Vasconcellos et al. described the correlation of cytotoxic lymphocyte gene expression (perforin, granzyme B, and Fas-ligand) and acute rejection (Vasconcellos et al. 1998), there are a wide range of studies evaluating gene expression of various effector molecules in diagnosis and prediction of rejection.

T-cell immunoglobulin mucin domain 3 (TIM-3) is a membrane glycoprotein expressed on Th1 cells, cytotoxic T-cells, natural killer cells, and Th17. It has a known role in inducing tolerance. TIM-3 binding to its ligand, galectin-9, results in reduction of cytotoxicity of $CD8^+$ T-cells. TIM-3 mRNA level is proposed as a biomarker of effector T-cell activation and was evaluated in 24 patients with acute rejection, 20 patients with ATN, and 18 patients with stable graft function by the means of RT-PCR. Peripheral blood cell TIM-3 mRNA was significantly higher among patients with acute rejection, and this increased level was not due to decreased GFR. At the threshold of 1.58, TIM-3 mRNA had 100 % sensitivity and 87.5 % specificity in discriminating acute rejection from ATN. The TIM-3 mRNA level did not differentiate refractory from responsive acute rejection (sensitivity of 66.7 % and specificity of 57.1 %). Despite encouraging results, a lack

of biopsy-proven acute rejection in all the cases and exclusion of infective causes of impaired renal function (CMV infection, UTI) brings up the need for further validation of the marker (Luo et al. 2011).

In order to bring biomarkers from bench to beside and assessing their clinical utilities and their limitations, recently the gene expression profiles of patients were studied.

In a large cohort, 367 blood samples from pediatric transplant patients, including 115 patients with biopsy-proven acute rejection, 180 cases with stable graft function, and 72 cases with other causes of graft dysfunction (chronic allograft injury, viral or bacterial infection, calcineurin inhibitor toxicity, and borderline acute rejection), microarray analysis and subsequent quantitative PCR led to the discovery of a five-gene panel. This gene panel consisted of DUSP1, MAPK9, NKTR, PBEF1, and PSEN1. The gene profile is representative of immunologic activity and injury: leukocyte recruitment; B-cell, T-cell, and monocyte activation; oxidative stress; apoptosis; IL-2 pathway activation; increased adhesion; and vascular smooth muscle cell injury. Except MAPK9 and NKTR, which were under-expressed, the remaining three genes were overexpressed in patients with acute rejection. The data was further validated in an independent cohort.

The five-gene model can discriminate acute rejection from those with stable graft function with a sensitivity of 91 % and specificity of 94 % and a NPV of 97 % (AUC 0.955). It also has the ability to separate acute rejection from other causes of graft dysfunction with 91 % and 90 % sensitivity and specificity, respectively. None of the confounding factors affected the results, and the high NPV in the setting of graft dysfunction might decrease the unnecessary biopsies. The downside of the five-gene profile is its inability in detecting borderline rejection and distinguishing humoral from cellular rejection (Li et al. 2012). Further validation for clinical utility in adult recipients is required.

To validate the five-gene panel (DUSP1, MAPK9, NKTR, PBEF1, and PSEN1) in Korean patients, Lee et al. conducted a study on 143 recipients. Patients with acute cellular rejection had significantly lower levels of MAPK9 and higher PSEN1 than controls. However, patients with acute antibody mediated had the similar profile with controls and those with other graft injuries (BK nephropathy, calcineurin inhibitor toxicity, glomerulonephritis, and ATN). Conversely, PSEN1 level was lower and MAPK9 level was higher in patients with other graft injuries. The two-gene set alone had 73.33 % sensitivity and 75 % specificity (AUC, 0.841) in discriminating acute cellular rejection from other causes of graft injury. However, the five-gene set in combination with clinical variables had 90 % sensitivity and specificity (AUC, 0.964) and PPV of 93.1 and NPV of 85.1. Therefore, this five-gene panel is a promising tool for diagnosis of acute cellular rejection from other causes of graft dysfunction (Lee et al. 2014).

Recently, Roedder et al. studied blood gene expression on 558 blood samples of 436 transplant patients both pediatric and adults in a multicenter study. Using real-time quantitative PCR (RT-qPCR), patients with acute rejection were compared with patients with other causes of graft dysfunction (chronic allograft injury, chronic calcineurin inhibitor toxicity, BK virus infection, and acute tubular nephritis).

They utilized the previously reported ten-gene panel (DUSP1, CFLAR, ITGAX, NAMPT, MAPK9, RNF130, IFNGR1, PSEN1, RYBP, and NKTR) (Li et al. 2012) and added seven genes (SLC25A37, CEACAM4, RARA, RXRA, EPOR, GZMK, RHEB). This 17-gene panel showed a significantly higher sensitivity (82.98 %) and specificity (90.63 %), with an AUC of 0.94 (95 % CI 0.91–0.98, $p < 0.001$). The 17-gene panel identified as the Kidney Solid Organ Response Test (kSORT) was validated in a 124 sample independent cohort, and a further cross-validation was performed on 100 samples. In the validation group, the mean predicted probability of acute rejection was significantly different between the two groups as reported in the training set. The kSORT is a sensitive and specific noninvasive test to detect acute rejection whether cellular or antibody mediated. Its high specificity and NPV (91.58 %) make it a valuable marker with a utility as a negative predictor of rejection. As most of the genes in the panel are related to monocyte activation, and monocyte activation is evident in both cellular- and antibody-mediated rejection, one of the limitations of kSORT is its inability to differentiate between these two types of rejection. In order to be used as a predictor of acute rejection, the group designed a longitudinal multicenter study and evaluated 191 blood samples before, at the time, and after acute rejection in an independent cohort. kSORT could predict clinical acute rejection in more than 60 % of samples up to 3 months before the clinical or histological event. After further validations, this panel might replace the invasive protocol biopsy in prediction of subclinical rejection. The group also created a risk score for acute rejection called kSAS (kSORT analysis suite). kSAS algorithm is able to categorize patients according to the risk of acute rejection: high risk for AR (risk score ≥ 9), low risk for AR (risk score ≤ -9), and indeterminate (risk score <9 and > -9) (Roedder et al. 2015).

It seems that after further validation in clinical trials, kSORT could be used as a diagnostic and predictive marker of acute rejection.

miRNAs

Like urine samples and tissue biopsies, peripheral blood samples could be assessed for the presence of miRNAs with the ability to diagnose acute rejection.

miRNAs were evaluated in 32 renal transplant patients including 11 patients with biopsy-proven acute rejection. Both intragraft and peripheral blood mononuclear cells (PBMCs) were evaluated for miRNA expression. miR-142-5p, miR-155, and miR-223 were overexpressed both in biopsy samples and in the peripheral blood (Angelicheau et al. 2009). The study showed correlation between tissue and serum markers, which could be the base for further investigations.

In a study on 12 transplant patients, eight of which had an episode biopsy-proven acute rejection, expression of miRNAs was analyzed in serum by qPCR. miR-223 and miR-10a were significantly reduced among patients with acute rejection. Although the results are encouraging, they must be interpreted keeping in mind the small number of cases (Betts et al. 2014). On the contrary, Lui et al. in their report on 12 transplant patients with acute rejection (in a cohort of 33 patients) demonstrated elevated levels of miR-223 in PBMCs at the time of rejection with a sensitivity of 92 % and specificity of 90 % in diagnosis of acute rejection

(Scian et al. 2013). Small number of cases and different study design may explain the discrepancies.

In a cohort of 112 transplant patients and 11 healthy controls, the miRNA profile of patients with chronic antibody-mediated rejection (CAMR) differed from that of acute rejection. Increased expression of miR-142-5p in PBMCs has been reported in CAMR. It was also reported to be able to discriminate CAMR from those with stable renal function (AUC, 0.74) (Danger et al. 2013).

As mentioned above, most of the miRNA studies are on urine samples, and the recent data opens new fields in biomarker studies in PBMCs or blood samples.

Overall, biomarker identification is a science in evolution, and there is a long way ahead in order to introduce a biomarker or a panel of biomarkers with accurate clinical utility to substitute the invasive gold standard "allograft biopsy."

Biomarkers of Tolerance

Allograft transplantation is the treatment of choice in patients with end-stage renal disease. The downside of transplantation is the long-term need for immunosuppression with infections, malignancies, and nephrotoxicity of drugs as the main side effects. Tolerance gives the opportunity to cease the immunosuppression or to minimize it. Attempts to induce tolerance were not a great success. In order to identify patients who are candidates for immunosuppression minimization or withdrawal, biomarkers of tolerance have been evaluated among patients with "clinical operational tolerance (COT)." COT is a state of tolerating the allograft in the absence of immunosuppressive drugs without pathologic evidences of rejection for at least 1 year. About 100 patients with kidney transplantation have been reported to be at the state of COT, mostly due to noncompliance or lymphoproliferative disorders (Orlando et al. 2010). The clinicians need an assay to guide them in safe reduction in immunosuppression in selected patients without increasing the risk of acute rejection; thus, most of the studies conducted on patients with COT. To evaluate biomarkers, a sample size of at least 200 is needed, and in order to compensate the lack of adequate sample size, studies were conducted on training set, validation set, and cross-validation sets. Data on urine biomarkers are rare. Most promising data come from gene expression studies in peripheral blood, although flow cytometry and ELISA methods also have been used (Gökmen and Hernandez-Fuentes 2013) (Table 4).

Gene Expression Studies

Gene expression microarray assays using RT-qPCR are valuable tools for biomarker discovery and extracting functional and biological role of the marker by the means of bioinformatics.

One of the earliest studies on biomarkers of tolerance was conducted by Brouard et al. They performed a microarray study on a group of 17 COT patients (5 in

Table 4 Biomarkers of tolerance

Biomarker set	Detection method	Reference
IGKV4-1, IGLL1, IGKV1D-13[a]	Multiplex real-time PCR	Newell et al. (2010)
CD79B, TCL1A, HS3ST1, SH2D1B, MS4A1, TLR5, FCRL1, PNOC, SLC8A1, FCRL2[a]	Microarray, RT-PCR	Sagoo et al. (2010)
Foxp3, CCL20, TLE4, CDH2, PARVG, SPON1, RAB30, BTLA, SMILE, SOX3, CHEK1, HBB, DEPDC1, CDC2[a]	Microarray, RT-qPCR	Brouard et al. (2007)
KLF6, BNC2, CYP1B1[a]	Microarray, qPCR	Roedder et al. (2015)
miR-142-3p	Microarray	Danger et al. (2012)
Urine CD20	RT-qPCR	Newell et al. (2010)

qPCR quantitative polymerase chain reaction, *RT-qPCR* real-time quantitative polymerase chain reaction
[a]Gene set as a biomarker

training group and 12 in test group) and compared the results with healthy controls and those with various graft statuses (chronic rejection, stable graft function on immunosuppressive therapy, and those on steroid monotherapy). A set of 49 genes was identified as the footprint of tolerance. Among these genes, 33 distinguished tolerance from chronic rejection with 86 % sensitivity and 99 % specificity. The identified genes were involved in costimulatory signaling and memory T-cell response. They also suggested a role for transforming growth factor-β pathways. If validated in larger cohorts, this panel could be used as a guide for immunosuppression reduction (Brouard et al. 2007).

In a cohort of 25 COT patients (off immunosuppressive drug for at least a year, 20 due to noncompliance), 33 patients with stable graft function, and 42 healthy controls, a microarray study conducted on whole-blood total RNA. A set of five genes were differentially expressed between COT and stable patients – TUBB2A, TCL1A, BRDG1, HTPAP, and PPPAPDC1B – all of which were involved in B-cell activation. The COT group had higher expression of CD20 transcript in urine sediment compared to those on immunosuppressive drugs. After performing multiplex RT-PCR, a 3-gene set found to predict tolerance – IGKV4-1, IGLL1, and IGKV1D-13 – with PPV of 83 % and NPV of 84 %. Whole-blood flow cytometry confirmed a significantly higher number of total B-cells, naïve B-cells, and transitional B-cells (CD19+CD38+CD24+IgD+) in COT patients than in those with stable graft function on drugs. Among the flow cytometry results, transitional B-cell had the highest predictive value for COT (85 % and 96 % PPV and NPV, respectively). These results pointed out the important role of B-cell in tolerance and introduced the 3-gene set as a predictive marker of tolerance (Newell et al. 2010).

Following this study, Sagoo et al. studied a cohort of 71 kidney transplant patients – 11 patients with COT, 11 patients on low-dose prednisolone only, 40 patients on full immunosuppression, and 9 patients with pathologic evidence of chronic rejection. Interestingly, the COT group had the highest degree of HLA mismatch but undetectable donor-specific anti-HLA antibodies. Microarray, RT-qPCR, and flow cytometry techniques were applied on peripheral blood monocyte cells (PBMCs). Recipients with COT (like healthy controls) had the highest B-cell-to-T-cell ratio, which was the result of an elevated number of B-cells rather than reduction of T-cell population. COT patients had decreased proportion of memory B-cells and activated T-cells and increased proportion of transitional B-cells as previously reported by Newell et al. Additionally, tolerant patients had a high ratio of Foxp3/α-1,2-mannosidase in peripheral blood. The microarray data and RT-qPCR resulted in a 10-gene set with diagnostic capability (See Table 4). The set could discriminate COT from non-tolerant transplant patients with 80.6 % sensitivity, 89 % specificity, and 93 % NPV (Sagoo et al. 2010).

Overall, these studies pointed out the significance of B-cell and natural killer cell (NK cell) expansion in tolerant patients. The B-cell signature of tolerance has been developed in two independent cohorts (increased number of naïve and transitional B-cells), and after further validation in larger cohorts, this could be used to choose patients for immunosuppression minimization or cessation. In a prospective observational study, Viklicky et al. tried to validate the abovementioned gene set as a guide in immunosuppression minimization. They compared operational tolerance-associated transcripts (MS4A1, CD79B, TCL1A, TMEM176B, Foxp3, TOAG-1, MAN1A1 (α-1,2-mannosidase), and TLR5) in patients with and without acute rejection in the first day posttransplantation. The expressions of MS4A1 (CD20), CD79B, TCL1A, and TOAG-1 as markers of naïve and immature B-cells were significantly higher in patients without acute rejection, as well as the Foxp3/α-1,2-mannosidase ratio. The expression of TLR5 was not different between the examined groups, and TMEM176B expression was higher in rejection group (Viklicky et al. 2013).

It seems that these seven genes have the capacity to be used as criteria to select patients who are still on immunosuppressive regimes for drug minimization or withdrawal.

In the most recent study, with the aim of providing a highly cross-validated COT gene signature in blood samples and estimating the frequency of the gene signature in patients on immunosuppressive drug, 571 peripheral blood samples were assessed by microarray, qPCR, and flow cytometric analysis (cross-platform) in a four-stage study design. The smallest gene set with the best performance in detection of COT was a three-gene set, KLF6, BNC2, and CYP1B1, with 84.6 % and 90.2 % sensitivity and specificity, respectively. Besides the strong B-cell signature in tolerance, the flow cytometric analysis results demonstrated decreased total number of T-cells and $CD4^{-}/CD3^{+}$ T-cells in COT patients. On the other hand, monocytes and dendritic cells were significantly increased in COT patients.

The study cross-validated the previous findings and introduced a highly validated assay to recognize patients with tolerance, which are still on immunosuppressive drugs and are targets of immunosuppression reduction. The frequency of predicted accommodation was 7.3 % by this assay (Roedder et al. 2015).

Briefly, all the abovementioned genomic studies and the cross-platform biomarkers of tolerance (gene expression, flow cytometry, anti-donor immune response, and anti-donor antibodies) are the first few steps in the long way of establishing personalized transplantation medicine.

Potential Applications to Prognosis, Other Diseases, or Conditions

Two major treats in the allograft patients are rejection and infection. In order to counteract these treats, balanced immunosuppression is needed. Determining the immunologic risk of every patient, and adjusting the immunosuppressive regime according to it, is the optimal way to face this issue.

In the light of novel biomarkers, physicians will be able to estimate the immunologic risk in the pretransplant period and prescribe initial immunosuppression in a personalized fashion rather than in a protocol-wise manner. Biomarkers of acute rejection after transplantation can be utilized to identify subclinical rejections and provide timely intervention before clinical and yet irreversible histological changes occur. The absence of these biomarkers helps in choosing appropriate patients for immunosuppressive withdrawal in the presence of the tolerance molecular signature.

Both the tolerance signatures and the rejection predictors are beneficial during the follow-up of patients with minimization or cessation of immunosuppressive agents. Figure 2 is a schematic plan of what would be the treatment approach if we have validated and easily performed biomarkers in future.

Summary Points

- This chapter is focused on novel biomarkers in diagnosis of acute rejection and tolerance among kidney transplant patients.
- Subclinical acute rejection could only be diagnosed by protocol biopsy, which is an invasive procedure.
- Biomarkers with the ability to diagnose subclinical rejection and guide therapy might improve long-term graft survival.
- High urinary granzyme A mRNA is able to differentiate patients with subclinical rejection and mild T-cell-mediated rejection from those with stable graft function. But CMV infection must be ruled out.
- Urine mRNA of CXCL-9 with its high negative predictive value is a useful biomarker in excluding acute rejection as the cause of impaired graft function.
- Urine mRNA of Foxp3 and OX40 predict occurrence of rejection.

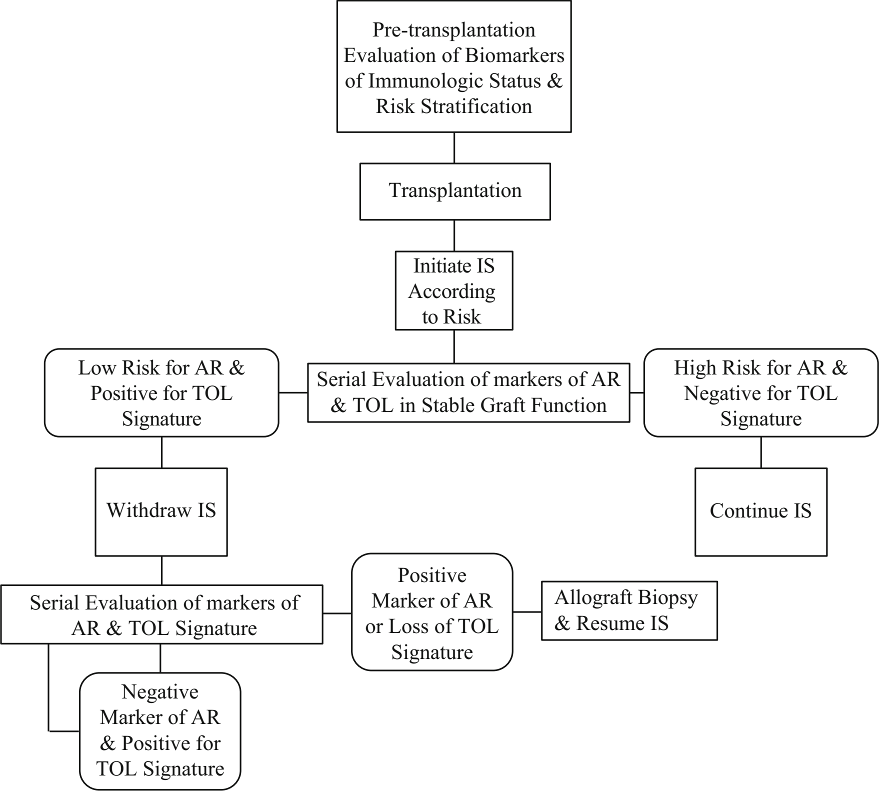

Fig. 2 The proposed clinical utility for biomarkers in individualization of immunosuppressive therapy. With the goal for individualization of IS therapy, biomarkers could be used in pretransplant period to identify high immunologic risk patients in need of strong IS regimes and low-risk patients who might benefit from withdrawal of IS. During the posttransplantation period, biomarkers of acute rejection help recognizing the subclinical rejections. If the patient has the TOL signature and devoid markers of rejection, he/she would be considered for IS withdrawal. After withdrawal serial assessment of biomarkers would be mandatory for timely diagnosis of loss of tolerance and predicting rejection. *AR* acute rejection, *IS* immunosuppression, *TOL* tolerance

- Urine proteomic and genomic (mRNA and miRNA) studies are in the path of evolution and soon be used clinically in predicting and detecting acute rejection.
- Posttransplant serum level of CD30 might be utilized as a marker of increased alloimmunity and a guide to immunosuppressive dose adjustment.
- The 17-gene panel identified as the Kidney Solid Organ Response Test (kSORT) is the best genomic marker of acute rejection till now.
- Increased expression of miR-142-5p in peripheral blood cells is diagnostic for chronic antibody rejection.
- Clinical operational tolerance is a state of tolerating the allograft in the absence of immunosuppressive drugs without pathologic evidences of rejection for at least 1 year.

- The tolerance signature was introduced based on genomic studies.
- Different cross-platform studies identified gene sets for diagnosis of tolerance.
- The identified gene sets pointed out the role of naïve and transitional B-cells and natural killer cells in maintenance of tolerance against allograft.

References

Afaneh C, Muthukumar T, Lubetzky M, et al. Urinary cell levels of mRNA for OX40, OX40L, PD-1, PD-L1 or PD-L2 and acute rejection of human renal allografts. Transplantation. 2010;90 (12):1381–7.

Anglicheau D, Sharma VK, Ding R, et al. MicroRNA expression profiles predictive of human renal allograft status. Proc Natl Acad Sci U S A. 2009;106(13):5330–5.

Aquino-Dias EC, Joelsons G, da Silva DM, et al. Non-invasive diagnosis of acute rejection in kidney transplants with delayed graft function. Kidney Int. 2008;73:877–84.

Bellos JK, Perrea DN, Theodoropoulou E, et al. Clinical correlation of nitric oxide levels with acute rejection in renal transplantation. Int Urol Nephrol. 2011;43(3):883–90.

Betts G, Shankar S, Sherston S, et al. Examination of serum miRNA levels in kidney transplant recipients with acute rejection. Transplantation. 2014;97(4):e28–30.

Brouard S, Mansfield E, Braud C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. PNAS. 2007; 104(39):15448–53.

Brown K, Wong W. Diagnostic value of regulatory T cells: a new facet of a much studied cell population. Transplantation. 2008;86:1485–91.

Chen R, Sigdel TK, Li L, et al. Differentially expressed RNA from public microarray data identifies serum protein biomarkers for cross-organ transplant rejection and other conditions. PLoS Comput Biol. 2010;6:e1000940.

Choy JC. Granzymes and perforin in solid organ transplant rejection. Cell Death Differ. 2010;17:567–76.

Danger R, Pallier A, Giral M, et al. Upregulation of miR-142-3p in peripheral blood mononuclear cells of operationally tolerant patients with a renal transplant. J Am Soc Nephrol. 2012;23:597–606.

Danger R, Paul C, Giral M, et al. Expression of miR-142-5p in peripheral blood mononuclear cells from renal transplant patients with chronic antibody-mediated rejection. PLoS One. 2013;8(4): e60702. doi:10.1371/journal.pone.0060702.

Delgado JC, Pavlov IY, Shihab FS. Post-transplant increased levels of serum sCD30 is a marker for prediction of kidney allograft loss in a 5-year prospective study. Transpl Immunol. 2009;22:1–4.

Ding R, Li B, Muthukumar T, et al. CD103 mRNA levels in urinary cells predict acute rejection of renal allografts. Transplantation. 2003;75:1307–12.

Ford ML, Adams AB, Pearson TC. Targeting co-stimulatory pathways: transplantation and autoimmunity. Nat Rev Nephrol. 2014;10:14–24.

Freue GV, Sasaki M, Meredith A, et al. Proteomic signatures in plasma during early acute renal allograft rejection. Mol Cell Proteomics. 2010;9(9):1954–67.

Gökmen R, Hernandez-Fuentes MP. Biomarkers of tolerance. Curr Opin Organ Transplant. 2013;18:416–20.

Hariharan S, Johnson CP, Bresnahan BA, et al. Improved graft survival after renal transplantation in the United States, 1988 to 1996. N Engl J Med. 2000;342(9):605–12.

Ho J, Rush DN, Karpinski M, et al. Validation of urinary CXCL10 as a marker of borderline, subclinical, and clinical tubulitis. Transplantation. 2011;92(8):878–82.

Hricik DE, Nickerson P, Formica RN, et al. Multicenter validation of urinary CXCL9 as a risk-stratifying biomarker for kidney transplant injury. Am J Transplant. 2013;13:2634–44.

Huang H, Xu X, Yao C, et al. Serum levels of CXCR3 ligands predict T cell-mediated acute rejection after kidney transplantation. Mol Med Rep. 2014;9(1):45–50.

Jackson JA, Kim EJ, Begley B, et al. Urinary chemokines CXCL9 and CXCL10 are noninvasive markers of renal allograft rejection and BK viral infection. Am J Transplant. 2011; 11(10):2228–34.

Lee A, Jeong JC, Choi YW, et al. Validation study of peripheral blood diagnostic test for acute rejection in kidney transplantation. Transplantation. 2014;98:760–5.

Li B, Hartono C, Ding R, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. N Engl J Med. 2001;344:947–54.

Li L, Khatri P, Sigdel TK, et al. A peripheral blood diagnostic test for acute rejection in renal transplantation. Am J Transplant. 2012;12:2710–8.

Ling XB, Sigdel TK, Lau K, et al. Integrative urinary peptidomics in renal transplantation identifies biomarkers for acute rejection. J Am Soc Nephrol. 2010;21(4):646–53.

Lodhi SA, Meier-Kriesche HU. Kidney allograft survival: the long and short of it. Nephrol Dial Transplant. 2011;26:15–7.

Loftheim H, Midtvedt K, Hartman A, et al. Urinary proteomic shotgun approach for identification of potential acute rejection biomarkers in renal transplant recipients. Transplant Res. 2012;1:9.

Lorenzen JM, Volkmann I, Fiedler J, et al. Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft recipients. Am J Transplant. 2011;11:2221–7.

Luo Y, Shi B, Qian Y, et al. Sequential monitoring of TIM-3 gene expression in peripheral blood for diagnostic and prognostic evaluation of acute rejection in renal graft recipients. Transplant Proc. 2011;43(10):3669–74.

Manfro RC, Aquino-Dias EC, Joelsons G, et al. Noninvasive Tim-3 messenger RNA evaluation in renal transplant recipients with graft dysfunction. Transplantation. 2008;86:1869–74.

Mas VR, Dumur CI, Scian MJ, et al. MicroRNAs as biomarkers in solid organ transplantation. Am J Transplant. 2013;13(1):11–9.

Masin-Spasovska J, Dohcev S, Stankov O, et al. Can an increased nitric oxide level be accepted as non-invasive marker for sub/acute rejection of the kidney allograft? Int J Artif Organs. 2013; 36(12):907–12.

Matignon M, Ding R, Dadhania DM, et al. Urinary cell mRNA profiles and differential diagnosis of acute kidney graft dysfunction. J Am Soc Nephrol. 2014;25(7):1586–97.

Metzger J, Chatzikyrkou C, Broecker V, et al. Diagnosis of subclinical and clinical acute T-cell-mediated rejection in renal transplant patients by urinary proteome analysis. Proteomics Clin Appl. 2011;5:322–33.

Muthukumar T, Dadhania D, Ding R, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. N Engl J Med. 2005;353:2342.

Nafar M, Farrokhi F, Vaezi M, et al. Pre-transplant and post-transplant soluble CD30 for prediction and diagnosis of acute kidney allograft rejection. Int Urol Nephrol. 2009;41:687–93.

Newell KA, Asare A, Kirk AD, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest. 2010;120(6):1836–47. doi:10.1172/JCI39933.

Nickel P, Presber F, Bold G, et al. Enzyme-linked immunosorbent spot assay for donor-reactive interferon-gamma-producing cells identifies T-cell presensitization and correlates with graft function at 6 and 12 months in renal-transplant recipients. Transplantation. 2004; 78(11):1640–6.

O'Riordan E, Orlova TN, Podust VN, et al. Characterization of urinary peptide biomarkers of acute rejection in renal allografts. Am J Transplant. 2007;7:930–40.

Orlando G, Hematti P, Stratta RJ, et al. Clinical operational tolerance after renal transplantation: current status and future challenges. Ann Surg. 2010;252(6):915–28.

Pelzl S, Opelz G, Wiesel M, et al. Soluble CD30 as a predictor of kidney graft outcome. Transplantation. 2002;73:3–6.

Peng W, Chen J, Jiang Y, et al. Acute renal allograft rejection is associated with increased levels of vascular endothelial growth factor in the urine. Nephrology (Carlton). 2008;13:73–9.

Roedder S, Li L, Alonso MN, et al. A three-gene assay for monitoring immune quiescence in kidney transplantation. J Am Soc Nephrol. 2015;26(8):2042–53 pii: ASN.2013111239.
Roelofs JJTH, Rowshani AT, van den Berg JG, et al. Expression of urokinase plasminogen activator and its receptor during acute renal allograft rejection. Kidney Int. 2003;64:1845–53.
Rush DN, Henry SF, Jeffery JR, et al. Histological findings in early routine biopsies of stable renal allograft recipients. Transplantation. 1994;57:208–11.
Sagoo P, Perucha E, Sawitzki B, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. J Clin Invest. 2010;120(6):1848–61. doi:10.1172/JCI39922.
Schaub S, Nickerson P, Rush D, et al. Urinary CXCL9 and CXCL10 levels correlate with the extent of subclinical tubulitis. Am J Transplant. 2009;9(6):1347–53.
Scian MJ, Maluf DG, Mas VR. MiRNAs in kidney transplantation, potential roles as new biomarkers. Expert Rev Mol Diagn. 2013;13(1):93–104.
Sigdel TK, Kaushal A, Gritsenko M, et al. Shotgun proteomics identifies proteins specific for acute renal transplant rejection. Proteomics Clin Appl. 2010;4(1):32–47.
Sigdel TK, Vitalone MJ, Tran TQ, et al. Rapid noninvasive assay for the detection of renal transplant injury. Transplantation. 2013;96:97–101.
Sigdel TK, Salomonis N, Nicora CD, et al. The identification of novel potential injury mechanisms and candidate biomarkers in renal allograft rejection by quantitative proteomics. Mol Cell Proteomics. 2014a;13(2):621–31.
Sigdel T, Ng Y, Lee S, et al. Perturbations in the urinary exosome in transplant rejection. Front Med. 2014b;1:57. doi:10.3389/fmed.2014.00057.
Srivastava M, Eidelman O, Torosyan Y, et al. Elevated expression levels of ANXA11, integrins beta3 and alpha3, and TNF-alpha contribute to a candidate proteomic signature in urine for kidney allograft rejection. Proteomics Clin Appl. 2011;5(5–6):311–21.
Süsal C, Döhler B, Sadeghi M, et al. Posttransplant sCD30 as a predictor of kidney graft outcome. Transplantation. 2011;91:1364–9.
Tian J, Shi WF, Zhang LW, et al. HLA class I (ABC) upregulation on peripheral blood CD3+/ CD8+ T lymphocyte surface is a potential predictor of acute rejection in renal transplantation. Transplantation. 2009;88(12):1393–7.
Ting YT, Coates PT, Marti HP, et al. Urinary soluble HLA-DR is a potential biomarker for acute renal transplant rejection. Transplantation. 2010;89(9):1071–8.
Van Ham SM, Heutinck KM, Jorritsma T, et al. Urinary granzyme A mRNA is a biomarker to diagnose subclinical and acute cellular rejection in kidney transplant recipients. Kidney Int. 2010;78:1033–40.
Vasconcellos LM, Schachter AD, Zheng XX, et al. Cytotoxic lymphocyte gene expression in peripheral blood leukocytes correlates with rejecting renal allografts. Transplantation. 1998; 66(5):562–6.
Viklicky O, Krystufkova E, Brabcova I, et al. B-cell-related biomarkers of tolerance are up-regulated in rejection-free kidney transplant recipients. Transplantation. 2013;95:148–54.
Xu X, Huang H, Cai M, et al. Combination of IL-1 receptor antagonist, IL-20 and CD40 ligand for the prediction of acute cellular renal allograft rejection. J Clin Immunol. 2013;33(1):280–7.
Yannaraki M, Rebibou JM, Ducloux D, et al. Urinary cytotoxic molecular markers for a noninvasive diagnosis in acute renal transplant rejection. Transpl Int. 2006;19:759–68.

Diagnostic Biomarkers of Acute Kidney Injury in Newborns

2

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Abstract

Acute kidney injury refers to the rapid loss of renal function. In newborns, although the precise incidence of acute kidney injury is unknown, research has shown that 8–24 % of all critically ill newborns in neonatal intensive care units may develop the condition. Although traditional markers of acute kidney injury lack sensitivity and specificity for early diagnosis in the neonatal period, several novel serum and urinary biomarkers are under intense scrutiny for their role as noninvasive indicators of early acute kidney injury. The most promising biomarkers are cystatin C, neutrophil gelatinase-associated lipocalin, interleukin-18, and kidney injury molecule-1.

Keywords

Acute kidney injury • Newborn • Neonatal period • Biomarkers • Diagnosis

Abbreviations

AKI	Acute kidney injury
CysC	Cystatin C
EGF	Epidermal growth factor
FGF-2	Fibroblast growth factor-2
GFR	Glomerular filtration rate
IL-18	Interleukin-18
KIM-1	Kidney injury molecule-1
NGAL	Neutrophil gelatinase-associated lipocalin
NICUs	Neonatal intensive care units
sCysC	Serum CysC
sNGAL	Serum neutrophil gelatinase-associated lipocalin
uCysC	Urinary CysC
uIL-18	Urinary interleukin-18
uKIM-1	Urine kidney injury molecule-1
uNGAL	Urinary neutrophil gelatinase-associated lipocalin

Key Facts of Diagnostic Biomarkers of Acute Kidney Injury in Newborns

- A major concern for neonatologists is the disturbance of renal function during the neonatal period, i.e., acute kidney injury.
- The diagnosis of acute kidney injury is not easy and is based mainly on urine production and various serum markers.
- The traditional markers are not so specific for early diagnosis of neonatal acute kidney injury.
- Research has shown that some novel biomarkers may be much better than the traditional.

- The novel biomarkers are cystatin C, neutrophil gelatinase-associated lipocalin, interleukin-18, and kidney injury molecule-1.
- Despite the encouraging data, further research is necessary in order to find the most reliable biomarker for timely detection of the condition.

Definitions

Apoptosis The process of programmed cell death.

Metabolomics The scientific study of chemical processes involving metabolites.

Necrosis The premature death of cells and living tissue.

Proteomics The large-scale study of proteins, particularly their structures and functions.

Proximal tubules The proximal tubule is the portion of the duct system of the nephron of the kidney which leads from Bowman's capsule to the loop of Henle.

Reperfusion injury The tissue damage caused when blood supply returns to the tissue after a period of ischemia or lack of oxygen.

Very low-birth-weight infants Infants who are born weighing less than 1,000 g.

Introduction

Acute kidney injury (AKI) is defined by an acute and reversible increase in serum creatinine levels associated or not with a reduction in urine output (Singbartl et al. 2012). It is an important cause of morbidity and mortality in newborns and is directly associated with poor outcomes (Singbartl and Kellum 2012; Siew and Deger 2012; Akcan-Arikan et al. 2007; Zappitelli et al. 2008). The overall incidence of AKI in critically ill newborns in NICUs is 8–24 %. In neonates with perinatal asphyxia, the incidence of AKI may increase to 56.3 % (Durkan and Alexander 2011). Other conditions that may lead to AKI in the perinatal period include prematurity, congenital diseases, sepsis, or administration of nephrotoxic agents (Andreoli 2004).

The newborn's kidneys are more susceptible to injury due to increased vulnerability to hypoperfusion, as well as due to low glomerular filtration rate, high renal vascular resistance, high plasma renin activity, decreased intercortical perfusion, and decreased reabsorption of sodium in the proximal tubules (Liborio et al. 2014). The prognosis of AKI in newborns depends on its etiology and on gestational age; however, 25–50 % of neonates with AKI die, and long-term problems may appear in the survivors (Andreoli 2004; Askenazi et al. 2009a; Agras et al. 2004; Abitbol et al. 2003).

Table 1 Classification and staging systems for acute kidney injury

System	Serum creatinine criteria	Urine output criteria
AKIN stage		
1	Serum creatinine increase ≥26.5 μmol/l (≥0.3 mg/dl) **or** increase to 1.5–2.0-fold from baseline	<0.5 ml/kg/h for 6 h
2	Serum creatinine increase >2.0–3.0-fold from baseline	<0.5 ml/kg/h for 12 h
3	Serum creatinine increase >3.0-fold from baseline **or** serum creatinine ≥354 μmol/l (≥4.0 mg/dl) with an acute increase of at least 44 μmol/l (0.5 mg/dl) **or** need for renal replacement therapy	Anuria for 12 h
RIFLE class		
Risk	Serum creatinine increase to 1.5-fold **or** glomerular filtration rate decrease >25 % from baseline	<0.5 ml/kg/h for 6 h
Injury	Serum creatinine increase to 2.0-fold **or** glomerular filtration rate decrease >50 % from baseline	<0.5 ml/kg/h for 12 h
Failure	Serum creatinine increase to 3.0-fold **or** glomerular filtration rate decrease >75 % from baseline **or** serum creatinine ≥354 μmol/l (≥4 mg/dl) with an acute increase of at least 44 μmol/l (0.5 mg/dl)	Anuria for 12 h

AKIN AKI Network; *RIFLE* Risk, Injury Failure, Loss, End-stage renal disease

On the other hand, diagnosis of neonatal AKI is highly challenging and is based on urine output, serum creatinine, and glomerular filtration rate (GFR) values (Table 1). Unfortunately, the diagnosis may be delayed due to abnormal or immature tubular function especially in premature neonates and the limitations in the use of serum creatinine as diagnostic marker; serum creatinine is not increased until 50 % of kidney function has been lost, while its levels are affected by renal and nonrenal factors (Argyri et al. 2013; Askenazi et al. 2009a; Bariciak et al. 2011; Druker and Guignard 2002).

Although traditional markers of AKI lack sensitivity and specificity for early diagnosis in the neonatal period, several novel serum and urinary biomarkers are under intense scrutiny for their role as noninvasive indicators of early AKI (Parikh and Devarajan 2008). These novel biomarkers will be presented in the present chapter, and their role in early diagnosis and prognosis of AKI in newborns will be discussed.

Biomarkers of Acute Kidney Injury

Several biomarkers have been so far associated with AKI in newborns (Alge et al. 2013). The most promising are cystatin C (CysC), neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18), and fibroblast growth factor-2 (FGF-2) in serum and CysC, NGAL, kidney injury molecule-1 (KIM-1), IL-18, and urinary epidermal growth factor (EGF) in urine (Table 2).

Table 2 Biomarkers associated with acute kidney injury in newborns

Serum	Urine
Cystatin C	Cystatin C
Neutrophil gelatinase-associated lipocalin	Neutrophil gelatinase-associated lipocalin
Interleukin-18	Kidney injury molecule-1
Fibroblast growth factor-2	Interleukin-18
	Urinary epidermal growth factor

Cystatin C

Cystatin C (CysC) is a low-molecular-weight (13 kDa) cysteine proteinase inhibitor expressed in all nucleated cells. It is produced at a constant rate and cleared exclusively by the kidney. Cystatin C does not cross the placenta and, thus, reflects the renal function of the neonates in early postnatal life regardless of body composition and size (Dinarello et al. 1998; Sharma et al. 2009). Due to its small size, CysC is normally filtered by the glomerulus and is completely reabsorbed and catabolized by the proximal tubule without significant secretion by the renal tubules (Westhuyzen 2006). Serum CysC (sCysC) levels increase in term newborns and decrease over the first 5 days of life, remaining stable up to day 28 of life (Plebani et al. 1997; Cataldi et al. 1999; Novo et al. 2011). Due to this, sCysC has been used as a biomarker of renal tolerance of ibuprofen treatment in very low-birth-weight infants (Gokmen et al. 2011). Serum CysC was measured in 62 preterm neonates with respiratory distress syndrome and 34 control neonates and was identified as an earlier marker of AKI before serum creatinine increase (Elmas et al. 2013). Moreover, sCysC has been used as a predictive biomarker of AKI in preterm very low-birth-weight infants who received indomethacin for duct closure followed by furosemide or placebo administration (Lee et al. 2010). In this study, infants of the furosemide group were more likely to develop AKI and had higher levels of serum creatinine and CysC than the placebo group.

Regarding its role as a biomarker in perinatal asphyxia, not only sCysC but also urinary CysC (uCysC) predicted AKI in neonates with a 5-min Apgar score <7 (Askenazi et al. 2012). Contrarily, sCysC was not a useful marker of renal function in neonates with sepsis (Maruniak-Chudek et al. 2012). Newborns with septic shock had lower levels of sCysC than those with sepsis. The uCysC was also predictive of AKI, in non-septic critically ill newborns (Li et al. 2012). Although other studies have evaluated CysC in older children, it is difficult to extend their results to the neonatal population, considering that normal values of uCysC decrease with tubular maturation.

Evidence has shown so far that sCysC is not more sensitive than serum creatinine as a marker of GFR in newborns (Treiber et al. 2006). Both sCysC and creatinine levels at birth and 3 days after birth have been reported to be independent of sex, gestational age, birth weight, bilirubin levels, and hydration state (Treiber et al. 2006; Armangil et al. 2008). In addition, sCysC was correlated with the degree of congenital renal abnormalities; a significant increase of sCysC was observed in neonates with bilateral congenital kidney anomalies in contrast to those with unilateral kidney abnormalities or without kidney malformation (Parvex et al. 2012).

Neutrophil Gelatinase-Associated Lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kDa protein of the lipocalin superfamily (Bolignano et al. 2008). NGAL is expressed in renal tubules after an ischemic or toxic insult and following transplantation (Mishra et al. 2003, 2004, 2006). Immediately after the onset of AKI, NGAL mRNA is rapidly upregulated in the loop of Henle and proximal tubules, causing an increase in the synthesis and excretion of NGAL into the urine by the proximal tubule (Gupta et al. 2005; Alge et al. 2013). However, NGAL is released in plasma by other injured tissues, and NGAL-based diagnosis of AKI may be challenging.

The possible use of NGAL in toxic AKI was studied both in animals and humans. In a rodent study, the authors administered an intraperitoneal dose of cisplatin (20 mg/kg) and reported that tubule cell necrosis and apoptosis, as well as expression of NGAL in renal tissue, occurred within 3 h, while NGAL was detected in urine in a dose- and duration-dependent manner (Mishra et al. 2003). In a human study, Wasilewska et al. reported that both serum and urinary NGAL levels increased during the course of cyclosporine treatment (Wasilewska et al. 2010). In this study, however, no firm conclusion could be drawn on urinary NGAL's (uNGAL) possible role as a predictor of cyclosporine nephropathy due to the small sample size. Despite this finding, NGAL has been recently associated with inflammation; expression of NGAL in trophoblast tissues of term placenta increased after intra-amniotic infection in vivo and after exposure to mediators of inflammation (Tadesse et al. 2011). Urinary NGAL was also increased in septic very low-birth-weight infants and predicted the renal function of approximately the first month of life in this age group (Parravicini et al. 2010; La Manna et al. 2011).

Of note, uNGAL has been characterized as the most promising AKI biomarker (Liborio et al. 2014). Urinary NGAL is the most strikingly upregulated gene and overexpressed protein in the kidney after ischemia and depends on birth weight and gestational age (Askenazi et al. 2009a; Mishra et al. 2004). In neonates, a 2-h serum NGAL (sNGAL) threshold of 100 ng/mL and a 2-h uNGAL of 185 ng/mL were the best cutoff values with improved sensitivity and specificity for early diagnosis of AKI compared with creatinine, whereas in children this threshold was set at 50 ng/Ml (Argyri et al. 2013). Nevertheless, a single measurement of sNGAL in neonates may be not sufficient for the prediction of AKI (Koch et al. 2011); the combination of NGAL with other biomarkers may be of value in the early diagnosis of AKI (Sarafidis et al. 2012). Askenazi et al. (2011) evaluated NGAL, IL-18, KIM-1, and CysC and reported that they may predict AKI and mortality in very low-birth-weight infants.

Interleukin-18

Interleukin-18 is a pro-inflammatory cytokine released by macrophages. It stimulates the synthesis of other inflammatory molecules and enhances the maturation of

T and natural killer cells, as well as the infiltration of neutrophils and macrophage accumulation (Dinarello et al. 1998). Interleukin-18 is cleaved by caspase-1 and is released in the proximal tubule after AKI (Melnikov et al. 2002); this biochemical behavior is responsible for its deleterious effects on renal function.

In an experimental model of ischemia-reperfusion injury, IL-18-deficient mice were protected from acute renal failure compared with wild-type controls (Melnikov et al. 2001). In the same study, the IL-18-deficient mice had attenuated production of pro-inflammatory molecules within the kidney at 24 h. Similar results were observed in neonatal septic mice, in which neutralization of IL-18 significantly increased mortality, while administration or recombinant IL-18 improved survival (Cusumano et al. 2004).

In humans, serum IL-18 was significantly increased in infected newborns compared with noninfected neonates, although IL-18 did not predict early onset neonatal sepsis (Bender et al. 2008). In this study, however, very few infants had septic shock, which might be the reason for the difference between the results. Li et al. enrolled 62 non-septic critically ill neonates of which they collected urine every 48–72 h during the first 10 days of life (Li et al. 2012). They reported that both urinary IL-18 (uIL-18) and uCysC were independently associated with AKI. Interestingly, uCysC levels may decrease with increasing renal maturity, but this may not occur with uIL-18 levels. Interleukin-18 has been reported to have a potential advantage of not changing its normal value with increasing renal maturity but can be influenced by sepsis, reducing its ability to detect AKI (Liborio et al. 2014).

Kidney Injury Molecule-1

KIM-1 is a type 1 transmembrane glycoprotein that is not detected in urine under normal conditions but is markedly increased after kidney ischemic or toxic injury, characterized by epithelial cell differentiation (Argyri et al. 2013). It has significant immunomodulatory properties as it confers the ability on epithelial cells to phagocytose dead cells and transforms them into phagocytes (Bonventre 2009). In kidneys, KIM-1 is shed from proximal tubule cells and secreted in urine.

In animals exposed to various nephrotoxic agents, as well as to gentamicin, S-(1,1,2,2-tetrafluoroethyl)-l-cysteine, and folic acid, urine KIM-1 (uKIM-1) has been reported to be a more sensitive and specific biomarker of AKI than creatinine.

In rats, it was reported to detect renal injury following ischemia-reperfusion (Vaidya et al. 2006). In humans, uNGAL predicted AKI and uKIM-1 correlated with mortality in very low-birth-weight infants, independent of gestational age and birth weight (Askenazi et al. 2011). In addition, newborns that developed AKI had higher levels of uKIM-1 and uNGAL than those without AKI, but this difference was not statistically significant (Askenazi et al. 2012). Moreover, the predictive value of uKIM-1 in premature neonates showed that the biomarker predicted aminoglycoside-induced nephrotoxicity (Argyri et al. 2013), while in contrast, a reduction in serum creatinine was also observed during treatment (McWilliam et al. 2011).

Fibroblast Growth Factor-2

Various studies have demonstrated that fibroblast growth factor-2 (FGF-2) may be a good urinary candidate biomarker for children with AKI secondary to renal endothelial injury (Ray et al. 1999, 2002, 2006). Recently, Hoffman et al. evaluated urinary FGF-2 as a marker of AKI in neonates and reported that the combination of NGAL and FGF-2 improved the specificity to identify newborns at risk of developing AKI. However, FGF-2 did not differentiate those with AKI among those at risk (Hoffman et al. 2013). Although the role of FGF-2 as a marker of AKI has not been fully established so far, it has a promising role as a late biomarker for detecting the recovery of renal function.

Urinary Epidermal Growth Factor

Urinary epidermal growth factor (EGF) may be a reliable biomarker to follow the outcome of infants with AKI (Askenazi et al. 2012; Soler-Garcia et al. 2009; Tsau et al. 1996; Chen and Liu 1997), while the urinary levels of EGF appear to be a predictor of renal function recovery in adults with AKI (Kwon et al. 2010). Considering that the urinary excretion of EGF is more dependent on its renal pool (Watanabe et al. 1989; Evans et al. 1986; Di Paolo et al. 1997), it may be a more specific biomarker to identify ongoing renal injury in critically ill neonates than serum creatinine.

Urine Metabolomic Profiling

In an experimental study on newborn rats, nephrotoxicity induced by gentamicin was associated with different urinary patterns of metabolites (Fanos et al. 2014). In total, glucose, galactose, *N*-acetylglucosamine, myoinositol, butanoic acid, pseudouridine, and 3-hydroxybutyrate were significantly increased following administration of gentamicin.

Atzori et al. investigated the possibility of kidney injuries being associated with a well-defined metabolic pattern using ^{1}H nuclear magnetic resonance spectroscopy technology (Atzori et al. 2011). They analyzed the urine metabolic profile of 21 children affected by nephropathies and compared it with that of 19 healthy controls. They reported that renal and urinary tract malformations were related to a specific metabolic profile belonging to the nuclear magnetic resonance spectroscopy regions [3.5–3.9], [4.1–4.4], and [8.2–8.6], suggesting the localization of the damage (cortexes 1, 2, and 3, medullary). In particular, multivariate analysis of urine spectral data revealed a clustered distribution with a significant separation of the term infant samples and preterm infant samples. This study is of major importance because data analysis enabled the identification of the main discriminating low-molecular-weight metabolites, suggesting that amino acid biosynthesis and metabolism are the key metabolic mechanisms underlying fetal and perinatal maturation processes. In

conclusion, ^{1}H-NMR metabolomic analysis of urine appears to be able to assess the metabolic status of preterm and term infants at birth. Nevertheless, the findings of Atzori et al. (2011) should be interpreted with caution because their results may be biased by variables such as maternal pathological conditions, mode of delivery, perinatal and neonatal disorders, as well as by iatrogenic factors. In either case, further large-scale prospective clinical metabolomic studies are needed in order to confirm the aforementioned findings. Particularly, research should focus on the investigation of selected groups of neonates affected by specific disorders or under particular conditions (Atzori et al. 2011).

Discussion

Until now, neither serum creatinine nor urine output meets the characteristics of the ideal biomarker for early diagnosis of AKI (Askenazi et al. 2009b; Bariciak et al. 2011; Drukker and Guignard 2002; Murray et al. 2002). The biomarkers discussed can be measured in serum and in urine. Each one of these biomarkers has some advantages.

On the other hand, sCysC does not reflect maternal values in contrast to serum creatinine (Plebani et al. 1997; Cataldi et al. 1999), but its role in estimating GFR in newborns still remains controversial (Treiber et al. 2006). NGAL seems to be the most promising biomarker of AKI in newborns. It increases earlier in ischemic AKI than other emerging markers (Krawczeski et al. 2011). Nevertheless, both serum and urinary NGAL are increased at 2 h after cardiopulmonary bypass in neonates, while sCysC, uKIM-1, and uIL-18 are markedly increased at 12–24, 6–12, and 4–6 h, respectively, in children undergoing the procedure (Krawczeski et al. 2010; Han et al. 2008; Parikh et al. 2006). In addition, both uNGAL and uKIM-1 seem to be promising biomarkers of toxic AKI (Mishra et al. 2004; Wasilewska et al. 2010; Vaidya et al. 2006; McWilliam et al. 2011; Zhou et al. 2008), while uKIM-1 and sIL-18 have been positively correlated with adverse clinical outcome (Argyri et al. 2013).

Despite the increase in evidence, further research and extensive comparison of each new marker with both traditional and other novel markers of AKI are necessary before any of these biomarkers can be used in everyday clinical practice for monitoring AKI. In addition, the range of baseline values of the aforementioned biomarkers has to be established. Given the fact that AKI is associated with high mortality, especially in very low-birth-weight infants (Koralkar et al. 2011), it is unquestionably important to establish biomarkers for early diagnosis. As none of the above biomarkers have all the characteristics required for a substance to be characterized as an ideal biomarker of AKI, their combination might be a better diagnostic and prognostic tool of the condition than each one separately (Argyri et al. 2013).

A combination of these biomarkers could make a “biomarker panel” for the early diagnosis and severity stratification of AKI in newborns with high sensitivity, specificity, and predictive value, especially when combined with the metabolomic profile. Recently, serum and urine samples have been analyzed by the “omics”

techniques. Not only proteomics but also metabolomics seems to be a promising tool in identifying diagnostic biomarkers, which in turn assist timely intervention (Lindon et al. 2004). However, although kidney disease is an appropriate field for mass spectrometry and ^{1}H nuclear magnetic resonance spectroscopy studies, the pathophysiology of kidney injury is still unclear (Pan and Raftery 2007; Weiss and Kim 2012).

Conclusions

Several biomarkers have been proposed as predictors of neonatal AKI. However, further prospective studies in large populations of neonates have to be conducted before any of these biomarkers are used in everyday practice. Comparison of each new marker with both traditional and other novel markers of AKI is necessary to find the most reliable biomarker for timely detection of the condition. A combination of the described biomarkers with metabolomics could make a "biomarker-metabolomic panel" for the early diagnosis and severity stratification of AKI in newborns with high sensitivity, specificity, and predictive value.

Potential Applications to Prognosis, Other Diseases, or Conditions

Critically ill newborns with acute kidney injury may develop hypertension, particularly those requiring intensive care. Hypertension increases the risk for early onset cardiovascular disease and requires careful diagnostic evaluation and prompt treatment. Moreover, acute kidney injury may evolve to multiple organ failure and death. Although in newborns, renal dysfunction did not seem to have a critical impact on mortality until now, in a recent study, the authors showed that severity of renal failure was strongly associated with mortality in infants who were treated with extracorporeal membrane oxygenation (Zwiers et al. 2013). This finding suggests that there should be a cutoff value at which the severity of acute kidney injury directly impacts mortality.

In general, acute kidney injury is an independent risk factor for poor outcomes in critically ill neonates, and prognosis is variable. Infants with prerenal acute kidney injury who receive prompt treatment for renal hypoperfusion have an excellent prognosis, while those with postrenal acute kidney injury related to congenital urinary tract obstruction have a variable outcome. Also, infants with intrinsic acute kidney injury have significant risks of morbidity and mortality.

Despite advantages in renal medicine, renal pathophysiology of critically ill newborn patients makes it difficult to interpret urine output and serum creatinine levels to diagnose acute kidney injury. Considering that renal dysfunction may be reversible if early diagnosed, improving our diagnostic methods with better acute kidney injury biomarkers will allow us to intervene much earlier in the disease course.

Summary Points

- Eight to twenty-four percent of all critically ill newborns in neonatal intensive care units may develop acute kidney injury.
- Diagnosis of neonatal acute kidney injury is highly challenging and is based on urine output, serum creatinine, and glomerular filtration rate values.
- Traditional markers of acute kidney injury lack sensitivity and specificity for early diagnosis in the neonatal period.
- Several novel serum and urinary biomarkers are under intense scrutiny for their role as noninvasive indicators of early acute kidney injury.
- The most promising biomarkers are cystatin C, neutrophil gelatinase-associated lipocalin, interleukin-18, and kidney injury molecule-1.
- A combination of the described biomarkers with metabolomics could make a "biomarker-metabolomic panel" for the early diagnosis and severity stratification of acute kidney injury in newborns with high sensitivity, specificity, and predictive value.

References

Abitbol CL, Bauer CR, Montané B, et al. Long-term follow-up of extremely low birth weight infants with neonatal renal failure. Pediatr Nephrol. 2003;18:887–93.

Agras PI, Tarcan A, Baskin E, et al. Acute renal failure in the neonatal period. Ren Fail. 2004;26:305–9.

Akcan-Arikan A, Zappitelli M, Loftis LL, et al. Modified RIFLE criteria in critically ill children with acute kidney injury. Kidney Int. 2007;71:1028–35.

Alge JL, Karakala N, Neely BA, SAKInet Investigators, et al. Urinary angiotensinogen and risk of severe AKI. Clin J Am Soc Nephrol. 2013a;8:184–93.

Alge JL, Karakala N, Neely BA, SAKInet Investigators, et al. Urinary angiotensinogen predicts adverse outcomes among acute kidney injury patients in the intensive care unit. Crit Care. 2013b;17:R69.

Andreoli SP. Acute renal failure in the newborn. Semin Perinatol. 2004;28:112–23.

Argyri I, Xanthos T, Varsami M, et al. The role of novel biomarkers in early diagnosis and prognosis of acute kidney injury in newborns. Am J Perinatol. 2013;30:347–52.

Armangil D, Yurdakök M, Canpolat FE, et al. Determination of reference values for plasma cystatin C and comparison with creatinine in premature infants. Pediatr Nephrol. 2008;23:2081–3.

Askenazi DJ, Ambalavanan N, Goldstein SL. Acute kidney injury in critically ill newborns: what do we know? What do we need to learn? Pediatr Nephrol. 2009a;24:265–74.

Askenazi DJ, Griffin R, McGwin G, et al. Acute kidney injury is independently associated with mortality in very low birthweight infants: a matched case-control analysis. Pediatr Nephrol. 2009b;24:991–7.

Askenazi DJ, Montesanti A, Hunley H, et al. Urine biomarkers predict acute kidney injury and mortality in very low birth weight infants. J Pediatr. 2011;159:907–12.

Askenazi DJ, Koralkar R, Hundley HE, et al. Urine biomarkers predict acute kidney injury in newborns. J Pediatr. 2012;161:270–5.

Atzori L, Antonucci R, Barberini L, et al. ^{1}H NMR-based metabolomic analysis of urine from preterm and term neonates. Front Biosci (Elite Ed). 2011;3:1005–12.

Bariciak E, Yasin A, Harrold J, et al. Preliminary reference intervals for cystatin C and beta-trace protein in preterm and term neonates. Clin Biochem. 2011;44:1156–9.

Bender L, Thaarup J, Varming K, et al. Early and late markers for the detection of early-onset neonatal sepsis. Dan Med Bull. 2008;55:219–23.
Bolignano D, Donato V, Coppolino G, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a marker of kidney damage. Am J Kidney Dis. 2008;52:595–605.
Bonventre JV. Kidney injurymolecule-1 (KIM-1): a urinary biomarker and much more. Nephrol Dial Transplant. 2009;24:3265–8.
Cataldi L, Mussap M, Bertelli L, et al. Cystatin C in healthy women at term pregnancy and in their infant newborns: relationship between maternal and neonatal serum levels and reference values. Am J Perinatol. 1999;16:287–95.
Chen L, Liu W. Effect of asphyxia on urinary epidermal growth factor levels in newborns. J Tongi Med Univ. 1997;17:144–6.
Cusumano V, Midiri A, Cusumano VV, et al. Interleukin-18 is an essential element in host resistance to experimental group B streptococcal disease in neonates. Infect Immun. 2004;72:295–300.
Di Paolo S, Gesualdo L, Stallone G, et al. Renal expression and urinary concentration of EGF and IL-6 in acutely dysfunctioning kidney transplanted patients. Nephrol Dial Transplant. 1997;12:2687–93.
Dinarello CA, Novick D, Puren AJ, et al. Overview of interleukin-18: more than an interferon-γ inducing factor. J Leukoc Biol. 1998;63:658–64.
Drukker A, Guignard JP. Renal aspects of the term and preterm infant: a selective update. Curr Opin Pediatr. 2002;14:175–82.
Durkan AM, Alexander RT. Acute kidney injury post neonatal asphyxia. J Pediatr. 2011;158: e29–33.
Elmas AT, Tabel Y, Elmas ON. Serum cystatin C predicts acute kidney injury in preterm neonates with respiratory distress syndrome. Pediatr Nephrol. 2013;28:477–84.
Evans NJ, Rutter N, Gregory H. Urinary excretion of epidermal growth factor in the newborn. Early Hum Dev. 1986;14:277–82.
Fanos V, Noto A, Caboni P, et al. Urine metabolomic profiling in neonatal nephrology. Clin Biochem. 2014;47:708–10.
Gokmen T, Erdeve O, Altug N, et al. Efficacy and safety of oral versus intravenous ibuprofen in very low birth weight preterm infants with patent ductus arteriosus. J Pediatr. 2011;158:549–54. e1.
Gupta BD, Sharma P, Bagla J, et al. Renal failure in asphyxiated neonates. Indian Pediatr. 2005;42:928–34.
Han WK, Waikar SS, Johnson A, et al. Urinary biomarkers in the early diagnosis of acute kidney injury. Kidney Int. 2008;73:863–9.
Hoffman SB, Massaro AN, Soler-García AA, et al. A novel urinary biomarker profile to identify acute kidney injury (AKI) in critically ill neonates: a pilot study. Pediatr Nephrol. 2013;28:2179–88.
Koch AM, Dittrich S, Cesnjevar R, et al. Plasma neutrophil gelatinase-associated lipocalin measured in consecutive patients after congenital heart surgery using point-of-care technology. Interact Cardiovasc Thorac Surg. 2011;13:133–6.
Koralkar R, Ambalavanan N, Levitan EB, et al. Acute kidney injury reduces survival in very low birth weight infants. Pediatr Res. 2011;69:354–8.
Krawczeski CD, Vandevoorde RG, Kathman T, et al. Serum Cystatin C is an early predictive biomarker of acute kidney injury after pediatric cardiopulmonary bypass. Clin J Am Soc Nephrol. 2010;5:1552–7.
Krawczeski CD, Woo JG, Wang Y, et al. Neutrophil gelatinase-associated lipocalin concentrations predict development of acute kidney injury in neonates and children after cardiopulmonary bypass. J Pediatr. 2011;158:1009–15. e1.
Kwon O, Ahn K, Zhang B, et al. Simultaneous monitoring of multiple urinary cytokines may predict renal and patient outcome in ischemic AKI. Ren Fail. 2010;32:699–708.
La Manna G, Galletti S, Capelli I, et al. Urinary neutrophil gelatinase-associated lipocalin at birth predicts early renal function in very low birth weight infants. Pediatr Res. 2011;70:379–83.

Lee BS, Byun SY, Chung ML, et al. Effect of furosemide on ductal closure and renal function in indomethacin-treated preterm infants during the early neonatal period. Neonatology. 2010;98:191–9.

Li Y, Fu C, Zhou X, et al. Urine interleukin-18 and cystatin-C as biomarkers of acute kidney injury in critically ill neonates. Pediatr Nephrol. 2012;27:851–60.

Libório AB, Branco KM, Torres de Melo Bezerra C. Acute kidney injury in neonates: from urine output to new biomarkers. Biomed Res Int. 2014;2014:601568.

Lindon JC, Holmes E, Bollard ME, et al. Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. Biomarkers. 2004;9:1–31.

Maruniak-Chudek I, Owsianka-Podleśny T, Wróblewska J, et al. Is serum cystatin C a better marker of kidney function than serum creatinine in septic newborns? Postepy Hig Med Dosw (Online). 2012;66:175–80.

McWilliam SJ, Antoine DJ, Turner M, et al. Urinary KIM-1 as a biomarker of aminoglycoside-induced nephrotoxicity in premature neonates. Pediatr Res. 2011;70:107.

Melnikov VY, Ecder T, Fantuzzi G, et al. Impaired IL-18 processing protects caspase-1-deficient mice from ischemic acute renal failure. J Clin Invest. 2001;107:1145–52.

Melnikov VY, Faubel S, Siegmund B, et al. Neutrophil-independent mechanisms of caspase-1- and IL-18-mediated ischemic acute tubular necrosis in mice. J Clin Invest. 2002;110:1083–91.

Mishra J, Ma Q, Prada A, et al. Identification of NGAL as a novel urine biomarker for ischemic injury. J Am Soc Nephrol. 2003;14:2534–43.

Mishra J, Mori K, Ma Q, et al. Neutrophil gelatinase-associated lipocalin: a novel early urinary biomarker for cisplatin nephrotoxicity. Am J Nephrol. 2004;24:307–15.

Mishra J, Ma Q, Kelly C, et al. Kidney NGAL is a novel early marker of acute injury following transplantation. Pediatr Nephrol. 2006;21:856–63.

Murray PT, Le Gall JR, Dos Reis Miranda D, et al. Physiologic endpoints (efficacy) for acute renal failure studies. Curr Opin Crit Care. 2002;8:519–25.

Novo AC, Sadeck LS, Okay TS, et al. Longitudinal study of Cystatin C in healthy term newborns. Clinics (Sao Paulo). 2011;66:217–20.

Pan Z, Raftery D. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal Bioanal Chem. 2007;387:525–7.

Parikh CR, Devarajan P. New biomarkers of acute kidney injury. Crit Care Med. 2008;36:S159–65.

Parikh CR, Mishra J, Thiessen-Philbrook H, et al. Urinary IL-18 is an early predictive biomarker of acute kidney injury after cardiac surgery. Kidney Int. 2006;70:199–203.

Parravicini E, Nemerofsky SL, Michelson KA, et al. Urinary neutrophil gelatinase-associated lipocalin is a promising biomarker for late onset culture-positive sepsis in very low birth weight infants. Pediatr Res. 2010;67:636–40.

Parvex P, Combescure C, Rodriguez M, et al. Is Cystatin C a promising marker of renal function, at birth, in neonates prenatally diagnosed with congenital kidney abnormalities? Nephrol Dial Transplant. 2012;27:3477–82.

Plebani M, Mussap M, Bertelli L, et al. Determination of blood cystatin C in pregnant women during labor and in their newborns. Pediatr Med Chir. 1997;19:325–9.

Ray PE, Liu XH, Xu L, et al. Basic fibroblast growth factor in HIV-associated hemolytic uremic syndrome. Pediatr Nephrol. 1999;13:586–93.

Ray P, Acheson D, Chitrakar R, et al. Basic fibroblast growth factor among children with diarrhea-associated hemolytic uremic syndrome. J Am Soc Nephrol. 2002;13:699–707.

Ray PE, Tassi E, Liu XH, et al. Role of fibroblast growth factor-binding protein in the pathogenesis of HIV-associated hemolytic uremic syndrome. Am J Physiol Regul Integr Comp Physiol. 2006;290:R105–13.

Sarafidis K, Tsepkentzi E, Agakidou E, et al. Serum and urine acute kidney injury biomarkers in asphyxiated neonates. Pediatr Nephrol. 2012;27:1575–82.

Sharma AP, Kathiravelu A, Nadarajah R, et al. Body mass does not have a clinically relevant effect on cystatin C eGFR in children. Nephrol Dial Transplant. 2009;24:470–4.

Siew ED, Deger SM. Recent advances in acute kidney injury epidemiology. Curr Opin Nephrol Hypertens. 2012;21:309–17.
Singbartl K, Kellum JA. AKI in the ICU: definition, epidemiology, risk stratification, and outcomes. Kidney Int. 2012;81:819–25.
Soler-Garcia AA, Rakhmanina NY, Mattison PC, et al. A urinary biomarker profile for children with HIV-associated renal diseases. Kidney Int. 2009;76:207–14.
Tadesse S, Luo G, Park JS, et al. Intra-amniotic infection upregulates neutrophil gelatinase-associated lipocalin (NGAL) expression at the maternal-fetal interface at term: implications for infection related preterm birth. Reprod Sci. 2011;18:713–22.
Treiber M, Pecovnik-Balon B, Gorenjak M. Cystatin C versus creatinine as a marker of glomerular filtration rate in the newborn. Wien Klin Wochenschr. 2006;118:66–70.
Tsau YK, Sheu JN, Chen CH, et al. Decreased urinary epidermal growth factor in children with acute renal failure: epidermal growth factor/creatinine ratio not a reliable parameter for urinary epidermal growth factor excretion. Pediatr Res. 1996;39:20–4.
Vaidya VS, Ramirez V, Ichimura T, et al. Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury. Am J Physiol Renal Physiol. 2006;290: F517–29.
Wasilewska A, Zoch-Zwierz W, Taranta-Janusz K, et al. Neutrophil gelatinase-associated lipocalin (NGAL): a new marker of cyclosporine nephrotoxicity? Pediatr Nephrol. 2010;25:889–97.
Watanabe K, Ono A, Hirata Y, et al. Maturational changes and origin of urinary human epidermal growth factor in the neonatal period. Biol Neonate. 1989;56:241–5.
Weiss RH, Kim K. Metabolomics in the study of kidney diseases. Nat Rev Nephrol. 2012;8:22–33.
Westhuyzen J. Cystatin C: a promising marker and predictor of impaired renal function. Ann Clin Lab Sci. 2006;36:387–94.
Zappitelli M, Parikh CR, Akcan-Arikan A, et al. Ascertainment and epidemiology of acute kidney injury varies with definition interpretation. Clin J Am Soc Nephrol. 2008;3:948–54.
Zhou Y, Vaidya VS, Brown RP, et al. Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in urine and kidney following acute exposure to gentamicin, mercury, and chromium. Toxicol Sci. 2008;101:159–70.
Zwiers AJ, de Wildt SN, Hop WC, et al. Acute kidney injury is a frequent complication in critically ill neonates on extracorporeal membrane oxygenation: a 14-year cohort study. Crit Care. 2013;17:R151.

Osmolal Gap as a Biomarker in Kidney Injury: Focusing on the Differential Diagnosis of Metabolic Acidosis

3

Jeonghwan Lee, Nam Ju Heo, and Jin Suk Han

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Abstract

Although numerous osmoles contribute to the measured value of osmolality in serum and urine, most clinical laboratories cannot measure these individually. The most part of osmolality of serum or urine is determined by five or six major effective osmoles. Formulae for calculating serum and urine osmolality derive from these osmoles. The difference between the measured and calculated osmolality in serum and urine is defined as the osmolal gap.

The serum osmolal gap is useful in the differential diagnosis of high anion gap metabolic acidosis and can be used as a screening test for intoxication with active osmoles, including ethanol or other toxic alcohols. In addition, serum osmolal gap can be increased in ketoacidosis, lactic acidosis, and advanced chronic kidney disease. The urine osmolal gap is correlated with ammonium (NH_4^+) excretion and is useful in the differential diagnosis of normal anion gap metabolic acidosis. A decreased urine osmolal gap can be observed in patients with distal renal tubular acidosis, early chronic renal failure, and aldosterone deficiency or resistance.

Keywords

Serum osmolal gap • Urine osmolal gap • Metabolic acidosis

Abbreviations

Cl^-	Chloride
H^+	Acid
HCO_3^-	Bicarbonate
K^+	Potassium
Na^+	Sodium
NH_4^+	Ammonium

Key Facts of the Osmolal Gap

- Although there are numerous osmoles that contribute to the actual measured value of osmolality in serum and urine, these cannot be measured individually in standard clinical laboratories.
- Since the osmolality of serum or urine is largely determined by five or six major effective osmoles, formulae for the calculation of serum and urine osmolality derive from these osmoles.
- The osmolal gap refers to the difference between the measured and calculated osmolality of serum or urine.
- The most common cause of an increased serum osmolal gap is the addition of osmotically active substances, including ethanol, toxic alcohols such as methanol, glycols, or drug metabolites.

- A decreased urine osmolal gap is associated with conditions including distal renal tubular acidosis, chronic renal failure, aldosterone deficiency, and aldosterone resistance; in these conditions, urine ammonium excretion or production is low due to defective renal acidification.

Definitions

Osmolarity The numbers of osmoles of solute per liter of solution (mmol or mOsmol/L).

Osmolality Osmoles of solute per kg of solvent, expressed as mmol or mOsmol/kgH_2O.

Measured osmolality Osmolality which can be measured directly by osmometry.

Calculated osmolality Osmolality which can be estimated by using formulae measuring the concentrations of the major effective solutes.

The osmolal gap Difference between the measured and calculated osmolality.

Metabolic acidosis Conditions with the gain of acid or loss of alkali due to the metabolic causes.

Introduction

The concentration of the osmotic particles (osmoles) of a solute is expressed as either osmolarity or osmolality. Osmolarity refers to the numbers of osmoles of solute per liter of solution (mmol or mOsmol/L), while osmolality refers to osmoles of solute per kg of solvent, expressed as mmol or mOsmol/kgH_2O. Most available osmometers measure the osmolality of a solution.

In serum or urine, there are numerous osmoles, which cannot be measured individually in standard clinical laboratories. In serum and urine, only five or six major effective (osmotically active) osmoles determine the osmolality, and some of these are routinely measured in the clinical laboratory. Once the concentration of the measurable principal osmoles is established, it is possible to approximately calculate the osmolality of serum or urine. The difference between the measured and calculated osmolality is defined as an osmolal gap. In serum, this refers to the concentration of trivial solutes, while in urine it comprises immeasurable major osmoles, namely, ammonium (NH_4^+).

An increase in the serum osmolal gap is associated with conditions in which abnormal solutes are either produced endogenously or gained exogenously. Heavy alcohol ingestion or toxic alcohol intoxication is one of the common causes of an increased serum osmolal gap.

A decreased urine osmolal gap is associated with conditions in which urinary ammonium excretion or production is low, due to defective renal acidification that occurred in distal renal tubular acidosis or chronic kidney disease.

Definition

Serum and urine osmolality can be measured by osmometry (the "measured osmolality") or estimated by using formulae measuring the concentrations of the major effective solutes of serum or urine (the "calculated osmolality"). The osmolal gap is the difference between the measured and calculated osmolality (Koga et al. 2004).

Estimation of the Osmolal Gap

Measured Osmolality

The osmolality of serum or urine is measured by an osmometer. The most commonly used osmometer is freezing point osmometry, which is useful for the majority of samples, which have an osmolality of approximately ~500 mOsmol (or mmol)/kgH_2O. Vapor pressure measurement is suitable for samples with osmolalities in the range of 100–3000 mOsmol (or mmol)/kgH_2O and not affected by sample viscosity or the presence of suspended particles (Walker et al. 1986; Sweeney et al. 1993.

Calculation of Osmolality

Calculated Serum Osmolality

The serum osmolality is estimated by the concentrations of the solutes in the serum. In normal subjects, total serum solute concentration is determined by the five major effective osmoles: sodium (Na^+), chloride (Cl^-), bicarbonate (HCO_3^-), glucose, and urea. Since sodium ions are counterbalanced by Cl^- and HCO_3^-, only Na^+, glucose, and urea need to be measured to calculate the serum osmolality (Rasouli and Kalantari 2005; Gennari 1984).

A variety of formulae have been proposed for use in the prediction of the serum osmolality. Most studies, however, have concluded that the calculated serum osmolality can be best estimated from the following formulae (Worthley et al. 1987; Purssel et al. 2001; Bhagat et al. 1984):

(a) **Calculated serum osmolality (mOsmol/kgH_2O)** $= \mathbf{2} \times [\mathbf{Na^+}] + [\mathbf{glucose}]/\mathbf{18} + [\mathbf{blood\ urea\ nitrogen}]/\mathbf{2.8}$
Note: in standard clinical laboratory, measured [Na^+] and [K^+] are in mmol/L, while [glucose] and [urea] are in mg/dL. The divisors 18 and 2.8 convert units of mg/dL into mOsmol/kg or mmol/L.

(b) **Calculated serum osmolality (mOsmol/kgH_2O)** $= \mathbf{2} \times [\mathbf{Na}] + [\mathbf{glucose}] + [\mathbf{blood\ urea\ nitrogen}]$ (in mmol/L)

Calculated Urine Osmolality

The calculation of the urine osmolality is possible by the concentrations of the solutes in the urine. In normal subjects, total urine solute concentration is determined by six major osmoles: Na^+, K^+, Cl^-, glucose, urea, and ammonium (NH_4^+).

Under normal physiologic conditions, there is a large amount of K^+, NH_4^+, and undetectable HCO_3^-. Nearly all of the filtered HCO_3^- is reabsorbed in the proximal tubules, and 1 mmol/kg of acid (H^+) per day should be excreted from the intercalated cells of the collecting ducts. The acid excreted is combined with luminal NH_3 and Cl^- (Figs. 1, 2). Since at least 1 mmol/kg/day of NH_4Cl is excreted in the urine, NH_4^+ becomes the major cation in urine. Given the abundance of K^+ in food, a mechanism is required to maintain serum concentration; the kidney is genetically adapted to excrete the excess K^+ into the urine. Together Na^+, NH_4^+, and K^+ which are counterbalanced by Cl^- are the major cations in urine. Since NH_4^+ cannot be measured easily in most standard clinical laboratory, only Na^+, K^+, glucose, and urea are measured to calculate urine osmolality.

The calculated urine osmolality can be estimated by the following formulae:

(a) **Calculated urine osmolality (mOsmol/kgH$_2$O) = 2 × {[Na$^+$] + [K$^+$]} + [glucose]/18 + [urea]/2.8**

Note: in standard clinical laboratory, measured [Na$^+$] and [K$^+$] are in mmol/L, while [glucose] and [urea] are in mg/dL. The divisors 18 and 2.8 convert units of mg/dL into mOsmol/kg or mmol/L.

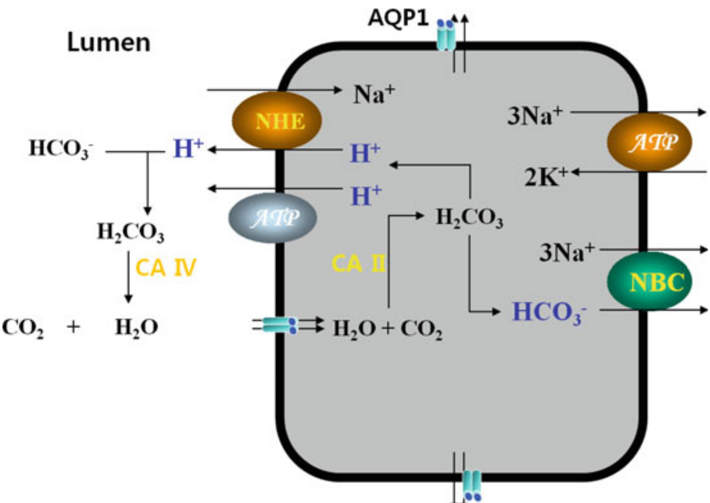

Fig. 1 Acidification in proximal tubule. Acid (H^+) is excreted into the lumen of the proximal tubule by Na^+-H^+ exchanger (NHE) or H^+-ATPase. In lumen, H^+ is combined to HCO_3^- forming dissolved CO_2 in water by carbonic anhydrase (CA). Dissolved CO_2 is reabsorbed by aquaporin (AQP) 1, water channel. In the proximal cell, it is cleaved to acid and HCO_3^- by CA. Along with Na^+, nearly all of HCO_3^- is reabsorbed to blood by Na^+-bicarbonate cotransporter (NBC)

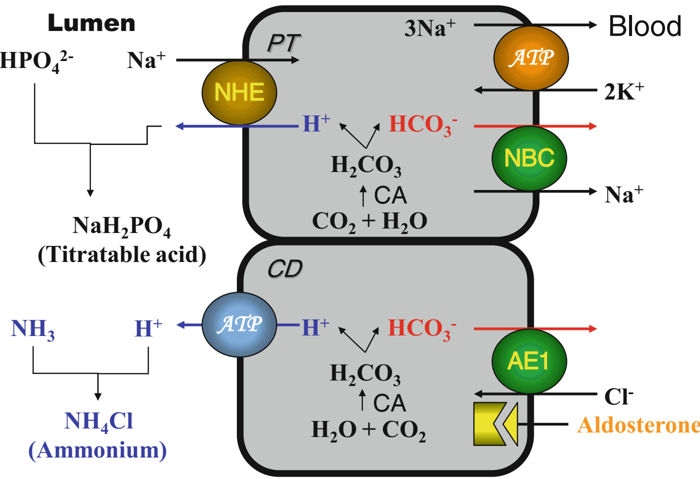

Fig. 2 Urine acidification by acid excretion and equimolar alkali (HCO_3^-) reclamation in the kidney. The remnant H^+ exceeding the limit that can be combined to HCO_3^- in the lumen of the proximal tubule should be combined to phosphate buffer forming titratable acidity. In the proximal tubule, nearly all of phosphate is reabsorbed by Na^+-phosphate transporter (NPT). In the collecting ducts, daily load of H^+ should be excreted by either H^+-ATPase or H^+-K^+-ATPase under the control of aldosterone. In the lumen, the excreted acid is combined to NH_3 and Cl^- forming NH_4Cl. Urine NH_4^+ reflects on the amount of acid excretion. In each process, H^+ excretion is accompanied by an equimolar reabsorption of HCO_3 by NBC and AE (anion exchanger)

(b) **Calculated urine osmolality (mOsmol/kgH_2O)** $= 2 \times$ **$\{[Na^+] + [K^+]\}$ + [glucose] + [urea]** (in mmol/L)

Serum or Urine Osmolal Gap

The serum osmolal gap refers to the concentration of trivial solutes in serum. In clinical practice, the normal serum osmolal gap is below 10–15 mOsmol/kgH_2O.

The urine osmolal gap refers to the concentration of immeasurable major osmoles, namely, NH_4^+, present in the urine (Fig. 3). In clinical practice, the normal urine osmolal gap is above 80–100 mOsmol/kgH_2O (Halperin et al. 1988).

Clinical Utility of the Serum and Urine Osmolal Gap

Serum Osmolal Gap

The mechanism of an increased serum osmolal gap is either the increase in measured osmolality or a decrease in calculated osmolality. An increase in unmeasured solutes in the serum results in an increase of the serum osmolality and an osmolal gap. In the case of pseudohyponatremia accompanying hyperlipidemia or hyperproteinemia,

$$\begin{aligned} &\text{Measured Osm} = \mathbf{2}\{[Na^+]+[K^+]+[\mathbf{NH_4^+}]\}+[\text{Urea}]+[\text{Glucose}]+\textbf{immeasurable Osm} \\ &-\text{Calculated Osm} = 2\{[Na^+]+[K^+]\} \quad +[\text{Urea}]+[\text{Glucose}] \\ \hline &\textbf{Osmolal gap} = \mathbf{2\,[NH_4^+]} + \textbf{immeasurable Osm} \end{aligned}$$

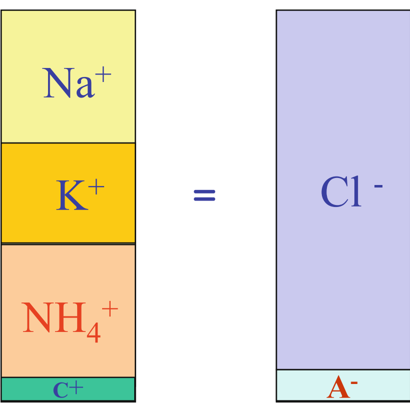

Fig. 3 **Compositions of urine osmoles and urine osmolal gap** (C^+ immeasurable cations, A^- immeasurable anions). In normal subjects, total urine solute concentration is determined by six major osmoles: Na^+, K^+, Cl^-, glucose, urea, and NH_4^+. The urine osmolal gap refers to the concentration of NH_4^+ present in the urine

Table 1 Serum osmolal gap. In normal subjects, total serum osmolality is determined by five major effective osmoles: sodium (Na^+), chloride (Cl^-), bicarbonate (HCO_3^-), glucose, and urea. Since sodium ions are counterbalanced by Cl^- and HCO_3^-, only Na^+, glucose, and urea need to be measured to calculate the serum osmolality. The osmolal gap is the difference between the measured and calculated osmolality (*Osm* osmolality)

$\text{Measured Osm}^* = 2\,[Na^+] + \frac{\text{Glucose}}{18} + \frac{\text{BUN}}{2.8} + \text{immeasurable Osm}$
$-\text{Calculated Osm} = 2\,[Na^+] + \frac{\text{Glucose}}{18} + \frac{\text{BUN}}{2.8}$
Osmolal gap = **Immeasurable Osm (<10–15 mOsmol/kgH$_2$O)**

*Osm osmolality

the calculated serum osmolality decreases, while the serum osmolal gap increases (Tables 1 and 2).

Estimation of the serum osmolal gap is useful in the differential diagnosis of high anion gap metabolic acidosis (Kraut and Xing 2011). The serum osmolal gap can be used as a rapid screening test for the detection of active osmoles, including ethanol or other toxic alcohols, such as methanol, ethylene glycol, diethylene glycol, propylene glycol, and isopropanol. Ethanol ingestion is one of the most common causes of an elevated serum osmolal gap (Shull and Rapoport 2010), and results in ketoacidosis with a high serum anion gap (Almaghamsi and Yeung 1997). The osmolality of ethanol can be calculated by the following formulae: (1) osmolal gap = ethanol

Table 2 Conditions with an increased serum osmolal gap. Increased osmolal gap is observed in several clinical conditions including intoxication of alcohol, toxin, or drugs

Alcohol
Ethanol
Toxic alcohols: methanol, (di)ethylene glycol, propylene glycol, isopropanol
Lactic acidosis, diabetic ketoacidosis
Hyperlipidemia, hyperproteinemia
Kidney disease (renal failure): acute, chronic
Drugs or metabolites: mannitol, radiocontrast dye, salicylate, acetaminophen
SIADH, diabetes insipidus
Trauma, crush injury
Circulatory collapse: severe heart failure, sepsis

Table 3 Causes of metabolic acidosis. The serum osmolal gap is high in patients with acidosis due to exogenous solutes gain. A low urine osmolal gap reflects on a low urinary excretion of ammonium (NH_4^+), in the patients with acidosis due to defective acid excretion of the kidney. This kind of acidosis can be easily detected by low urine osmolal gap (*GI gastrointestinal)

I. Acid gain (production)
1. Endogenous
Lactic acidosis, diabetic ketoacidosis, renal failure
2. Exogenous: *high serum osmolal gap*
Methanol, *ethanol*, *ethylene glycol*, *paraldehyde*
II. Alkali (HCO_3^-) loss
1. Direct loss
1. GI* loss: *diarrhea*, *pancreatic/biliary drainage*, *short bowel*, *ileostomy*
2. Renal loss: *proximal renal tubular acidosis*
2. Indirect loss: *low urine osmolal gap*
Defective HCO_3^- reclamation due to defective H^+ excretion
Distal renal tubular acidosis, *hypoaldosteronism*, Gordon syndrome, *mild chronic renal failure*

**GI* gastrointestinal

(mg/dL)/3.7–0.35 or (2) 1.25 × ethanol (mmol/L) – 0.35 (Purssell et al. 2001). A high anion gap metabolic acidosis with a high osmolal gap may also be caused by the ingestion of toxic alcohol/glycols, such as methanol and ethylene glycols (Kraut et al. 2013). A serum osmolal gap >10 mOsmol/kgH_2O can be used as a reliable screening test for methanol or ethylene glycol ingestion (Lynd et al. 2008). Other alcohol derivatives, such as diethylene glycol and propylene glycol, can cause a high anion gap metabolic acidosis with a high serum osmolal gap. In contrast, isopropyl alcohol (isopropanol or rubbing alcohol), which is metabolized to acetone, results in a high osmolal gap without metabolic acidosis (Slaughter et al. 2014; Table 3).

In ketoacidosis, lactic acidosis, and advanced chronic kidney disease (renal failure), the serum osmolal gap may be slightly increased; a typical osmolal gap in

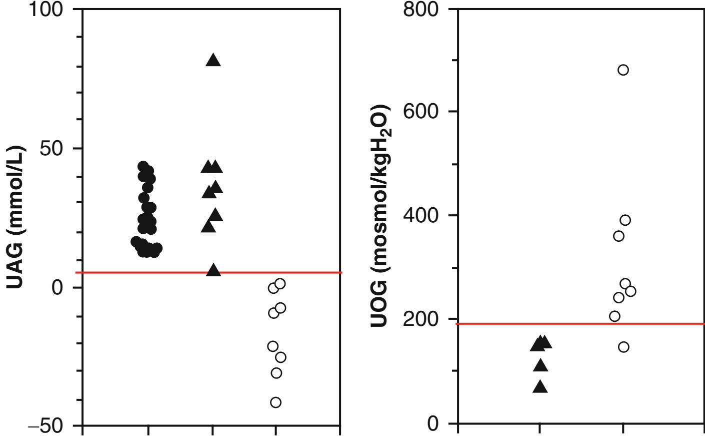

Fig. 4 Urine anion gap and osmolal gap in chronic renal failure and distal renal tubular acidosis patients and in acid-loaded normal controls. Patients with chronic renal failure (*solid circle*) and distal renal tubular acidosis (*triangle*) had defects in acid excretion in the collecting ducts. The urine osmolal gaps were very low in those patients due to decreased NH_4^+ excretion in urine compared to the acid-loaded normal controls (*open circle*) (Kim et al. 1996)

these conditions is in the region of 10 mOsmol/kgH_2O, and it is rarely >20 mOsmol/kgH_2O (Dursun et al. 2007).

Estimation of the serum osmolal gap can serve a variety of purposes. It is a useful indicator for the initiation and termination of dialysis in methanol intoxication (Hunderi et al. 2006). The increased serum osmolal gap is predictive of contrast-induced acute kidney injury (Kim et al. 2012; Ford et al. 2013). An increased osmolal gap (>10 mOsmol/kgH_2O) has been reported in patients with a wide variety of conditions including acute renal failure (associated with sepsis, abdominal surgery, or acute pancreatitis), abdominal sepsis with liver damage, severe heart failure, syndrome of inappropriate antidiuretic hormone (SIADH), acquired diabetes insipidus, trauma or crushing injuries, lactic acidosis, and ingestion of drugs including salicylate, acetaminophen, and lorazepam (Tomey 1997; Shull 1978).

Urine Osmolal Gap

In clinical practice, the urine osmolal gap is useful in the differential diagnosis of normal anion gap metabolic acidosis.

Normal individuals excrete over 75 mmol/L (≥150 mOsmol/kgH_2O) of urine NH_4^+ per day, and this increases further in metabolic acidosis. The reference value of the urine osmolal gap for the individual with metabolic acidosis whose acid excretion of the kidney is normal is up to 150–200 mOsmol/kgH_2O (Kim et al. 1996; Halperin et al. 2004); (Fig. 4). A urine osmolal gap below normal limits indicates that urinary NH_4^+ excretion is low. A decreased urinary NH_4^+ excretion may result

from defective renal acid excretion which will be observed in patients with distal renal tubular acidosis, early chronic renal failure, and resistance to or deficiency of aldosterone. Impaired urine acidification results in a decrease in urinary NH_4^+ and net acid excretion (NAE). Distal renal tubular acidosis, early chronic renal failure, or defective response to aldosterone with impaired urinary acid excretion is associated with a high urine anion gap and a low urine osmolal gap.

In patients with a high anion gap metabolic acidosis, the urine osmolal gap would be high due to urinary loss of organic acid anions (i.e., ketones in diabetic ketoacidosis).

Limitations to the Clinical Utility of the Osmolal Gap

Various Causes of an Osmolal Gap

An elevated serum osmolal gap can occur in a variety of clinical settings, other than metabolic acidosis. Causes of an osmolal gap include sick cell syndrome in patients with multiorgan failure (Guglielminotti et al. 2002), absorption of glycine, and intravenous infusion of hypertonic mannitol (Dorman et al. 1990). After ingestion or intoxication, toxic alcohol/glycols are metabolized from uncharged active osmoles to charged molecules such as formate or glycolate; the latter molecules have no effect on osmolality or the osmolal gap. Therefore, the diagnostic usefulness of the serum osmolal gap is limited in the acute phase of intoxication with ethanol or toxic alcohols.

Limitations of Methods of Osmometry

Accurate measurements of osmolality using osmometry require that the solutions have specific characteristics. Freezing point osmometry cannot be performed on samples containing particles, which can act as crystallization nuclei, or on samples with viscosities that are different to water. Vapor pressure osmometry is less useful in certain circumstances, particularly for samples containing volatile solutes (Walker et al. 1986; Sweeney et al. 1993).

Summary Points

- This chapter focuses on the clinical usefulness of the serum or urine osmolal gap in the differential diagnosis of metabolic acidosis.
- Metabolic acidosis is the process of acid (H^+) gain or alkali (HCO_3^-) loss.
- In metabolic acidosis, the associated anions are increased in the blood due to acid gain.
- Acids are converted from endogenous solutes or exogenously inflowing solutes.
- Acids and associated anions converted from endogenous solutes or exogenously inflowing solutes act as osmoles.

- In the case of metabolic acidosis, there is an additional increase in osmoles in the serum due to ingestion of toxins or drugs (exogenous solutes).
- Decreased renal excretion of acid results in a low urinary excretion of ammonium NH_4^+ and defective urinary acidification: distal renal tubular acidosis, early chronic renal failure, and resistance to or deficiency of aldosterone.
- Since the majority of the urine osmolal gap consists of NH_4^+, a low urine osmolal gap may be useful in screening for defective urinary acidification.

References

Almaghamsi AM, Yeung CK. Osmolal gap in alcoholic ketoacidosis. Clin Nephrol. 1997;48(1):52–3.

Bhagat CI, Garcia-Webb P, Fletcher E, et al. Calculated vs measured plasma osmolalities revisited. Clin Chem. 1984;30(10):1703–5.

Dorman HR, Sondheimer JH, Cadnapaphornchai P. Mannitol-induced acute renal failure. Medicine. 1990;69(3):153–9.

Dursun H, Noyan A, Cengiz N, et al. Changes in osmolal gap and osmolality in children with chronic and end-stage renal failure. Nephron Physiol. 2007;105(2):19–21.

Ford JB, Amiri-Davani NC, Diercks DB, et al. Effect of low-osmolality intravenous contrast on serum osmolal gap in adults. J Emerg Med. 2013;45(1):53–6.

Gennari FJ. Current concepts. Serum osmolality. Uses and limitations. N Engl J Med. 1984;310(2):102–5.

Guglielminotti J, Pernet P, Maury E, et al. Osmolar gap hyponatremia in critically ill patients: evidence for the sick cell syndrome? Crit Care Med. 2002;30(5):1051–5.

Halperin ML, Margolis BL, Robinson LA, Halperin RM, West ML, Bear RA. The urine osmolal gap: a clue to estimate urine ammonium in “hybrid” types of metabolic acidosis. Clin Invest Med. 1988;11(3):198–202.

Halperin ML, David MR, Kamel K. Interpretation of urine electrolyte and acid-base parameters. In Brenner BM, editor. Brenner & Rectors’ the kidney. 7th ed. Philadelphia: Saunders;2004. pp.1151–1181.

Hunderi OH, Hovda KE, Jacobsen D. Use of the osmolal gap to guide the start and duration of dialysis in methanol poisoning. Scand J Urol Nephrol. 2006;40(1):70–4.

Kim GH, Han JS, Kim YS, et al. Evaluation of urine acidification by urine anion gap and urine osmolal gap in chronic metabolic acidosis. Am J Kidney Dis. 1996;27(1):42–7.

Kim S, Sung J, Kang WC, et al. Increased plasma osmolar gap is predictive of contrast-induced acute kidney injury. Tohoku J Expl Med. 2012;228(2):109–17.

Koga Y, Purssell RA, Lynd LD. The irrationality of the present use of the osmole gap: applicable physical chemistry principles and recommendations to improve the validity of current practices. Toxicol Rev. 2004;23(3):203–11.

Kraut JA, Xing SX. Approach to the evaluation of a patient with an increased serum osmolal gap and high-anion-gap metabolic acidosis. Am J Kidney Dis. 2011;58(3):480–4.

Lynd LD, Richardson KJ, Purssell RA, et al. An evaluation of the osmole gap as a screening test for toxic alcohol poisoning. BMC Emerg Med. 2008;8:5.

Purssell RA, Pudek M, Brubacher J, et al. Derivation and validation of a formula to calculate the contribution of ethanol to the osmolal gap. Ann Emerg Med. 2001;38(6):653–9.

Rasouli M, Kalantari KR. Comparison of methods for calculating serum osmolality: multivariate linear regression analysis. Clin Chem Lab Med. 2005;43(6):635–40.

Slaughter RJ, Mason RW, Beasley DM, et al. Isopropanol poisoning. Clin Toxicol. 2014;52(5):470–8.

Sweeney TE, Beuchat CA. Limitations of methods of osmometry: measuring the osmolality of biological fluids. Am J Physiol. 1993;264(3 Pt 2):R469–80.

Shull RM. The value of anion gap and osmolal gap determination in veterinary medicine. Vet Clin Pathol. 1978;7(3):12–4.

Shull PD, Rapoport J. Life-threatening reversible acidosis caused by alcohol abuse. Nat Rev Nephrol. 2010;6(9):555–9.

Tormey WP. Are the increasing clinical demands for osmolality measurements and their associated elelctrolytes appropriate? Ir J Med Sci. 1997;166(2):75–9.

Walker JA, Schwartzbard A, Krauss EA, et al. The missing gap. A pitfall in the diagnosis of alcohol intoxication by osmometry. Arch Intern Med. 1986;146(9):1843–4.

Worthley LI, Guerin M, Pain RW. For calculating osmolality, the simplest formula is the best. Anaesth Intensive Care. 1987;15(2):199–202.

Peritoneal Effluent Biomarker Discovery in Peritoneal Dialysis: The Omics Era

4

Deirisa Lopes Barreto and Dirk G. Struijk

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Abstract

One of the main renal replacement treatment modalities for patients with end-stage renal diseases is peritoneal dialysis (PD). In PD therapy, the peritoneum is used as an intracorporeal dialysis system. The monitoring of intraperitoneal events is hampered by the absence of serial peritoneal biopsies. However, the acquisition of peritoneal effluent is simple and usually occurs after a predefined dwell or if possible after a standardized peritoneal function test. This peritoneal effluent is composed of several proteins and metabolites, which modifies accordingly due to intraperitoneal events. To date, peritoneal effluent biomarker discovery is evolving with a holistic perspective. The rise of applying suffix -omics technologies within PD therapy introduced a more exploratory approach for the

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identification of candidate effluent biomarkers. The application of genomics, metabolomics, and proteomics with the peritoneal effluent as biospecimen is however still in its infancy.

The emerging field of omics techniques as tools for peritoneal effluent biomarker discovery is presented in this chapter. The high sensitivity of omics technologies requires stringent conditions, and therefore methodological precautions must be undertaken on laboratory technical level, appropriate selection of study design and population, as well as data analysis. For this reason, methodological considerations for conducting omics-based PD research and the current developments with regard to the usage of these disciplines are addressed. Lastly, a summary is given on the available literature concerning the usage of omics techniques with the peritoneal effluent as a liquid biopsy within PD therapy.

Keywords

Biomarker • Discovery • Effluent biomarker • Genomics • Metabolomics • Peritoneal dialysate • Peritoneal dialysis • Peritoneal effluent • Proteomics

Abbreviations

2D-DIGE	Two-dimensional difference gel electrophoresis
Biobank	Biological bank
Biomarker	Biological marker
CAPD	Continuous ambulatory peritoneal dialysis
CKD	Chronic kidney disease
CRP	C-reactive protein
CV	Coefficient of variation
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immuno assay
EPS	Encapsulating peritoneal sclerosis
GN	Glomerulonephritis
GWAS	Genome-wide association studies
Ig	Immunoglobulin
IL-6	Interleukin-6
MS	Mass spectrometry
NECOSAD	Netherlands Cooperative Study on the Adequacy of Dialysis
NMR	Nuclear magnetic resonance
NRI	Net reclassification index
PD	Peritoneal dialysis
RNA	Ribonucleic acid
ROC curve	Receiver operating characteristic curve
SNPs	Single nucleotide polymorphisms
SOP	Standard operating procedures
VEGF	Vascular endothelial growth factor

Definitions

Biological bank (biobank) An archive containing human biospecimens that may include blood, effluent, serum, or tissue samples. The storage of these samples occurs preferably under standardized conditions within a single or multicenter study cohort.

Encapsulating peritoneal sclerosis (EPS) EPS is the most devastating complication of PD therapy that occurs in 3–7 % of PD patients. EPS is characterized by a dense cocoon of fibrous tissue that covers the abdomen. High mortality rates and severe morbidity are present for patients diagnosed with EPS.

Free water transport (FWT) Aquaporin-1 mediated transport of water, without dissolved solutes and electrolytes. FWT is one of the peritoneal transport parameters that become impaired in long-term PD patients.

Genomics Laboratory strategy that investigates genes and their functions. Utilized methods comprise DNA sequencing and fingerprinting. Genomics is furthermore subdivided into various disciplines such as epigenomics and metagenomics and functional genomics and structural genomics.

Metabolomics Laboratory method for the identification and quantification of metabolite levels. Chromatography is one of the primary steps used as a separation technique. Thereafter, the metabolites may be detected by means of mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy.

Peritoneal dialysis (PD) therapy One of the renal replacement therapies for end-stage renal disease patients for over almost five decades. The proportion of PD patients in the worldwide dialysis population covers 11 %, and the 5-year survival has increased to 41 %. In many countries, PD therapy is presented as primary dialysis modality choice, as there is a survival benefit over hemodialysis in the first 3 years.

Peritoneal function test A standardized peritoneal test to assess the function status of the peritoneal membrane. Additionally, the tests provide insight into the magnitude of small-solute removal, fluid transport, and ultrafiltration capacity. Several forms of peritoneal function tests are available such as a standardized peritoneal permeability analysis or (modified) peritoneal equilibration test. However, all of them are characterized by a predefined dwell time with or without intermediate sampling of the peritoneal effluent.

Peritoneal membrane The peritoneal membrane is described to be a semipermeable membrane that consists of three main layers. Firstly, the mesothelial cell layer is encountered, followed by the interstitium in which the peritoneal capillaries are imbedded. Long-term and continuous exposure of PD solutions to this membrane may lead to functional and morphological alterations.

Proteomics Laboratory discipline for analyzing structure, conformation, and biological function of proteins. In brief, proteomic analyses encompass separation of the proteins by gel electrophoresis methods, followed by MS-based analyses.

Systems biology A holistic point of view for gaining insight into biological mechanisms within cells, organisms, or species. Systems biology has been applied from the early 1900. Genomics, metabolomics, and proteomics are disciplines within systems biology that seek to qualify and quantify the genome, metabolome, or proteome, respectively.

Introduction

The field of omics is expanding at a rapid pace as novel instruments for biological marker (biomarker) discovery and unraveling pathophysiological mechanisms. Within peritoneal dialysis (PD) therapy, the application of high-throughput technologies is still in its infancy. Especially, the peritoneal effluent as biospecimen, which contains a variety of proteins either due to transperitoneal transport or local production, has not yet been explored extensively (Table 1). Numerous biomarker consortia, working groups, and scientists contributed to the diversity of existing biomarker definitions with different classifications and applications (Atkinson et al. 2001;

Table 1 Key facts of peritoneal dialysis and peritoneal effluent biomarkers

In PD therapy, the peritoneum serves as a biological semipermeable dialysis membrane. Through this membrane, toxic waste products and excess fluid are removed from the circulation into the peritoneal cavity. During the day, 3–5 daily exchanges of PD solutions take place. These exchanges vary in dose and duration of the dwell. After a predefined dwell time, the infused dialysis solution is drained. This drained fluid is called the peritoneal effluent or peritoneal dialysate, which contains several substances
Proteomic profiling and characterization of the peritoneal effluent indicated that the substances represent merely extracellular proteins. In addition, the circulation is found to be responsible for the presence of a majority of peritoneal effluent constituents. Substances or proteins in the peritoneal effluent can only be eligible as effluent biomarker within PD if they are locally produced within the peritoneal cavity
Unfortunately, no direct visualization of the peritoneal membrane is possible without invasive procedures. Furthermore, computed tomography scans are unable to detect anatomical modifications timely. Peritoneal effluent biomarkers are considered as noninvasive instruments for screening or diagnostic purposes. They would allow uncomplicated monitoring of the integrity of the peritoneal membrane. For this reason, the peritoneal effluent is regarded as the most clinically relevant specimen within PD therapy. However, the implementation of the more established peritoneal effluent biomarkers in routine PD patient care is fairly small
The discovery of peritoneal effluent biomarkers was previously based on pathophysiological knowledge and hypothesis driven. The first omics-conducted research within PD therapy originates from 2007. Effluent biomarker discovery through omics technologies is aimed at providing proxies for specific anatomical alterations. This holistic approach is expected to deliver insight into the sequence of intraperitoneal events. Nevertheless, current omics studies within PD require further calibration and standardization

PD peritoneal dialysis

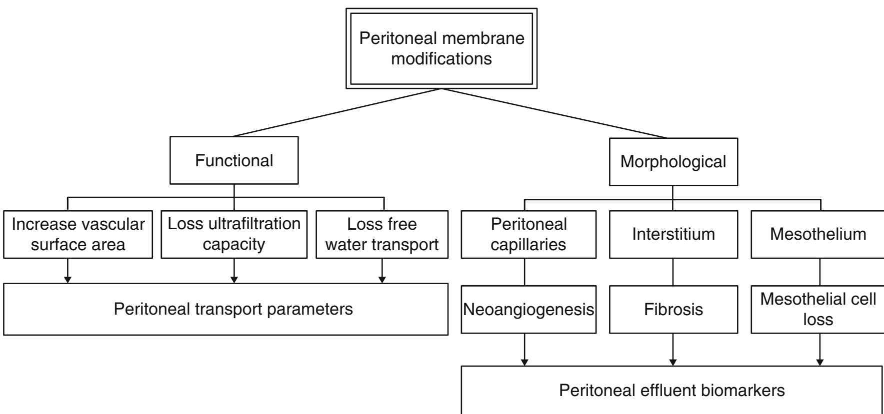


Fig. 1 Peritoneal membrane modifications. The peritoneal membrane consists of three main layers: mesothelium, interstitium, and peritoneal capillaries. In all of these layers, morphological modifications are observed for which effluent biomarkers could be utilized for diagnostic or prognostic purposes. However, not all of these layers are considered as prominent barriers to the peritoneal membrane transport. The increase in perfused peritoneal capillaries leads to a rapid dissipation of glucose and consequently may evolve in a decreased ultrafiltration capacity. In a progressive stage, the fluid transport through the water channels, e.g., aquaporins, may become impaired

Hulka et al. 1990; Perera and Weinstein 2000; Colburn 2000; Jain 2010). Overall, the essence of molecular biomarkers is to provide noninvasive, cost-effective tools for screening or diagnostic purposes and disease prognosis and surveillance. Additionally, molecular biomarkers may also be intended to assess therapeutic responsiveness or offer novel intervention strategies (Atkinson et al. 2001).

The main purpose of PD therapy as renal replacement treatment modality is to remove toxins and excess fluid from the body. For this intention, a permanent catheter is inserted into the peritoneal cavity through which dialysis solutions can be instilled. The removal of these toxic waist products and excess fluid, from the circulation into the peritoneal cavity, occurs mainly by means of diffusion through the peritoneal membrane. Therefore, the efficacy of PD therapy is highly dependent on the biological condition of the peritoneal membrane. The continuous exposure of dialysis solutions to the peritoneal membrane causes however several functional and morphological modifications (Fig. 1). The most encountered functional changes are the rapid dissipation of the osmotic gradient and a decrease in ultrafiltration capacity. Furthermore, some of the long-term PD patients also present with an impairment of free water transport. Especially in patients who develop EPS, free water transport appears to be the only peritoneal transport parameter, which can distinguish patients with this severe complication from patient with a long PD therapy duration (Lopes Barreto et al. 2014). The functionality of the peritoneal membrane can be monitored incessantly by peritoneal permeability tests (Coester et al. 2009), whereas the progression of morphological modifications remains unrevealed because no serial peritoneal biopsies can be performed. More importantly, the functionality of the peritoneal membrane is not inherent to the observed morphologic modifications. In

Table 2 Prerequisites for a peritoneal effluent biomarker

1.	Detectable in peritoneal effluent by means of omics technologies or other protein detection methods
2.	Computational assessment of local release or production within the peritoneal cavity
3.	Involvement in pathological pathway of the peritoneal membrane
4.	Good measures of diagnostic accuracy for PD-related outcomes (e.g., high sensitivity/specificity)

this perspective, more emphasis is placed on potential markers that are present in the peritoneal effluent and mirror the integrity of peritoneal tissues. Thus, promising peritoneal effluent substances have to meet several prerequisites before the acknowledgment of a peritoneal effluent biomarker is given to them (Table 2). Prior to the rise of suffix -omics technologies, effluent biomarker discovery in PD was merely based on hypothesis-driven research. Even though this is still valid within these translational scientific disciplines, at present the discovery of effluent biomarkers is evolving with a more global and exploratory perspective. The application of high-throughput laboratory techniques offers a great opportunity to gain insight in the peritoneal alterations that occur over time due to PD treatment and identify clinically relevant effluent substances that may serve as biomarkers. The vast majority of high-dimensional methodologies applied to the peritoneal effluent consist of genomics, metabolomics, and proteomics. Previous reviews have acknowledged the potential use of proteomics for effluent biomarker discovery and elucidation of pathophysiological processes of the peritoneal membrane (Brewis and Topley 2010; Thongboonkerd 2010). The present chapter highlights the evolving landscape of omics, in which the human peritoneal effluent is regarded as central specimen. Methodological considerations in effluent biomarker discovery are provided, and an overview is given to illustrate the application and progression of omics-conducted research within PD therapy.

Methodological Considerations

As a consequence of the large amount of data that is acquired by suffix -omics analyses, several methodological precautions have to be taken. These preventative measures include the optimization of analytical validity, well-designed study and selection of study population, as well as proper data analysis. This section describes these aforementioned considerations and provides examples in PD-conducted omics studies.

Analytical Validity

In biomarker discovery studies, three main phases are encountered on a laboratory technical level that might alter research findings and thwart the interpretation of the acquired data: pre-analytical, analytical, and post-analytical.

The pre-analytical phase evolves sample handling and has a direct influence on the accuracy and reproducibility. This is especially of importance when investigating the proteome, metabolites, or candidate effluent biomarkers, which are influenced by structural peritoneal membrane modifications over time. One of the bias-introducing factors could be the effect of storage on the assembled effluent biospecimens. DNA extraction and genotyping from frozen peritoneal effluent samples up to 7 years at −20 °C indicated no influence of storage duration (Gillerot et al. 2004). However, it is not known whether long-term storage of the effluent at lower temperatures is superior to temperatures of at least −20 °C. Moreover, the stability of metabolites and proteins present in peritoneal effluent and the effect of repeated freeze-thaw cycles have not been investigated yet. To preserve the quality of the effluent and reduce the amount of variability due to incorrect or unbalanced sample handling, standard operating procedures (SOPs) are needed. Included elements in a SOP for local or multicenter biorepositories should at least cover the amount and volume of aliquots, storage conditions, e.g., minimal temperature of −20 °C, mechanical freezer, or directly frozen by liquid nitrogen, and the necessity to document the number of freeze-thaw cycles. From the omics studies within the discipline of PD, only some of the articles provided detailed information on sample collection and archiving comprising momentum of effluent withdrawal, storage temperature, and time frame of sample processing. For comparability and assessment of study quality, reporting this information is essential.

Intra-analytical inaccuracies may contribute to random or systematic errors. These errors occur during assaying of effluent constituents, which could be influenced by sampling handling of the laboratory personnel, measurement apparatus, or reagents. By performing the experiments in dupli- or triplicate, the degree of precision can be assessed. Also, the range of standard reference curves should be wisely chosen in order to determine the appropriate detection limit of an assay. Typically acceptable coefficients of variation (CV) lie beneath 20 % for which one should strive for CVs of 5 %. Furthermore, in the validation phase of biomarker discovery by means of enzyme-linked immunosorbent assays (ELISAs) or Western blots, one could reduce intra- and inter-variability by even distribution of the study groups within and throughout batches. Preferably, the laboratory technician should be blinded for the outcome of interest as well. Recently, a methodological article proposed an optimal high-resolution effluent protein separation technique by two-dimensional gel electrophoresis (2D-DIGE) intended for proteomic analysis (Zhang et al. 2013). The authors investigated five precipitation methods followed by constraining abundant proteins of the peritoneal effluent. Such attempts are in favor of reducing sample preparation heterogeneity and enhance critical appraisal of the used high-throughput methodologies across studies analyzing the peritoneal effluent.

Incorrect post-analytical inferences may lead to differential misclassification bias. For example, if threshold values of the peritoneal effluent are inappropriately determined, the contrast between groups could be augmented or diminished. As a consequence, the results could indicate nonexisting differences between the group with the outcome of interest and those without. Eventually, this nonrandom measurement error may contribute to biased estimates of association.

Study Designs and Population

The majority of the omics studies with the peritoneal effluent follow a cross-sectional design. Generally, in these studies, the profiles of PD patients with the characteristic or clinical endpoint of interest are compared to PD patients with a stable condition. Omics studies have to be well designed, especially with regard to the study subjects where one should strive for a homogeneous population, as the peritoneal metabolome and proteome of PD patients are likely to be susceptive to posttranslational modifications due to patient-related and external factors. Therefore, the selection of patients as well as a priori specification of a validation subset is of similar or even of greater importance when compared to other epidemiologic studies. Unfortunately, often clinical and demographic data of the study population or independent validation sample is lacking in omics-conducted PD research. The sparse number of cohort and nested case-control studies is presumably the result of impracticable specimen collection or cost related. This is however a great loss, as the presence of a local biological bank with repetitive effluent specimens would enable trend analyses. In addition, the sample size is relatively small in the majority of the omics studies with PD therapy. Lastly, it is questionable whether the follow-up duration in a number of studies is sufficient to measure difference in peritoneal membrane alterations. Especially, since the factual peritoneal membrane dysfunction is usually observed after a therapy duration of at least 2 years.

Another important factor of the peritoneal effluent is its origin. As the peritoneal effluent can be obtained right after a regular PD exchange or after a standardized peritoneal function test, it follows from this that the biological variability increases concordantly. Therefore, the withdrawal of effluent samples in studies should be harmonized within or between centers. Regrettably, not all omics studies within PD report whether the peritoneal effluent is derived from a regular dwell of after a standardized peritoneal function test.

Omics Data Analysis

An effluent biomarker can be an outcome to monitor the progression of PD therapy as well as a prognostic factor of various PD-related complications. When a biomarker is intended as a diagnostic or prognostic instrument, receiver operating characteristic (ROC) curve and C-statistics are used in order to evaluate the discriminative power. Additionally, optimal threshold values can be estimated based on the sensitivity and specificity of a biomarker. However, these measures are highly dependent on the base study population, and misclassification may arise due to patient demographics, laboratory measurement errors, and the degree of the exposure to dialysis solutions. Hence, one has to be cautious with the definition and selection of a correct clinical endpoint in biomarker research. Nevertheless for that reason, the net reclassification index (NRI) was introduced suggesting a method to gain prognostic accuracy of a biomarker (Wilson et al. 2008). The interpretation of omics-derived findings usually requires the use bioinformatics or the application of molecular epidemiology. In general, the sample

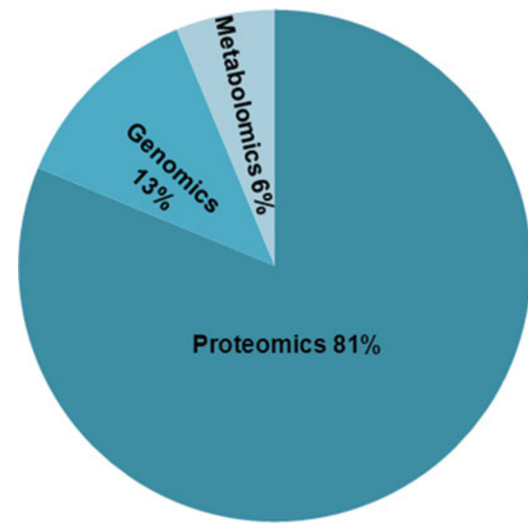


Fig. 2 Proportion of omics studies with the human peritoneal effluent as biospecimen. The number of studies applying omics technique with the human peritoneal effluent as central biospecimen is still limited. Proteomics attributed to 81 % of all studies followed by genomics (13 %) and metabolomics (6 %)

sizes of the current omics studies in PD are small. However, omics studies generate enormous amount of data that are subjective to false-positive or false-negative results when not handled as appropriate. Therefore, correction for multiple comparisons or adjustments with regard to p-value thresholds should be applied.

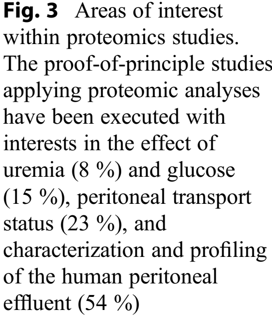
Current Developments

Proteomics is the main applied high-throughput technology followed by genomics and metabolomics for peritoneal effluent biomarker discovery (Fig. 2). These fields have the potential to elucidate underlying molecular mechanisms that are involved in the pathophysiology of the peritoneum. Moreover, they can empirically provide novel diagnostic and therapeutic biomarkers based on genome, metabolite, or protein profiles of PD patients. Nevertheless, the number of studies is still modest; a summary is given on the recent developments and main findings.

Proteomics

In nephrology, proteomics is merely applied on plasma, serum and urinary samples, or renal tissue. The number of proteomics studies with the peritoneal effluent as sample type is rising, and investigations have been executed from various perspectives other than the identification of novel biomarkers for PD (Fig. 3). The main obstacle for the discovery of effluent biomarkers includes the dynamic range in proteomic analyses.

Uremia in itself is suggested to alter the functional and structural organization of the peritoneal membrane. Therefore, the effect of a uremic environment was investigated



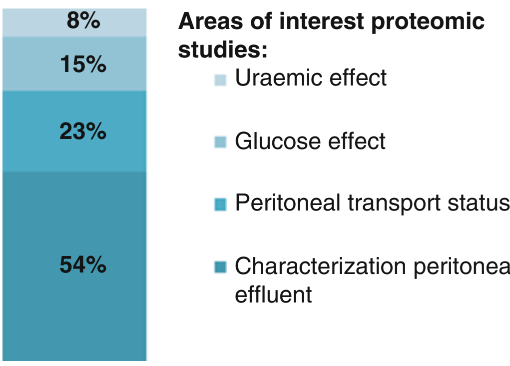


Fig. 3 Areas of interest within proteomics studies. The proof-of-principle studies applying proteomic analyses have been executed with interests in the effect of uremia (8 %) and glucose (15 %), peritoneal transport status (23 %), and characterization and profiling of the human peritoneal effluent (54 %)

by proteomic analysis in chronic kidney disease patients (stage five) versus patients with normal renal function (Wang et al. 2012). A number of protein alterations were found including elevated levels of vascular endothelial growth-A (VEGF-A).

The majority of proteomics research is aimed at the characterization and profiling of the peritoneal effluent in order to identify potential biomarkers. This discovery-based approach has been adapted in the peritoneal effluent of incident and prevalent adult PD patients (Wu et al. 2013; Cuccurullo et al. 2011). The study by Wu et al. included a number of ten incident PD patients for whom three patients were used for validation. The peritoneal effluent was assembled at start of PD therapy and once again after 1 year. Validation by Western blots showed elevated levels of immunoglobulin (Ig) μ chain, fibrinogen γ chain, and C-reactive protein (CRP) at baseline and Ig δ, α-1 antitrypsin, histidine-rich glycoprotein, apolipoprotein A1, and serum amyloid P-component after 1 year of PD therapy duration. The authors speculated that elevation in the protein levels that were measured after 1 year could indicate markers for early peritoneal membrane injury. The second study consisted of 15 prevalent PD patients with varying PD therapy duration ranging from 1 to 84 months. A subgroup was additionally defined to study the effect of glucose in PD solutions. Profiling and characterization of the peritoneal effluent has also been investigated in nine pediatric PD patients (Raaijmakers et al. 2008). A number of 88-shared proteins were identified. All of the abovementioned studies indicated that the effluent of PD patients is merely from systematic origin and reflects extracellular proteins. Characterization of the human effluent has furthermore been performed with regard to diabetic PD patients (Yang et al. 2013; Wang et al. 2010) and before and after a peritonitis episode (Lin et al. 2008; Tyan et al. 2013). Validation by Western blots was performed within the same study populations. The latter study identified up to 41 proteins with shared alterations in haptoglobin expression and revealed in an area under the receiver operating characteristic curve of 0.92.

Higher levels of glucose and osmolarity in PD solutions are known to induce a greater removal of excess fluid and toxic waste products. However, continuous exposure of these PD solutions high in glucose leads to damage to the peritoneal membrane. In this respect, proteomic analyses have been performed as well. An

increased appearance of advanced glycosylation end products was shown when patients were infused with higher percentages of glucose-based dialysis solutions (Pešić et al. 2011). Moreover, a number of nonredundant proteins including cystatin C, collagen, fibronectin, matrix metalloproteinase-2, plasminogen, and vitronectin were identified (Cuccurullo et al. 2011; Pešić et al. 2011). Additionally, an under-expression was found for α-1 antitrypsin, apolipoprotein A-IV, fibrinogen β-chain, and transthyretin in patients treated with the highest glucose concentration (4.25 %) of PD solutions when compared to those treated with 1.5 % or 2.5 % glucose-containing PD solutions (Cuccurullo et al. 2011).

Due to the large interindividual variation in peritoneal transport status at initiation of PD therapy, comparative analyses of the peritoneal effluent in PD patients with different peritoneal transport characteristics have been studied as well. In these studies, the authors found increased protein losses for PD patients with a fast transport status as compared to patients characterized by slow peritoneal transport rates (Wen et al. 2013; Sritippayawan et al. 2007). External validation by ELISA confirmed elevated levels of complement 4A and IgG in the fast transporters (Sritippayawan et al. 2007). Overall, the heterogeneity in study populations and practical laboratory techniques contributes to the variety of identified proteins and complexes inferences throughout studies.

Genomics and Metabolomics

Genomic biomarkers have not yet been identified, but intriguing single nucleotide polymorphisms (SNPs) have been found in the C/C genotype on the interleukin-6 (IL-6)-174G/C loci (Verduijn et al. 2012). The base population for this study originated from a Dutch multicenter cohort, also known as the Netherlands Cooperative Study on the Adequacy of Dialysis, archiving peritoneal effluent and serum of incident dialysis patients. Additionally, two external cohorts were used for independent data replication. A significant increased risk for mortality was associated with this IL-6 gene variant in adult patients who survived PD treatment over a period of at least 2 years. Albeit external cohorts were defined to authenticate these findings, further validation is necessary. Polymorphisms in the promoter region of VEGF have also been associated with an increased risk for mortality (Szeto et al. 2004). This was found in a prospective cohort study in 135 continuous ambulatory PD (CAPD) patients who had a follow-up duration of 1 year. The effluent was obtained within 2 months after start of PD and after 12 months. Furthermore, effluent levels of VEGF measured by ELISA showed a tendency toward lower levels in patients with the CC genotype when compared to those with an AA/AC genotype. In contrast, messenger RNA expression was significantly lower in PD patients with the CC genotype.

The GLOBAL Fluid Study group is a prospective longitudinal worldwide biobank within PD that serially collects peritoneal effluent alongside serum samples. The effluent samples within the cohort are all obtained at the end of a 4-h peritoneal

equilibration test in incident PD patients and repeated thereafter at predefined intervals. This group recently published a study in which an attempt was made to identify metabolic profiles specific for PD patients who developed EPS (Dunn et al. 2012). As no previous study investigated metabolites within the peritoneal effluent, this study secondly aimed to provide optimal strategies for analyzing the metabolome alongside the identification of differences in effluent composition. The authors found that the peritoneal effluent consists grossly of low molecular weight metabolites. Moreover, prior to the diagnosis of EPS, modifications in several amino and short-chain fatty acids and its derivates were present. The abovementioned studies demonstrate the capability and importance of longitudinal (multicenter) study cohorts containing effluent specimens.

Potential Applications to Prognosis, Other Diseases, or Conditions

Within the discipline of nephrology, suffix -omics technologies are widely applied. The earliest studies originate from 1997 with an increasing tendency in the number of published articles ever since. Genome-wide association studies (GWASs) have contributed greatly to the understanding of chronic kidney diseases (CKDs) as well as the main kidney failure diseases: renovascular disease, diabetic nephropathy (DN), and glomerulonephritis (GN) (Atzler et al. 2014; Kottgen 2010). The reviews on the recent developments of genomic and metabolomic analyses uncovered that over 98 % of the estimated heritability within nephrological diseases remains to be revealed. From this, it can be concluded that a majority of the pathophysiological mechanisms of kidney diseases and function still warrants elucidation. Proteomics have been applied widely as well with similar intentions such as biomarker discovery, to reveal causal pathways of kidney diseases and to indicate potential therapeutic targets (Bonomini et al. 2012). Proteomic studies within nephrology have focused on urinary biomarkers for DN, IgA nephropathy, lupus nephritis, and rejection of renal transplants (Papale et al. 2010; Rocchetti et al. 2008; Zhang et al. 2008; Metzger et al. 2011). However, harmonization of the analytical procedures is warranted. Another commonly mentioned issue encompasses the study designs, which are merely represented by transversal research rather than longitudinal cohorts investigating the course of events. Moreover, the sample sizes of some studies are relatively small. These tendencies are in line with the current advances observed in omics-conducted PD research.

The utilized biospecimens within nephrology range from blood and serum samples to urine and renal tissue. Within nephrology, these various specimens have been investigated extensively. However, the clinical application of potential biomarkers still fails to appear despite their promising results. One of the aspects that also should not be overseen is the collaboration between the clinicians, epidemiologists, (laboratory) scientists, and healthcare funding organizations to enable a more rapid integration and implementation of biomarkers.

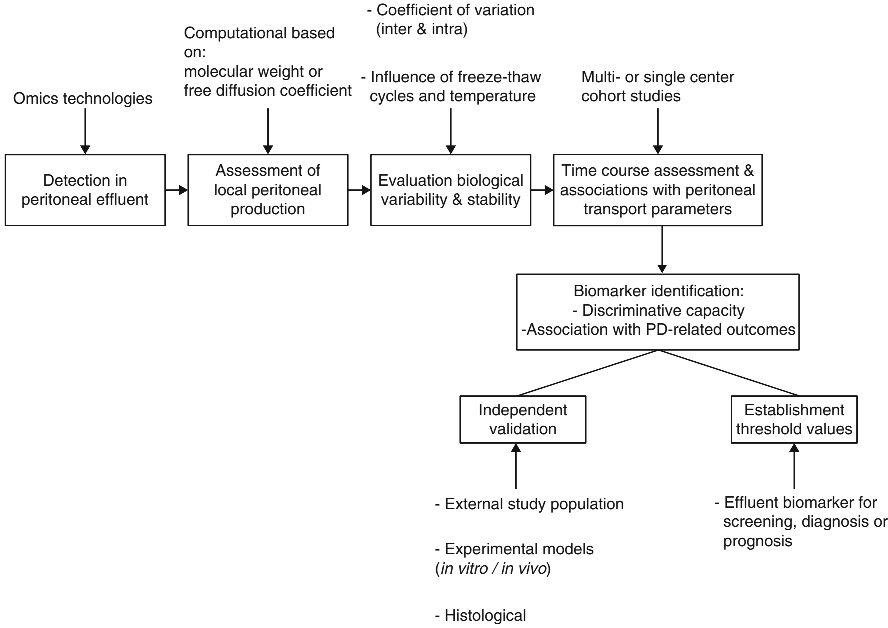


Fig. 4 Suggested flowchart for peritoneal effluent biomarker discovery. A flowchart is suggested for effluent biomarker discovery that are detected by means of omics technologies. Some of the suggested phases are based on the requirements of a peritoneal effluent biomarker. These comprise the detection of the substance in peritoneal effluent, which should be locally produced within the peritoneal cavity. Furthermore, the candidate peritoneal effluent marker is involved in the pathology of peritoneal membrane and related to peritoneal dialysis-related clinical outcomes

Future Directions

As blood, serum, and urine samples are not representative for intraperitoneal events, the central emphasis remains to be on the peritoneal effluent. PD treatment induces a complex and multifactorial pathogenesis of the peritoneal membrane. The deficiency of this non-defined common pathway contributes to the difficulties in effluent biomarker discovery. These challenges can possibly be overcome with the unbiased field of omics technologies, where associations between clinical and expression data from confirmatory investigations may lead to the perception of underlying biological processes preceding peritoneal injury and novel biomarker identification. A suggested flowchart for effluent biomarkers discovery is depicted in Fig. 4 in which their prerequisites are integrated. To our knowledge, unfortunately no peritoneal tissue of patients treated with PD has been investigated by means of omics technologies. The presence of serial peritoneal tissue alongside peritoneal effluents of PD patients would be of great additive value and a prerequisite for omics-conducted PD investigation. Nevertheless, it is doubtful that an individual peritoneal

effluent biomarker possesses the ability to mirror or predict all of these processes. More likely is that a synergism of effluent biomarkers will eventually be identified in order to predict clinically relevant PD outcomes. Thus, large multicenter biobanks with preferably longitudinal data would contribute significantly to the discovery of novel effluent biomarkers and their validation. The number of omics-based research in PD is still limited, and the early phase of high-throughput technologies warrants standardization and calibration. Essential in the conductance of omics studies is systematic collection and storage of peritoneal effluent. To date, the absence of longitudinal effluent biobanks including peritoneal tissue and a small sample size has prevented the analysis of modifications in proteomic profiles over time. However, longitudinal multicenter studies such as the GLOBAL Fluid Study or within center biobanks will hopefully bridge this gap. Furthermore, collaborations are necessary to facilitate independent replication and validation of candidate effluent biomarkers.

In summary, peritoneal effluent biomarker discovery is moving toward system biology where a holistic approach may eventually lead to personalized-guided medicine within PD. Nevertheless, the challenge remains, and the actual application and implementation of omics-discovered effluent biomarkers is a process that may encompass decades.

Summary Points

- This chapter focuses on the emerging field of omics technologies within peritoneal dialysis (PD) therapy for the discovery of peritoneal effluent biomarkers.
- The peritoneal effluent can be regarded as a noninvasive liquid biopsy within PD therapy that is easily acquired after a (standardized) predefined PD exchange.
- The peritoneal effluent contains clinically relevant proteins and substances such as lymphocytes, macrophages, and a variety of proteins that mirror intraperitoneal events.
- The peritoneal effluent is susceptible to posttranslational modifications.
- Methodological precautions on a laboratory level as well as computational techniques are essential for proper assessment and interpretation of omics-derived data.
- The number of studies that apply high-throughput laboratory techniques with the peritoneal effluent as biospecimen within PD therapy is still limited.
- Peritoneal effluent biomarker discovery is moving toward systems biology.

References

Atkinson AJ, Colburn WA, DeGruttola VG, et al. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Therm. 2001;69:89–95.

Atzler D, Schwedhelm E, Zeller T. Integrated genomics and metabolomics in nephrology. Nephrol Dial Transplant. 2014;29:1467–74.

Bonomini M, Sirolli V, Magni F, et al. Proteomics and nephrology. J Nephrol. 2012;25:865–71.

Brewis IA, Topley N. Proteomics and peritoneal dialysis: early days but clear potential. Nephrol Dial Transplant. 2010;25:1749–53.

Coester AM, Smit W, Struijk DG, et al. Peritoneal functions in clinical practice: the importance of follow-up and its measurement in patients. Recommendations for patient information and measurement of peritoneal function. NDT Plus. 2009;2:104–10.

Colburn WA. Optimizing the use of biomarkers, surrogate endpoints and clinical endpoints for more efficient drug development. J Clin Pharmacol. 2000;40:1419–27.

Cuccurullo M, Evangelista C, Vilasi A, et al. Proteomic analysis of peritoneal fluid of patients treated by peritoneal dialysis: effect of glucose concentration. Nephrol Dial Transplant. 2011;26:1990–9.

Dunn WB, Summers A, Brown M, et al. Proof-of-principle study to detect metabolic changes in peritoneal dialysis dialysate in patients who develop encapsulating peritoneal sclerosis. Nephrol Dial Transplant. 2012;27:2502–10.

Gillerot G, Debaix H, Devuyst O. Genotyping: a new application for the spent dialysate in peritoneal dialysis. Nephrol Dial Transplant. 2004;19:1298–301.

Hulka BS, Wilcosky TC, Griffith JD. Biological markers in epidemiology. New York: Oxford University Press; 1990.

Jain KK. The handbook of biomarkers. New York: Humana Press; 2010.

Kottgen A. Genome-wide association studies in nephrology research. Am J Kidney Dis. 2010;56:743–58.

Lin WT, Tsai CC, Chen CY, et al. Proteomic analysis of peritoneal dialysate fluid in patients with dialysis-related peritonitis. Ren Fail. 2008;30:772–7.

Lopes Barreto D, Sampimon DE, Coester AM, et al. The value of osmotic conductance and free water transport in the prediction of encapsulating peritoneal sclerosis. Adv Perit Dial. 2014;30:21–6.

Metzger J, Chatzikyrkou C, Broecker V, et al. Diagnosis of subclinical and clinical acute T-cell-mediated rejection in renal transplant patients by urinary proteome analysis. Proteomics Clin Appl. 2011;5:322–33.

Papale M, Di Paolo S, Magistroni R, et al. Urine proteome analysis may allow noninvasive differential diagnosis of diabetic nephropathy. Diabetes Care. 2010;33:2409–15.

Perera FP, Weinstein IB. Molecular epidemiology; recent advances and future directions. Carcinogenesis. 2000;21:517–24.

Pešić I, Dihazi GH, Müller GA, et al. Short-term increase of glucose concentration in PDS results in extensive removal and high glycation level of vital proteins during continuous ambulatory peritoneal dialysis. Nephrol Dial Transplant. 2011;26:2674–83.

Raaijmakers R, Pluk W, Schröder CH, et al. Proteomic profiling and identification in peritoneal fluid of children treated by peritoneal dialysis. Nephrol Dial Transplant. 2008;23:2402–5.

Rocchetti MT, Centra M, Papale M, et al. Urine protein profile of IgA nephropathy patients may predict the response to ACE-inhibitor therapy. Proteomics. 2008;8:206–16.

Sritippayawan S, Chiangjong W, Semangoen T, et al. Proteomic analysis of peritoneal dialysate fluid in patients with different types of peritoneal membranes. J Proteome Res. 2007;6:4356–62.

Szeto CC, Chow KM, Poon P, et al. Genetic polymorphism of VEGF: impact on longitudinal change of peritoneal transport and survival of peritoneal dialysis patients. Kidney Int. 2004;65:1947–55.

Thongboonkerd V. Proteomics in extracorporeal blood purification and peritoneal dialysis. J Proteomics. 2010;73:521–6.

Tyan YC, Su SB, Ting SS, et al. A comparative proteomics analysis of peritoneal dialysate before and after the occurrence of peritonitis episode by mass spectrometry. Clin Chim Acta. 2013;420:34–44.

Verduijn M, Maréchal C, Coester AM, et al. The −174G/C variant of IL6 as risk factor for mortality and technique failure in a large cohort of peritoneal dialysis patients. Nephrol Dial Transplant. 2012;27:3516–21.
Wang HY, Tian YF, Chien CC, et al. Differential proteomic characterization between normal peritoneal fluid and diabetic peritoneal dialysate. Nephrol Dial Transplant. 2010;25:1955–63.
Wang HY, Lin CY, Chien CC, et al. Impact of uremic environment on peritoneum: a proteomic view. J Proteomics. 2012;75:2053–63.
Wen Q, Zhang L, Mao HP, et al. Proteomic analysis in peritoneal dialysis patients with different peritoneal transport characteristics. Biochem Biophys Res Commun. 2013;30(438):473–8.
Wilson PW, Pencina M, Jacques P, et al. C-reactive protein and reclassification of cardiovascular risk in the Framingham Heart Study. Circ Cardiovasc Outcome. 2008;1:92–7.
Wu HY, Liao AC, Huang CC, et al. Comparative proteomic analysis of peritoneal dialysate from chronic glomerulonephritis patients. Biomed Res Int. 2013. doi:10.1155/2013/863860.
Yang MH, Wang HY, Lu CY, et al. Proteomic profiling for peritoneal dialysate: differential protein expression in diabetes mellitus. Biomed Res Int. 2013. doi:10.1155/2013/642964.
Zhang X, Jin M, Wu H, et al. Biomarkers of lupus nephritis determined by serial urine proteomics. Kidney Int. 2008;74:799–807.
Zhang L, Wen Q, Mao HP, et al. Developing a reproducible method for the high-resolution separation of peritoneal dialysate proteins on 2-D gels. Protein Expr Purif. 2013;89:196–202.

Metabolomics and Stages of Chronic Kidney Disease

5

Toshihiro Kobayashi

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Abstract

The kidneys are critical for the secretion of cytokines and hormones, the excretion of waste metabolites, and the homeostasis of electrolytes. Chronic kidney disease (CKD) is a major epidemiologic problem and a risk factor for cardiovascular events and cerebrovascular accidents. At present, renal function is generally evaluated by measuring estimated glomerular filtration rate (eGFR). However, this method has low sensitivity during the early stages of CKD. A new biomarker that can detect CKD during its early stages is eagerly awaited: mass spectrometry (MS), an effective technology for the discovery of biomarkers due to its high sensitivity to detect many compounds, seems to fit these conditions.

Metabolomics using mass spectrometry is a powerful strategy for profiling metabolites and can be used to effectively explore unknown compounds that

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change in abundance with respect to disease condition. Recently, many researchers have endeavored to apply metabolomics techniques to diagnose various diseases, including CKD. Some metabolites that can serve as biomarkers for CKD severity have been discovered, thanks to their efforts.

This chapter reviews metabolomics techniques and their potential to be applied to CKD diagnosis.

Keywords
Chronic kidney disease • Metabolomics • Biomarker • Multivariate statistics • Mass spectrometry

Abbreviations

CE-MS	Capillary electrophoresis-mass spectrometry
CKD	Chronic kidney disease
CysC	Cystatin C
DMSO	Dimethyl sulfoxide
ESRD	End-stage renal disease
FT-ICR-MS	Fourier transform-ion cyclotron resonance-mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
GFR	Glomerular filtration rate
HMDB	Human metabolome database
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS	Liquid chromatography-mass spectrometry
MS/MS	Tandem mass spectrometry
PLS	Partial least squares
SFC-MS	Supercritical fluid chromatography-mass spectrometry
TOC	Total organic carbon
TOF-MS	Time-of-flight-mass spectrometry

Key Facts About Metabolomics and Stages of Chronic Kidney Disease

- Chronic kidney disease (CKD) is a concept that was advocated for the first time in the United States in 2002.
- A lifestyle-related disease, CKD has attracted concern and has entered the medical consciousness worldwide.
- Left untreated, CKD aggravates irreversibly and finally degenerates into end-stage renal disease (ESRD), requiring either dialysis therapy or renal transplantation.
- Dialysis therapy is effective in removing uremic toxins from the blood but has no effect on anemia or electrolyte disturbance, resulting in impaired quality of life.
- ESRD also is a risk factor of cardiovascular disease.
- Thus, early detection and treatment of CKD is not only an important issue for the health of individuals but also a wider problem in the medical economy.

Definitions

Chronic kidney disease (CKD) and glomerular filtration rate (GFR) CKD is defined by the following two criteria. (1) Kidney damage confirmed by urine tests, imaging tests, and blood tests. Especially, albuminuria is a typical symptom. (2) Reduced glomerular filtration rate (GFR) less than 60 mL/min/1.73 m^2 over 3 months.

CKD is classified into six stages according to GFR (see table below) (Levey et al. 2011). Stage 1 and Stage 2 do not equate to CKD if kidney damage is not comorbid. Stage 5 is equivalent to end-stage renal disease.

GFR (mL/min/1.73 m^2)	CKD stage
90>	1
60–89	2
45–59	3a
30–44	3b
15–29	4
15<	5

Estimated glomerular filtration rate (eGFR) Since direct measurements of GFR are invasive and time-consuming, GFR is typically estimated using blood creatinine, age, and gender. There are various formulas for calculating eGFR: the Cockcroft–Gault equation, the Modification of Diet in Renal Disease (MDRD) Study equation, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, the Japanese Society of Nephrology Chronic Kidney Disease Initiative (JSN-CKDI) equation, and so on. Because blood creatinine is proportional to the muscle mass, the formula used differs depending on the patient's race and gender.

Introduction

Mass spectrometry (MS), which is capable of quantitative and qualitative analyses with high sensitivity, is a useful technique for determining the presence of various trace metabolites in body fluids like blood or urine. Taking advantage of MS's utility, many researchers have attempted to perform definitive diagnosis or early detection of diseases using it, including metabolic abnormalities, cancers in various organs, and chronic kidney disease (CKD). In fact, mass spectrometry is already used to find metabolic abnormalities in neonatal mass screening programs.

Because healthy kidneys have a large reserve capacity, the early stages of CKD are difficult to identify by subjective symptoms. CKD is frequently detected by symptoms of significantly reduced glomerular filtration rate (GFR) or increased albumin concentration in urine (Levey et al. 2011).

Though glomerular filtration rate can be measured rigorously in terms of inulin clearance according to international criteria, the method is very intricate and difficult

to routinely use clinically. Thus, creatinine clearance and estimated GFR (eGFR) are generally used as surrogate markers of inulin clearance.

However, creatinine is known to be produced by the degradation of the muscle and secreted from the proximal renal tubule. Therefore, in aged people, low levels of blood creatinine may instead be a result of low muscle mass, which creates the problem of consequent overestimation of renal function. To avoid this confusion, cystatin C (CysC) has been frequently used as an alternative clinical index for creatinine in recent years.

CysC is not affected by muscle mass or dietary protein but is affected by hyperthyroidism, obesity, and steroid therapy (Inker et al. 2012). Thus, CysC alone is not a sufficient index for GFR, and further studies are needed to determine whether to include this measurement in standard criteria (Peralta et al. 2011b).

Given this background, some researchers are exploring biomarkers that reflect renal function loss with the use of mass spectrometry and have proposed some candidate markers (Niwa 1997, 2009; Toyohara et al. 2010; Kikuchi et al. 2010; Sato et al. 2011; Kobayashi et al. 2014a).

This chapter outlines search methods for biomarkers that are concerned with renal function loss using LC-MS and evaluation methods for CKD severity using these biomarkers.

Why Use Metabolomics to Diagnose CKD

CKD is definitively diagnosed by the following symptoms: abnormally low glomerular filtration rate, existence of albuminuria, and apparent nephropathy as determined by imaging or pathological examination. These are not preventive indices of CKD, but definitive criteria.

Representative evaluative indices for GFR are serum creatinine, CysC concentrations, and inulin clearance (Peralta et al. 2011a, b; Horio et al. 2013). However, serum creatinine and CysC have a so-called blind area in that their concentrations are not increased immediately in the early stages of CKD, which causes difficulty in detecting CKD in the early stages using creatinine and CysC as indices of CKD (Herget-Rosenthal et al. 2007; Tangri et al. 2011). While inulin clearance is the gold standard method for determining GFR (Levey et al. 2005), the procedure for measuring it is invasive and cumbersome, and so it is not suitable for screening use.

Therefore, from the viewpoint of early discovery and prevention of CKD, it is necessary to employ indicators that more sensitively reflect decreased renal function than these CKD diagnostic criteria commonly adopted today. Because healthy kidneys have a large reserve capacity, even if the concentration of metabolites in vivo fluctuates due to the pathology of CKD, it is reasonable to believe that the degree of fluctuation is negligible in its initial stages. Therefore, the use of MS has been promoted in order to detect very slight changes in the concentrations of compounds in samples.

The relatively new field of metabolomics has been developed in recent years, stimulated by the spread of mass spectrometry (Kell 2004). At first, it was proposed

as a means to comprehensively analyze the various metabolic products present in the living body. Many attempts to assess renal function using metabolomics techniques have been made to date (Niwa 1997, 2009; Toyohara et al. 2010; Kikuchi et al. 2010; Sato et al. 2011; Kobayashi et al. 2014a, b).

Using Mass Spectrometry for Metabolite Exploration

Since LC-MS has high sensitivity, the results of metabolomics analysis might be affected by even slight changes in conditions. Thus, conditions surrounding the sample vial, sample handling, and analytic method need to be kept constant as much as possible. These measurement methods, including sample collection and pretreatment, must be examined beforehand in house with respect to procedures detailed in previous studies and should not be changed during a series of experiments.

Pollution of the LC-MS probe and mass detector during repeated analysis, including by biological samples and contaminants, causes poor peak detection and high background noise. In addition, column deterioration and slight compositional changes or flow rate fluctuation of the solvent affect peak retention times. In order to obtain reliable results, because contaminants in water are a major cause of noise peaks, the total organic carbon (TOC) in a solvent used for LC-MS should be lower than 1 ppb if using an in-house ultrapure water generator. In addition, although it is a very basic precaution, moving phase solvents should be obtained from reliable reagent suppliers, and once reagent bottles are opened, it is better to consume them as soon as possible.

Moreover, in the case of performing a quantitative analysis, it is necessary to analyze specimens with respect to reference standards every time and determine the concentration of each compound of interest based on its calibration curve.

LC-MS can analyze a wide variety of metabolites, including refractory or thermolabile substances, if they are soluble in the solvent. This is an advantage in LC-MS, but not in GC-MS. An additional concern is that even when using a LC-MS device at the same settings as in a previous study, the retention time of metabolites is often not consistent, for various reasons, e.g., differences in the plumbing head of the devices, in the state of the separation column, and in the purity of solvent. Therefore, even if a given peak has the same mass unit and the same retention time as a known compound as indicated in a previous study or databases, these compounds cannot be said to be identical purely on that basis. It is necessary to confirm the spectrum of the reference standards with the same device used for that analysis and to acquire spectral data in house.

GC-MS is characterized by a high compatibility between devices compared with LC-MS or CE-MS. Since commercial and open-access databases of GC-MS libraries (i.e., compound databases) are well developed, it is advantageous to identify metabolites by qualitative analyses. However, in profiling metabolites exhaustively using body fluid samples such as blood or urine, a derivatization step is necessary to vaporize metabolites, which creates difficulties because of differences in volatility of

the constituent compounds. Because the derivatization efficiency of all compounds is not constant, quantitative discussions should be careful when interpreting results.

More recently, newer analyses using CE-MS and SFC-MS have become possible with advances in analytic devices; some researchers have reported studies that employ these cutting-edge systems (Toyohara et al. 2010; Taguchi et al. 2014). CE-MS shows promise in effectively analyzing ionic metabolites that are hardly retained on the reverse-phase columns widely used in LC-MS, whereas using it to analyze lipophilic and neutral metabolites is difficult in principle. On the other hand, SFC-MS is feasible for and capable of analyzing refractory and heat-labile compounds that GC-MS can hardly detect and also boasts higher throughput than LC-MS. These new devices will be powerful tools when widely used in the future.

However, even among the analytical methods described above, it is impossible to find all metabolites comprehensively in a sample using a single analytical instrument. It is thus necessary to select the device according to the purpose of a study or to use multiple devices. Prerequisites for metabolite identification using MS have been discussed in some review papers (Zhao 2013; Sumner et al. 2007; Yoshida et al. 2012).

In general, target compounds explored by LC-MS-based metabolomics are low-molecular-weight compounds of less than 1000 Da. Typical target biological samples – serum, plasma, and urine – require pretreatment steps to remove inhibitory components such as proteins and minerals.

Preprocessing methods are mainly divided into methods to denature proteins using solvents (such as methanol, chloroform, and acetonitrile) and methods to separate the sample physicochemically by solid-phase extraction. Neither trifluoroacetic acid nor DMSO is unequivocally superior for use as a solvent in LC-MS pretreatment, because the former inhibits the ionization process and the latter causes background noise peaks. Additionally, ultrafiltration is also available, but it seems to not be used widely owing to its high cost compared with solvent extraction methods.

Solvent extraction methods are widely used because of their low cost and simplicity, but they still have disadvantages: centrifugation and collection of supernatant are necessary, and working time increases in proportion to the number of samples. In addition, the pretreatment procedure for biological fluids such as plasma needs a large amount of organic solvent, and thereby the extracted volume will be increased above the starting volume, diluting the concentration of target metabolites.

Solid phase extraction methods are profitable because they can analyze a large number of samples and are able to concentrate the target metabolites by controlling elution volume (Mallet et al. 2004); however, they demand higher costs than solvent extraction methods do and suffer from the possibilities that some kinds of metabolites can be absorbed or, conversely, hardly retained by the column and that conditions in the housing unit of the column can affect following analyses.

In other words, each kind of method has advantages and disadvantages. In addition, for both methods, the recovery efficiency changes depending on the solvent and column selected. The important thing is that users must refer to a pretreatment method used by a previous study and examine its applicability in advance by using

their own MS. Sharp signal peaks, large signal/noise ratio (e.g., S/N >10), and low background peaks are good criteria for this prior examination.

Metabolites known to reflect disease severity by alterations in their blood levels, such as indoxyl sulfate in the case of CKD (Barreto et al. 2009; Kikuchi et al. 2010), can be used as a positive control when analyzed quantitatively. Thus, when considering the pretreatment method, one good strategy would be to carry out the procedure with the addition of such an authentic sample and to select an extraction method in which the recovery rate is close to 100 %.

After determining the pretreatment method, it is necessary to confirm the dynamic range of the device using an authentic sample of the compounds targeted for detection before performing quantitative analysis. Usually, it is necessary to select two or more kinds of targeted compounds. Even for metabolites whose blood or urine levels change according to CKD severity, their concentration is not in all cases regulated directly by CKD. For example, kynurenine is known as a metabolite whose concentration in the blood increases with CKD exacerbation (Saito et al. 2000; Kobayashi et al. 2014b), but its increase is also known to reflect staging of colon cancer (Nishiumi et al. 2012). In addition, plasma kynurenine has been reported to be elevated in major depression patients (Dahl et al. 2015). Thus, estimating CKD stages using only one metabolite is prone to low reliability. Since LC-MS is able to detect multiple compounds at the same time in a single injection, the time cost is almost the same for one target metabolite as for two or more. In fact, in many previous studies, four or more metabolites with different masses and retention times were selected (Toyohara et al. 2010; Sato et al. 2011; Kobayashi et al. 2014b).

Because a biological sample such as the plasma, serum, and urine contains various compounds, some sorts of contaminants will remain even after pretreatment. These contaminants sometimes inhibit ionization of target metabolites (the so-called matrix effect). Because each metabolite has a different ionization efficiency, internal standard samples of targeted metabolites are needed when doing quantitative analysis. Stable isotope-labeled compounds have equal ionization efficiency to their non-labeled equivalents, making them the most reasonable internal standards for quantitative analysis. They can be employed effectively by adding a known concentration of labeled compounds to samples prior to pretreatment procedures. It is preferable to analyze corresponding labeled compounds for every targeted metabolite; however, when obtaining some compounds is difficult, the labeled compound should be prepared at as many concentrations as possible, with the remainder quantified using a calibration curve method for the non-labeled standard while paying attention to quantitative accuracy.

For this calibration curve method, first, the compounds are dissolved in solvent and mixed in one vial. Then, sequential dilutions are prepared from this vial. In doing so, researchers should be careful to reconcile the solvent component of the diluent with that of the pretreated specimens. When analyzing the dilution series, obtained peaks should be manually checked for good separation, sharp shape, and minimal tailing. Next, the peak area is plotted for the prepared diluents at measured concentrations, followed by construction of a calibration curve. The range of the

calibration curve should be linear; the range over which the linearity of the calibration curve is preserved indicates the concentrations at which metabolites can be analyzed quantitatively.

Nontarget Analysis and Target Analysis

Biomarker analysis using MS is useful for disease exploration and medical checkups. To conduct it, two ways are possible: one is revealing previously unknown metabolites that can serve as disease markers (nontarget analysis), and the other is quantitative analysis of predefined metabolites and estimating disease severity based on them (target analysis or multi-target analysis).

When using nontarget analysis methods, unknown metabolites are detected in the biological samples in addition to well-known metabolites. Accurate mass measurements are necessary when detecting unreported, new metabolites and many kinds of known metabolites alike: thus, high-resolution time of flight-MS (TOF-MS) or fourier transform-ion cyclotron resonance-MS (FT-ICR-MS) is essential for nontarget analysis.

Since the main purpose of nontarget analysis is the screening and detection of unknown metabolites, the most important factors are mass accuracy and spectral resolution. Therefore, the concentration data of metabolites are typically shown in terms of relative quantitation. On the other hand, when using the multi-target analysis approach, the target compounds are limited to several metabolites. Thus, multi-target analysis is usually performed using quantitative analysis with a stable isotope-labeled compound or authentic sample as the internal standard.

Nontarget Analysis

Since a meaningful spectrum must be selected for nontarget analysis – that is, a spectrum in which background and noise peaks are excluded and large quantities of mass spectrum data are accurately profiled – a software that can help extract significant peaks and construct matrix data is necessary. Unlike GC-MS, a universal mass spectral library is insufficient for LC-MS, because it often causes undetectable peaks to appear when the matrix data were derived from values obtained by LC-MS/MS analysis. In order to solve this problem, many mass spectra databases have been established, including MassBank, HMDB, KNApSAcK, and METLIN, which are able to predict (or identify) constituent metabolites based on LC-MS/MS spectra. An outline of the nontarget analysis procedure is shown in Fig. 1. In the metabolite identification step, the following requirements must be met: the sample and reference standards must be analyzed under the same conditions, and the retention time and mass spectrum used for each analyte must be ensured to be identical (Sumner et al. 2007).

To date, many studies have reported alterations in the concentrations of various blood metabolites, along with a decline in renal function, in outpatients with CKD (Niwa 2011; Toyohara et al. 2010), hemodialysis patients (Niwa 2011; Saito

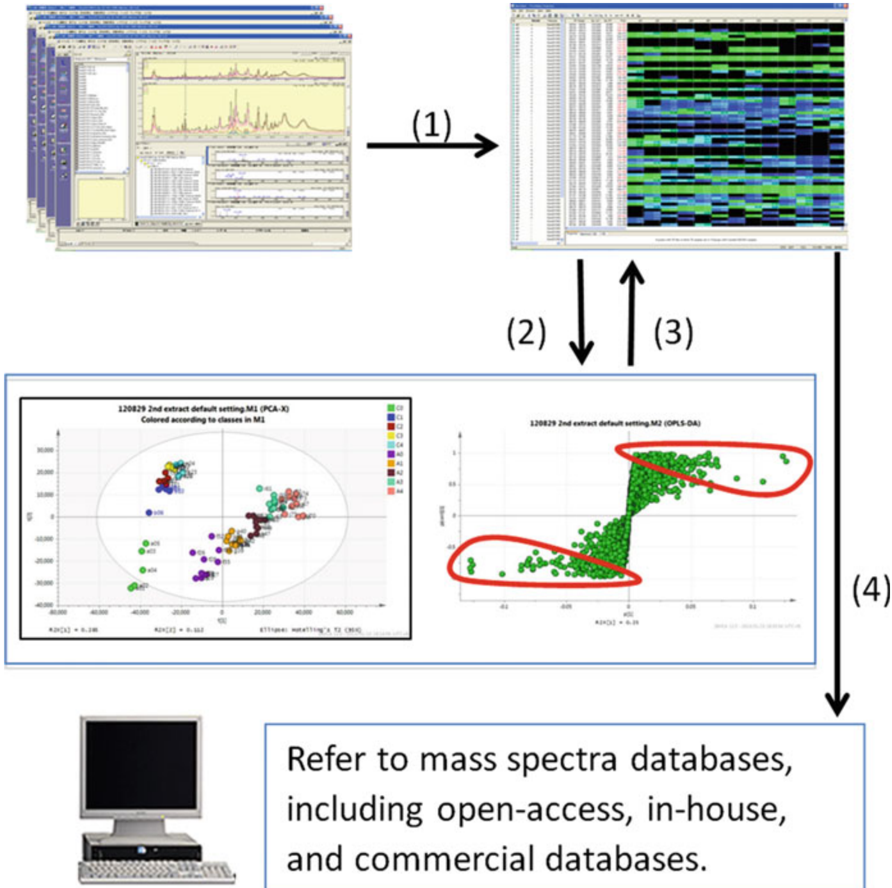

Fig. 1 Outline of nontarget analysis: Searching for early detection markers of CKD. *1* LC-MS/MS analysis of plasma samples, peak selection, and matrix generation (by software to analyze device data, additional purchase may be required). *2* Multivariate analysis (by commercial software) and selection of candidate peaks for markers (part of the red circle). *3* Evaluation of chronological change of the candidate peaks. *4* Identification of metabolite(s) using analyzed data: mass number, retention time, and MS/MS spectrum

et al. 2000; Sato et al. 2011), rats with adenine-induced CKD (Zhao et al. 2012; Kobayashi et al. 2014a), and partially nephrectomized rats (Saito et al. 2000; Kikuchi et al. 2010), suggesting that the concentrations of several of these metabolites can be used to construct a quantitative index of renal function. Metabolomics approaches are most suitable for targeting low-molecular-weight compounds (approx. <1000 Da) whose molecular structures and characters are the same between animal species. This is one advantage of exploring metabolites versus genes or proteins. Thus, common MS programs can be used even when animal species are different. However, the human metabolic system is nevertheless different from those of other experimental animals. As a simple example, hyperuricemia,

which is speculated to be related with CKD, shows the following features. Since *Homo sapiens* and some other primates do not have uricase (Yeldandi et al. 1991), the end product of adenosine and guanosine metabolic systems is a urate. Low urate excretion from the kidney or excess intake of purine bases from diets causes hyperuricemia in *Homo sapiens* and some other primates. In contrast, some other mammals, typified by rodents, have active urate oxidase that metabolizes uric acid to allantoin (Ackroyd 1914). Thus, these animals are able to excrete excess uric acid, and blood uric acid levels are not easily affected by experimental CKD induction. This observation shows the importance of understanding metabolic pathways when applying the results of animal experiments to human samples. In this respect, it is best to use open databases such as the KEGG pathway website where many useful pathway maps for *Homo sapiens* and other animal species are published.

In order to explore disease biomarkers by the use of nontarget analysis, namely, through metabolomics, understanding of metabolic systems in vivo and knowledge of mass spectrometry and bioinformatics are also necessary. In addition, the mass spectrometer employed should have high enough resolving power to measure mass accurately. Thus, it might be difficult for novices to conduct metabolomics studies. In part, however, how to perform data mining and extract useful peaks from the sea of spectral data obtained by MS to identify new metabolites and how to predict the onset of a disease by observing the layout of mass spectra are processes that depend on the ideas of individual researchers. The field of metabolomics is awaiting breakthroughs from new researchers; this field could take a quantum leap forward by their contributions.

Target Analysis (Multi-target Analysis)

In target analysis, metabolites to be monitored are selected prior to the analyses. If the target metabolite has been previously identified, its peak can be detected during the analysis with mass spectrometer based on its retention time and mass number. The peaks, which were considered meaningful for the study, can be selected as an examination target, even if their corresponding compound is unidentified at first. However, for practical purposes, determining which peaks is derived from what kind of metabolite is an essential objective.

Uremic toxins, which are metabolites that are excreted under normal circumstances, accumulate in vivo because of renal function loss and have been inferred to harm many tissues (Vanholder et al. 2003; Richard and Shaul 2012). Therefore, uremic toxins of low molecular weight can be adopted as candidate biomarkers that reflect renal function loss in metabolomics studies. However, the extent by which a uremic toxin increases in the blood according to CKD stage differs among toxins. Candidate biomarkers should exhibit more remarkably altered blood levels in the early stages of CKD than those of existing biomarkers (i.e., creatinine and CysC) and have high sensitivity and reproducibility. Otherwise, they have limited utility (although knowledge about such compounds is important). This is because the existing biochemical indices such as creatinine and CysC have already spread

widely, and biomarkers that do not fulfill the conditions above cannot compete with them to be employed as standards in analysis techniques using the MS because of issues of measurement cost, convenience, and specificity.

In addition, metabolites that exhibit decreased blood levels according to the extent of renal function loss (Toyohara et al. 2010) could be used as CKD biomarkers in the same way as uremic toxins.

Though several specified metabolites are quantified in a target analysis as mentioned above, in most cases, the concentrations of a plurality of metabolites that can reflect the severity of CKD do not change according to the same ratio. Thus, in many cases, a quantified concentration of given metabolites is not informative or useful for predicting severity of CKD directly. By using multivariate analyses, a speculative regression equation can be constructed based on the prospective contribution ratios of the metabolites identified in a multi-target analysis. In this process, logistic regression or partial least squares (PLS) regression are often used, and the derived equation should correlate with CKD stage as tightly as possible (Kobayashi et al. 2014b). Next, unused sample sets should be used as the validation set to validate the accuracy of the derived equation. The validation set should be input into the equation, and the CKD stage estimated. By this process, whether the equation is able to estimate CKD stage can be validated before it is used.

Future of Metabolomics in the Diagnosis of Disease

There are several reports on new biomarkers for estimating CKD severity that have been found through metabolomics studies (Zhao 2013; Rhee et al. 2013; Shah et al. 2013; Duranton et al. 2014; Kobayashi et al. 2014b). The predictive formula and combination of metabolites used differ variously between each report. In other words, these are method development studies, and their methods have not yet been validated sufficiently for use in clinical settings. Progress in metabolomics techniques and parameters would enable the establishment of reliable biomarkers to predict CKD stage, as well as the discovery of CKD in earlier stages.

This chapter introduced metabolomics in the context of evaluating CKD stage. There are other studies that have established diagnostic methods for other diseases using metabolomics as well (Nishiumi et al. 2010; Soga et al. 2011; Ikeda et al. 2012; Nishiumi et al. 2012; Maekawa et al. 2013; Yokokura et al. 2014). Since diagnostic strategies for various diseases using metabolomics are currently in development, metabolomics techniques are not fully refined. Recently, new researchers who start metabolomics studies as well as research reports have been increasing every year. In addition, the performance of mass spectrometry, which plays an important role in metabolite identification, is improving day by day. Accordingly, metabolomics is a field that is expected to be developed more and more into the future. Through its development, after biomarkers are established by which practitioners are able to detect many diseases in their early stages, including CKD, collecting one drop of blood and analyzing it using mass spectrometry would enable the diagnosis of many critical illness all at once.

Potential Applications to Prognosis and Other Diseases and Conditions

Though this chapter described applications of metabolomics to CKD diagnosis, diseases that can potentially be targeted are not limited to CKD. Metabolomics is used to study various other diseases as well, including gastrointestinal cancer (Ikeda et al. 2012), colorectal cancer (Nishiumi et al. 2012), pancreatic cancer (Nishiumi et al. 2010), inflammation (Yokokura et al. 2014), dilated cardiomyopathy (Maekawa et al. 2013), and different forms of liver disease (Soga et al. 2011).

Summary Points

- CKD is the irreversible loss of kidney function, often progressing to ESRD and resulting in impaired quality of life.
- Although estimated GFR, a commonly used marker for CKD is convenient and useful for the initiation of dialysis, the indicator cannot detect CKD in its early stages because of its low sensitivity.
- Metabolites indicative of CKD have been identified using metabolomics.
- CKD stages can be estimated by metabolite concentrations after carrying out statistical processing.
- Metabolomics shows promise as a novel method for identifying patients with early stage CKD.
- In addition, metabolomics can be applied not only to CKD but to various other diseases.

References

Ackroyd H. On the purine metabolism of rats. Biochem J. 1914;8:434–7.

Barreto FC, Barreto DV, Liabeuf S, et al. Serum indoxyl sulfate is associated with vascular disease and mortality in chronic kidney disease patients. Clin J Am Soc Nephrol. 2009;4:1551–8.

Dahl J, Andreassen OA, Verkerk R, et al. Ongoing episode of major depressive disorder is not associated with elevated plasma levels of kynurenine pathway markers. Psychoneuroendocrinology. 2015;56:12–22.

Duranton F, Lundin U, Gayrard N, et al. Plasma and urinary amino acid metabolomic profiling in patients with different levels of kidney function. Clin J Am Soc Nephrol. 2014;9:37–45.

Herget-Rosenthal S, Bökenkamp A, Hofmann W, et al. How to estimate GFR-serum creatinine, serum cystatin C or equations? Clin Biochem. 2007;40:153–61.

Horio M, Imai E, Yasuda Y, et al. Performance of GFR equations in Japanese subjects. Clin Exp Nephrol. 2013;17:352–8.

Ikeda A, Nishiumi S, Shinohara M, et al. Serum metabolomics as a novel diagnostic approach for gastrointestinal cancer. Biomed Chromatogr. 2012;26:548–58.

Inker LA, Schmid CH, Tighiouart H, et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. N Engl J Med. 2012;367:20–9.

Kell DB. Metabolomics and systems biology: making sense of the soup. Curr Opin Microbiol. 2004;7:296–307.

Kikuchi K, Itoh Y, Tateoka R, et al. Metabolomic search for uremic toxins as indicators of the effect of an oral sorbent AST-120 by liquid chromatography/tandem mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2010;878:2997–3002.

Kobayashi T, Matsumura Y, Ozawa T, et al. Exploration of novel predictive markers in rat plasma of the early stages of chronic renal failure. Anal Bioanal Chem. 2014a;406:1365–76.

Kobayashi T, Yoshida T, Fujisawa T, et al. A metabolomics-based approach for predicting stages of chronic kidney disease. Biochem Biophys Res Commun. 2014b;445:412–6.

Levey AS, Eckardt KU, Tsukamoto Y, et al. Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int. 2005;67:2089–100.

Levey AS, de Jong PE, Coresh J, et al. The definition, classification, and prognosis of chronic kidney disease: a KDIGO controversies conference report. Kidney Int. 2011;80:17–28.

Maekawa K, Hirayama A, Iwata Y, et al. Global metabolomic analysis of heart tissue in a hamster model for dilated cardiomyopathy. J Mol Cell Cardiol. 2013;59:76–85.

Mallet CR, Lu Z, Mazzeo JR. A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. Rapid Commun Mass Spectrom. 2004;18:49–58.

Nishiumi S, Shinohara M, Ikeda A, et al. Serum metabolomics as a novel diagnostic approach for pancreatic cancer. Metabolomics. 2010;6:518–28.

Nishiumi S, Kobayashi T, Ikeda A, et al. A novel serum metabolomics-based diagnostic approach for colorectal cancer. PLoS One. 2012;7:e40459.

Niwa T. Mass spectrometry in the search for uremic toxins. Mass Spectrom Rev. 1997;16:307–32.

Niwa T. Recent progress in the analysis of uremic toxins by mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2009;877:2600–6.

Niwa T. Update of uremic toxin research by mass spectrometry. Mass Spectrom Rev. 2011;30:510–21.

Peralta CA, Katz R, Sarnak MJ, et al. Cystatin C identifies chronic kidney disease patients at higher risk for complications. J Am Soc Nephrol. 2011a;22:147–55.

Peralta CA, Shlipak MG, Judd S, et al. Detection of chronic kidney disease with creatinine, cystatin C, and urine albumin-to-creatinine ratio and association with progression to end-stage renal disease and mortality. JAMA. 2011b;305:1545–52.

Rhee EP, Clish CB, Ghorbani A, et al. A combined epidemiologic and metabolomic approach improves CKD prediction. J Am Soc Nephrol. 2013;24:1330–8.

Richard JG, Shaul GM. Uremic toxins: an integrated overview of definition and classification. In: Niwa T, editor. Uremic toxins. Hoboken: Wiley; 2012. p. 3–12.

Saito K, Fujigaki S, Heyes MP, et al. Mechanism of increases in L-kynurenine and quinolinic acid in renal insufficiency. Am J Physiol Ren Physiol. 2000;279:F565–72.

Sato E, Kohno M, Yamamoto M, et al. Metabolomic analysis of human plasma from haemodialysis patients. Eur J Clin Invest. 2011;41:241–55.

Shah VO, Townsend RR, Feldman HI, et al. Plasma metabolomic profiles in different stages of CKD. Clin J Am Soc Nephrol. 2013;8:363–70.

Soga T, Sugimoto M, Honma M, et al. Serum metabolomics reveals gamma-glutamyl dipeptides as biomarkers for discrimination among different forms of liver disease. J Hepatol. 2011;55:896–905.

Sumner LW, Amberg A, Barrett D, et al. Proposed minimum reporting standards for chemical analysis. Metabolomics. 2007;3:211–21.

Taguchi K, Fukusaki E, Bamba T. Determination of niacin and its metabolites using supercritical fluid chromatography coupled to tandem mass spectrometry. Mass Spectrom (Tokyo). 2014;3: A0029.

Tangri N, Stevens LA, Griffith J, et al. A predictive model for progression of chronic kidney disease to kidney failure. JAMA. 2011;305:1553–9.

Toyohara T, Akiyama Y, Suzuki T, et al. Metabolomic profiling of uremic solutes in CKD patients. Hypertens Res. 2010;33:944–52.

Vanholder R, De Smet R, Glorieux G, et al. Review on uremic toxins: classification, concentration, and interindividual variability. Kidney Int. 2003;63:1934–43.
Yeldandi AV, Yeldandi V, Kumar S, et al. Molecular evolution of the urate oxidase-encoding gene in hominoid primates: nonsense mutations. Gene. 1991;109:281–4.
Yokokura Y, Isobe Y, Matsueda S, et al. Identification of 14,20-dihydroxy-docosahexaenoic acid as a novel anti-inflammatory metabolite. J Biochem. 2014;156:315–21.
Yoshida M, Hatano N, Nishiumi S, et al. Diagnosis of gastroenterological diseases by metabolome analysis using gas chromatography-mass spectrometry. J Gastroenterol. 2012;47:9–20.
Zhao YY. Metabolomics in chronic kidney disease. Clin Chim Acta. 2013;422:59–69.
Zhao YY, Cheng XL, Wei F, et al. Serum metabolomics study of adenine-induced chronic renal failure in rats by ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. Biomarkers. 2012;17:48–55.

Hypoxia as a Biomarker of Kidney Disease

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Abstract

All established (e.g., serum creatinine, albuminuria) and emerging (e.g., neutrophil gelatinase-associated lipocalin, cystatin C) biomarkers of kidney disease suffer from the disadvantage that they are markers of damage to the kidney or loss of renal function. Tissue hypoxia is believed to be an initiating factor, in both chronic kidney disease (CKD) and acute kidney injury (AKI), so may provide a physiological biomarker for early diagnosis of both conditions. Currently blood oxygen dependent magnetic resonance imaging (BOLD MRI) appears to have little diagnostic value in human CKD. On the other hand, the measurement of urinary oxygen tension (PO_2) has potential as a biomarker of risk of AKI in a hospital setting because: (i) Hypoxia in the renal medulla plays a central role in AKI of multiple causes; (ii) The vasa recta are closely associated with collecting ducts in the medulla so that pelvic urinary PO_2 would be expected to equilibrate with medullary tissue PO_2; (iii) The PO_2 of urine in both the renal pelvis and the bladder varies in response to stimuli that would be expected to alter medullary tissue PO_2; and (iv) New fibre-optic methods make it feasible to measure bladder urine PO_2 in patients with a bladder catheter. But translation of this approach to hospital practice requires: (i) A quantitative understanding of the impact of oxygen transport across the epithelium of the ureter and bladder on urinary PO_2 measured from the bladder, (ii) confirmation that changes in urinary PO_2 parallel those in medullary PO_2 in physiology and pathology, and (iii) Studies of the prognostic utility of urinary PO_2 in hospital settings associated with risk of AKI, such as in patients undergoing cardiac surgery with cardiopulmonary bypass, those at risk of sepsis, and those undergoing imaging procedures requiring administration of radiocontrast agents.

Keywords

Acute kidney injury • BOLD MRI • Cardiopulmonary bypass • Chronic kidney disease • Intensive care • Sepsis • Urinary oxygen tension

Abbreviations

AKI	Acute kidney injury
BOLD	Blood oxygen dependent
CKD	Chronic kidney disease
CPB	Cardiopulmonary bypass

GFR	Glomerular filtration rate
MRI	Magnetic resonance imaging
NGAL	Neutrophil gelatinase-associated lipocalin
RBF	Renal blood flow

Key Facts of Kidney Oxygenation

- Although renal blood flow represents a large proportion (~25 %) of cardiac output, the kidneys are susceptible to hypoxia.
- Oxygenation of kidney tissue is determined by the balance between oxygen delivery and oxygen consumption.
- Oxygen is delivered to the kidney through renal blood flow. Blood flow to the renal medulla is in part regulated independently of blood flow to the bulk of the renal cortex. Blood flow per unit tissue volume is much less in the renal medulla than in the renal cortex.
- Oxygen delivery to kidney tissue is limited by diffusive oxygen shunting in the renal cortex and medulla. In the medulla, oxygen delivery to the thick ascending limbs of the loop of Henle is also limited by their anatomical position, at the periphery of the vascular bundles
- Approximately 80 % of renal oxygen consumption is used to power tubular reabsorption of sodium. The proximal tubules and thick ascending limbs of the loop of Henle are the major sites of renal sodium reabsorption.

Definitions

Acute kidney injury (AKI) Some loss of function of the kidney, usually assessed initially as a reduction in glomerular filtration rate. It can be relatively mild, in which case complete recovery can occur in as little as a few days, or be so severe that renal replacement therapy (dialysis or a kidney transplant) is required (end-stage renal disease).

Cardiopulmonary bypass (CPB) To perform open heart surgery, in most cases the heart must be stopped and the patient’s circulation supported by a ‘bypass circulation’ incorporating a pump to do the job of the heart and an oxygenator to do the job of the lungs.

Chronic kidney disease (CKD) A condition where the function of the kidney gradually declines over many years. In some cases, this leads to end stage renal disease. Chronic kidney disease is usually first diagnosed when glomerular filtration rate is found to be less than the normal range.

Clearance This is a technique used for measurement of certain kidney functions. It relies on the ability to measure the concentrations of a marker in the plasma and urine and measurement of the amount of urine produced over a given time period.

Cortex The outer part of an organ (e.g., kidney cortex).

End-stage renal disease A condition in which the kidneys are no longer able to function sufficiently to maintain the patients overall health. In such cases renal replacement therapy is required, in the form of dialysis therapy or kidney transplantation.

Fibrosis Many disease conditions, including chronic kidney disease, are characterized by the build-up of connective tissue fibres. In the kidney, fibrosis reduces the ability of oxygen to get to cells.

Fluorescence lifetime oximetry A technique for measuring the partial pressure of oxygen which relies on the ability of oxygen to quench the fluorescence emitted by certain molecules.

Glomerular filtration rate The amount of plasma filtered by the kidney each minute.

Haemoglobin The oxygen carrying molecules that are packed inside red blood cells. When haemoglobin is not carrying oxygen it is called deoxyhaemoglobin.

Hypoxia Low levels of oxygen.

Ischaemia Inadequate blood supply to an organ or part of the body.

Magnetic resonance imaging (MRI) A medical imaging technique that can produce three dimensional images of anatomy. It can also be used to map functional indices such as the concentration of deoxyhaemoglobin (blood oxygen level dependent – BOLD MRI).

Medulla The inner part of an organ (e.g., kidney medulla).

PO_2 The partial pressure of oxygen. This is how we measure the concentration of dissolved oxygen in biological fluids and tissue, in the units of millimetres of mercury (mmHg).

Radiocontrast An agent which is administered to patients undergoing some x-ray procedures to assist visualisation of body structures.

Renal replacement therapy Patients who suffer from end-stage renal disease require renal replacement therapy, either in the form of renal dialysis therapy or a kidney transplant.

Risk factor A risk factor is anything that is found to be associated with the development of disease. Some of these are non-modifiable (e.g., age, gender) while others are potentially modifiable (e.g., smoking, alcohol use etc.).

Sepsis An inflammatory condition that results from severe bacterial infection.

Ureter The tube in which urine travels from the kidney to the urinary bladder.

Vasa recta A series of straight blood vessels in the kidney medulla.

Introduction

Kidney disease is broadly classified into two major types; acute kidney injury (AKI) and chronic kidney disease (CKD). CKD is characterized by a gradual decline in glomerular filtration rate (GFR) over many years. AKI, on the other hand, is characterized by a rapid decline in GFR over a 24–48 h period. These two forms of kidney disease share some similarities. Both are traditionally diagnosed by measurement of serum concentrations of creatinine, or by the renal clearance of creatinine, to give estimates of GFR. As we will outline in detail herein, renal hypoxia is also a common feature of both AKI and CKD (Mimura and Nangaku 2010; Heyman et al 2012; Evans et al. 2013). However, the challenges for early diagnosis and accurate prognosis for these two conditions contrast strikingly.

Early diagnosis of CKD may provide an opportunity for early intervention with therapies to slow disease progression. However, this is made more difficult by the fact that measurable deficits in GFR occur well into disease progression (Fig. 1a). This is because, as nephrons are lost during the disease process, filtration in the remaining nephrons increases (Fong et al. 2014). Thus, as CKD progresses GFR can remain stable because reserve filtration capacity is consumed as single nephron GFR increases. It is only when these compensatory processes are exhausted that clinically significant deficits in GFR can be detected. Thus, early diagnosis of CKD requires minimally invasive methods to assess renal function at the single nephron level, and/or methods to detect the upstream events that damage nephrons. In this context, it is worth considering the evidence (vide infra) that renal tissue hypoxia precedes and drives progression of CKD. Thus, minimally invasive methods for assessing kidney oxygenation may provide a means for early diagnosis of CKD.

In AKI, the challenge is to reach a diagnosis before the onset of irreversible damage (Fig. 1b). The ideal would be to identify patients who are at risk of developing AKI and intervene to manage their kidney health before injury occurs. The traditional biomarker serum creatinine, which is used to estimate GFR, can show that a patient has developed AKI, but not that they are currently at risk of developing AKI. Other plasma and urinary biomarkers, including neutrophil gelatinase-associated lipocalin (NGAL) and cystatin C, have shown promise in the early detection of AKI (Haase et al. 2010), but they are markers of renal damage, so diagnosis still lags hours behind the initiating insult. As with CKD, assessment of kidney oxygenation may provide a method for early diagnosis of AKI. Importantly, in the hospital setting, including during surgery, a measure of kidney oxygenation could provide an approach to identify patients at risk of developing AKI, so that steps can be taken to reduce that risk.

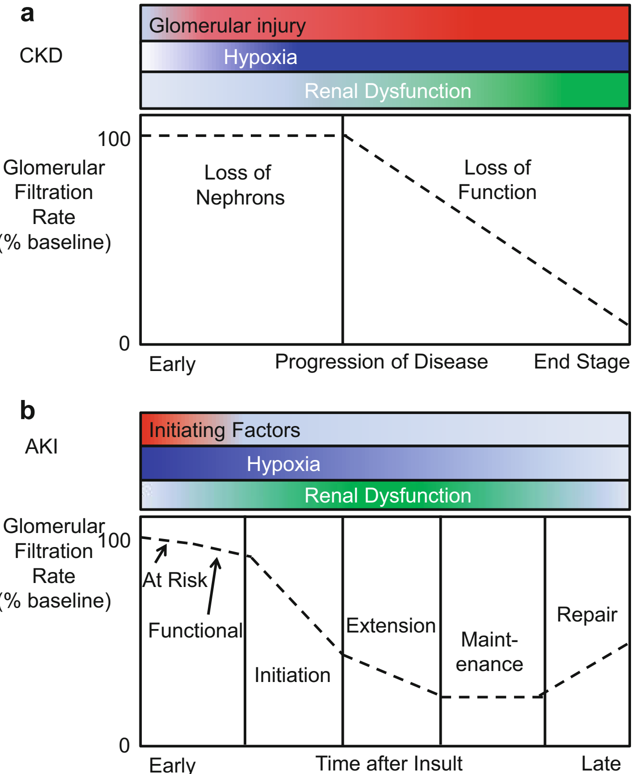

Fig. 1 Proposed timelines in the initiation and progression of chronic kidney disease (*CKD*) and acute kidney injury (*AKI*). (**a**) According to the chronic hypoxia hypothesis, proposed initially by Fine and colleagues (1998) and Fine and Norman (2008), in CKD primary glomerular injury leads to insufficiency of post glomerular capillary blood flow, and thus tubulointersitial hypoxia. Tubulointerstitial hypoxia in turn drives fibrosis, capillary rarefaction and tubular atrophy, and thus progressive glomerular injury. However, because surviving nephrons hyperfilter, a deficit in glomerular filtration is not observed until the disease process is well advanced. (**b**) Initiating factors such as cardiopulmonary bypass, sepsis or nephrotoxins lead to renal medullary ischaemia and/or hypoxia, which in turn may contribute to loss of renal functional capacity. This concept is related to the established clinical phases of AKI (Adapted from Okusa et al. (2013)). In both CKD and AKI it has been proposed that hypoxia occurs before, and contributes to, loss of renal function. Thus, hypoxia may be a useful biomarker for kidney disease

This chapter presents the argument that assessment of kidney oxygenation, and thus detection of renal hypoxia, may provide a means for early diagnosis of AKI and CKD. Early diagnosis would allow more reliable prognosis and improved management of renal health. The optimal diagnostic approach is likely to differ between CKD and AKI. For CKD, blood oxygen dependent (BOLD) magnetic resonance imaging (MRI) has been proposed as a useful tool for early diagnosis. This approach is non-invasive so can be applied periodically in out-patients. Unfortunately, despite promising observations in animal models of CKD, available evidence indicates that

this approach has little clinical utility. For AKI, a method for continuous assessment of kidney oxygenation, in the operating theatre or bedside, is required for rapid identification of patients at risk of developing AKI. Continuous measurement of urinary PO_2 may provide this information. But the reader must first be convinced that renal hypoxia is a common feature of both CKD and AKI, and that it precedes, and perhaps even drives, the disease process.

Chronic Kidney Disease

The Role of Hypoxia in the Pathogenesis of Chronic Kidney Disease

Renal tissue hypoxia has been observed in all experimental forms of CKD examined to date, including those initiated by renovascular disease (Evans and O'Connor 2014), diabetic nephropathy (Hansell et al. 2013), five sixths nephrectomy (the remnant kidney model) (Manotham et al. 2004), polycystic kidney disease (Ow et al. 2014), and CKD after long-term recovery from ischaemia-reperfusion injury (Basile et al. 2003).

Fine and colleagues first developed the chronic hypoxia hypothesis regarding the initiation and progression of CKD more than 15 years ago (Fine et al. 1998; Fine and Norman 2008). In brief, they propose that primary injury to glomeruli results in increased metabolic work in remaining nephrons to reabsorb the filtered load of sodium. The resultant hypoxia initiates inflammatory and fibrotic pathways, which in turn drive rarefaction of the microcirculation, so exacerbating hypoxia (Fig. 1a). Evidence in support of this hypothesis includes the observation of tubulointerstitial hypoxia preceding evidence of nephropathy in the remnant kidney model (Manotham et al. 2004) and in diabetes (dos Santos et al. 2007; Rosenberger et al. 2008). Furthermore, CKD can be induced by treatments that promote renal hypoxia, even in the absence of confounding effects such as oxidative stress (Friederich-Persson et al. 2013).

The Clinical Problem: Early Diagnosis and Instigation of Therapy

CKD cannot yet be cured. Current therapies are palliative, so aimed at slowing the progression of disease rather than reversing it (Lambers Heerspink and de Zeeuw 2013). The major flaw of the current diagnostic approach is that deficits in GFR are only observed once disease processes are well advanced (Fig. 1a). Diagnostic methods capable of detecting the disease process in its early stages would represent a significant advance, as this would support early intervention to prevent progression to end-stage renal disease. Could diagnosis of kidney hypoxia provide such an advance?

Assessment of Kidney Oxygenation by BOLD MRI in Chronic Kidney Disease

There has recently been an explosion of interest in the use of BOLD MRI for assessment of kidney oxygenation in patients with CKD. BOLD MRI provides a

measure of deoxygenated haemoglobin, so is an indirect measure of tissue oxygenation (Evans et al. 2008b). Nevertheless, changes in the BOLD signal have been shown to follow changes in more direct measures of renal tissue oxygenation in response to multiple physiological manoeuvres in multiple species of healthy animals (Pedersen et al. 2005; Pohlmann et al. 2013; Zhang et al. 2014). Furthermore, renal hypoxia has been detected using BOLD MRI in the early stages of diabetic nephropathy (dos Santos et al. 2007; Prasad et al. 2010) and in severe renovascular disease (Gloviczki et al. 2011) in humans. Yet two relatively recent and large clinical studies failed to detect differences in the BOLD signal, in either the cortex or medulla, between patients with CKD and controls at rest (Michaely et al. 2012; Pruijm et al. 2014). In both studies, which included a total of 400 (Michaely et al. 2012) and 195 (Pruijm et al. 2014) patients respectively, no relationship could be detected between BOLD signals and GFR estimated from creatinine clearance or serum creatinine concentration at rest (Michaely et al. 2012). Inoue and colleagues were also unable to demonstrate a relationship between the BOLD signal and GFR in 43 patients with diabetic nephropathy, although they could detect a relationship in 76 subjects with CKD chiefly due to glomerulonephritis or hypertensive nephrosclerosis (Inoue et al. 2011). Furthermore, in a relatively small study, hypoxia could not be detected using BOLD MRI in patients with moderate renal artery stenosis (Gloviczki et al. 2010).

How can the disparity in the experimental and clinical findings described above be explained? One possibility is that established CKD in humans, unlike in experimental animals, is not associated with renal tissue hypoxia. This possibility cannot be completely discounted, since reductions in GFR would be expected to lead to reduced renal oxygen consumption, as the major source of oxygen demand in the kidney is for generation of ATP for tubular Na/K-ATPase activity (Evans et al. 2014a). For example, reduced renal oxygen demand likely explains why the BOLD MRI signal reflects increased renal oxygenation in chronic allograft rejection (Djamali et al. 2007). Part of the explanation might also lie with the heterogeneous nature of CKD, and the way in which renal oxygen supply and demand change over the course of development of the various forms of CKD (Neugarten 2012). However, it seems likely that much of the problem lies with the indirect nature of the BOLD signal in relation to tissue oxygenation. The BOLD signal is heavily weighted towards venous blood, because it chiefly provides a measure of deoxyhaemoglobin concentration. Furthermore, renal tissue oxygenation does not necessarily reflect renal blood oxygenation, in part because of the phenomenon of arterial-to-venous oxygen shunting (O'Connor et al. 2006a; Evans et al. 2008a). Indeed, renal venous PO_2 and renal tissue PO_2 can change independently in the kidney, providing direct evidence that changes in the BOLD MRI signal do not necessarily reflect changes in tissue oxygenation (O'Connor et al. 2006a; Evans et al. 2008a). Regardless, available evidence indicates that measurement of baseline BOLD signals provide little diagnostic or prognostic information in patients with CKD.

A more promising approach may be to use BOLD MRI in conjunction with a physiological challenge to kidney function. Recently it was demonstrated, in a

cohort of 195 subjects, that the response of the medullary BOLD signal to furosemide is markedly blunted in CKD (Pruijm et al. 2014). Furosemide inhibits sodium reabsorption, and thus oxygen consumption, in the loop of Henle. Thus, in normal subjects, renal oxygenation (as assessed by BOLD MRI and more direct methods) increases markedly after furosemide administration. The fact that this response is blunted in patients with CKD provides a diagnostic opportunity. The critical unresolved question is whether this change in the response to furosemide occurs before, or only after CKD can be diagnosed by conventional methods. This question could only be answered through prospective studies.

Acute Kidney Injury

The Role of Hypoxia in the Pathogenesis of Acute Kidney Injury

The kidneys receive a quarter of the cardiac output, so are very highly perfused in relation to their demand for oxygen (Evans et al. 2008a). It is rather paradoxical, then, that they are susceptible to hypoxic damage in both CKD (Fine and Norman 2008) and AKI (Heyman et al. 2012). A number of factors conspire to render the medulla particularly susceptible to hypoxia. Firstly, blood flow per unit volume of tissue is considerably less in the medulla than the cortex (Evans et al. 2013). Secondly, oxygen delivery to medullary tissue is limited by counter-current shunting of oxygen, between arteries and veins in the cortex (Evans et al. 2013) and between descending and ascending vasa recta in the medulla (Zhang and Edwards 2002). Thirdly, in the outer medulla the thick ascending limbs of Henle's loop, which require oxygen to reabsorb sodium, are located at the periphery of the vascular bundles (Chen et al. 2009a, b). This topographic arrangement limits oxygen delivery from vasa recta to the thick ascending limbs (Chen et al. 2009a, b). These factors likely explain why the outer medullary thick ascending limbs are often found to be damaged in human AKI (Heyman et al. 2010).

Tissue hypoxia has been proposed to be a common pathway in the pathogenesis and progression of AKI (Aksu et al. 2011; Heyman et al. 2012). It is consistently observed in AKI of multiple aetiologies (Heyman et al. 2012; Evans et al. 2013). Furthermore, hypoxia can initiate signalling pathways that drive renal tissue dysfunction and damage (Heyman et al. 2012; Evans et al. 2013). These pathways include those driven by transforming growth factor β and Smads (inflammation and fibrosis), depletion of cellular ATP (apoptosis and necrosis) and oxidative stress. Tissue hypoxia is then further exacerbated as a consequence of initiation of these pathways. Hypoxia inducible factors can provide some protection against mild and/or brief hypoxia, but not when hypoxia is profound and/or prolonged (Heyman et al. 2012). Hypoxia in the renal medulla may initiate a vicious cycle of damage and dysfunction, which drives further hypoxia. Early disruption of these processes could be beneficial by preventing renal medullary hypoxia. In order to prevent medullary hypoxia, a means to evaluate it is required.

The Clinical Problem: Prevention of Acute Kidney Injury in the Hospital Setting

Approximately 5 % of hospitalized patients develop AKI, so it is a major burden on health systems (Mehta and Chertow 2003). AKI is chiefly an iatrogenic illness. It is often associated with situations in which the patient and/or their kidneys are exposed to hypoxia (Evans et al. 2013).

Cardiac Surgery

AKI is a prevalent complication of major surgery. After cardiac surgery, up to one third of patients experience some degree of AKI (Karkouti et al. 2009). Even mild AKI following surgery performed on cardiopulmonary bypass (CPB) is prognostically important, being associated with more than a fourfold increase in the risk of in-hospital death as well as extended hospitalisation (Karkouti et al. 2009). Mortality rate exceeds 60 % when AKI is so severe that renal replacement therapy is required (in 1–2 % of patients after CPB) (Lenihan et al. 2013). The scale of the problem is receiving growing recognition, with the most recent evidence showing that the problem is becoming worse rather than better (Lenihan et al. 2013).

There are currently no effective pharmacological approaches for preventing AKI after CPB (Patel et al. 2011b). Thus, the best approach to prevention is avoidance of known modifiable risk factors in at-risk patients. Consequently, AKI risk scoring models have been developed (Huen and Parikh 2012). These tools are used in practice to help identify at-risk patients, but they only have limited predictive efficacy (Huen and Parikh 2012). They also provide little guidance for the medical team to know what actions are needed before, during and after surgery to ameliorate the risk of AKI.

Clinical findings indicate that prevention of kidney ischaemia and hypoxia during CPB should reduce the risk of AKI. Fundamental to this argument is the observation that most potentially modifiable risk factors for AKI after CPB are associated with reduced renal oxygen delivery (Karkouti et al. 2009). For example, a haematocrit of less than 21 % and whole body oxygen delivery less than 262 ml/min/m^2 are risk factors for AKI following cardiac surgery (Rosner et al. 2008). This clinical finding accords with the prediction that pre-operative anaemia and intraoperative haemodilution reduce blood oxygen carrying capacity and so reduce renal oxygen delivery (Evans et al. 2013). Chronic obstructive pulmonary disease causes systemic hypoxaemia (and thus reduced renal oxygen delivery) and is also a risk factor for AKI in patients undergoing cardiac surgery (Rosner et al. 2008). During CPB, the autoregulatory capacity of the renal circulation is blunted, so that renal blood flow (RBF) and thus renal oxygen delivery is limited by the level of pump flow, and thus the level of arterial pressure (Andersson et al. 1994). Indeed, computational modelling indicates that the combined effects of haemodilution and non-pulsatile blood flow during CPB render the kidney susceptible to hypoxia if arterial pressure falls below 50 mmHg (Sgouralis et al. 2014), a relatively common occurrence in clinical practice (Rosner et al. 2008). It is also salient to consider that patients with heart disease will often have marked activation of the renin-angiotensin and sympathetic nervous systems, which will further promote renal vasoconstriction and thus reduced

renal oxygen delivery (Evans et al. 2013). Furthermore, anaesthesia itself tends to reduce RBF, and thus renal oxygen delivery (Ullman et al. 2001).

It is not currently feasible to directly assess kidney oxygenation in patients on CPB, but this has been achieved in experimental animals. In pigs, medullary and urinary hypoxia (PO_2 1–5 mmHg for both) were observed during CPB (Stafford-Smith and Grocott 2005) and profound medullary hypoxia was observed 24 h after CPB (Patel et al. 2011a, c). Treatments that ameliorated medullary hypoxia ameliorated AKI (Patel et al. 2011a, c). Medullary hypoxia was also observed in rats during CPB (Darby et al. 2013). Critically, as discussed in detail later in this chapter, data from humans show that low intra-operative urinary PO_2 and a poor recovery of urinary PO_2 after CPB is associated with increased post-operative serum creatinine and thus reduced GFR (Kainuma et al. 1996).

A unique aspect of CPB as a cause of AKI is that the surgical team, including perfusionists and anaesthetists, have the means to modify systemic and renal haemodynamic function when the patient is at risk of developing AKI. The fundamental problem is that different tissues have different perfusion needs. Consequently a single group of macro-cardiovascular parameters may not provide suitable rates of perfusion for all tissues. The optimal choice of these macro-cardiovascular parameters is not clear because, unfortunately, surgical teams currently have little tissue specific information upon which to base these important decisions.

Sepsis

Ten to 50 % of patients with sepsis develop AKI. Indeed, sepsis is responsible for approximately 50 % of cases of AKI in patients who are critically ill (Zarjou and Agarwal 2011). Furthermore, outcomes are often worse for patients with septic AKI than for those with non-septic AKI (Parmar et al. 2009).

It is difficult to predict which patients with sepsis will go on to develop AKI. Furthermore, despite recent advances in urinary and plasma biomarkers, once patients have developed sepsis the only established read-outs clinicians have to determine the impact of the therapies they institute on renal function are systemic haemodynamics, creatinine clearance and urine flow (Zarjou and Agarwal 2011). The ‘real time’ evaluation of interventions to raise arterial pressure such as volume resuscitation and vasoconstrictor therapy (Mori et al. 2010), is thus mainly limited to assessment of their effects on systemic haemodynamics and blood chemistry, rather than organ-specific effects on perfusion and oxygenation. Thus, septic AKI is another example in which a continuous measure of kidney health should allow considerable improvement in the management of patients at risk of, or who have already developed, AKI. Could renal hypoxia be a useful biomarker in this respect?

Renal hypoxia has been observed in most, although not all, models of sepsis in experimental animals (Evans et al. 2013). The potential mechanisms that drive renal hypoxia in sepsis have recently been reviewed in detail (Aksu et al. 2011). In brief, evidence from rodent models indicates that the process is initiated by hypoperfusion, hypoxia, and/or inflammatory activation. The delicate balance between the bioavailability of oxygen, nitric oxide and oxygen radical species is then disrupted, resulting

in reduced oxygen delivery to tissue and inappropriately high renal oxygen consumption. However, there is currently considerable controversy as to the relative merits of the various animal models of sepsis. Most models in rodents are associated with renal ischaemia, so the presence of renal hypoxia comes as little surprise. It has been argued that sepsis in humans is often associated with a hyperdynamic circulation, so that renal blood flow is maintained or even increased from baseline (Langenberg et al. 2005). Recently, medullary hypoxia and ischaemia was observed in an ovine model of septic AKI in which total renal blood flow is actually increased during sepsis (Calzavacca et al. 2015). These observations provide further support for the notion that medullary hypoxia contributes to the maintenance and progression of septic AKI. Thus, a real time measure of renal medullary oxygenation would potentially provide a method for early diagnosis and better management of septic AKI, with potential for limiting the progression of septic AKI.

Radiocontrast Agents and Other Nephrotoxins

Radio-contrast administration is the third most common cause of hospital acquired AKI (11 % of cases) (McCullough 2008). It adds considerably to both in-hospital costs and the expenses patients face after they are discharged (McCullough 2008). Perhaps most concerning is the fact that radio-contrast induced AKI is associated with a four to sixfold increase in the risk of death during and after hospitalization (McCullough 2008). AKI can also arise in (and outside) a hospital setting due to the nephrotoxicity of a range of drugs (Perazella 2009). The countercurrent exchange mechanisms in the renal medulla may concentrate contrast agents and other nephrotoxins, and thus exacerbate the risk of AKI.

Radiocontrast-induced AKI probably progresses via multiple pathways. Nevertheless, one of these pathways is likely to be medullary hypoxia (Heyman et al. 2008). Medullary hypoxia has been observed after radiocontrast administration in anaesthetized rats (Heyman et al. 1991; Liss et al. 1998; Prasad et al. 2001) and humans (Hofmann et al. 2006). Hypoxia is driven by a range of factors, including activation of vasoconstrictor factors, the haemodynamic consequences of the viscosity of contrast agents, and increased medullary tissue oxygen consumption due to the combined effects of increased solute delivery to the distal nephron segments and oxidative stress (Heyman et al. 2008; Evans et al. 2013).

Standard practice is to volume load patients thought to be at risk of AKI, with isotonic saline, prior to radiocontrast administration (McCullough 2008). This is the only prophylactic treatment that has been conclusively shown to be effective.

As is the situation with CPB and sepsis, a major challenge for management of renal health in patients receiving radiocontrast agents or other potential nephrotoxins, is the development of methods for real-time assessment of kidney health. In the following section, the argument is developed that continuous measurement of urinary PO_2, to monitor changes in medullary oxygenation, would provide the critical information needed to manage patients at risk of developing of AKI in a hospital setting.

Urinary PO_2 as a Diagnostic Biomarker in Acute Kidney Injury

The evidence that (bladder) urinary PO_2 can provide prognostic information regarding kidney health in a hospital setting has recently been reviewed in detail (Evans et al. 2014b). The argument relies on five lines of evidence: that (i) the anatomy of the renal medulla promotes diffusion of oxygen between the collecting ducts and the vasa recta, so that (ii) oxygen tension in urine in the renal pelvis is in equilibrium with that in medullary tissue, (iii) that oxygen diffusion across the epithelium of the ureter and bladder only partially confounds the relationship between medullary PO_2 and the PO_2 of urine in the bladder, (iv) that bladder urine PO_2 provides prognostically useful information, and (v) that new fibre optic methods make continuous measurement of urinary PO_2 in patients with a bladder catheter relatively straight forward and risk free. Each of these lines of argument is described in turn.

(i) ***Renal medullary anatomy promotes diffusion of oxygen between the collecting ducts and the vasa recta***: Detailed anatomical information regarding the arrangement of various vascular and tubular elements within the rat renal medulla indicates that, in the inner medulla, four ascending vasa recta usually abut each collecting duct (Pannabecker and Dantzler 2006). This intimate association likely promotes oxygen diffusion between collecting ducts and ascending vasa recta, making it likely that urinary oxygen content equilibrates with inner medullary interstitial oxygen, as shown in three human subjects (Leonhardt et al. 1965) (Fig. 2).

(ii) ***Oxygen in the renal pelvis is in equilibrium with that in medullary tissue***: In the In the 1950s it was shown in anaesthetized dogs that the PO_2 of pelvic urine is rather low; certainly much lower than that of renal venous blood (Reeves et al. 1957; Rennie et al. 1958). Later studies in anaesthetized dogs demonstrated that experimental manoeuvres aimed at altering oxygen delivery to the medulla, or medullary oxygen consumption, could alter the PO_2 of pelvic urine (Aukland and Krog 1961; Aukland 1962; Washington and Holland 1966). In humans, it was shown that diuresis could increase pelvic urinary PO_2, while infusion of hypertonic saline reduced it. Furthermore, pelvic urinary PO_2 fell in response to vasopressin administration (Leonhardt and Landes 1965), a treatment associated with reduced medullary perfusion and oxygenation (O'Connor et al. 2006b). The response of human pelvic urinary PO_2 to inspiration of 100 % oxygen was also studied (Leonhardt and Landes 1965). This response was found to be altered in a number of disease states, including chronic pyelonephritis, hydronephrosis, essential hypertension, arteriolar nephrosclerosis and renal artery stenosis. The authors argued that breathing 100 % oxygen would increase medullary oxygen delivery but not oxygen consumption. Thus, the degree to which urinary PO_2 increases upon breathing 100 % oxygen, and the rate at which it increases, may provide an indirect measure of medullary perfusion.

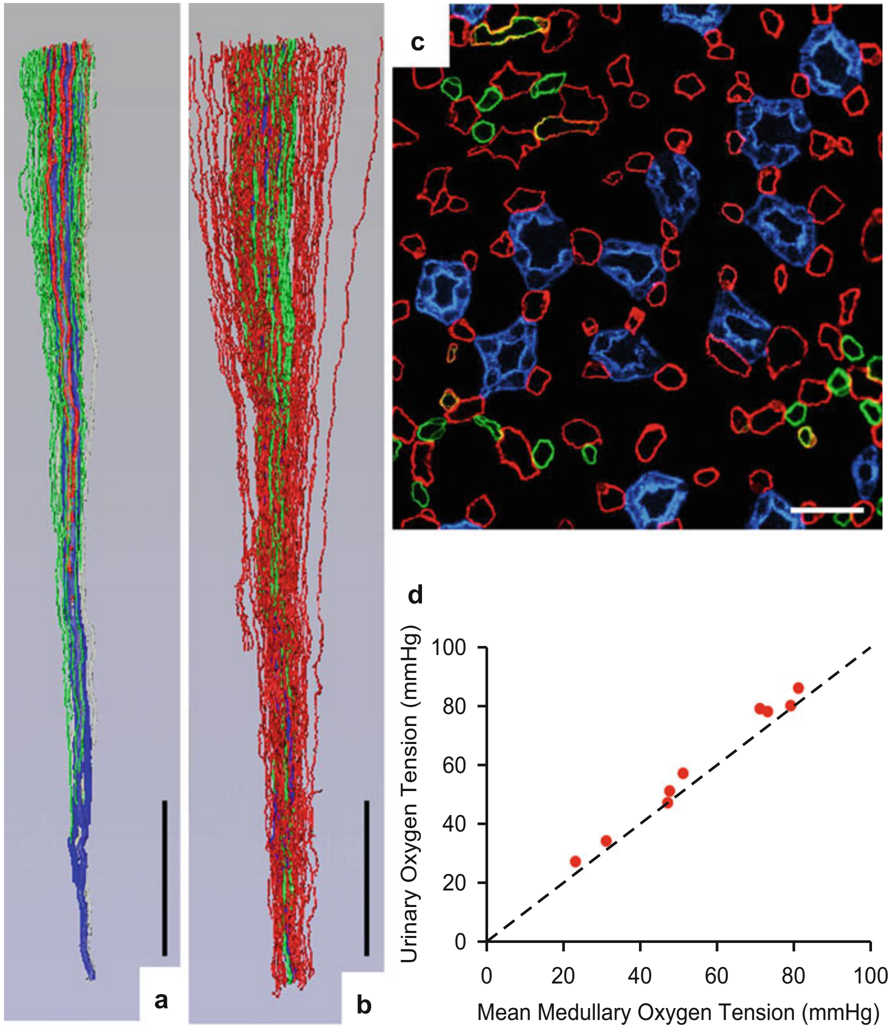

Fig. 2 The relationship between oxygen tension (PO_2) in the renal medulla and in pelvic urine. Panels (**a–c**), originally reproduced from Pannabecker and Dantzler (2006) (with permission from the American Physiological Society), show the close spatial relationships between ascending vasa recta) and the collecting ducts. (**a**, **b**) Three dimensional reconstructions of the inner medulla of the rat. Scale bars = 500 μm. In (**a**), descending vasa recta are shown in *green*, descending thin limbs of the loop of Henle are shown in *red* or *grey*, and collecting ducts are shown in *blue*. In (**b**), collecting ducts are shown in *blue*, ascending vasa recta are shown in *red* and ascending thin limbs are shown in *green*. (**c**) Shows a transverse section near the base of the inner medulla. Ascending vasa recta, shown in *red*, are seen to be positioned around collecting ducts which are shown in *blue*. Descending vasa recta are shown in *green*. Scale bar = 30 μm. Panel (**d**) was redrawn from Leonhardt et al. (1965). It shows nine paired measurements of urinary PO_2 and mean medullary PO_2 made in a total of three human subjects. The *dashed line* is the line of identity. The entire figure is reproduced with permission from the American Physiological Society (Evans et al. 2014b)

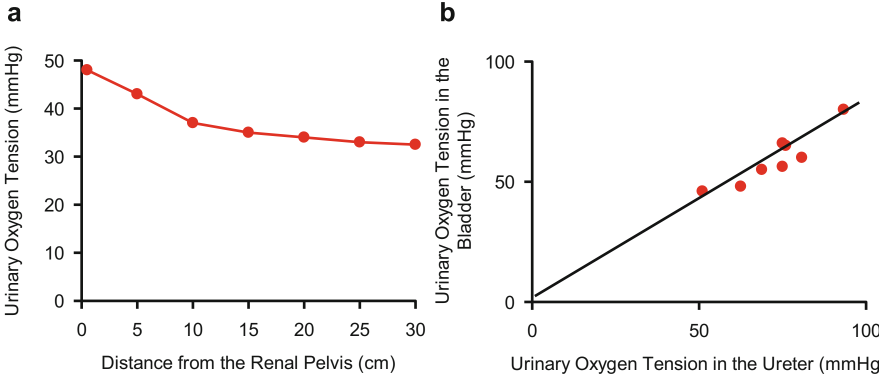

Fig. 3 Changes in urinary oxygen tension along the upper urinary tract. (**a**) Mean ($n = 5$) oxygen tension of ureteral urine at various distances from the renal pelvis in human subjects (Data redrawn from Leonhardt and Landes (1963)). (**b**) Scattergram with line of best fit for measures of oxygen tension in the ureter and bladder in two anaesthetized dogs in which renal blood flow was altered by infusion of dobutamine and propranolol (Figure redrawn from Kitashiro et al. (1995))

Taken collectively, the studies described in the paragraph above provide indirect evidence that the PO_2 of pelvic urine provides a useful index of medullary oxygenation. Nevertheless, there is limited direct evidence to support this proposition. The only published observations are data from three patients (Leonhardt et al. 1965), in which there was excellent agreement between pelvic urinary PO_2 and medullary tissue PO_2 (Fig. 2). Thus, there remains a need for studies in experimental animals to better define the relationship between medullary tissue PO_2 and pelvic urinary PO_2 in the healthy state and in disease.

(iii) ***Oxygen tension of urine in the bladder***: There are a number of factors that might confound measurement of urinary PO_2 in the bladder. These include the presence of reducing agents such as ascorbic acid, thiols and polyphenols that can lead to oxygen consumption (Rennie et al. 1958). Diffusion of oxygen between the urine and the epithelium of the ureter and bladder might also be a confounding factor (Rennie et al. 1958). However, experiments in both experimental animals and humans have provided evidence and optimism that these confounding effects do not invalidate the use of bladder urine PO_2 as a measure of medullary tissue PO_2. For example, the PO_2 of urine in the bladder of anaesthetised dogs was consistently less (12 ± 3 mmHg) than the PO_2 of urine in the ureter (Kitashiro et al. 1995). Nevertheless, when these animals were subjected to treatments that altered cardiac output, the PO_2 of urine in the bladder was found to correlate closely with the PO_2 of urine in the ureter (Fig. 3). Similarly, the PO_2 of urine was found to fall in humans, from ~48 mmHg at the level of the renal pelvis to ~33 mmHg in the bladder (Leonhardt and Landes 1963; Fig. 3). Nevertheless, in the same individuals the PO_2 of voided urine consistently increased in response to hydration and fell in response to dehydration (Leonhardt and Landes 1963).

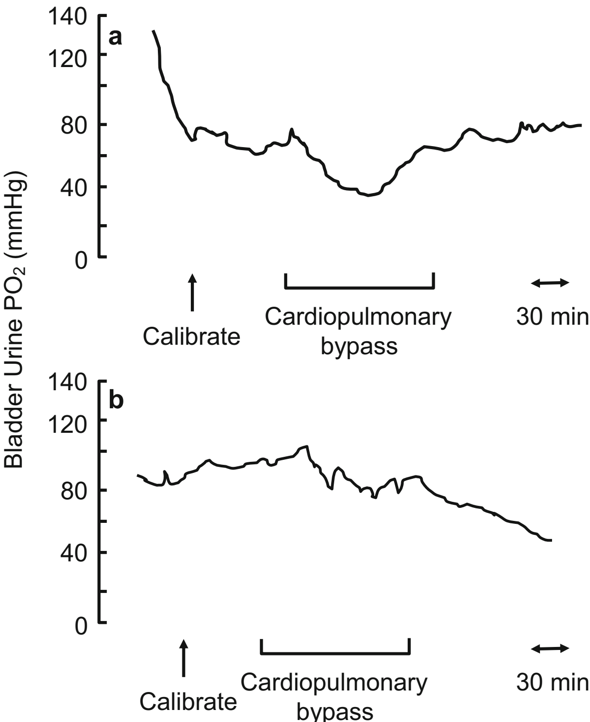

Fig. 4 Oxygen tension in the urine of two patients undergoing cardiac surgery and cardiopulmonary bypass. In (**a**), note the fall in urinary oxygen tension after bypass commenced and the gradual increase when the patient was weaned from bypass. In (**b**), note the fall in urinary PO_2 after weaning from bypass (Redrawn from Kainuma et al. (1996))

The PO_2 of urine from the bladder of patients with circulatory shock was also found to be low (10–15 mmHg), but could be increased by fluid loading. Thus, although oxygen transport between urine and the epithelium of the ureter and bladder alters urinary PO_2, changes in the PO_2 of bladder urine do appear to reflect those in pelvic urine (Fig. 3).

(iv) ***Prognostic value of urinary oxygen tension***: In patients, bladder urine PO_2 has been shown to fall during surgical procedures associated with renal dysfunction (Koivusalo et al. 1998), but not during surgical procedures associated with well-maintained renal function (Laisalmi et al. 2001). It has also been shown to increase in response to blood transfusion in anaemic patients (Valente et al. 2008), and when tubular oxygen consumption is reduced by diuretic therapy (Morelli et al. 2003). In some patients undergoing cardiac surgery requiring CPB, urinary PO_2 progressively decreased after the start of CPB and then partially recovered during weaning from CPB (Kainuma et al. 1996; Fig. 4). In others, it was found to be well maintained during CPB but to then fall after the patient was weaned from CPB. Crucially, post-operative peak serum creatinine was higher in patients whose bladder urine PO_2 decreased following CPB (Fig. 5). Importantly, post-operative recovery of urinary PO_2 predicted post-operative renal function (Fig. 5) better than pre-operative serum creatinine,

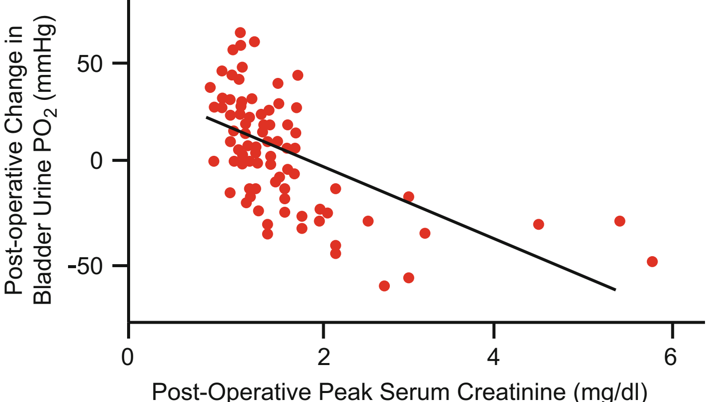

Fig. 5 Scatterplot and line of best fit for the relationship between the peak serum creatinine concentration versus the change in PO_2 of bladder urine in the post-operative period after cardio-pulmonary bypass (Redrawn from Kainuma et al. (1996))

blood urea-nitrogen, duration of CPB, cardiac index or urine flow after CPB. In another study, a fall in the PO_2 of bladder urine was also observed during CPB (Farahani et al. 2010). Thus, there is considerable evidence that bladder urine PO_2 can provide prognostically useful information in the setting of surgery. We are not aware of any measurements of urinary PO_2 in patients with septic AKI or AKI induced by radiocontrast agents or other nephrotoxins.

(v) ***New methods for measurement of urinary*** PO_2: Most previous clinical and experimental studies in which urinary PO_2 has been measured have employed either fragile polarographic electrodes (Aukland and Krog 1961; Leonhardt and Landes 1965; Washington and Holland 1966; Kainuma et al. 1990; Kitashiro et al. 1995; Morelli et al. 2003) or collection of urine for measurement of PO_2 using a standard blood gas analyser (Valente et al. 2008). MRI could be used to measure urinary PO_2 in a non-invasive manner (Wang et al. 2008), but is not feasible for continuous monitoring of patients.

Fluorescence lifetime oximetry provides an opportunity for continuous measurement of urinary PO_2 via a fibre optic probe. The probe can be inserted into a bladder catheter and advanced to its tip, so as to be in direct contact with bladder urine. These optical fibres can be very thin, so risk of obstruction of the bladder catheter is minimal (Fig. 6). Other advantages of this approach are that the fibre optic probes are sturdy; they come pre-calibrated by the manufacturer, and can be operated with complete electrical isolation from the patient. They have been extensively validated (Evans et al. 2008b) and applied to in vivo measurement of the PO_2 in a range of tissues including the kidney (Evans et al. 2011) and in body fluids (e.g., oviductal fluid (Rafferty et al. 2013) and blood (Abdelkader et al. 2014)). However, they are currently not approved for human use.

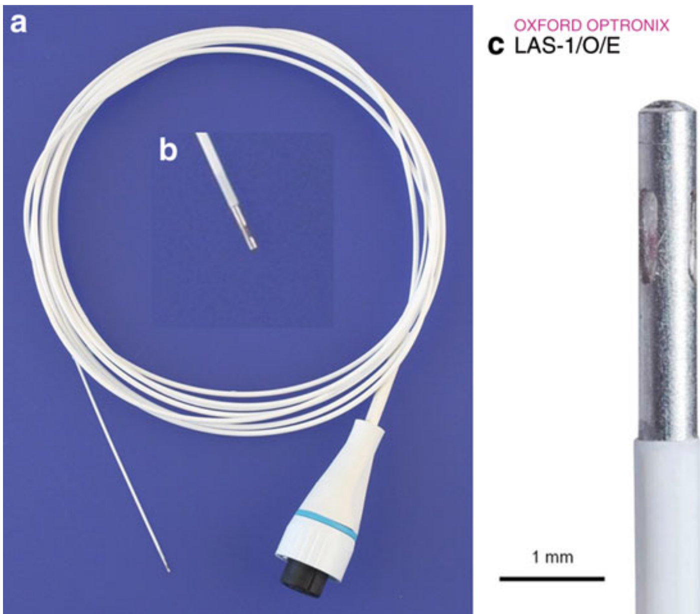

Fig. 6 A fibre optic probe for fluorescence lifetime oximetry. (**a**) Coiled probe with *inset* (**b**) showing the tip of the probe. (**c**) Close up of the probe tip. This particular probe (NX-LAS-1/O/E) is commercially available (Oxford Optronix Milton Park, United Kingdom; http://www.oxford-optronix.com). Note that these probes are not approved for human use (Images were provided courtesy of Oxford Optronix and are reproduced with their permission)

Potential Applications to Prognosis, Other Disease and Conditions

The potential approaches to the use of hypoxia as a biomarker for kidney disease will depend on the specific situation. CKD develops slowly, so a method that can be applied to outpatients and provides relatively good reproducibility within subjects would be ideal. BOLD MRI has these characteristics, but has proved so far to be rather disappointing as a marker of CKD in human subjects. As things currently stand, the balance of evidence does not support the use of BOLD MRI as a diagnostic and prognostic tool in CKD. It is also impractical for continuous monitoring of patients in a hospital setting.

AKI often occurs in a hospital setting. Because of its rapid onset, and because bladder catheterization is standard practice in many of the clinical situations in which patients are at risk of AKI, continuous measurement of urinary PO_2 by fluorescence lifetime oximetry has the potential to provide real time information regarding the risk of AKI. Continuous measurement of urinary PO_2 might be particularly useful in

patients undergoing cardiac surgery requiring CPB and those in the intensive care unit. This information could inform clinical decisions, particularly those regarding renal or haemodynamic support. They could also be incorporated into a computational model of the kidney during surgery (Sgouralis et al. 2014). Such an approach would allow real-time feedback-driven control of circulatory function to optimise management of renal health. However, at least three gaps exist in the evidential basis for such an approach. Firstly, understanding of the roles of tissue hypoxia in the initiation and progression of AKI remains rudimentary (Evans et al. 2013). Secondly, there is limited information regarding the relationship between the PO_2 of urine in the bladder and renal medullary PO_2 (Evans et al. 2014b). Thirdly, although available information regarding the prognostic value of urinary PO_2 are promising, they are limited to a single study of 98 patients undergoing cardiac surgery while on CPB (Kainuma et al. 1996).

Summary Points

- Current methods for diagnosis of chronic kidney disease and acute kidney injury detect disease only once it is well progressed.
- There is evidence that renal hypoxia, a hallmark of both chronic kidney disease and acute kidney injury, both initiates and drives progression of renal disease.
- Blood oxygen level dependent magnetic resonance imaging provides a measure of the deoxyhaemoglobin content of renal tissue, so an indirect measure of renal hypoxia.
- In recent clinical studies, the blood oxygen level dependent magnetic resonance imaging signal has been found to correlate poorly with the degree of renal impairment in chronic kidney disease, so is unlikely to be useful for early diagnosis of chronic kidney disease.
- There is strong evidence that oxygen in urine in the renal pelvis is in equilibrium with oxygen in the renal medulla.
- Despite oxygen exchange between urine and the epithelium of the ureter and bladder, changes in the oxygen tension of urine in the bladder appear to reflect changes in the oxygen tension of pelvic urine, and thus medullary tissue.
- In patients undergoing cardiac surgery requiring cardiopulmonary bypass, post-operative recovery of urinary oxygen tension predicted post-operative renal function better than pre-operative serum creatinine or blood urea-nitrogen, duration of cardiopulmonary bypass, or cardiac index and urine flow after cardiopulmonary bypass.
- Fibre-optic probes using fluorescence lifetime oximetry provide a safe and technically simple method for continuous measurement of the oxygen tension of bladder urine in patients at risk of acute kidney injury in a hospital setting.
- Continuous measurement of urinary oxygen tension may aid in management of patients at risk of developing acute kidney injury, particularly in surgical and intensive care settings.

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References

Abdelkader A, Ho J, Ow CP, et al. Renal oxygenation in acute renal ischemia-reperfusion injury. Am J Physiol Renal Physiol. 2014;306:F1026–38.

Aksu U, Demirci C, Ince C. The pathogenesis of acute kidney injury and the toxic triangle of oxygen, reactive oxygen species and nitric oxide. Contrib Nephrol. 2011;174:119–28.

Andersson LG, Bratteby LE, Ekroth R, et al. Renal function during cardiopulmonary bypass: influence of pump flow and systemic blood pressure. Eur J Cardiothorac Surg. 1994;8:597–602.

Aukland K. Urine oxygen tension; lack of correlation to some renal functions. Acta Physiol Scand. 1962;55:362–75.

Aukland K, Krog J. Influence of various factors on urine oxygen tension in the dog. Acta Physiol Scand. 1961;52:350–65.

Basile DP, Donohoe DL, Roethe K, Mattson DL. Chronic renal hypoxia after acute ischemic injury: effects of L-arginine on hypoxia and secondary damage. Am J Physiol Renal Physiol. 2003;284: F338–48.

Calzavacca P, Evans RG, Bailey M, Bellomo R, May CN. Cortical and medullary tissue perfusion and oxygenation in experimental septic acute kidney injury. Crit Care Med 2015;43:e431–9.

Chen J, Edwards A, Layton AT. A mathematical model of O_2 transport in the rat outer medulla. II. Impact of outer medullary architecture. Am J Physiol Renal Physiol. 2009a;297:F537–48.

Chen J, Layton AT, Edwards A. A mathematical model of O_2 transport in the rat outer medulla. I. Model formulation and baseline results. Am J Physiol Renal Physiol. 2009b;297:F517–36.

Darby PJ, Kim N, Hare GM, et al. Anemia increases the risk of renal cortical and medullary hypoxia during cardiopulmonary bypass. Perfusion. 2013;28:504–11.

Djamali A, Sadowski EA, Muehrer RJ, et al. BOLD-MRI assessment of intrarenal oxygenation and oxidative stress in patients with chronic kidney allograft dysfunction. Am J Physiol Renal Physiol. 2007;292:F513–22.

dos Santos EA, Li LP, Ji L, Prasad PV. Early changes with diabetes in renal medullary hemodynamics as evaluated by fiberoptic probes and BOLD magnetic resonance imaging. Invest Radiol. 2007;42:157–62.

Evans RG, O'Connor PM. Blood flow, oxygenation and oxidative stress in the post-stenotic kidney. In: Lerman LO, Textor SC, editors. Renal vascular disease. London: Springer; 2014. p. 151–71.

Evans RG, Gardiner BS, Smith DW, O'Connor PM. Intrarenal oxygenation: unique challenges and the biophysical basis of homeostasis. Am J Physiol Renal Physiol. 2008a;295:F1259–70.

Evans RG, Gardiner BS, Smith DW, O'Connor PM. Methods for studying the physiology of kidney oxygenation. Clin Exp Pharmacol Physiol. 2008b;35:1405–12.

Evans RG, Goddard D, Eppel GA, O'Connor PM. Factors that render the kidney susceptible to tissue hypoxia in hypoxemia. Am J Physiol Regul Integr Comp Physiol. 2011;300: R931–40.

Evans RG, Ince C, Joles JA, et al. Haemodynamic influences on kidney oxygenation: clinical implications of integrative physiology. Clin Exp Pharmacol Physiol. 2013;40:106–22.

Evans RG, Harrop GK, Ngo JP, Ow CP, O'Connor PM. Basal renal oxygen consumption and the efficiency of oxygen utilization for sodium reabsorption. Am J Physiol Renal Physiol. 2014a;306:F551–60.

Evans RG, Smith JA, Wright C, Gardiner BS, Smith DW, Cochrane AD. Urinary oxygen tension: a clinical window on the health of the renal medulla? Am J Physiol Regul Integr Comp Physiol. 2014b;306:R45–50.

Farahani J, Mohammadi M, Naseri SMH, Ghiasi SMS. Urine oxygen pressure measurement as an early renal function assessment in patients undergoing open heart surgery. Kowsar Med J. 2010;15:167–9.
Fine LG, Norman JT. Chronic hypoxia as a mechanism of progression of chronic kidney diseases: from hypothesis to novel therapeutics. Kidney Int. 2008;74:867–72.
Fine LG, Orphanides C, Norman JT. Progressive renal disease: the chronic hypoxia hypothesis. Kidney Int Suppl. 1998;65:S74–8.
Fong D, Denton KM, Moritz KM, Evans R, Singh RR. Compensatory responses to nephron deficiency: adaptive or maladaptive? Nephrology. 2014;19:119–28.
Friederich-Persson M, Thorn E, Hansell P, Nangaku M, Levin M, Palm F. Kidney hypoxia, attributable to increased oxygen consumption, induces nephropathy independently of hyperglycemia and oxidative stress. Hypertension. 2013;62:914–9.
Gloviczki ML, Glockner JF, Lerman LO, et al. Preserved oxygenation despite reduced blood flow in poststenotic kidneys in human atherosclerotic renal artery stenosis. Hypertension. 2010;55:961–6.
Gloviczki ML, Glockner JF, Crane JA, et al. Blood oxygen level-dependent magnetic resonance imaging identifies cortical hypoxia in severe renovascular disease. Hypertension. 2011;58:1066–72.
Haase M, Bellomo R, Haase-Fielitz A. Novel biomarkers, oxidative stress, and the role of labile iron toxicity in cardiopulmonary bypass-associated acute kidney injury. J Am Coll Cardiol. 2010;55:2024–33.
Hansell P, Welch WJ, Blantz RC, Palm F. Determinants of kidney oxygen consumption and their relationship to tissue oxygen tension in diabetes and hypertension. Clin Exp Pharmacol Physiol. 2013;40:123–37.
Heyman SN, Brezis M, Epstein FH, Spokes K, Silva P, Rosen S. Early renal medullary hypoxic injury from radiocontrast and indomethacin. Kidney Int. 1991;40:632–42.
Heyman SN, Rosen S, Rosenberger C. Renal parenchymal hypoxia, hypoxia adaptation, and the pathogenesis of radiocontrast nephropathy. Clin J Am Soc Nephrol. 2008;3:288–96.
Heyman SN, Rosenberger C, Rosen S. Experimental ischemia-reperfusion: biases and myths-the proximal vs. distal hypoxic tubular injury debate revisited. Kidney Int. 2010;77:9–16.
Heyman SN, Evans RG, Rosen S, Rosenberger C. Cellular adaptive changes in AKI: mitigating renal hypoxic injury. Nephrol Dial Transplant. 2012;27:1721–8.
Hofmann L, Simon-Zoula S, Nowak A, et al. BOLD-MRI for the assessment of renal oxygenation in humans: acute effect of nephrotoxic xenobiotics. Kidney Int. 2006;70:144–50.
Huen SC, Parikh CR. Predicting acute kidney injury after cardiac surgery: a systematic review. Ann Thorac Surg. 2012;93:337–47.
Inoue T, Kozawa E, Okada H, et al. Noninvasive evaluation of kidney hypoxia and fibrosis using magnetic resonance imaging. J Am Soc Nephrol. 2011;22:1429–34.
Kainuma M, Kimura N, Shimada Y. Effect of acute changes in renal arterial blood flow on urine oxygen tension in dogs. Crit Care Med. 1990;18:309–12.
Kainuma M, Yamada M, Miyake T. Continuous urine oxygen tension monitoring in patients undergoing cardiac surgery. J Cardiothorac Vasc Anesth. 1996;10:603–8.
Karkouti K, Wijeysundera DN, Yau TM, et al. Acute kidney injury after cardiac surgery: focus on modifiable risk factors. Circulation. 2009;119:495–502.
Kitashiro S, Iwasaka T, Sugiura T, et al. Monitoring urine oxygen tension during acute change in cardiac output in dogs. J Appl Physiol. 1995;79:202–4.
Koivusalo AM, Kellokumpu I, Scheinin M, Tikkanen I, Makisalo H, Lindgren L. A comparison of gasless mechanical and conventional carbon dioxide pneumoperitoneum methods for laparoscopic cholecystectomy. Anesth Analg. 1998;86:153–8.
Laisalmi M, Koivusalo AM, Valta P, Tikkanen I, Lindgren L. Clonidine provides opioid-sparing effect, stable hemodynamics, and renal integrity during laparoscopic cholecystectomy. Surg Endosc. 2001;15:1331–5.
Lambers Heerspink HJ, de Zeeuw D. Novel drugs and intervention strategies for the treatment of chronic kidney disease. Br J Clin Pharmacol. 2013;76:536–50.

Langenberg C, Bellomo R, May C, Wan L, Egi M, Morgera S. Renal blood flow in sepsis. Crit Care. 2005;9:R363–74.

Lenihan CR, Montez-Rath ME, Mora Mangano CT, Chertow GM, Winkelmayer WC. Trends in acute kidney injury, associated use of dialysis, and mortality after cardiac surgery, 1999 to 2008. Ann Thorac Surg. 2013;95:20–8.

Leonhardt KO, Landes RR. Oxygen tension of the urine and renal structures. Preliminary report of clinical findings. N Engl J Med. 1963;269:115–21.

Leonhardt KO, Landes RR. Urinary oxygen pressure in renal parenchymal and vascular disease. Effects of breathing oxygen. JAMA. 1965;194:345–50.

Leonhardt KO, Landes RR, McCauley RT. Anatomy and physiology of intrarenal oxygen tension: preliminary study of the effects of anesthetics. Anesthesiology. 1965;26:648–58.

Liss P, Nygren A, Erikson U, Ulfendahl HR. Injection of low and iso-osmolar contrast medium decreases oxygen tension in the renal medulla. Kidney Int. 1998;53:698–702.

Manotham K, Tanaka T, Matsumoto M, et al. Evidence of tubular hypoxia in the early phase in the remnant kidney model. J Am Soc Nephrol. 2004;15:1277–88.

McCullough PA. Radiocontrast-induced acute kidney injury. Nephron Physiol. 2008;109:61–72.

Mehta RL, Chertow GM. Acute renal failure definitions and classification: time for change? J Am Soc Nephrol. 2003;14:2178–87.

Michaely HJ, Metzger L, Haneder S, Hansmann J, Schoenberg SO, Attenberger UI. Renal BOLD-MRI does not reflect renal function in chronic kidney disease. Kidney Int. 2012;81:684–9.

Mimura I, Nangaku M. The suffocating kidney: tubulointerstitial hypoxia in end-stage renal disease. Nat Rev Nephrol. 2010;6:667–78.

Morelli A, Rocco M, Conti G, et al. Monitoring renal oxygen supply in critically-ill patients using urinary oxygen tension. Anesth Analg. 2003;97:1764–8.

Mori T, Shimizu T, Tani T. Septic acute renal failure. Contrib Nephrol. 2010;166:40–6.

Neugarten J. Renal BOLD-MRI and assessment for renal hypoxia. Kidney Int. 2012;81:613–4.

O'Connor PM, Anderson WP, Kett MM, Evans RG. Renal preglomerular arterial-venous O_2 shunting is a structural anti-oxidant defence mechanism of the renal cortex. Clin Exp Pharmacol Physiol. 2006a;33:637–41.

O'Connor PM, Kett MM, Anderson WP, Evans RG. Renal medullary tissue oxygenation is dependent on both cortical and medullary blood flow. Am J Physiol Renal Physiol. 2006b;290:F688–94.

Okusa MD, Jaber BL, Doran P, et al. Physiological biomarkers of acute kidney injury: a conceptual approach to improving outcomes. Contrib Nephrol. 2013;182:65–81.

Ow CPC, Abdelkader A, Hilliard LM, Phillips JK, Evans RG. Determinants of renal tissue hypoxia in a rat model of polycystic kidney disease. Am J Physiol Regul Integr Comp Physiol. 2014;307: R1207–15.

Pannabecker TL, Dantzler WH. Three-dimensional architecture of inner medullary vasa recta. Am J Physiol Renal Physiol. 2006;290:F1355–66.

Parmar A, Langenberg C, Wan L, May CN, Bellomo R, Bagshaw SM. Epidemiology of septic acute kidney injury. Curr Drug Targets. 2009;10:1169–78.

Patel NN, Lin H, Toth T, et al. Reversal of anemia with allogenic RBC transfusion prevents post-cardiopulmonary bypass acute kidney injury in swine. Am J Physiol Renal Physiol. 2011a;301: F605–14.

Patel NN, Rogers CA, Angelini GD, Murphy GJ. Pharmacological therapies for the prevention of acute kidney injury following cardiac surgery: a systematic review. Heart Fail Rev. 2011b;16:553–67.

Patel NN, Toth T, Jones C, et al. Prevention of post-cardiopulmonary bypass acute kidney injury by endothelin A receptor blockade. Crit Care Med. 2011c;39:793–802.

Pedersen M, Dissing TH, Morkenborg J, et al. Validation of quantitative BOLD MRI measurements in kidney: application to unilateral ureteral obstruction. Kidney Int. 2005;67:2305–12.

Perazella MA. Renal vulnerability to drug toxicity. Clin J Am Soc Nephrol. 2009;4:1275–83.

Pohlmann A, Cantow K, Hentschel J, et al. Linking non-invasive parametric MRI with invasive physiological measurements (MR-PHYSIOL): towards a hybrid and integrated approach for investigation of acute kidney injury in rats. Acta Physiol. 2013;207:673–89.
Prasad PV, Priatna A, Spokes K, Epstein FH. Changes in intrarenal oxygenation as evaluated by BOLD MRI in a rat kidney model for radiocontrast nephropathy. J Magn Reson Imaging. 2001;13:744–7.
Prasad P, Li LP, Halter S, Cabray J, Ye M, Batlle D. Evaluation of renal hypoxia in diabetic mice by BOLD MRI. Invest Radiol. 2010;45:819–22.
Pruijm M, Hofmann L, Piskunowicz M, et al. Determinants of renal tissue oxygenation as measured with BOLD-MRI in chronic kidney disease and hypertension in humans. PLoS One. 2014;9: e95895.
Rafferty AR, Evans RG, Scheelings TF, Reina RD. Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. Am Nat. 2013;181:245–53.
Reeves RB, Rennie DW, Pappenheimer JR. Oxygen tension of urine and its significance. Fed Proc. 1957;16:693–6.
Rennie DW, Reeves RB, Pappenheimer JR. Oxygen pressure in urine and its relation to intrarenal blood flow. Am J Physiol. 1958;195:120–32.
Rosenberger C, Khamaisi M, Abassi Z, et al. Adaptation to hypoxia in the diabetic rat kidney. Kidney Int. 2008;73:34–42.
Rosner MH, Portilla D, Okusa MD. Cardiac surgery as a cause of acute kidney injury: pathogenesis and potential therapies. J Intensive Care Med. 2008;23:3–18.
Sgouralis I, Evans RG, Gardiner BS, Smith JA, Fry BC, Layton AT. Renal hemodynamics, function and oxygenation during cardiac surgery performed on cardiopulmonary bypass: a modeling study. Physiol Rep. 2014;3:e12260.
Stafford-Smith M, Grocott HP. Renal medullary hypoxia during experimental cardiopulmonary bypass: a pilot study. Perfusion. 2005;20:53–8.
Ullman J, Eriksson S, Rundgren M. Losartan increases renal blood flow during isoflurane anesthesia in sheep. Acta Anaesthesiol Scand. 2001;45:1168–75.
Valente A, Sorrentino L, La Torre G, Draisci G. Post-transfusional variation in urinary oxygen tension in surgical patients. Clin Exp Pharmacol Physiol. 2008;35:1109–12.
Wang ZJ, Joe BN, Coakley FV, Zaharchuk G, Busse R, Yeh BM. Urinary oxygen tension measurement in humans using magnetic resonance imaging. Acad Radiol. 2008;15:1467–73.
Washington 2nd JA, Holland JM. Urine oxygen tension: effects of osmotic and saline diuresis and of ethacrynic acid. Am J Physiol. 1966;210:243–50.
Zarjou A, Agarwal A. Sepsis and acute kidney injury. J Am Soc Nephrol. 2011;22:999–1006.
Zhang W, Edwards A. Oxygen transport across vasa recta in the renal medulla. Am J Physiol Heart Circ Physiol. 2002;283:H1042–55.
Zhang JL, Morrell G, Rusinek H, et al. Measurement of renal tissue oxygenation with blood oxygen level-dependent MRI and oxygen transit modeling. Am J Physiol Renal Physiol. 2014;306: F579–87.

MicroRNAs in Kidney Diseases

7

Grazia Serino, Fabio Sallustio, and Francesco Paolo Schena

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Abstract

MicroRNAs (miRNAs) play an important role in physiological and pathological condition in human organs including kidneys. Their dysregulation on one hand can induce the onset of a specific disease and on the other hand may represent potential biomarkers for the diagnosis and therapy. In this chapter, the miRNA functions and modulations are described in several kidney diseases such as polycystic kidney disease, primary and secondary glomerulonephritides and renal transplantation. After the overview on the approach to identify and study miRNAs in nephrology, the miRNA meaning in renal physiology is illustrated. Also, the attractive perspectives of the use of miRNAs as diagnostic tools or for the specific treatment of kidney disease are taken into consideration.

Keywords

MicroRNA • Kidney physiology • Polycystic kidney disease • Glomerulonephritis • Kidney transplantation

Abbreviations

AGO	Argonaute
AKI	Acute kidney injury
ANA	Autoantibodies to nuclear antigen
ASO	Antisense oligonucleotide
Bicc1	Bicaudal C. homolog 1
CAMR	Chronic antibody mediated rejection
Cdc25A	Cell division cycle 25A
cDNA	Complementary DNA
DGCR8	RNA-binding protein DiGeorge syndrome critical region gene 8
DGF	Delayed graft function
EMT	Epithelial-to-mesenchymal transition
FPC	Fibrocystin
GTP	Guanosine triphosphate
HBEGF	Heparin binding epidermal growth factor
HCV	Hepatitis C virus
HIVAN	HIV associated nephropathy
IF	Interstitial fibrosis
IFN	Interferon
IRAK1	IL-1 receptor associated kinase1
IRI	Ischemia-reperfusion injury

LN	Lupus nephritis
miRNA	MicroRNA
MRE	MiRNA recognition elements
OREB	Osmotic response element binding protein
PAZ	Piwi-Argonaute-Zwille
PBMCs	Peripheral blood mononuclear cells
PC	Polycystin
PKD	Polycystic kidney disease
pri-miRNA	Primary miRNA transcript
RISC	RNA-induced silencing complex
shRNA	Small hairpin RNA
SLE	Systemic lupus erythematosus
TA	Tubular atrophy
TAL	Thick ascending limb
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRAF6	Tumor necrosis factor receptor-associated factor 6
TRBP	Transactivator RNA binding protein
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VLP	Virus like particles
WMLK	With-no-lysine kinase

Key Facts

Key Facts of Renal Physiology

- The juxtaglomerular apparatus, located in the wall of the afferent arterioles of glomeruli, regulates the blood flow at renal level and controls the blood pressure by the release of renin.
- Acute kidney injury is the consequence of reduced or fallen arterial blood pressure caused by hemorrhagic shock or of toxic effect of agents or drugs.
- Chronic renal failure is the consequence of chronic kidney diseases characterized by persistent hypoxic injury that causes renal fibrosis.
- Ischemia/reperfusion injury is a process occurring in kidney transplantation. The hypoxic injury followed by fluid reperfusion leads to the activation of innate and adaptive immune responses and to the delivery of cytokines and growth factors.
- Glomerular filtration is the process of blood ultrafiltration at the level of glomerular membrane that is formed by three layers: (i) endothelial cells; (ii) capillary basement membrane; (iii) visceral epithelial cells (podocytes). Filtration is determined by the molecular size and charge of proteins.

- The entire tubular part of nephron provides the salt and water reabsorption and electrolyte excretion by regulating the electrolyte homeostasis.
- The medullary part of the kidney modulates the osmoregulation response and water handling under physiological and pathological conditions.

Key Facts of PKD

- The autosomal dominant polycystic kidney disease (ADPKD) is characterized by cysts in kidneys and sometimes in liver and/or other organs (pancreas, arachnoid membrane). This common genetic disorder clinically manifests in adulthood, often leading to end-stage kidney disease with renal replacement therapy (dialysis or kidney transplantation).
- The autosomal recessive polycystic kidney disease (ARPKD) is a rare cystic disease that affects the renal tubules and the biliary tract. This rare genetic disorder typically begins in utero and it is associated with congenital hepatic fibrosis.
- The mechanosensory organelle localized in the apical surface of the cilia in tubular cells has the important role in regulating the fluid flow and in signaling the intracellular calcium.
- In the tubular cells of PKD there is the dysregulation of some intracellular pathways like MAPK, ERK and AKT/mTOR leading to abnormality in cell proliferation and cyst expansion.

Key Facts of Primary and Secondary Glomerulonephritis

- IgA Nephropathy (IgAN) is characterized by mesangial proliferative lesions associated with diffuse deposition of IgA1-IgG immune complexes. The clinical symptoms are recurrent episodes of gross hematuria in concomitance of upper respiratory tract infections or permanent benign microscopic hematuria.
- Minimal change disease (MCD) is a glomerular lesion characterized by fusion of epithelial cell foot processes that is responsible for an abnormal filtration of albumin and transferrin through the filtration barrier. It is the principal cause of nephrotic syndrome in children younger than 10 years. May be corticosteroid responsive, non-responsive or dependent.
- Focal Segmental Glomerulosclerosis (FSGS) is characterized by a glomerulo-sclerotic lesion that involves a minority of glomeruli located in deep section of renal cortex and a portion of the glomerular tuft. For this reason the renal lesion is defined focal and segmental. It is more frequent in adults than in children.
- Membranous glomerulonephritis (MGN) is an immune complex disease in which deposits of immunoglobulins and complement are located in the subepithelial surface of the glomerular capillary wall. The increasing thickness of the glomerular basement membrane causes proteinuria with development of nephrotic syndrome. It is more frequent in adults than in children.

- C3-glomerulopathy is a glomerular disease characterized by deposits of C3 alone associated with mesangial and endocapillary proliferation and crescents. The clinical manifestations are proteinuria and microscopic hematuria, but in two thirds of patients is present nephrotic syndrome.
- Crescentic glomerulonephritis (CGN) is characterized by abnormal formation of crescents (more than 50 %) that may occur in different forms of glomerulonephritis. The crescentic lesion is formed by epithelial proliferation of the Bowman capsule delimiting the glomerular tuft.
- Lupus nephritis (LN) is the kidney involvement of systemic lupus erythematosus caused by glomerular deposition of circulating immune complexes. The polymorphism of renal lesions and the different stages of the disease have been included in the international classification of LN for the reading of the renal biopsy. This approach is necessary for diagnosis, prognosis and therapy.
- HIV-associated nephropathy is a collapsing glomerulopathy characterized by involvement of glomeruli (proliferation and dysregulation of podocytes, and glomerular collapse), tubules (microcystic changes) and interstitium (chronic inflammation and fibrosis). Nephrotic syndrome and failure of the renal function are the clinical manifestations.

Key Facts of Kidney Transplantation

- Ischemia-reperfusion injury (IRI) is a damage in the tissue due to the blood supply return to the tissue after a period of ischemia or lack of oxygen.
- Delayed graft function is a form of acute renal failure resulting in low output of urine after transplantation.
- Interstitial fibrosis and tubular atrophy (IF/TA) is a common histological abnormality of kidney transplants in which an expanded interstitial space replaces normal cortical structures.
- Chronic antibody mediated rejection (CAMR) is an important cause of allograft dysfunction and graft loss.

Key Facts of miRNA Therapy

- The antimiR oligonucleotides are chemically modified molecules in which sugar modifications are included for increasing the duplex melting temperature and the nuclear resistance.
- The locked nucleic acid is a bicyclic RNA analogue in which the introduced 2′-O, 4′-C methylene locks the ribose.
- Morpholino oligomer is a six-numbered morpholine ring that replaces the sugar in the oligo nucleotide thus increasing the binding affinity with the cognate miRNA.
- MiRNA mimic is composed of an antisense (guide) that is identical to the cognate miRNA, whereas the sense strand (passenger) is modified and linked to a macromolecule to enhance the uptake.

Definitions

Antisense oligonucleotide inhibitors Antisense oligonucleotide inhibitors are unmodified or chemically modified single-stranded molecules that specifically bind mature miRNAs and inhibit their function.

Argonaute Argonaute is a family of proteins involved in RNA silencing process. They are components of RNA-induced silencing complex (RISC) in which have the role to cleave the target mRNA strand complementary to the bound miRNA.

Dicer Dicer is a large protein that contains several domains: ATPase/RNA helicase, a DUF283 (Domain of unknown function) domain, a PAZ (Piwi, Argonaut and Zwille) domain, two catalytic RNase III domains (RIIIa and RIIIb), and a C-terminal double-stranded RNA-binding domain (dsRBD) is an enzyme with endonuclease activity that cleaves the precursor-miRNA in double strand miRNA.

Locked nucleic acid (LNA) Locked nucleic acid (LNA) is an oligonucleotide that contains one or more RNA modified nucleotides in which the ribose moiety is modified with an extra methylene bridge that connects the $2'$ oxygen and $4'$ carbon. This type of oligonucleotide offers an increased affinity for its complementary strand.

miRNAsponges miRNA sponges are transcripts with repeated miRNA antisense sequences that contain complementary binding sites to a miRNA of interest. As with most miRNA target genes, sponge's binding sites are specific to the miRNA seed region, which allows them to block a whole family of related miRNAs.

Ran-GTP Ran (RAs-related Nuclear) protein is a small nuclear protein that is required for the translocation of RNA and/or proteins through the nuclear membrane. Ran binds either GTP and GDP, in particular nuclear Ran is mainly in the GTP form and cytoplasmic Ran is in the GDP-bound form.

Transfection Transfection is a process that permits the introduction of nucleic acids into cells.

Introduction: Biogenesis of miRNAs

MicroRNAs (miRNAs) are a class of small non-coding single strand RNAs (19–25 nucleotides) that regulate gene expression at the post-transcriptional level by targeting messenger RNAs for translation repression or, less frequently, degradation (Ambros 2004). MiRNAs represent the 1–5 % of all genes and are present in the genome as independent genes or in intron regions of coding genes. The high sequence conservation of many miRNAs among distantly organisms suggests strong evolutionary pressure and participation in essential processes.

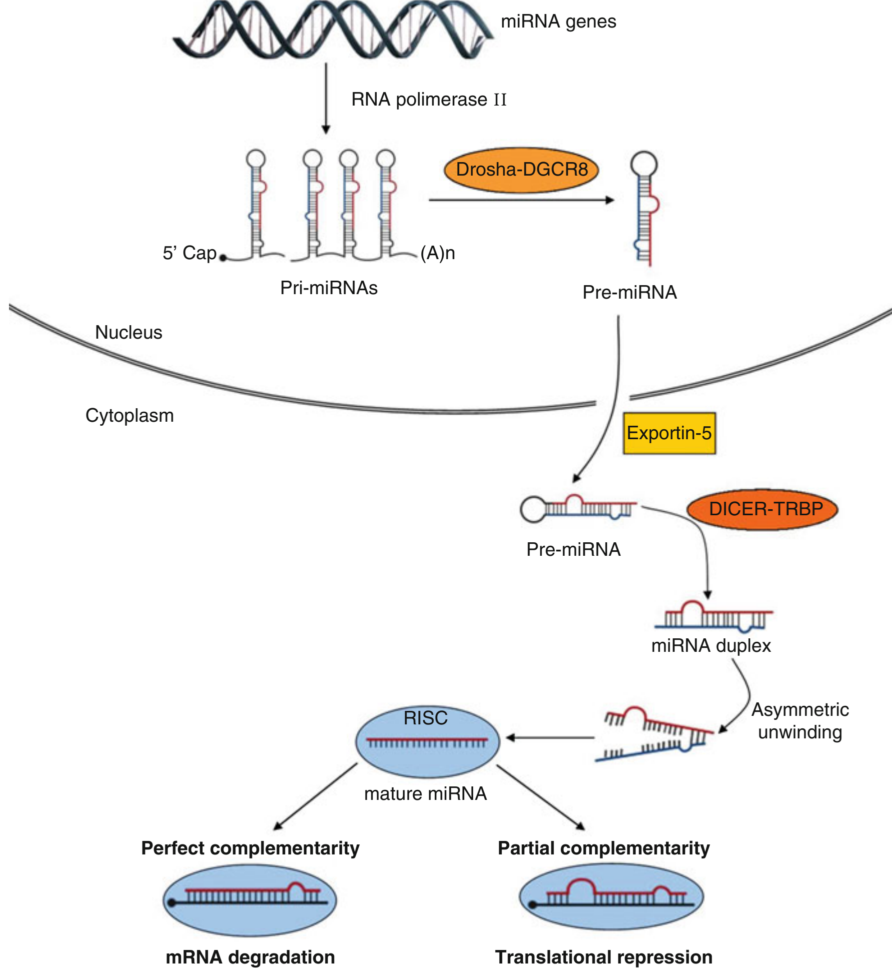

Fig. 1 Biogenesis of miRNAs

The biogenesis of miRNAs starts from the transcription of miRNA genes in the nucleus (Fig. 1). MiRNA genes are transcribed by RNA polymerase II in primary miRNA transcript (pri-miRNA); some miRNAs are transcribed by RNA polymerase III in repetitive regions of genome. Pri-miRNAs contain a stem loop structure that encodes the functional miRNA sequences in the stem. This stem loop structure is precisely cut by the nuclear RNAse III type enzyme called Drosha, as part of a complex called Microprocessor that also contains the RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8). DGCR8 recognizes and interacts with the hairpin structure in the pri-miRNAs and recruits Drosha. These pri-miRNAs are processed in fragments of RNA composed of ~70 nucleotides, called precursor miRNAs or pre-miRNAs. The pre-miRNAs are transported from the

nucleus to the cytoplasm by Exportin-5, a nuclear export factor that specifically binds to the pre-miRNA in a Ran-GTP dependent manner. Within the cytoplasm, a second cleavage event occurs via the RNAse III enzyme Dicer, which forms the mature double stranded miRNA composed of ~22-nucleotides. Like Drosha, Dicer is associated with dsRNA-binding domain containing a protein partner known as Loquacious/TRBP (transactivator RNA binding protein). In addition to its involvement in mature miRNA generation, the Dicer-containing protein complex regulates also the assembly of mature miRNA into the effector complex called RNA-induced silencing complex (RISC)-like ribonucleoprotein particle (miRNP).

After strand separation of the duplexes, the mature single-stranded miRNA (guide strand) is incorporated into RISC, whereas the other strand is often degraded. The main constituents of the RISC are members of the Argonaute (AGO) family, which in humans include eight members, of which four are ubiquitously expressed and are associated with siRNA and miRNA, and four seem restricted to expression in the germ line, whose function is poorly understood. Argonaute is a basic protein consisting of a Piwi-Argonaute-Zwille (PAZ) domain that mediates nucleic acid binding, a middle domain that seems to be critical association between the RNA and AGO, and a PIWI domain that contains endonuclease activity. This complex inhibits mRNA translation or reduces mRNA stability following imperfect binding of the guide strand to miRNA recognition elements (MRE) within the 3′-untraslated region (UTR) of target genes. MiRNAs bind their target RNAs in the 3′UTR through RNA-RNA base pairing that involves not only the Watson-Crick pairs but also the G:U pair. The miRNA-binding sequence in the target is mediated by the “seed” region localized at residues 2–8 at the 5′-end, although it also appears to be influenced by additional factors such as the presence and cooperation between multiple MREs, the spacing between MREs, the proximity to the stop codon, position within the 3′UTR, AU composition, and target mRNA secondary structure. Since the specificity between miRNA and mRNA is mainly determined by Watson-Crick base pairing, each miRNA can potentially regulates the translation of a large number of different mRNAs, and each mRNA can possess multiple binding sites for a single or for many different miRNAs.

New Strategies for Detection of Renal miRNA

The approach to identify and study miRNAs in kidney physiology and renal diseases is summarized in Fig. 2. The experimental procedure can be divided into three steps: (i) detection of miRNAs, (ii) individuation of miRNA target genes and (iii) identification of cellular processes affected by specific miRNAs and functional studies. These experimental methods have been extensively used to identify miRNA regulation in renal and other pathophysiological conditions.

The first step of miRNA research is their detection. Since the expression of miRNA genes does not correspond to the expression level of mature miRNAs, only the quantification of the mature miRNA level allows to determine whether a miRNA is present and/or is regulated in a cell or a tissue. After the first miRNA discovery, the quantification of miRNA sequences has been challenged; nowadays,

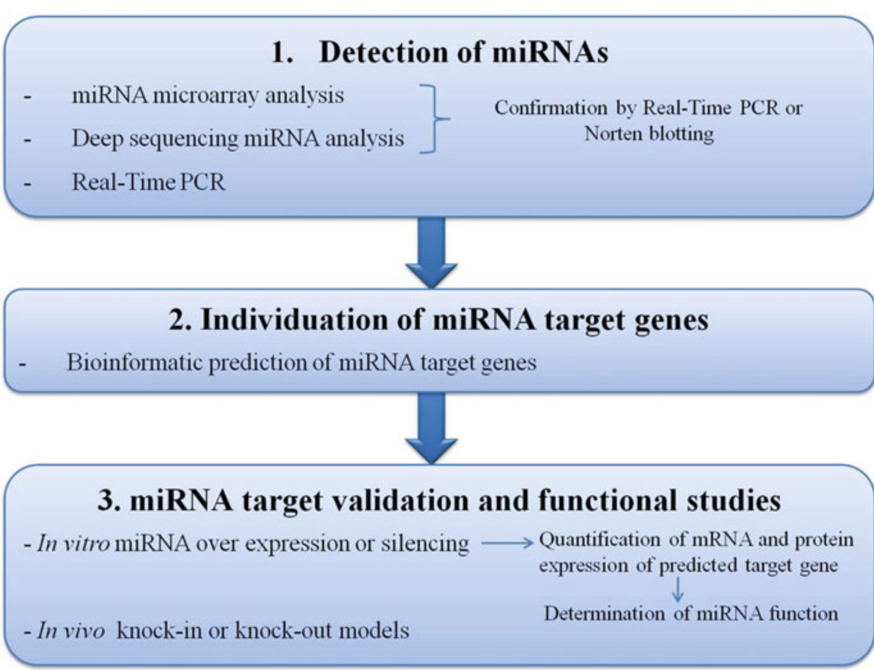

Fig. 2 Flow-chart that describes the steps to study miRNA in renal physiology and kidney diseases. The experimental procedure can be divided into three sections: detection of miRNAs, individuation of miRNA target genes and identification of cellular processes affected by specific miRNAs and functional studies

advanced technologies facilitate their detection. The expression profiles of different miRNAs in parallel can be measured by microarray analysis or deep sequencing, whereas quantitative real-time RT-PCR and Northern blotting can be used to determine the level of individual miRNAs.

Microarray technology is a high-throughput method that allows the parallel screening of thousands of miRNAs in one sample. Although miRNA microarray offers a good tool to examine the miRNA that are expressed or deregulated in a cell or tissue of interest, these data should be considered as a guide and should be validated by other methods.

In addition to using microarray analysis, in the last years, next generation sequencing platforms allow the sequencing of small RNA molecules, including miRNAs. Deep sequencing utilizes massively parallel sequencing, generating millions of small RNA sequence reads from a given sample. The sensitivity of deep sequencing offers several advantages over microarray technology. First, unlike microarray, sequencing is not limited to detecting miRNAs that correspond to existing sequences, but this allows the discovery of novel miRNAs and could reveal variations in the sequence. Second, sequencing has very low background noise and cross hybridization; however, since deep-sequencing experiments produce a lot of sequence data, many bioinformatic skills are necessary for their analysis.

To date the common method to detect the level of specific miRNAs is real-time RT-PCR that has high sensitivity, specificity and it is cheap. There are two different approaches for this reaction; the first one comprises double-stranded DNA intercalating molecules, such as SYBR Green I and EvaGreen; the second one includes fluorophore-labeled oligonucleotides. A miRNA real-time reaction starts with RNA that is reverse transcribed into cDNA. The short length of the mature miRNA, the lack of a common sequence like a poly(A) tail, and the fact that the mature miRNA sequence is also present in the pri- and pre-miRNA transcript mean that the reaction is different from the classical reverse transcription. Currently, two methods are used for the reverse transcription: miRNA-specific and universal reverse transcription. In the first method, individual miRNAs in tissue or in cells are reverse transcribed specifically by using stem-loop-specific reverse transcription primers. Stem-loop primers are designed to have a short single-stranded region that is complementary to the known sequence on the 3′ end of the miRNA, a double-stranded part (the stem), and the loop that contains the universal primer-binding sequence. The resulting cDNA is then used as a template for the real-time RT-PCR with a miRNA-specific primer and a second universal primer. The second approach initially adds a poli-A tail to all miRNAs and then reverse transcribes all miRNAs by means of a universal primer. A primer consisting of an oligo(dT) sequence with a universal primer-binding sequence is then used to amplify the specific miRNA in the real-time RT-PCR.

More recently, real time PCR has been adapted to quantify also precursors and primary transcripts. Moreover, miRNA real-time PCR array has been developed for the screening of a small number of miRNAs especially in samples from body fluids (blood and urine). The miRNA profiles depict the possibility to discover disease biomarkers that can easily measured in samples collected in a non-invasively manner and can promote the monitoring of the disease outcome.

An approach to visualize specific mature miRNAs and pre-miRNAs is the Northern blotting technique. It involves the use of electrophoresis to separate RNA samples by size and detection with a hybridization probe complementary to miRNA sequence.

The profile of miRNAs varies greatly between tissues (organ-specific), cells (cell-specific) and during the phase of activity in a cell (phase-specific). The average half-life of miRNA is approximately 5 days.

MiRNAs have been also studied in renal biopsy (frozen or formalin-fixed paraffin-embedded renal tissue) through combined approach of "in situ" hybridization and immunohistochemistry. Mostly biopsy samples exist in formalin-fixed paraffin-embedded archives and the possibility to use this enormous resource could permit to organize mechanistic studies in humans. Another interesting approach is the study of miRNA expression in isolated glomeruli or tubules obtained by laser-captured kidney tissue.

After the individuation of a list of miRNAs that differentiate two conditions (e.g. normal and disease), the most important step is to predict and then to confirm the miRNA target genes (Fig. 2). First, several bioinformatic programs or databases, as miRBase (http://microrna.sanger.ac.uk), TargetScan (http://www.targetscan.org), PicTar (http://pictar.org), and miRWalk (http://www.ma.uni-heidelberg.de) are available to predict the potential target genes for each miRNA. Since each miRNA can regulate the translation of numerous mRNAs, and each mRNA possess multiple

binding sites for a single or for many different miRNAs, these algorithms generate numerous putative target genes (Brodersen and Voinnet 2009). Second, after finding potential targets for miRNAs, it is essential to validate biologically the relationship between a miRNA and a mRNA. To deepen the study of the pathophysiological role of an identified miRNAs, it is possible to up-regulate specific miRNA levels in cells or knockdown specific miRNA activity (Fig. 2). There are two main approaches to study experimentally the regulation of a specific gene by miRNA: the use of a miRNA mimic or a miRNA inhibitor. MiRNA mimics are small, chemically modified double-stranded RNAs that mimic mature endogenous miRNAs after transfection into cells. Instead, miRNA inhibitors are antisense oligonucleotides, chemically synthesized, which specifically inhibit endogenous miRNA function after transfection into cells. After transfection, the miRNA target gene must be measured at mRNA and protein levels.

Once the target protein has been biologically validated, it is important to identify its functional role in the pathophysiological conditions in order to provide a comprehensive view of the pathogenesis and leads to the development of novel therapeutic approaches.

MiRNAs in Renal Physiology

The expression profile of miRNAs is different in organs. Kidney compared to other organs has high expression of miR-192, miR-194, miR-204, miR-215 and miR-216 (Sun et al. 2004); however other miRNAs expressed in other organs are present at lower level. Tian et al. (2008) demonstrated a different distribution of miRNAs in renal cortex and medulla of Sprague Dawley rats; these miRNAs have different target genes and explain the different function in the sections of kidney.

In this section the different expression of miRNAs that contribute to the normal renal physiology like blood flow, glomerular filtration, tubular reabsorption and excretion, and interstitial osmolarity are described.

Blood Flow and Oxygen Supply

Blood pressure is modulated at renal level by the juxtaglomerular cell apparatus that produces renin. Sequeira-Lopez et al. (2010) studied the participation of miRNAs in hypertensive kidney disease by inducing the ablation of the Dicer enzyme in the juxtaglomerular cells (Fig. 3). They obtained a reduced number of juxtaglomerular cells with low expression of renin genes (Ren1 and Ren2), decreased plasma renin and low blood pressure in the knockout Dicer mouse that developed nephrovascular abnormalities and corticomedullary fibrosis.

Kidney hypoxia is characterized by a reduction of oxygen in site. It occurs either in the acute process of ischemia (duration: seconds/minutes) leading to the acute kidney injury or in the chronic process (duration: hours/days) leading to chronic renal failure and end-stage kidney disease. Many investigators have demonstrated that intracellular signaling pathways involved in hypoxia are modulated by miRNAs

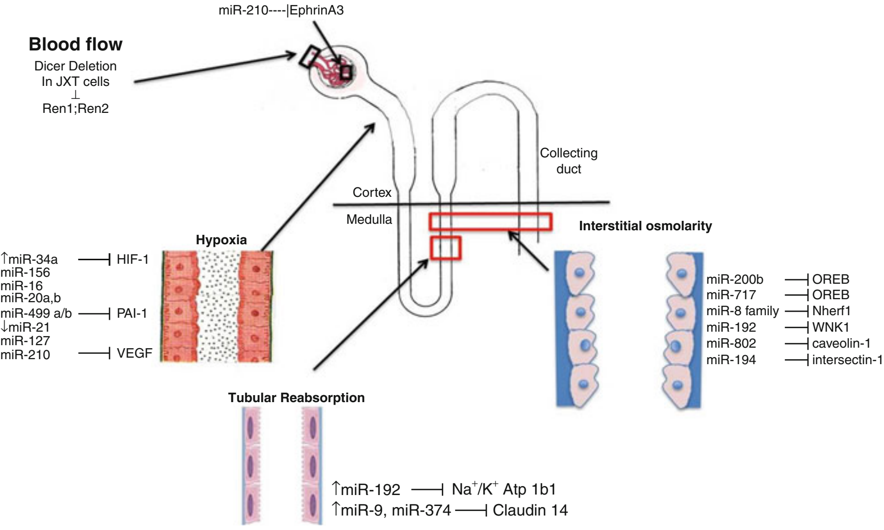

Fig. 3 Participation of miRNAs in modulating the kidney functions

(Fig. 3). Hypoxia causes renal damage because the activation of HIF-1 signal in epithelial tubular cells under low oxygen induces downregulation of miR-34a expression and activation of Notch signaling pathway leading to epithelial-mesenchymal transition process and renal fibrosis (Du et al. 2012).

The participation of miRNAs in hypoxic injury followed by subsequent reperfusion has been studied in kidneys of C57BL/6 mice by Godwin et al. (2010). They found a miRNA pattern characterized by 9 miRNAs differently expressed. Among these, miR-21, abnormally expressed in mice and in tubular epitelial cells, was defined an important player in protecting cells from death.

A protective effect of miRNAs in the ischemia/reperfusion injury has been described by some investigators. Aguado-Fraile et al. (2013) demonstrated that miR-127 is a regulator of the proximal tubular cell response to the injury (prevention of the focal adhesion complex disassembly and tight junction disruption). Recently, Bijkerk et al (2014) showed the protective role of miR-216 in mice that, before the ischemia/reperfusion injury, received bone marrow transplantation containing hematopoietic cells with overexpression of this miRNA. MiR-216 promoted progenitor cell mobilization, vascular integrity and recovery of the renal damage after the ischemia/reperfusion injury.

Another miRNA, modulated by hypoxia, is miR-210 that is involved in the process of renal angiogenesis during the acute ischemia/reperfusion injury (Liu et al. 2012). This miRNA mediates the activation of VEGF signaling pathway.

Finally, Wei et al. (2010) showed that Dicer deletion of proximal tubular cells protected against renal ischemia-reperfusion injury. They used a proximal tubule specific Dicer KO mice model that was resistant to hypoxia showing better renal function, reduced tissue damage, lower apoptosis of tubular cells and higher survival

rate. During the 12–48 h of reperfusion there was a change of miR-132, miR-362, miR-379, miR-668 and miR-687.

Glomerular Filtration

MiRNAs modulate the development and function of podocytes and consequently the glomerular filtration. The ablation of Dicer enzyme in podocytes of mice caused significant proteinuria by 2 weeks after birth and death within 4 weeks (Harvey et al. 2008; Ho et al. 2008; Shi et al. 2008).

Tubular Reabsorption and Excretion

Salt and fluid reabsorption at the thick ascending limb (TAL) level of Henle loop are modulated by miR-192 that targets the Na+/k+-ATPase beta1 subunit gene (Atp1b1) through the 5′UTR (Fig. 3). High salt intake increases the expression of this miRNA thus suppressing the Atp1b1 gene function and promoting diuresis (Mladinov et al. 2013).

Claudin-14 is localized in the TAL and it is important for the Ca2+ reabsorption in the kidney. MiR-9 and miR-374 recognize the 3′UTR of claudin-14 mRNA. High intake of Ca2+ downregulates the expression levels of these two miRNAs in TAL cells that in turns induce an increase of claudin 14 expression. Claudin 14, included in the complex claudin 16–11 proteins, inhibits the reabsorption of urinary Ca2+ in the TAL of nephron (Gong et al. 2012). Recently, the same group demonstrated that treatment with histone deacetylase inhibitors stimulated miR-9 and miR-374, that downregulated the renal Claudin 14 mRNA. These findings suggest a novel approach for treating hypercalciuria (Gong et al. 2014).

Interstitial Osmolarity

Osmotic response element binding protein (OREB) is a transcription factor that regulates the cellular osmoregulation in kidneys. Huang et al. (2011) demonstrated that in mice miR-200b and miR-717 modulated the common gene target OREB (Fig. 3). In fact, the depletion of these miRNAs by knocking-down Dicer increased significantly OREB causing more renal tonicity, whereas their overexpression decreased the hypertonicity.

The osmolarity has also been studied in zebrafish embryos. Flynt et al. (2009) showed that the miR-8 family regulated the osmoregulation because these miRNAs are expressed in ionocytes where they modulate the expression of Nherf1, a regulator of apical trafficking of transmembrane ion transporters. Deletion of the miR-8 family caused an inability of response to the osmotic stress and blocked the properties of the transmembrane glycoproteins at the apical surface of ionocytes.

The distal nephron, formed by the distal convoluted tubule, the connecting tubule and the collecting duct, plays an important role in modulating the interstitial

osmolarity. In this area two miRNAs participate to the regulation of sodium and potassium ion transport. MiR-192 modulates the target gene serine-threonine kinase WNK1 that is also regulated by aldosterone, thus it contributes to the sodium reabsorption (Elvira-Matelot et al. 2010). MiR-802 modulates the caveolin-1 gene expression that regulates the renal outer medullary channel located in the collecting duct and involves the urinary potassium secretion (Lin et al. 2011). Therefore, high intake of potassium rich diet induces upregulation of miR-802 which decreases its gene target (caveolin-1) leading more excretion of potassium ions. Recently, Lin et al. (2014) discovered another miRNA, miR-194, that regulates the renal outer medullary potassium channel activity by targeting the intersectin-1 and modulating the With-No-Lysine Kinase (WMLK)-induced endocytosis at cellular level.

MiRNAs in Polycystic Kidney Disease

Polycystic kidney disease (PKD) is a congenital disorder characterized by the presence of numerous cysts arising from the tubules in the renal parenchyma. PKD may be transmitted as autosomal dominant (ADPKD) or autosomal recessive (ARPKD) disease caused by mutations of three genes like PKD1 (located on chromosome 16p13.3), PKD2 (located on chromosome 4q21) and PKHD1 (located on chromosome 6) that encode the corresponding proteins, polycystin-1 (PC-1), polycystin-2 (PC-2) and fibrocystin (FPC), respectively. These proteins are present in the primary cilium that is a sensory organelle localized in the apical surface of the tubular cells (Fig. 4). The abnormal function of these proteins induces abnormal cell division and proliferation leading to the formation of cysts in kidneys and liver (ARPKD). Polycystins and other proteins constitute a complex mechanoreceptor that senses fluid flow in lumen of tubules, triggering Ca2+ influx through the TRFP2 channel, thus affecting the fluid secretion. The development of renal cysts is the result of a process based on two hits. The first one is the inheritance of the germline mutation; the second one is the somatic mutation that inactivates PKD1 or PKD2 allele gene in tubular cells thus reducing the expression levels of polycystin. This hypothesis is supported by studies carried out in genetically engineered mouse models with targeted disruption of PKD1 and PKD2 genes. However, the inactivation of PKD genes in tubular epithelial cells is not sufficient to initiate the cyst formation, therefore an additional factor may be required (third hit) and it may be the renal injury or miRNAs described in this section. In conclusion, several pathways including cell polarity, fluid secretion, cell proliferation and apoptosis participate in the cyst formation.

Pandey et al. (2008) used a microarray-based approach to study the mRNA and miRNA expression patterns in PKD of Hannover rat (Han: Sprague Dawley – cy/+ rat) that develops by 8 weeks an ADPKD characterized by cyst formation and progressive renal failure. The combinatorial approach of mRNA and miRNA study demonstrated 935 dysregulated genes, 29 downregulated miRNAs and the upregulated miR-21 (Fig. 4). Several dysregulated genes were targets of some miRNAs like miR-21, miR-31, miR-128, miR-147 and miR-217. Later, Pandey

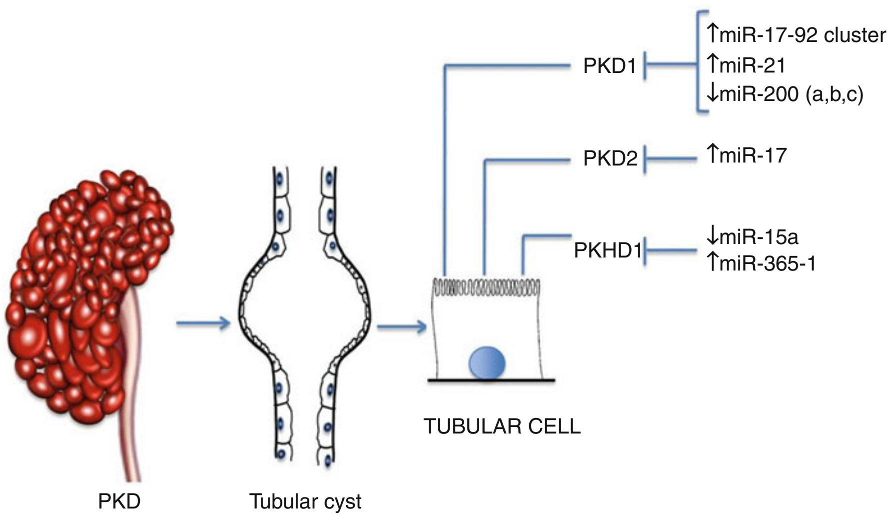

Fig. 4 Modulation of PKD genes by miRNAs in polycystic kidney disease (PKD)

et al. (2011) examined the global gene-expression profiling of renal cyst formation and growth in embryonic kidneys of PKD-null type mice at days 14.5 and 17.5. They, using computational analyses, predicted miRNAs that targeted the differently expressed mRNAs, confirmed by quantitative real-time PCR analysis. At days 14.5 miR-204 and miR-488 were downregulated whereas miR-10a, miR-30a-5p, miR-126-5p, miR-182, miR-200a and miR-429 were significantly upregulated. At days 17.5 miR-10a, miR-126-5p and miR-425 were significantly downregulated while miR-96, miR-182, miR-30a-5p were upregulated. These findings indicate that several miRNAs are involved in the development of cysts. Since each miRNA targets more genes and each gene is targeted by more than one miRNA the investigators found a cascade of dysregulated pathways (MAPK, JAK-STAT, Ca2+ signaling and WnT) leading to renal failure in PKD null type mice.

Dweep et al. (2013) studied the regulatory role of miRNAs in another model of PKD/Mhn (cy/+) rat used for understanding the biological processes in cyst formation in ADPKD. The profile of abnormal expression of mRNA and miRNA revealed 3,333 deregulated genes and 8 upregulated miRNAs (miR-214, miR-34a, miR-199a-5p, miR-146b, miR-503, miR-31, miR-132, and miR-21). These miRNA regulated 23 gene pathways that participate in the cyst formation and expansion.

Patel et al. (2012) generated a transgenic mouse with the ablation of the Dicer enzyme in miRNA for studying the renal tubular maturation. This model had the targeted deletion of the enzyme only in the section of nephros generating renal tubules and collecting ducts. The Dicer mutant mice developed cysts at tubular level and the microarray analysis of the kidneys showed a downregulation of miR-200 family (miR-200a, miR-200b and miR-200c) and an upregulation of PKD1 gene (Fig. 4). These findings were confirmed by in vitro study on cultured renal epithelial

cells in which the inhibition of miR-200 dysrupted tubulogenesis and upregulated PKD1. In conclusion, PKD1 gene is a target of miR-200 in the process of renal tubule maturation and dowregulation of this miRNA causes cyst initiation and formation. Recently, same group of investigators (Patel et al. 2013) demonstrated the upregulation of the miR-17-92 cluster (miR-17, miR-18 and miR-20a) in a mouse model of PKD; its inactivation reduced the kidney cyst growth, improved renal function and prolonged survival (Fig. 4). This miRNA cluster promotes cell proliferation through the post-transcriptional repression of PKD1 and PKD2 genes and the hepatocyte nuclear factor-1β.

Two independent groups of investigators (Sun et al. 2010; Tran et al. 2010) have demonstrated the translational repression of PKD2 gene by miR-17 (Fig. 4); the ectopic expression of this miRNA can promote the proliferation of HEK cells by targeting PKD2. After bioinformatics analysis miR-17 was found to be a regulator of PKD2 gene expression. Sun et al. (2010), used stable cell lines with overexpression of miR-17 for demonstrating that this miRNA modulates PKD2 gene in 3′UTR thus repressing the expression of mRNA and promoting cell proliferation. Therefore, miR-17 may be considered an important player in the mechanism of cystogenesis and at the same time a therapeutic target for the growth of cyst and progression of renal damage in ADPKD. The gene Bicaudal C. homolog 1 (Bicc 1), located on chromosome 10 may have some mutations in mouse that can be responsible for renal cyst formation; Tran et al. (2010) demonstrated that Bicc1, a key regulator of embryonic development, acts as a post-transcriptional regulator upstream of PKD2, thus regulating the stability of PKD2 mRNA, and its translation efficiency. In addition, Bicc1 antagonizes the repressive activity of the miR-17 on the 3′UTR of PKD2 mRNA. Therefore, mouse with Bicc1 mutants develop cysts in organs like kidneys, liver and pancreas.

Lee et al. (2008), using microarray on RNA isolated from cholangiocyte cell lines of PKD rats (a model of ARPDK), demonstrated a decreased expression of miR-15a associated with upregulation of its target, the cell-cycle regulator cell division cycle 25A (Cdc25A). The depression of this miRNA in normal rat caused accelerated cell proliferation and hepatic cystic formation while overexpression of miR-15a in PKD-CCL cells decreased Cdc25A, inhibited cell proliferation and reduced cyst growth (Fig. 4). These results indicate that the dysregulation of miR-15c/Cdc25A complex may be an important player in the hepatic cyst growth and proliferation. Recently, Duan et al. (2012) identified by bioinformatics analysis the miR-365-1 as a regulator of the 3′UTR of PKHD1 gene of which some mutations contribute to the development of ARPKD (Fig. 4). The expression of PKHD1, post transcriptionally regulated by miR-365-1, was validated in cell lines; miR-365-1 modulated PKHD1 gene suppressing cell-cell adhesion through E-cadherin. This implies that dysregulation of this miRNA may be relevant for the ARPKD onset.

In conclusion, data reported in the literature support the hypothesis that abnormal expression of some miRNAs may alter the function of PKD genes thus contributing to the cyst development and growth in kidneys and/or liver.

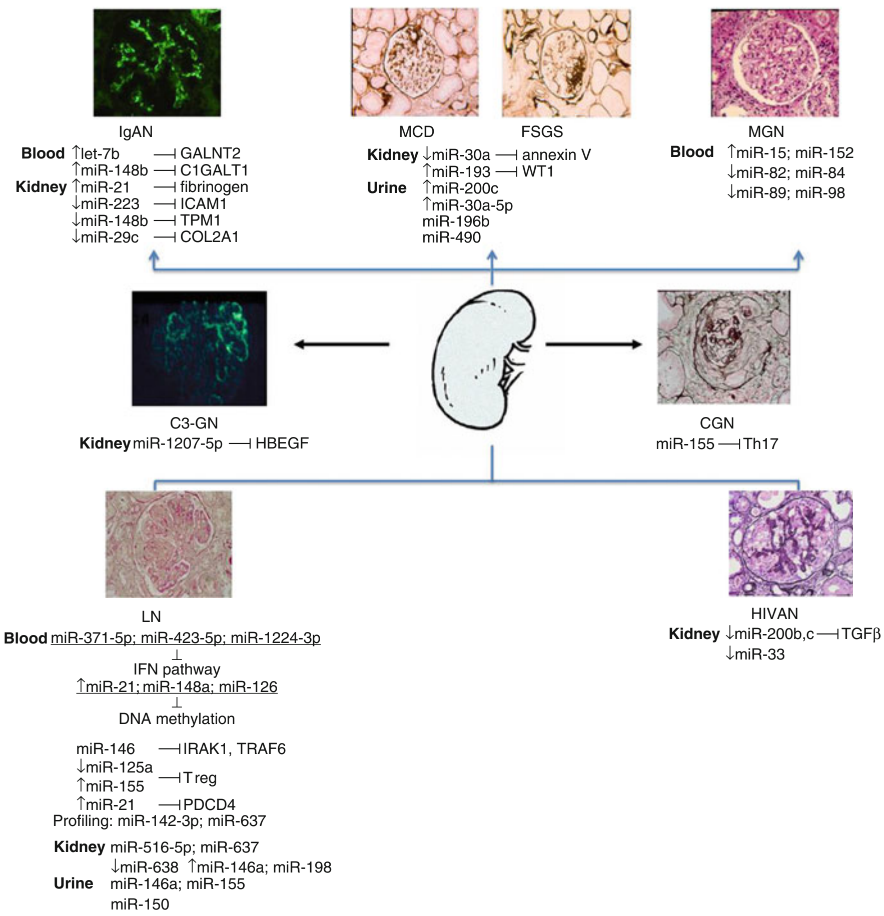

Fig. 5 MiRNAs involved in the pathogenesis of primary and secondary glomerulonephritides. HIVAN histology picture has been kindly given by A. Fogo, Dept of Pathology, Vanderbilt University

MiRNAs in Primary and Secondary Glomerulonephritis

This section describes the role of miRNAs involved in different forms of glomerulonephritis (Fig. 5).

IgA Nephropathy (IgAN) is the most common primary glomerulonephritis in the world. It is characterized by deposition of immune complexes (IgA1-anti IgA1) or polymeric IgA1 in the mesangial area of glomeruli. IgA1 in its deglycosylated form is the first hit of this disease because it is recognized like a non-self antigen by the immune system that produces IgG or IgA antibodies against the deglycosylated

IgA1. Serino et al. (2012, 2015) demonstrated that two miRNAs modulate the process of IgA1 glycosylation, let7 and miR-148b, that have as gene targets the enzyme N-acetylgalactosamyltransferase 2 (GALNT2) and core 1, β1, 3-galactosyltransferase1 (C1GALT1), respectively (Fig. 5). The overexpression of these miRNAs reduced the activity of the two enzymes which participate in the process of IgA1 glycosylation.

Bao et al. (2014a, b) demonstrated that polymeric IgA obtained from serum of IgAN patients stimulated in vitro human mesangial cells to produce abnormal amount of TGFβ and TNFα that upregulated miR-21 that was found at glomerular and tubular level in kidney biopsies of IgAN patients. In addition, a downregulation of miR-223 in glomerular endothelial cells was responsible of high expression of ICAM-1 and monocyte-endothelial adhesion that participate in the progression of renal damage. The downregulation of other miRNAs, like miR-148b and miR-29c caused the high expression of some molecule targets (TPM1, COL2A1), that participate in production of collagen and sequential glomerular sclerosis and interstitial fibrosis.

Minimal change disease (**MCD**) and **focal segmental glomerulosclerosis** (**FSGS**) occur more frequently in children and boys. This disease is characterized by an extensive flattening of podocyte foot processes in the first phase (MCD), then glomerular sclerosis, tubular atrophy and interstitial fibrosis appear in FSGS. A downregulation of the miR-30 family has been found in microdissected glomeruli of renal biopsies from patients with FSGS; this alteration explains the abnormal function and structure of podocytes (Wu et al. 2014; Fig. 5). An important role of miR-193a has been shown in the destabilization of podocyte foot processes (Gebeshuber et al. 2013). The upregulation of this miRNA suppressed the transcription factor WT1 that participates in the maturation process of podocytes, thus causing downregulation of podocalyxin, nephrin and podocin with collapse of the podocyte structure.

After the first report of Cai et al. (2013), who described high serum levels of miR-192 and miR-205 in patients with FSGS, a recent retrospective study of Zhang et al. (2014) has shown high values of miR-125b, miR-186 and miR-193a-3p in the plasma of patients with disease in active phase. MiR-125b and miR-186 declined markedly in patients who were responsive to corticosteroid therapy and miR-186 correlated with the degree of proteinuria.

The evaluation of miRNAs in the urine of patients with FSGS has been done by Wang et al. (2013) who found increased expression of miR-200c in the urinary cells but only a few cases were analyzed and data were not conclusive. Recently, Zhang et al. (2014) identified a panel of four urinary miRNAs (miR-155, miR-196a, miR-30a-5p and miR-490) that were significantly higher in patients with active FSGS than patients in remission. In addition, three combined miRNAs (miR-30a-5p, miR-196a and miR-490) formed a signature with a discriminating AUC of 95 %. This pattern was validated in a prospective study that indicated this signature as biomarker to discriminate patients with FSGS in active phase from those in remission. Finally, urinary miR-30a-5p levels significantly reduced after corticosteroid therapy thus predicting the positive response to treatment.

Membranous glomerulonephrits (MGN) is characterized by a thickening of the capillary wall in the glomeruli after deposition of immune complexes. Recently, Chen et al. (2014) studied the miRNA profiling in peripheral blood mononuclear cells (PBMCs) of 30 patients with MGN. They found a list of 40 miRNAs with the highest fold change in expression of which 20 downregulated and 20 upregulated. Furthermore, they reported four novel miRNAs downregulated (miR-82, miR-84, miR-89 and miR-98) and two novel miRNAs upregulated (miR-15 and miR-152) (Fig. 5). The authors concluded that these miRNAs for their higher difference in expression may be involved in the pathogenesis of the disease.

SNPs can occur in the sequential phases of miRNA biogenesis (pri-miRNA, pre-miRNA and mature miRNA) causing the deregulation of the correlated gene targets and development of a disease. This situation has been observed in **C3-glomerulopathy**, that is characterized by a common exon 2–3 heterozygous duplication of CFHR5 gene. Papagregoriou et al. (2012) evidenced the important role of miR-1207-5p that regulates the heparin binding epidermal growth factor (HBEGF), expressed in the podocytes, as gene target (Fig. 5). This regulation was abolished by the presence of the C 1936 T SNP in the miR-1207-5p. This variant was evaluated in a cohort of 78 patients with biopsy-proven C3 glomerulopathy and it indicated the progression to chronic renal failure in the long-term follow-up.

Autoimmunity is an interesting field that involves many investigators because many renal diseases have an autoimmune origin. Recently, Krebs et al. (2013) have studied the role of miR-155 in a mouse model of **crescentic glomerulonephritis** (CGN) induced by sheep globulins. They demonstrated that this miRNA drives renal injury through the recruitment of nephritogenetic Th17 cells (Fig. 5). The potential role of miR-155 was confirmed in nephritis in induced miR$^{-/-}$ mice in which the systemic and renal nephritogenic Th17 immune response was markedly decreased and mice developed a less severe nephritis. These results suggest that miR-155 may be considered a potential therapeutic target in autoimmune nephritis; in fact, preventive treatment of wild-type mice with a miR-155 antagonist decreased the recruitment of Th17 at renal level and reduced the renal damage. Finally, the investigators studied the expression of miR-155 in renal biopsies of patients with ANCA-associated glomerulonephritis and found high expression of this miRNA that correlated with severity of renal damage.

Lupus nephritis (LN) is the manifestation of renal damage occurring in systemic lupus erythematosus (SLE) that is an autoimmune disorder characterized by the loss of immune tolerance to nuclear self antigens with consequent formation of circulating immune complexes that deposit in kidneys. Innate and adaptive immunity are involved in this disease and miRNAs participate as potential modulators of this immune response.

First, Dai et al. (2007) described the miRNA pattern in PBMCs of patients with SLE and identified 16 miRNAs of which 9 upregulated and 7 downregulated. Later, Te et al. (2010) described the miRNA pattern of LN (miR-371-5p, miR-423-5p and miR-1224-3p). Bioinformatic analysis demonstrated that the IFN signaling pathway was modulated by these miRNAs.

MiRNA participate in DNA hypomethylation of CD4+ T cells in this disease; this phenomenon is responsible for T cell autoreactivity. Three upregulated miRNAs (miR-21, miR-148a and miR-126) inhibited the DNA methylation process in CD4+ T cells of SLE patients (Pan et al. 2010; Zhao et al. 2011). It has been demonstrated that a genetic variant (rs57095329) in the promoter region of miR-146 gene is responsible for a reduced expression of this miRNA that targets some genes of the innate immunity like IL-1 receptor associated kinase1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) that are two signal transducers in the NFkB pathway.

Other dysregulated miRNAs contribute to the development of SLE, like a downregulation of miR-125a (Zhao et al. 2010) and an overexpression of miR-21 (Stagakis et al. 2011) and miR-155 (Divekar et al. 2011). They modulate the activity of T cells, mainly T reg.

The presence of miRNAs in serum or plasma of patients with SLE has also been studied by two groups of Chinese investigators. The Szeto's group (Wang et al. 2010b, c) proposed the downregulated miR-200 family, miR-205, miR-192, miR-146a and miR-155 as biomarkers of disease activity. In addition, an improvement of miR-146a was observed after administration of calcitriol that may be considered an immune modulator because the low expression of miR-146a promoted the type 1 IFN pathway activation.

The Dai's group (Wang et al. 2012) identified the circulating miR-126 specifically higher in SLE patients and other 3 miRNAs (miR-125a-3p, miR-155 and miR-146a) downregulated. They suggested this miRNA signature as a potential biomarker for monitoring SLE. Since bioinformatic analysis identified a number of significant pathways, they considered these miRNAs an additional step for studying the pathogenetic mechanisms of the disease (Sui et al. 2014).

Recently, Carlsen et al. (2013) reported a miRNA signature (miR-142-3p, miR-106a, miR-17 and miR-20a) as a classifier for analyzing the SLE risk. Interestingly, these results were validated in two independent cohorts of SLE patients from Denmark and Sweeden, respectively. The target genes of these miRNAs are involved in the inflammatory pathogenesis of the disease.

Reports of scientists on the miRNA expression in kidney biopsies of SLE patients are very few (Fig. 5). The transcriptomic analysis of Dai et al. (2009; Sui et al. 2014) indicated two validated miRNAs (miR-516-5p and miR-637) whereas Lu et al. (2012) found a downregulated expression of miR-638 at glomerular level and an upregulation of miR-146a and miR-198 in the glomerular and tubulointerstitial compartments. Fibronectin and CXCR3 were found as the target genes of these miRNAs.

Szeto's group (Wang et al. 2010b) reported additional data of miRNAs in urine of patients with SLE. This team of investigators indicated two urinary miRNAs (miR-146a and miR-155) as expression of the disease because they are regulators of the immune system. Finally, Zhou et al. (2013) indicated the upregulated miR-150 expression at renal level as a potential biomarker for evaluating the renal outcome in SLE patients.

The role of miRNAs has been studied in the pathogenesis of **HIV associated nephropathy** (HIVAN), a collapsing focal segmental glomerulosclerosis with microcystic dilatation of tubules, by Cheng et al. (2013a) in the HIV-1 transgenic mouse. Thirteen miRNAs belonging to 11 miRNA families were found downregulated in kidney sections. They were validated in vitro in HIV-1 transduced human podocytes and a notable downregulation of miR-200b and c and miR-33 expression was found in podocytes. The authors concluded that these miRNAs contribute to the development of the renal damage because miR-200 could inhibit TGFβ- induced EMT at tubular level. Later, the same investigators (Cheng et al. 2013b) evaluated the effect of rapamycin administration on miRNA expression pattern in HIVAN mice. They observed an attenuation of renal lesions because rapamycin reversed the expression of downregulated miRNAs, mainly those of miR-200 family. In conclusion, this agent attenuates the renal cell EMT through the modulation of miR-200 expression and these results could be taken into consideration for improving the outcome of HIVAN.

MiRNAs in Kidney Transplantation

MiRNAs can participate to several physiopathological mechanisms that characterize the kidney transplantation: the ischemia-reperfusion injury (IRI), the acute rejection and the chronic allograft dysfunction (Table 1).

The IRI is one of the main causes of acute kidney injury (AKI) in kidney transplantation. It is due to a decreased blood supply followed by a re-establishment of the normal bloodstream that led to hypoxia, vascular dysfunction and immune response activation. IRI have a major role in the development of delayed graft function (DGF) following kidney transplantation (Jang et al. 2009; Eltzschig and Eckle 2011).

One of the miRNAs more involved in the IRI is miR-21. It has been found upregulated in murine kidney injury model, performed by unilateral ureteral obstruction, at 3 and 7 days after IRI. The ureteral obstruction caused a renal fibrosis whereas the in vivo inhibition of miR-21 led to a decrease of expression of TGF-β, α-SMA, PAI-1, col1A1, col1A2 and fibronectin (Godwin et al. 2010; Shapiro et al. 2011; Zarjou et al. 2011). Also the macrophage infiltration was reduced and further miRNAs were found modulated in this process: miR-142–3p, miR-142–5p, miR-214, miR-223, miR-101a, miR-193 and miR-218 (Shapiro et al. 2011). Most of these miRNAs did not regulate processes involving lymphocytes since they are regulated also in immunodeficient mice (Godwin et al. 2010). In addition to these miRNAs, also miR-155 and miR-18a have been found upregulated in rat kidneys following IRI-induced tubular injury, even if in blood and urine they resulted at the same time downregulated (Saikumar et al. 2012), suggesting a specific pattern of expression, depending on the type of cells.

To understand how the miRNAs influence the injured human kidney, a prospective cohort study investigated zero-hour and protocol allograft biopsies from

Table 1 miRNAs involved in kidney transplantation

Pathology	Modulated miRNA	Involved pathway	Expressed in
Ischemia-reperfusion injury	miR-142–3p, miR-142–5p, miR-214, miR-223, miR-101a, miR-193, miR-218 miR-155 and miR-18a	Modulation of TGF-β, α-SMA, PAI-1, collagens and fibronectin	Renal tissue/cells
Acute rejection	miR-10a, miR-10b and miR-210, miR-142–5p, miR-155, miR-223, miR-10b, miR-30a-3p and let-7C, miR-10a, miR-10b and miR-210	/	Urinary cell pellets; PBMCs; renal tissue/cells
Chronic allograft dysfunction with IF/TA	miR-204, miR-21, miR-142-5p, miR-142-3p, miR-506 miR-30b and miR-30c	Smad /TGFB signalling	Renal tissue; PBMCs
Allograft chronic dysfunction	miR-125b, miR-203miR-142-3p, miR-204, miR-211	Regulation of inflammation and fibrosis development	Renal tissue
CAMR with IF/TA	miR-142-5p	Immune-regulation	Renal tissue; PBMCs
AKI	miR-182-5p and miR-21-3p	Homeostasis of cells of the immune system; apoptosis and proliferation	Renal tissue

166 patients. Eight cases with AKI and ten matched allografts without pathology, used as control group, were followed-up within the first 12 days after engraftment. In these samples miRNA and mRNA profiles were analyzed and, following the baseline adjustment for zero-hour biopsy expression levels, a specific molecular AKI signature of 20 mRNAs and 2 miRNAs (miR-182-5p and miR-21-3p) were identified. These miRNAs could describe the evolution of events during acute injury. The miR-182-5p seems be the main controller of the kidney tissue injury; it can be activated by IL-2 and STAT5 and it inhibits FOXO1 expression that regulates homeostasis of cells of the immune system such as T-cells, B-cells and neutrophils. MiR-182-5p could regulate also molecular processes related to the AKI, as apoptosis and proliferation (Wilflingseder et al. 2013, 2014).

The screening of miRNAs in acute rejection biopsies revealed three most important miRNAs that were overexpressed (miR-142-5p, miR-155 and miR-223) and three miRNAs downregulated (miR-10b, miR-30a-3p and let-7c). The three miRNAs upregulated in the renal tissue were also highly expressed in PBMCs and the stimulation with the mitogen phytohaemagglutinin led to increased levels of miR-155 and lower levels of miR-223 and let-7c. Moreover, intragraft levels of miR-142-5p or miR-155 can accurately predict the acute rejection (100 % sensitivity and 95 % specificity) (Anglicheau et al. 2009). Therefore, these miRNAs could be potentially considered as non-invasive diagnostic biomarkers of acute rejection.

In patients with acute rejection, the levels of miR-10a, miR-10b and miR-210 have been found modulated in urinary cell pellet. Specifically, miR-10a was

upregulated, whereas miR-10b and miR-210 were downregulated (Lorenzen et al. 2011). However, the data on miR-210 are discordant considering another study on the expression of miRNAs isolated from plasma of patients with AKI, in which it was found upregulated (Lorenzen et al. 2011). This inconsistency can be ascribed to the different type of samples or conditions.

A study of the miRNA signature in chronic allograft dysfunction has been performed comparing patients with interstitial fibrosis (IF) and tubular atrophy (TA) with patients with normal allografts and correlating miRNA profiles derived from allograft biopsies with IF/TA with urinary profiles. Three miRNAs, miR-142-3p, miR-204 and miR-21, were found differentially expressed in tissue and urine and were confirmed in an independent set of samples (Scian et al. 2011). Another study has been carried out on eight human kidney allograft biopsies, four IF/TA and four normal biopsies, and confirmed on further ten IF/TA and eight normal samples. Ben-Dov et al. (2012) found miR-21, miR-142-5p, miR-142-3p and miR-506 upregulated and miR-30b and miR-30c downregulated, compared with normal kidneys.

Even if the studies on miRNAs expression in the renal tissue can be useful to understand the process correlated to the rejection or fibrosis subsequent the transplantation, they assume a greater clinical significance when are performed on body fluids as plasma or urine because the investigated miRNAs could be used as non-invasive biomarkers.

Several studies have investigated this issue. Twenty-two miRNAs have been found able to discriminate between renal allograft recipients with chronic dysfunction and well-functioning controls. This study was performed on urinary cell pellets on a total of 191 samples. The identified miRNAs are involved in the regulation of inflammation and fibrosis development pathways and the panel of urine miRNAs, if further validated, could be useful in allograft for monitoring graft function and for the prediction of progression to chronic allograft dysfunction (Maluf et al. 2014).

Interestingly, the miR-142-5p, previously described as upregulated in allograft biopsies with IF/TA (Ben-Dov et al. 2012) has been found overexpressed also in a further study both in PBMCs and in renal tissue of patients with chronic antibody mediated rejection (CAMR) as well as in a rodent model of CAMR. The miR-142-5p was not modulated by immunosuppressive therapy, suggesting that its expression was not induced by treatment. Moreover, when PBMCs of those patients were activated with phytohemagglutinin A, the miR-142-5p expression decreased and was not increased in the blood of patients with acute rejection. This miRNA may be considered an excellent CAMR biomarker because it has been validated in PBMCs of an independent cohort of patients discriminating those with CAMR (AUC $= 0.74$; $p = 0.0056$) (Danger et al. 2013). Moreover, this miRNA modulates the expression of genes belonging to the category of immune-regulation and could be used as a marker of tolerance in B-cells of operationally tolerant patients (Danger et al. 2012).

Taking into account all these studies, we can conclude that the analysis of the expression of certain miRNAs is a promising methodology to impact clinical decisions. The miRNAs, in addition to provide further discernment into specific pathophysiological mechanisms, are good candidate molecular biomarkers since

they are stable overtime and can be detected in body fluids and in sample sources with degraded RNA.

Potential Applications to Prognosis and Other Diseases or Conditions

Since some diseases are characterized by an abnormal miRNAs expression, the identification of miRNA signature for specific disease will be helpful in early and differential diagnosis. There are some studies that provide evidence on the potential use of circulating miRNAs in different body fluids as biomarkers for disease state and progression. The application of miRNAs in diagnosis and prognosis of disease are described in detail below in paragraph “MiRNAs as diagnostic tools in blood and urine.”

Moreover, miRNAs have some properties that make them useful as targets for therapeutic applications. First, miRNA signature differentiates various disease; this allow the identification of specific miRNAs that could be manipulated to control gene regulation. Second, miRNAs, as small entities, are able to be in vivo easily delivered. Finally, miRNAs have multiple gene targets, some of that work simultaneously to control a common pathway or biological process. However, this could also be a disadvantage due the “off-target” side effects. The application of miRNAs in therapy and the description of different approaches used are well explained below in paragraph “New approaches for miRNA therapy.”

MiRNAs as Diagnostic Tools in Blood and Urine

In the last years, a particular attention was given to the identification of novel and reliable biomarkers for renal diseases. Recent studies, mainly in the cancer field, have provided evidence on the potential use of miRNAs as new diagnostic tool. As described above, miRNA studies in renal diseases have demonstrated that miRNAs have both an important role in the pathogenesis and in the diagnosis of many renal diseases.

The first evidence regarding the extraction and determination of cell-free miRNA content in body fluids was shown by Chen et al. (2008). Cell-free miRNAs in body fluids are stable under not easy conditions including boiling, low/high pH, extended storage, multiple freeze-thaw cycles. Moreover, they are resistant to endogenous RNase for their small size and perhaps for packaging inside lipid or lipoprotein complexes such as microvesicles/microparticles or exosomes (Mitchell et al. 2008). It has been also hypothesized that circulating miRNAs have a role in cell-to-cell communication; in fact, they could transport information from a donor to a recipient cell. Instead, in urine, miRNAs may be filtered and excreted by, or directly from, the kidney and/or urinary tract.

All these characteristics and the non-invasive nature make blood- and urine-circulating miRNAs as ideal and potential biomarkers to detect or monitor various human diseases.

Since the levels of circulating miRNAs are very low, the real-time RT-PCR is well adapted for the analysis of circulating miRNA profiles for its sensitivity. However, one of the major challenge in the analysis of circulating miRNAs is the suitable method of normalization. To date, an established housekeeping gene to normalize the expression of circulating miRNAs is lacking. Consequently, researchers have proposed other methods of normalization. Some researchers have added spiked-in control miRNAs during the RNA purification process in order to resolve differences in recovery during the purification procedure and amplification efficiency. Synthetic C. elegans miRNAs are usually used as spiked-in control miRNAs. Other groups have used different miRNAs as normalizing controls such as miR-16 or miR-17. However, one needs to be mindful that miRNAs used as normalizers are highly and equally expressed in the samples analyzed and they are stable in different disease conditions. In fact, levels of miR-16 have been shown to be increased in critical limb ischemia patients (Spinetti et al. 2013), miR-17 is reduced in patients with systemic lupus erythematosus (Carlsen et al. 2013).

In conclusion, although circulating miRNAs potentially could be used as disease biomarker, for their introduction in clinical practice, a method that replaces the time-consuming RNA isolation, reverse transcription and quantitative PCR analysis is needed. Moreover, currently studies published on circulating miRNAs in kidney diseases have enrolled a low number of patients and the use of miRNAs as prognostic biomarkers is still limited. Future studies involving larger cohorts of patients and different ethnic groups will be useful.

New Approaches for miRNA Therapy

Therapeutic Approaches

MiRNA dysregulation occurs in many kidney diseases. The main therapeutic approach is to normalize miRNA expression values in blood, kidney or urine. There are two ways for modulating the dysregulated miRNAs: (i) inhibition of the upregulated miRNAs; (ii) restoration of the activity in downregulated miRNAs (Table 2).

(i) miRNA antagonists

First, the inhibition of miRNA activity can be obtained using small interfering RNA (siRNA) against the sequential components of miRNA biogenesis that are the two enzymes, Drosha and Dicer/DGCR8, and the RISC-miRNA complex. The ablations of Dicer, Drosha or RISC in animal models have generated kidney malformations or genetic diseases.

Second, the inhibition of upregulated miRNA activity can be obtained by several methods like chemically modified antisense oligonucleotide inhibitors (antagomirs or anti-miRs) or introducing a tandem miRNA-binding site repeats

Table 2 Strategies to modulate miRNAs in the clinic

MiRNA antagonists
Small interfering RNAs (siRNAs)
Antagomirs/anti-miRs (ASO): 2′-O-methyl RNA (2′-O-Me)
2′-O-methoxyethyl (2′-MOE)
2′-fluoromodification
Sponge/decoy miRNAs locked-nucleic acid
Phosphorotionate linkage
MiRNA erasers
MiRNA target occupiers
MiRNA mimics
MiRNA sharing
Small hairpin RNAs (ShRNAs)
Scaffold miRNAs
Artificial miRNAs

(Sponge or decoy miRNAs). The antisense oligonucleotide (ASO) has a complementary sequence to the mature miRNA thus it binds and sequesters the cognate miRNA; this approach has been used in cell cultures for repressing the mRNA gene target. The antagomirs may have different chemical modifications such as 2′-*O*-methylRNA, 2′-fluomodification, LNA (locked-nucleic acid) modifications, phosphorotionate linkage. The LNA modifications provide long-term and efficient suppression of miRNAs. MiRNA sponges consist of multiple tandem binding sites into the 3′UTR of cognate endogenous miRNAs. Other approaches are the miRNA erasers and the miRNA target occupiers.

(ii) miRNA mimics

The restoring of downregulated miRNAs can be obtained introducing miRNA mimics that share the structure of miRNA or employing small hairpin RNA (shRNA) or using miRNA scaffolds and artificial miRNAs.

MiRNAs as Potential Therapeutic Strategies

(i) Animal models

The ablation of Dicer or Drosha or the introduction of siRNA against these two enzymes can cause death of animals or kidney malformations. This means that miRNAs play an important role in embryo development and organ formation. Details of these experimental studies can be found in the section "miRNAs and kidney development."

Transgenic mice with ablation of a miRNA gene or introduction of overexpressed miRNA gene have been realized for studying the biological properties of a cognate miRNA.

Manipulation of the downregulated miR-146a has been done in lupus-prone BXSB mice by Pan et al. (2012) administering virus like particles (VLP) containing miR146a. The 20-week-old BXSB mice showed an increase of the cognate miRNA expression in PBMCs, kidney, spleen and lung associated with reduction of

anti-dsDNA antibodies and ANA. Furthermore, the investigators observed reduced expression of inflammatory cytokine SLE-related (IFNα, IL-1β, IL-6). These results suggest a therapeutic approach for mice predisposed to develop SLE.

Since miR-192 is overexpressed in streptozotocin (STZ)-induced diabetic mice (type 1 Diabetic Nephropathy) and in diabetic db/db mice (type 2 DN), Putta et al. (2012) evaluated the efficacy of the anti-miR-192 in C57BL/6 diabetic mice. They observed a decreased expression of extracellular matrix associated with reduced expression of profibrotic genes (TGFβ, CTGF, fibronectin, COL 1α2 and COL 4α1). Furthermore, they found reduced albuminuria in diabetic mice.

Qin et al. (2011) administered a double-stranded miRNA mimics like miR-29b in a rat model of obstructive nephropaty to block renal fibrosis. The ultrasound-mediated gene delivery of miR-29b blocked the progression of renal fibrosis by inhibiting the Smad 3/TGF-β pathway.

(ii) Human diseases

The use of miRNA blockers or enhancers in humans with non-renal diseases is entering in a crucial phase of development and application. An extensive review on this topic has recently been published by Van Rooij E and Kauppinen S (2014). We have focused in this section only the recent progresses on the therapeutic application of Miravisen in patients with hepatitis C virus (HCV) infection because these individuals could potentially develop secondary cryoglobulinemic glomerulonephritis. This locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide sequesters the oligomeric miR-122-HCV complex that protects the HCV genome from nucleolytic degradation.

Two drug companies, Santaris Pharma and Regulus Therapeutics, are currently moving to phase III studies with Miravisen in patients with HCV infection after the recent published data of Janssen et al. (2013) obtained from the first phase II study. Thirty-six patients received Miravisen subcutaneously at doses of 3, 5 or 7 mg/kg or placebo for a total of 5 weeks. Treatment with this antimiR-122 produced a dose-dependent and long-lasting anti-viral activity with reduction of HCV RNA levels. Remission was obtained in a large number of treated patients with only rare mild side-effects. The therapeutic results are promising but remain to be validated in large cohorts of patients.

Challenges and Future Perspectives

Despite the research on miRNAs in renal pathophysiology is very interesting, it is highly challenging. Firstly, it is not completely understood the mechanism of regulation of miRNA biogenesis. In fact a lot of miRNAs are located within the introns of genes, but their expression regularly doesn't correlate with that of host gene demonstrating additional regulatory mechanisms after the miRNA transcription. For many miRNAs several gene targets are unknown. Bioinformatic analyses have predicted many thousands of target genes for each miRNA but only a small number of these has been validated biologically. MiRNAs have a tissue and cell-specific expression,

but kidneys are constituted by different types of cells which could be diversely affected in various renal diseases. So, to validate experimentally the role of a miRNA, it is necessary to identify the cell types in which a miRNA is expressed in a particular pathologic condition. As described in detail above, the main system to study experimentally the link between a miRNA and a target gene is the transfection of miRNA mimics and inhibitors. Sometimes cell lines are difficult to transfect and the introduced oligonucleotides may have non-specific effect when used in the in vivo models.

Although several challenges exist in miRNA research, progress in technological advances will help to overcome these difficulties and permit us to understand the effect of miRNAs on pathogenesis of renal diseases. In the future, animal models that enhance or silence a particular miRNA could provide the best model to study the function of miRNAs. Moreover, the detection of circulating miRNAs could help the clinicians in the diagnosis, prognosis and therapeutic response of kidney diseases. Finally, the most attractive perspective is the use of miRNAs for the specific treatment of kidney diseases even if the development of safe and reliable organ and cell-specific delivery systems, the limitation of toxicity derived from off-target effects and from the activation of immune response are needed.

Summary Points

- This chapter focuses on the role of microRNAs (miRNAs) in kidneys.
- MiRNAs are small RNA molecules that can regulate gene expression at post-transcriptional level.
- MiRNAs play an important role in essential biological processes of renal physiology.
- MiRNA dysregulations are involved in the pathogenesis of several kidney diseases such as polycystic kidney disease, primary and secondary glomerulonephritides and renal transplantation.
- Thus, they represent an important tool for the diagnosis of renal diseases and could be used as new targets for the treatment of kidney diseases.

References

Aguado-Fraile E, Ramos E, Conde E, et al. MicroRNAs in the kidney: novel biomarkers of acute kidney injury. Nefrologia. 2013;33:826–34.

Ambros V. The function of animal microRNAs. Nature. 2004;431:350–5.

Anglicheau D, Sharma VK, Ding R, et al. MicroRNA expression profiles predictive of human renal allograft status. Proc Natl Acad Sci U S A. 2009;106:5330–5.

Bao H, Chen H, Zhu X, et al. MiR-223 downregulation promotes glomerular endothelial cell activation by upregulating importin α4 and α5 in IgA nephropathy. Kidney Int. 2014a;85:624–35.

Bao H, Hu S, Zhang C, et al. Inhibition of miRNA-21 prevents fibrogenic activation in podocytes and tubular cells in IgA nephropathy. Biochem Biophys Res Commun. 2014b;444:455–60.

Ben-Dov IZ, Muthukumar T, Morozov P, et al. MicroRNA sequence profiles of human kidney allografts with or without tubulointerstitial fibrosis. Transplantation. 2012;94:1086–94.
Bijkerk R, van Solingen C, de Boer HC, et al. Hematopoietic microRNA-126 protects against renal ischemia/reperfusion injury by promoting vascular integrity. J Am Soc Nephrol. 2014;25:1710–22.
Brodersen P, Voinnet O. Revisiting the principles of microRNA target recognition and mode of action. Nat Rev Mol Cell Biol. 2009;10:141–8.
Cai X, Xia Z, Zhang C, et al. Serum microRNAs levels in primary focal segmental glomerulosclerosis. Pediatr Nephrol. 2013;28:1797–801.
Carlsen AL, Schetter AJ, Nielsen CT, et al. Circulating microRNA expression profiles associated with systemic lupus erythematosus. Arthritis Rheum. 2013;65:1324–34.
Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18:997–1006.
Chen W, Lin X, Huang J, et al. Integrated profiling of microRNA expression in membranous nephropathy using high-throughput sequencing technology. Int J Mol Med. 2014;33:25–34.
Cheng K, Rai P, Plagov A, et al. MicroRNAs in HIV-associated nephropathy (HIVAN). Exp Mol Pathol. 2013a;94:65–72.
Cheng K, Rai P, Plagov A, et al. Rapamycin-induced modulation of miRNA expression is associated with amelioration of HIV-associated nephropathy (HIVAN). Exp Cell Res. 2013b;319:2073–80.
Dai Y, Huang YS, Tang M, et al. Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. Lupus. 2007;16:939–46.
Dai Y, Sui W, Lan H, et al. Comprehensive analysis of microRNA expression patterns in renal biopsies of lupus nephritis patients. Rheumatol Int. 2009;29:749–54.
Danger R, Pallier A, Giral M, et al. Upregulation of miR-142-3p in peripheral blood mononuclear cells of operationally tolerant patients with a renal transplant. J Am Soc Nephrol. 2012;23:597–606.
Danger R, Paul C, Giral M, et al. Expression of miR-142-5p in peripheral blood mononuclear cells from renal transplant patients with chronic antibody-mediated rejection. PLoS ONE. 2013;8:e60702.
Divekar AA, Dubey S, Gangalum PR, et al. Dicer insufficiency and microRNA-155 overexpression in lupus regulatory T cells: an apparent paradox in the setting of an inflammatory milieu. J Immunol. 2011;15:924–30.
Du R, Sun W, Xia L, et al. Hypoxia-induced down-regulation of microRNA-34a promotes EMT by targeting the Notch signaling pathway in tubular epithelial cells. PLoS ONE. 2012;7:e30771.
Duan J, Huang H, Lv X, et al. PKHD1 post-transcriptionally modulated by miR-365-1 inhibits cell-cell adhesion. Cell Biochem Funct. 2012;30:382–9.
Dweep H, Sticht C, Kharkar A, et al. Parallel analysis of mRNA and microRNA microarray profiles to explore functional regulatory patterns in polycystic kidney disease: using PKD/Mhm rat model. PLoS ONE. 2013;8:e53780.
Eltzschig HK, Eckle T. Ischemia and reperfusion – from mechanism to translation. Nat Med. 2011;17:1391–401.
Elvira-Matelot E, Zhou XO, Farman N, et al. Regulation of WNK1 expression by miR-192 and aldosterone. J Am Soc Nephrol. 2010;21:1724–31.
Flynt AS, Thatcher EJ, Burkewitz K, et al. miR-8 microRNAs regulate the response to osmotic stress in zebrafish embryos. J Cell Biol. 2009;185:115–27.
Gebeshuber CA, Kornauth C, Dong L, et al. Focal segmental glomerulosclerosis is induced by microRNA-193a and its downregulation of WT1. Nat Med. 2013;19:481–7.
Godwin JG, Ge X, Stephan K, et al. Identification of a microRNA signature of renal ischemia reperfusion injury. Proc Natl Acad Sci U S A. 2010;107:14339–44.
Gong Y, Renigunta V, Himmerkus N, et al. Claudin-14 regulates renal Ca^{++} transport in response to CaSR signalling via a novel microRNA pathway. EMBO J. 2012;31:1999–2012.

Gong Y, Himmerkus N, Plain A, et al. Epigenetic regulation of microRNAs controlling CLDN14 expression as a mechanism for renal calcium handling. J Am Soc Nephrol. 2014; pii: ASN.2014020129.
Harvey SJ, Jarad G, Cunningham J, et al. Podocyte-specific deletion of dicer alters cytoskeletal dynamics and causes glomerular disease. J Am Soc Nephrol. 2008;19:2150–8.
Ho J, Ng KH, Rosen S, Dostal A, Gregory RI, Kreidberg JA. Podocyte-specific loss of functional microRNAs leads to rapid glomerular and tubular injury. J Am Soc Nephrol. 2008;19:2069–75.
Huang W, Liu H, Wang T, et al. Tonicity-responsive microRNAs contribute to the maximal induction of osmoregulatory transcription factor OREBP in response to high-NaCl hypertonicity. Nucleic Acids Res. 2011;39:475–85.
Jang HR, Ko GJ, Wasowska BA, et al. The interaction between ischemia-reperfusion and immune responses in the kidney. J Mol Med. 2009;87:859–64.
Janssen HL, Reesink HW, Lawitz EJ, et al. Treatment of HCV infection by targeting microRNA. N Engl J Med. 2013;368:1685–94.
Krebs CF, Kapffer S, Paust HJ, et al. MicroRNA-155 drives TH17 immune response and tissue injury in experimental crescentic GN. J Am Soc Nephrol. 2013;24:1955–65.
Lee SO, Masyuk T, Splinter P, et al. MicroRNA15a modulates expression of the cell-cycle regulator Cdc25A and affects hepatic cystogenesis in a rat model of polycystic kidney disease. J Clin Invest. 2008;118:3714–24.
Lin DH, Yue P, Pan C, et al. MicroRNA 802 stimulates ROMK channels by suppressing caveolin-1. J Am Soc Nephrol. 2011;22:1087–98.
Lin DH, Yue P, Zhang C, et al. MicroRNA-194 (miR-194) regulates ROMK channel activity by targeting intersectin 1. Am J Physiol Renal Physiol. 2014;306:F53–60.
Liu F, Lou YL, Wu J, et al. Upregulation of microRNA-210 regulates renal angiogenesis mediated by activation of VEGF signaling pathway under ischemia/perfusion injury in vivo and in vitro. Kidney Blood Press Res. 2012;35:182–91.
Lorenzen JM, Kielstein JT, Hafer C, et al. Circulating miR-210 predicts survival in critically ill patients with acute kidney injury. Clin J Am Soc Nephrol. 2011a;6:1540–6.
Lorenzen JM, Volkmann I, Fiedler J, et al. Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft recipients. Am J Transplant. 2011b;11:2221–7.
Lu J, Kwan BC, Lai FM, et al. Glomerular and tubulointerstitial miR-638, miR-198 and miR-146a expression in lupus nephritis. Nephrology. 2012;17:346–51.
Maluf DG, Dumur CI, Suh JL, et al. The urine microRNA profile may help monitor post-transplant renal graft function. Kidney Int. 2014;85:439–49.
Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105:10513–8.
Mladinov D, Liu Y, Mattson DL, et al. MicroRNAs contribute to the maintenance of cell-type-specific physiological characteristics: miR-192 targets Na+/K+-ATPase β1. Nucleic Acids Res. 2013;41:1273–83.
Pan W, Zhu S, Yuan M, et al. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. J Immunol. 2010;184:6733–81.
Pan Y, Jia T, Zhang Y, et al. MS2 VLP-based delivery of microRNA-146a inhibits autoantibody production in lupus-prone mice. Int J Nanomedicine. 2012;7:5957–67.
Pandey P, Brors B, Srivastava PK, et al. Microarray-based approach identifies microRNAs and their target functional patterns in polycystic kidney disease. BMC Genomics. 2008;9:624.
Pandey P, Qin S, Ho J, et al. Systems biology approach to identify transcriptome reprogramming and candidate microRNA targets during the progression of polycystic kidney disease. BMC Syst Biol. 2011;5:56.
Papagregoriou G, Erguler K, Dweep H, et al. A miR-1207-5p binding site polymorphism abolishes regulation of HBEGF and is associated with disease severity in CFHR5 nephropathy. PLoS ONE. 2012;7:e31021.

Patel V, Hajarnis S, Williams D, et al. MicroRNAs regulate renal tubule maturation through modulation of Pkd1. J Am Soc Nephrol. 2012;23:1941–18.

Patel V, Williams D, Hajarnis S, et al. miR-17~92 miRNA cluster promotes kidney cyst growth in polycystic kidney disease. Proc Natl Acad Sci U S A. 2013;110:10765–70.

Putta S, Lanting L, Sun G, et al. Inhibiting microRNA-192 ameliorates renal fibrosis in diabetic nephropathy. J Am Soc Nephrol. 2012;23:458–69.

Qin W, Chung AC, Huang XR, et al. TGF-β/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. J Am Soc Nephrol. 2011;22:1462–74.

Saikumar J, Hoffmann D, Kim TM, et al. Expression, circulation, and excretion profile of microRNA-21, -155, and -18a following acute kidney injury. Toxicol Sci. 2012;129:256–67.

Scian MJ, Maluf DG, David KG, et al. MicroRNA profiles in allograft tissues and paired urines associate with chronic allograft dysfunction with IF/TA. Am J Transplant. 2011;11:2110–22.

Sequeira-Lopez ML, Weatherford ET, Borges GR, et al. The microRNA-processing enzyme dicer maintains juxtaglomerular cells. J Am Soc Nephrol. 2010;21:460–7.

Serino G, Sallustio F, Cox SN, et al. Abnormal miR-148b expression promotes aberrant glycosylation of IgA1 in IgA nephropathy. J Am Soc Nephrol. 2012;23:814–24.

Serino G, Sallustio F, Curci C, et al. Role of let-7b in the deregulation process of IgA1 glycosylation in IgA nephropathy. Nephrol Dial Transplant. 2015;30:1132–9.

Shapiro MD, Bagley J, Latz J, et al. MicroRNA expression data reveals a signature of kidney damage following ischemia reperfusion injury. PLoS One. 2011;6:e23011.

Shi S, Yu L, Chiu C, et al. Podocyte-selective deletion of dicer induces proteinuria and glomerulosclerosis. J Am Soc Nephrol. 2008;19:2159–69.

Spinetti G, Fortunato O, Caporali A, et al. MicroRNA-15a and microRNA-16 impair human circulating proangiogenic cell functions and are increased in the proangiogenic cells and serum of patients with critical limb ischemia. Circ Res. 2013;112:335–46.

Stagakis E, Bertsias G, Verginis P, et al. Identification of novel microRNA signatures linked to human lupus disease activity and pathogenesis: miR-21 regulates aberrant T cell responses through regulation of PDCD4 expression. Ann Rheum Dis. 2011;70:1496–506.

Sui W, Liu F, Chen J, et al. Microarray technology for analysis of microRNA expression in renal biopsies of lupus nephritis patients. Methods Mol Biol. 2014;1134:211–20.

Sun Y, Koo S, White N, et al. Development of a micro-array to detect human and mouse microRNAs and characterization of expression in human organs. Nucleic Acids Res. 2004;32: e188.

Sun H, Li QW, Lv XY, et al. MicroRNA-17 post-transcriptionally regulates polycystic kidney disease-2 gene and promotes cell proliferation. Mol Biol Rep. 2010;37:2951–8.

Te JL, Dozmorov IM, Guthridge JM, et al. Identification of unique microRNA signature associated with lupus nephritis. PLoS One. 2010;5:e10344.

Tian Z, Greene AS, Pietrusz JL, Matus IR, Liang M. MicroRNA-target pairs in the rat kidney identified by microRNA microarray, proteomic, and bioinformatic analysis. Genome Res. 2008;18:404–11.

Tran U, Zakin L, Schweickert A, et al. The RNA-binding protein bicaudal C regulates polycystin 2 in the kidney by antagonizing miR-17 activity. Development. 2010;137:1107–16.

van Rooij E, Kauppinen S. Development of microRNA therapeutics is coming of age. EMBO Mol Med. 2014;6:851–64.

Wang G, Kwan BC, Lai FM, et al. Intrarenal expression of microRNAs in patients with IgA nephropathy. Lab Invest. 2010a;90:98–103.

Wang G, Tam LS, Li EK, et al. Serum and urinary cell-free MiR-146a and MiR-155 in patients with systemic lupus erythematosus. J Rheumatol. 2010b;37:2516–22.

Wang G, Tam LS, Li EK, et al. Serum and urinary free microRNA level in patients with systemic lupus erythematosus. Lupus. 2010c;20:493–500.

Wang H, Peng W, Ouyang X, et al. Circulating microRNAs as candidate biomarkers in patients with systemic lupus erythematosus. Transl Res. 2012;160:198–206.

Wang G, Kwan BC, Lai FM, et al. Urinary sediment miRNA levels in adult nephrotic syndrome. Clin Chim Acta. 2013;418:5–11.

Wei Q, Bhatt K, He HZ, Mi QS, Haase VH, Dong Z. Targeted deletion of Dicer from proximal tubules protects against renal ischemia-reperfusion injury. J Am Soc Nephrol. 2010;21:756–61.

Wilflingseder J, Regele H, Perco P, et al. miRNA profiling discriminates types of rejection and injury in human renal allografts. Transplantation. 2013;95:835–41.

Wilflingseder J, Sunzenauer J, Toronyi E, et al. Molecular pathogenesis of post-transplant acute kidney injury: assessment of whole-genome mRNA and miRNA profiles. PLoS One. 2014;9: e104164.

Wu J, Zheng C, Fan Y, et al. Downregulation of microRNA-30 facilitates podocyte injury and is prevented by glucocorticoids. J Am Soc Nephrol. 2014;25:92–104.

Zarjou A, Yang S, Abraham E, Agarwal A, et al. Identification of a microRNA signature in renal fibrosis: role of miR-21. Am J Physiol Renal Physiol. 2011;301:F793–801.

Zhang W, Zhang C, Chen H, et al. Evaluation of microRNAs miR-196a, miR-30a-5P, and miR-490 as biomarkers of disease activity among patients with FSGS. Clin J Am Soc Nephrol. 2014;9:1545–52.

Zhao X, Tang Y, Qu B, et al. MicroRNA-125a contributes to elevated inflammatory chemokine RANTES levels via targeting KLF13 in systemic lupus erythematosus. Arthr Rheum. 2010;62:3425–35.

Zhao S, Wang Y, Liang Y, et al. MicroRNA-126 regulates DNA methylation in CD4+ T cells and contributes to systemic lupus erythematosus by targeting DNA methyltransferase 1. Arthr Rheum. 2011;63:1376–86.

Zhou H, Hasni SA, Perez P, et al. MiR-150 promotes renal fibrosis in lupus nephritis by downregulating SOCS1. J Am Soc Nephrol. 2013;24:1073–87.

Serum Creatinine Trajectories in Kidney Disease

8

Macaulay Onuigbo, Nneoma Agbasi, Ogonna Oguejiofor, Emmanuel Okocha, Chinawaeze Aneke, and Charles Odenigbo

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Abstract

Creatinine is the end product of the metabolism of creatine phosphate, a by-product of skeletal muscle metabolism. It is excreted mainly via the kidneys, primarily by glomerular filtration. It is therefore the most widely used blood assay to measure the presence and progression of chronic kidney disease. The real-time monitoring of serum creatinine translations at the individual patent level, the so-called serum creatinine trajectories, offers a fascinating methodology of the study of kidney function and disease. In this review, we have examined and analyzed the serum creatinine trajectories in kidney disease, ranging from acute kidney injury (AKI) with its multifarious rainbow spectrum of renal outcomes in AKI, through the titillating vicissitudes of the different patterns of CKD to ESRD progression including a description of the syndrome of rapid-onset end-stage renal disease (SORO-ESRD) and the syndrome of late-onset renal failure from angiotensin blockade (LORFFAB) to the serum creatinine trajectories of some specific renal syndromes including adult polycystic kidney disease, HIV nephropathy, and sickle cell disease. These patients represent representative cases of the named renal states as managed at the Renal Unit of the Mayo Clinic Health System, Eau Claire, Northwestern Wisconsin, USA, and the Renal Clinic of Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria. We surmise that the study of individual patient-level serum creatinine trajectories, an evolving area of current nephrology practice, can indeed provide additional diagnostic and prognostic insights in the management of the nephrology patient.

Keywords

Acute kidney injury (AKI) • Chronic kidney disease (CKD) • End-stage renal disease (ESRD) • Late-onset renal failure from angiotensin blockade (LORFFAB) • Renal replacement therapy (RRT) • Renoprevention • Serum creatinine • Serum creatinine trajectories • Syndrome of rapid-onset end-stage renal disease (SORO-ESRD)

Key Facts

- Serum creatinine is a measure of kidney function in patients with kidney disease.
- With kidney disease and the resulting failing kidney function, the serum creatinine concentration rises.

- The estimated glomerular filtration rate (eGFR) derived from serum creatinine using prespecified formulas gives GFR values equivalent to specific serum creatinine concentrations for a particular patient at any point in time and is expressed in ml/min/1.73 m^2 BSA.
- The graphical representation and analysis of the real-time translations in serum creatinine at the individual patient level constitute the science of serum creatinine trajectories.
- Serum creatinine trajectories can provide exciting and scintillating new insights into renal disease diagnosis and prognostication.
- Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are the most studied pharmaceutical agents which by blocking the renin angiotensin aldosterone system are able to slow down the progression of kidney disease especially in proteinuric CKD patients, diabetic and nondiabetic.
- There are recent reports of a potential nephrotoxic effect of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers especially at higher doses in older (>65-year-old) later (>CKD III) patients, as typified in the newly described syndrome of late-onset renal failure from angiotensin blockade (LORFFAB).

Definitions

Acute kidney injury (AKI) This is any disease state or condition that results in an acute new-onset injury to the kidneys, usually leading to an increase in measured serum creatinine concentration.

Chronic kidney disease (CKD) This describes a chronic and sometimes often stable state of chronic disease affectation of the kidneys by medical conditions such as hypertension and diabetes mellitus and is characteristically categorized into five stages, CKD I, II, III, IV, and V, according to prespecified eGFR cutoff ranges with CKD V being the worst group with eGFR of <15 ml/min/1.73 m^2 BSA.

Creatinine This is the end product of skeletal muscle creatine phosphate metabolism that enters the blood stream and is excreted from the body via the kidneys in the urine; hence the serum concentration of creatinine is a measure of kidney function in health and in disease.

End-stage renal disease (ESRD) This is the terminal state of usually irreversible kidney disease resulting from disease conditions such as hypertension and diabetes mellitus that require renal replacement therapy.

Renal replacement therapy (RRT) This refers to alternative lifesaving interventions used to prolong life in patients with advanced kidney failure and include hemodialysis, peritoneal dialysis, and kidney transplantation.

Renoprevention This is the practice of calculated and preemptive avoidance or minimization of nephrotoxic exposure in CKD patients undergoing major surgery,

prior to iodinated contrast administration and during critical illness, and the agents to avoid include nephrotoxic antimicrobials such as aminoglycosides, NSAIDs and cox II inhibitors, diuretics, and angiotensin-converting enzyme inhibitors or angiotensin receptor blockers ("triple whammy" medications).

Quadruple whammy This is the name of a newly described syndrome of postoperative AKI occurring in CKD patients on concurrent "triple whammy" medications.

Syndrome of rapid-onset end-stage renal disease (SORO-ESRD) This is a newly described syndrome of rapid unanticipated acute-onset yet irreversible kidney failure requiring renal replacement therapy indefinitely, following episodes of acute kidney injury from medical and surgical factors.

Introduction

Creatinine: Metabolism, Chemical Structure, and Excretion in the Urine

Creatinine is the end product of the breakdown of creatine phosphate in muscle metabolism. It is usually produced at a fairly constant rate by the body, depending on the individual's muscle mass. It is produced via a biological system involving creatine, phosphocreatine (also known as creatine phosphate), and adenosine triphosphate (ATP, the body's immediate energy supply). Creatine is synthesized primarily in the liver from the methylation of glycocyamine (guanidinoacetate, synthesized in the kidney from the amino acids, arginine, and glycine) by S-adenosylmethionine. It is then transported through the blood to the other organs, muscle, and the brain, where, through phosphorylation, it becomes the high-energy compound phosphocreatine (Taylor 1989). During the reaction, creatine and phosphocreatine are catalyzed by creatine kinase, and a spontaneous nonenzymatic conversion process to creatinine may occur (Allen 2012). Degradation consists of cyclization of creatine to form creatinine (Wyss and Kaddurah-Daouk 2000). The degradation process is dependent on both pH and temperature. If creatine is in the presence of basic solutions, it will remain as creatine, if the temperature remains low (Wyss and Kaddurah-Daouk 2000). Conversely, if creatine is in the presence of an acidic solution with high temperatures, it will be converted to creatinine. Creatinine is eventually excreted from the body through the urine. It is removed from the blood chiefly by the kidneys, primarily by glomerular filtration, but also by proximal tubular secretion. Little or no tubular reabsorption of creatinine occurs. Thus, the level of the serum creatinine in a subject is a general reflection of the level of kidney function. With kidney disease and loss of nephrons, the level of serum creatinine would therefore show an upward trend. Conversely, with improving kidney function, say following acute kidney injury (AKI), the level of serum creatinine will then trend downwards. Accordingly, serum creatinine is the most widely used assay to measure the presence and progression of chronic kidney disease (Levey 1990).

Serum Creatinine Trajectories in Kidney Disease

Serum Creatinine Trajectories in Acute Kidney Injury (AKI): The Rainbow Spectrum of Renal Outcomes Following AKI in CKD Patients

Whereas nephrologists and physicians are generally conversant with the common diagnosis of acute kidney injury (AKI) in patients with chronic kidney disease (CKD), the so-called phenomenon of acute-on-chronic renal disease (AKI-on-CKD), nevertheless, the common consensus is that the impact of AKI on renal function is usually short-lived and fleeting, with typical expected recovery of renal function in most instances (Ponte et al. 2008; Wald et al. 2009; Ishani et al. 2009; Onuigbo and Achebe 2013). Nonetheless, mutually anecdotal as well as objective evidence in the nephrology literature support a contrarian notion that quite often, much less renal recovery follows these AKI on CKD events (Onuigbo and Achebe 2013). Indeed, there is new and cumulative evidence in the AKI literature demonstrating that AKI not only leads to and propagates CKD but that AKI could also directly lead to irreversible ESRD and the need for permanent renal replacement therapy, the so-called newly described syndrome of rapid-onset end-stage renal disease (Onuigbo 2010).

The following are four case reports with representative graphs of serum creatinine trajectories of patients seen and managed at the Renal Unit of the Mayo Clinic Health System in Northwestern Wisconsin, USA, who typify the varying spectrum of renal outcomes following AKI in patients with CKD.

Rapid and Full Recovery of Renal Function Following AKI on CKD

A 48-year-old obese hypertensive Caucasian male patient, with previously stable stage II CKD, with a baseline serum creatinine of 1.2 mg/dL and estimated GFR (eGFR) of >60 ml/min/1.73 m^2 BSA in January 2013, developed AKI on CKD following fever of unknown origin (FUO) complicating methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia (Onuigbo and Achebe 2013). ACE inhibition with benazepril was discontinued due to AKI presentation, whereas amlodipine was continued for hypertension control. He had iodinated contrast administration as part of work-up of the bacteremia. He received multiple courses of different parenteral antibiotics including nafcillin, vancomycin, and Cubicin, as prescribed by Infectious Disease Consultation. A kidney biopsy revealed acute interstitial nephritis, without evidence for contrast-induced nephropathy, nor glomerulonephritis. Serum creatinine peaked at 3.3 mg/dL (eGFR of 20 ml/min/1.73 m^2 BSA) (Fig. 1). He was treated conservatively and did not need renal replacement therapy (RRT). He rapidly improved and exhibited full recovery of his renal function within a month (Fig. 1).

Partial Recovery of Renal Function Following AKI on CKD

An 83-year-old white woman with hypertension and otherwise stable CKD III underwent a right hemicolectomy procedure together with the resection of about

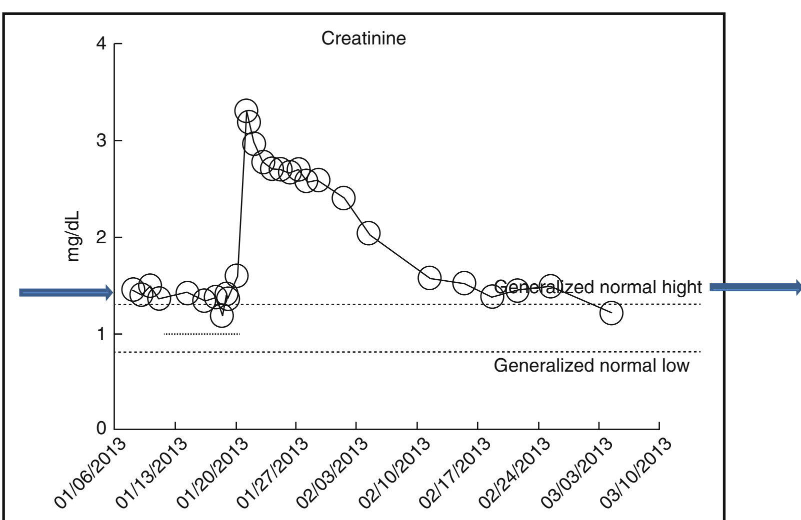

Fig. 1 Serum creatinine trajectory showing rapid full recovery of renal function following AKI on CKD

150 cm of her small intestine with end-to-end ileocolic anastomosis for colon cancer in October 2011. This was complicated by diarrhea and dehydration. Furthermore, there was the need for a second laparoscopic procedure in November 2011. She experienced AKI in October 2011 following the initial surgical intervention. She experienced yet a second AKI episode the following month in November 2011 from hypovolemic dehydration with peak creatinine values as shown in Fig. 2. She partially recovered kidney function and since December 2011 has maintained a higher new baseline serum creatinine. She otherwise remains asymptomatic and continues to feel great at her current age of 85 years. Her previous baseline serum creatinine was 1.2 mg/dL in January 2006, 1.3–1.4 mg/dL in 2010–2011 (CKD stage III, eGFR approximately 36–38 mL/min per 1.73 m^2 BSA). Between December 2011 and March 2014, the timeline for this review, she has maintained a new higher but stable baseline serum creatinine of approximately 2.0–2.2 mg/dl (eGFR 20–25 mL/min per 1.73 m^2 BSA), CKD stage IV (Fig. 2).

Rapid-Onset Yet Irreversible ESRD or the Syndrome of Rapid-Onset ESRD (SORO-ESRD) Following AKI on CKD in a Patient with Native Kidneys

An 81-year-old type 2 diabetic hypertensive Caucasian female patient on losartan 50 mg daily for hypertension control was evaluated for acute worsening dyspnea in December 2004 (Onuigbo and Achebe 2013). This was associated with a large right-sided pleural effusion. Critical Care Pulmonary Medicine consultation ordered a contrast-enhanced chest CT examination to rule out pulmonary embolism. She did not have pulmonary embolism. An echocardiogram showed stable left ventricular ejection fraction of 45–50 %, unchanged from a previous examination completed

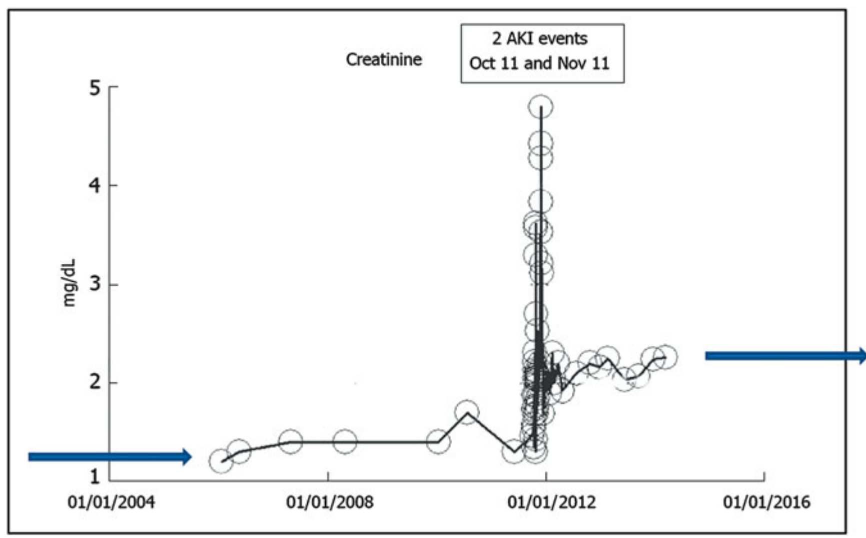

Fig. 2 Serum creatinine trajectory showing partial recovery of renal function following postoperative AKI on CKD in an 83-year-old Caucasian woman

earlier in April 2003. However, the patient very rapidly, within 24 h, following iodinated contrast exposure, developed oliguric AKI on CKD. Serum creatinine had more than doubled, from a baseline of 1.6 mg/dL, quickly up to 3.7 mg/dL, in association with acutely worsening new-onset dipstick proteinuria. Serum creatinine continued to rise with falling urine output mandating the initiation of RRT (Onuigbo and Achebe 2013). She was started on hemodialysis, just 3 days following iodinated contrast exposure. She remained oligoanuric and was on maintenance hemodialysis up until October 2012, nearly 8 years later. She died in October 2012, at the age of 89 years, serum creatinine about 4 mg/dL, from hypotensive shock following urosepsis, overall cachexia, and failure to thrive. This picture of precipitate acute unanticipated but yet irreversible AKI resulting in ESRD needing permanent RRT was termed the syndrome of rapid-onset ESRD (SORO-ESRD), a newly described syndrome that we first reported in 2010 (Onuigbo 2010; Onuigbo et al. 2013d, 2014).

Rapid-Onset Yet Irreversible ESRD or the Syndrome of Rapid-Onset ESRD (SORO-ESRD) Following AKI on CKD in a Renal Transplant Recipient

A 53-year-old Caucasian female patient, with type 1 diabetes mellitus, hypertension, and diabetic gastroparesis, had received a simultaneous pancreas-kidney transplantation (SPK) in 2000 for ESRD. She was on maintenance of immunosuppression with tacrolimus, mycophenolate mofetil, and prednisone. Through 2010, she had maintained a baseline serum creatinine of 1.6–1.8 mg/dL, eGFR ~34 ml/min/1.73 m^2 BSA, consistent with stable renal allograft stage III CKD (Onuigbo

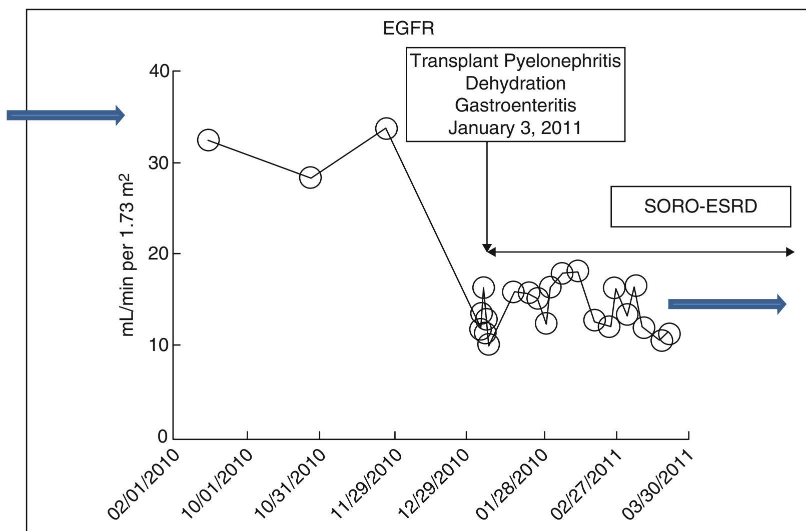

Fig. 3 eGFR trajectory in a renal transplant recipient demonstrating rapid-onset yet irreversible ESRD following AKI on CKD requiring permanent RRT following transplant pyelonephritis and concomitant dehydration

and Achebe 2013). In January 2011, she presented with symptomatic acute transplant pyelonephritis from *Escherichia coli*, further complicated by dehydration following 1 week of nausea, vomiting, and diarrhea (Onuigbo 2013d; 2014). She quickly developed worsening AKI on CKD with worsening oliguria. Serum creatinine quickly increased within days to 5.16 mg/dL (Fig. 3). She soon needed the initiation of RRT for progressive oliguric AKI with anorexia and volume overload. Emergent RRT was started as hemodialysis via a tunneled central dialysis catheter on January 8, 2011. She was then referred to Mayo Clinic, Rochester, for continued care. Renal allograft biopsy, carried out the following week at Mayo Clinic, Rochester, revealed acute tubular necrosis and chronic transplant glomerulopathy, but without rejection (Onuigbo 2013; Onuigbo et al. 2014). She remained on maintenance outpatient in-center, three times weekly, hemodialysis, for oliguric irreversible ESRD, for 1 year. In January 2012, exactly 1 year since the AKI on CKD event which led to the rapid-onset ESRD or SORO-ESRD, she received a second living-related renal allograft from her then 32-year-old son, again at Mayo Clinic, Rochester.

Remarkably, throughout all of these events, her pancreas allograft, part of the SPK from 2000, has nevertheless remained perfectly functional. She has continued to maintain excellent renal allograft function, with current baseline serum creatinine in May 2014 of 0.88 mg/dL, eGFR >60 ml/min/1.73 m^2 BSA and a current A1c of 4.8 %. Again, this picture of precipitate acute unanticipated but yet irreversible AKI resulting in ESRD needing permanent RRT was termed the syndrome of rapid-onset ESRD (SORO-ESRD), a newly described syndrome that we first reported in 2010 (Onuigbo 2010; Onuigbo et al. 2013, 2014).

Serum Creatinine Trajectories in Adult Polycystic Kidney Disease (ADPKD): An Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria Renal Clinic Experience

Case I

A 50-year-old obese Nigerian female patient, with a 4-year history of uncontrolled hypertension, on four different antihypertensive drugs, was evaluated in the Renal Clinic of Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria, in July 2008, following the development of early morning facial puffiness. Her initial serum creatinine was 1.6 mg/dL, eGFR of 46 ml/min/1.73 m^2 BSA, CKD stage III. There was a strong family history of hypertension and an older brother had died of a hemorrhagic stroke. She had hepatomegaly of 8 cm. Both kidneys were ballottable. Chest radiograph showed features of hypertensive heart disease, EKG revealed left ventricular hypertrophy, and abdominal ultrasound demonstrated enlarged kidneys with multiple cysts as well as hepatic cysts. A brain CT scan was normal. There was 2+ proteinuria. A diagnosis of autosomal dominant polycystic kidney disease (ADPKD) was made. Her antihypertensive drugs were adjusted to include lisinopril 20 mg daily. Serum creatinine remained stable. After 13 months follow-up, in August 2009, she was admitted with complicated acute pyelonephritis. Urine culture showed coliform organisms, sensitive to ciprofloxacin. She experienced AKI with serum creatinine peaking at 5.2 mg/dL (Fig. 4). She improved and was discharged after 6 days. Kidney function improved and serum creatinine decreased to 3.0 mg/dL a month later (Fig. 4).

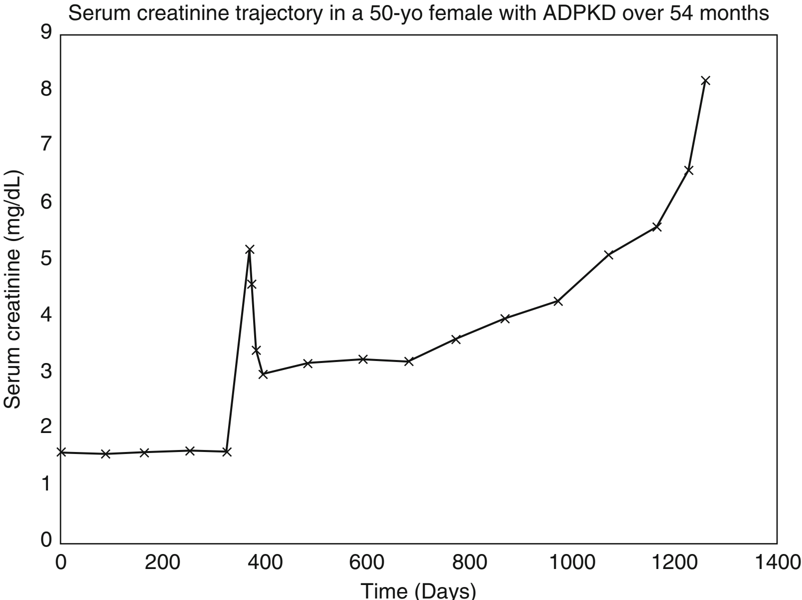

Fig. 4 Serum creatinine trajectory in a 50-year-old female with ADPKD over 54 months

Afterward, for almost a year, serum creatinine remained stable at 3.0–3.2 mg/dL, equivalent to eGFR of 21 ml/min/1.73 m^2 BSA, consistent with otherwise stable CKD IV (Fig. 4). Nevertheless, for unknown reasons, in late 2010, she developed progressively worsening azotemia and anemia, without clinical evidence of fluid overload. Serum creatinine had risen to 8.2 mg/dL by January 2012 (Fig. 4). She started renal replacement therapy in January 2012, but died after just 6 months on hemodialysis.

Case II

A 62-year-old grandmother presented to the Renal Clinic, Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria, in February 2014, with a history of loin pain and gross hematuria following a fall while on a visit to her daughter's house. She had been hypertensive for the preceding 6 years and had been compliant with her antihypertensive medications. In previous months, prior to her presentation, her blood pressure had been poorly controlled despite continuing her usual antihypertensive agents. Family history of hypertension was present in both parents. She had tender ballotable kidneys but no other palpable abdominal organs. Chest radiograph was normal, but EKG showed changes of the left ventricular hypertrophy. Serum creatinine was normal, at 0.8 mg/dL, eGFR of 92 ml/min/1.73 m^2 BSA, consistent with CKD stage I. Renal ultrasound showed bilateral multiple cysts in both kidneys. The liver, pancreas, and other abdominal organs were otherwise normal. Again, a diagnosis of ADPKD was made. She was admitted for bed rest, analgesics, and intravenous fluids. She made an uneventful recovery and has since continued her outpatient visits and her serum creatinine has remained otherwise stable (Fig. 5).

Autosomal dominant polycystic kidney disease is the most common inherited renal cystic disease. It occurs worldwide, in all races, but appears less common in black individuals (Yersin et al. 1997). Although, it has been suggested that ADPKD is rare in Africans, the reality is that the paucity of reports from Africa is most likely attributable to a low index of suspicion and inadequate diagnosis (Fary Ka et al. 2010). The first sonographic family study of ADPKD in a Nigerian family was described in 1991, following the introduction of the first functional ultrasound unit in Southeastern Nigeria in Enugu (Onuigbo et al. 1991). This was the first Nigerian publication on a family study of ADPKD that emphasized the need and foundational relevance of ultrasonographic family screening to increase the premorbid diagnosis of ADPKD among Nigerians (Onuigbo et al. 1991). With the increasing availability of ultrasound and other imaging methods, more cases are clearly now being recognized in Africa. A recent report from Ilorin in Northern Nigeria showed that 8 % of renal cases visiting the renal clinics over a 10-year period have ADPKD (Chijioke et al. 2010).

Hypertension occurs in about 50 % of young adults with ADPKD and normal renal function. Its frequency increases as renal function deteriorates and almost approaches 100 % of patients at ESRD (Kelleher et al. 2004). The development of renal failure is highly variable. In most patients renal function is maintained within normal range, despite relentless cyst growth until the fourth to sixth decade of life. By the time decline in renal function begins, cyst growth is extensive with little recognizable renal parenchyma. When it starts, the rate of decline of renal function is about 4.4–5.9 ml/min/year (Klahr et al. 1995). Risk factors for decline in renal

function include PKD1 gene, male sex, black race, first episode of hematuria before 30 years, onset of hypertension before 35 years, hyperlipidemia, low HDL concentration, and sickle cell trait (Johnson and Gabow 1997).

The first case of ADPKD presented in this review of serum creatinine trajectories in kidney disease clearly demonstrated the various vicissitudes of serum creatinine translations in CKD patients (Fig. 4) (Onuigbo and Agbasi 2014). Earlier on, the patient was able to maintain a stable CKD stage III status, with serum creatinine of about 1.6 mg/dL, eGFR of 46 ml/min/1.73 m^2 BSA. However, following complicated acute coliform pyelonephritis, she experienced AKI on CKD, serum creatinine peaked at 5.2 mg/dL. After antibiotic therapy, she had experienced partial recovery from the AKI episode, with a new albeit higher baseline serum creatinine of 3.0 mg/dL (Onuigbo and Agbasi 2014) (Fig. 4). Nevertheless, for almost a year following this, she maintained stable serum creatinine of 3.0–3.2 mg/dL, eGFR ~21 ml/min/1.73 m^2 BSA, consistent with otherwise stable CKD IV (Onuigbo and Agbasi 2014). However, after mid-2010, for unclear reasons, she now developed slowly but relentlessly progressive kidney failure, inexorably reaching ESRD and the need for RRT in January 2012, with serum creatinine reaching 8.2 mg/dL (Fig. 4).

The second case of ADPKD, on the other hand, despite several years of hypertension, on antihypertensive agents, often uncontrolled, and despite an episode of gross hematuria following a fall in February 2014, had continued to maintain stable excellent renal function, in November 2014, with a serum creatinine of 0.86 mg/dL, eGFR of 92 ml/min/1.73 m^2 BSA, CKD stage I (Fig. 5).

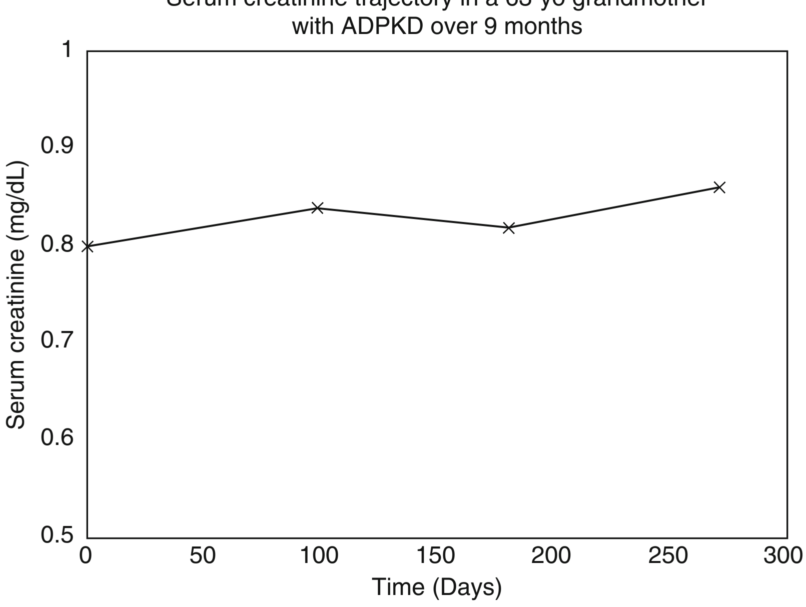

Fig. 5 Serum creatinine trajectory in a 63-year-old female with ADPKD over 9 months

Serum Creatinine Trajectories in Chronic Kidney Disease: The NKF KDOQI CKD Staging Paradigm Revisited: CKD Prediction Is an Inexact Science: The Novel Concept of CKD "Progressors" and CKD "Nonprogressors"

The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) expert committee in 2002 instituted and established new guidelines that established a novel chronic kidney disease (CKD) staging paradigm (NKF K/DOQI 2002; Levey et al. 2003). In this CKD prototype model, using ranges of prespecified estimated glomerular filtration rates (eGFR), CKD was characterized into five stages: I, II, III, IV, and V (NKF K/DOQI 2002; Levey et al. 2003). Clearly, the principle behind this NKF K/DOQI CKD staging archetype solely rests on the absolute assumption that serum creatinine and eGFR trajectories in CKD patients generally follow a linear, predictable, smoothly progressive, and time-dependent curve to advance through the increasing CKD stages I through V before inexorably reaching ESRD and the need for renal replacement therapy (RRT) (NKF K/DOQI 2002; Levey et al. 2003; Chiu et al. 2008; Onuigbo and Agbasi 2014). Nonetheless, it must be recognized that such proposition of predictable, linear, time-dependent progressive step-wise decline in kidney function, with mathematically linear falling eGFR over time, and with eGFR methodically marching through these incremental projected CKD stages I through V, and finally inexorably ending in symptomatic ESRD and the need for RRT, is unproven, untested, and potentially flawed (Ballardie et al. 1983; Walser et al. 1989; Shah and Levey 1992; Onuigbo 2009a, 2013; Onuigbo et al. 2013; Onuigbo and Agbasi 2014, 32). Moreover, a 2011 Canadian retrospective analysis, which analyzed the longitudinal changes during a 1.1-year observation period of eGFR and CKD stages, demonstrated CKD stage variability (defined by changes in CKD stages) among 1262 patients, mean age 71.25 years, drawn from two large Canadian renal clinics (Sikaneta et al. 2012). This study reported that CKD stage changed in 40 % of the cohort (including 7.4 % in whom CKD stage improved), whereas CKD stage remained static in 762 (60.4 %) patients, the majority of this CKD cohort (Sikaneta et al. 2012). Another earlier Canadian study examined 4231 CKD IV patients characterized by an index eGFR of <30 mL/min per 1.73 m^2 BSA, with at least three subsequent eGFR values available for analysis, and no <4 months of follow-up between January 2000 and January 2004 (Levin et al. 2008). The conclusion was that the clinical course of patients with CKD stage 4 was unpredictably variable (Levin et al. 2008). Furthermore, a 2012 retrospective report from South Korea examined 347 CKD III patients, enrolled between January 1997 and December 1999, who were followed up through June 2010, a period of 10 years (Baek et al. 2012). One hundred and sixty-seven patients (48.1 %) did not progress, 60 (17.3 %) progressed to stage 4, and 120 (34.6 %) progressed to stage 5, with 91 (26.2 %) starting dialysis (Baek et al. 2012). Besides, recently, French investigators examined 406 patients in the NephroTest cohort with measured glomerular filtration rates (mGFRs) measured by 51Cr-EDTA clearance at least three times during at least 2 years of follow-up (Weis et al. 2013). The individual examination of mGFR trajectories by four independent nephrologists classified patients as "improvers," defined as those showing a sustained

mGFR increase, or "nonimprovers" (Weis et al. 2013). Measured GFR improved over time in 62 patients (15.3 %). Their median mGFR slope was + 1.88 (IQR, 1.38, 3.55) mL/min per year; it was 22.23 (23.9, 20.91) for the 332 "nonimprovers." The conclusion from this French study was that GFR improvement is possible in CKD patients at any CKD stage through stages 4–5 (Weis et al. 2013). In a previous 2013 review and in a more recent wide-ranging review article published in 2014, we have exhaustively reexamined these phenomena regarding CKD behavior and again proposed the nomenclature of CKD "progressors" and CKD "nonprogressors" (Onuigbo et al. 2013; Onuigbo and Agbasi 2014).

We shall now describe some selected CKD patients seen and managed at the Mayo Clinic Health System Renal Unit in Northwestern Wisconsin who showed stable and unchanged kidney functional states at CKD stages III, IV, and V, respectively, over several years with no perceptible changes in eGFR, despite the advanced ages of the majority of these patients, mostly >75 years of age, the so-called CKD nonprogressors (Onuigbo et al. 2013; Onuigbo and Agbasi 2014).

Stable CKD V Over 7 Years in a Now 78-Year-Old Caucasian Hypertensive Diabetic Male

As at February 2014, a now 78 year-old obese hypertensive type II diabetic white male has over the last 7 years, between 2006 and 2013, despite a serum creatinine of 4.5–5.5 mg/dL, eGFR 8–11 mL/min per 1.73 m^2 BSA, stage V CKD, remained otherwise asymptomatic (Fig. 6) (Onuigbo and Agbasi 2014). He has continued for the past 4 years on alternate monthly courses of prophylactic short-duration oral

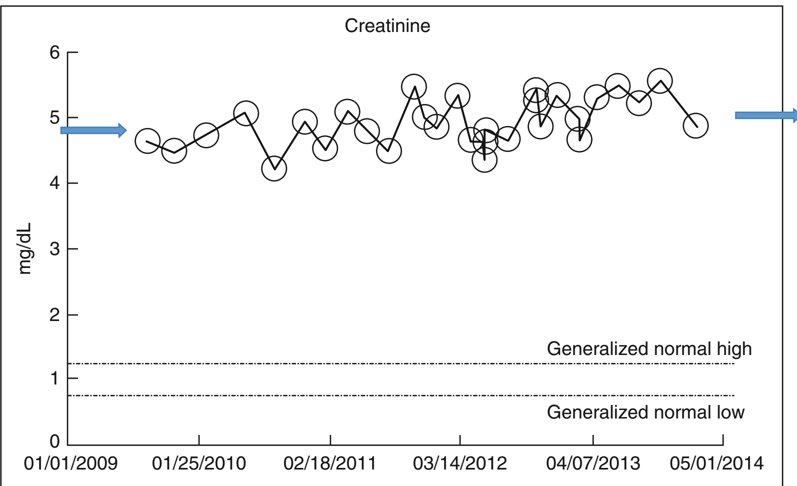

Fig. 6 Serum creatinine trajectory in a now 78-year-old Caucasian hypertensive diabetic male with stable CKD V between 2006 and 2013, serum creatinine of 4.5–5.5 mg/dL, eGFR 8–11 mL/min per 1.73 m^2 BSA

levofloxacin for recurrent UTI prophylaxis. He has not progressed any further in the last 7 years and has not needed renal replacement therapy.

Stable CKD IV Over 8 Years in a Now 78-Year-Old Caucasian Hypertensive Diabetic Male

A then 69-year-old Caucasian male was diagnosed with Wegener's granulomatosis complicated by AKI on CKD in 2005 (Fig. 7). Serum creatinine then had increased from 2.0 mg/dL to 3.5–4.0 mg/dL (Onuigbo and Agbasi 2014). The Wegener's granulomatosis was treated with standard immunosuppressive therapy using prednisone. He had remained in remission on low-dose prednisone since 2006, albeit with a new higher baseline serum creatinine of 3.5–4.0 mg/dL, eGFR 16–22 mL/min per 1.73 m^2 BSA, stage IV CKD. He, now aged 78 years in 2014, has remained otherwise an asymptomatic CKD IV patient for 8 years (Fig. 7).

Stable CKD III Over Nearly 10 Years in a Now 87-Year-Old Caucasian Hypertensive Male

An 87-year-old obese Caucasian hypertensive male patient, with a history of a cerebrovascular accident following right carotid surgery in 2001, has generally maintained a baseline serum creatinine of 1.6–2.0 mg/dL, eGFR 31–40 ml/min/ 1.73 m^2 BSA, CKD stage III, between October 2005 and September 2014, the only period that we have available serum creatinine values.

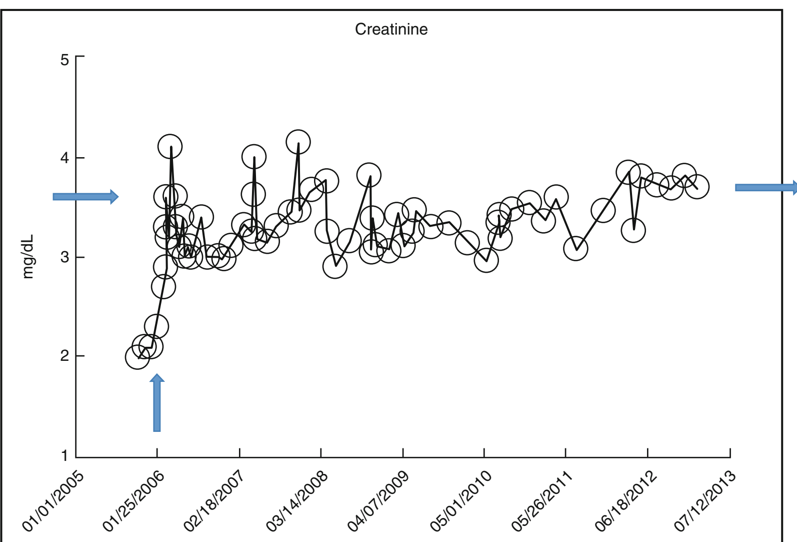

Fig. 7 Serum creatinine trajectory in a now 78-year-old Caucasian male with Wegener's granulomatosis in remission, otherwise stable CKD IV between 2006 and 2014, serum creatinine of 3.5–4.0 mg/dL, eGFR 16–22 mL/min per 1.73 m^2 BSA

Two Divergent Patterns of CKD to ESRD Progression: The "Classic" Pattern and the Pattern of the Syndrome of Rapid-Onset End-Stage Renal Disease (SORO-ESRD)

Generally, it is a commonly held consensus opinion among practicing nephrologists, in particular, and physicians, in general, that the propagation of CKD to ESRD is that of a predictable, linear, progressive, relentless, time-dependent, and knowable decline in renal function, with predictably increasing serum creatinine or falling eGFR, leading inexorably to ESRD and the need for RRT (Rutherford 1977; NKF K/DOQI 2002; Levey et al. 2003; Chiu et al. 2008). This universally accepted paradigm of CKD-ESRD progression will be referred to in this review as the "classic" pattern of CKD to ESRD progression (Onuigbo and Agbasi 2014). Nevertheless, various observations and reports in the nephrology literature, to the contrary, have demonstrated that quite often, the path from a priori stable CKD to irreversible ESRD can be precipitate, acute, and unpredictable (Merino et al. 1975; Bonomini et al. 1984; Bhandari and Turney 1996; Firth 1996; Onuigbo 2009, 2010, 2013; Onuigbo and Onuigbo 2011, 2012; Onuigbo et al. 2013, 2014). In 2010, we first described the previously unrecognized syndrome of rapid-onset end-stage renal disease or SORO-ESRD in the journal, *Renal Failure* (Onuigbo 2010). SORO-ESRD, the syndrome of rapid-onset ESRD, is the unpredictable, unanticipated, and accelerated progression from a priori stable CKD to irreversible ESRD, requiring permanent RRT, following a new episode of AKI precipitated by antecedent new medical/surgical events, with the interval between AKI and the need for RRT represented by a period of often <2 weeks, usually measured only in days following surgically induced AKI (Onuigbo 2010, 2013; Onuigbo et al. 2014; Onuigbo and Agbasi 2014).

In the following section, we would present selected case reports demonstrating the features of "classic" CKD-ESRD progression pattern and the CKD-ESRD progression pattern of the syndrome of rapid-onset ESRD.

"Classic" Pattern of CKD-ESRD Progression in a Hypertensive Caucasian Male

A 52-year-old Caucasian hypertensive male with low ejection fraction ischemic cardiomyopathy, and hypothyroidism, had developed progressively worsening renal failure with predictable linear increases in serum creatinine after November 2007 when serum creatinine was 1.9 mg/dL through to December 2010 when serum creatinine exceeded 7.0 mg/dL, and he developed features of uremia and started renal replacement therapy in the form of in-center outpatient hemodialysis (Onuigbo and Agbasi 2014) (Fig. 8). He has continued on maintenance hemodialysis through March 2014, when this review was completed.

"Classic" Pattern of CKD-ESRD Progression in a Caucasian Hypertensive Diabetic Male

An 82-year-old Caucasian man with past medical history for hypertension, low ejection fraction ischemic cardiomyopathy, type II diabetes mellitus, coronary

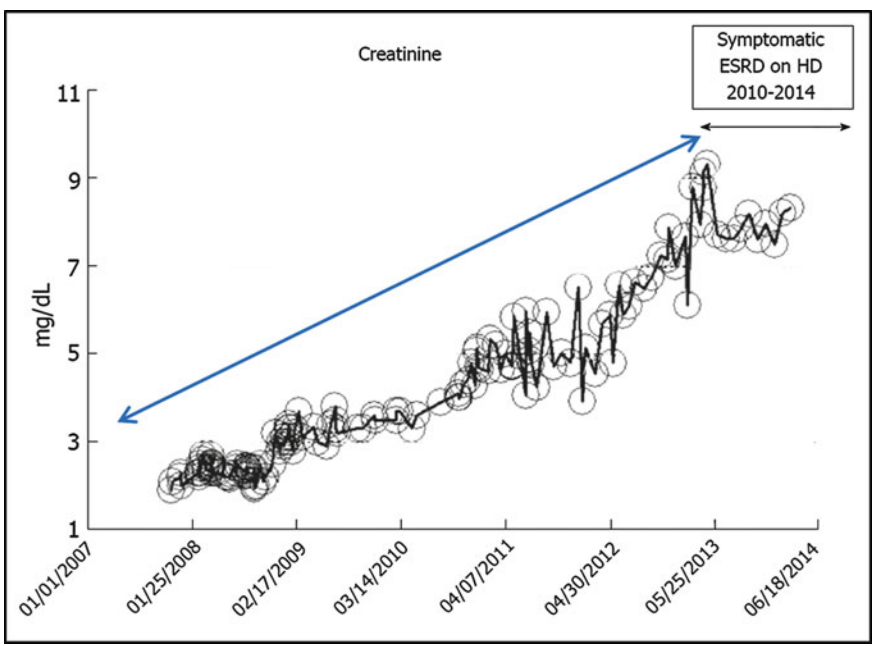

Fig. 8 Serum creatinine trajectory in a 52-year-old Caucasian hypertensive male who developed predictable linear and progressive time-dependent CKD to ESRD, 2007–2010, and has remained on maintenance hemodialysis 2010–2014

artery disease, obesity, and hypothyroidism developed progressively worsening renal failure with a time-dependent predictable linear increase in serum creatinine from May 2006. Serum creatinine was 2.6 mg/dL in May 2006 and progressively increased through to January 2010 when serum creatinine exceeded 6.0 mg/dL and he had then developed features of uremia. He therefore initiated renal replacement therapy in the form of in-center outpatient hemodialysis in January 2010. He however died in early 2014, while still on maintenance hemodialysis, from failure to thrive. Regrettably, there were no laboratory data for serum creatinine available for this patient before May 2006.

Pattern of Syndrome of Rapid-Onset ESRD in a Caucasian Hypertensive Diabetic Male

A 73-year-old obese hypertensive type II diabetic male patient with a stable baseline serum creatinine of approximately 1.7 mg/dL, eGFR 43 ml/min/1.73 m^2 BSA, stable CKD stage III, between 2010 and 2012, on concurrent ACE inhibition with lisinopril 40 mg daily, was admitted to the coronary care unit in February 2012 with acute decompensated heart failure (Onuigbo and Agbasi 2014). Acute coronary syndrome was ruled out by investigation, and the patient subsequently underwent minimally invasive aortic valve replacement for symptomatic aortic

stenosis. A 25 mm St. Jude Epic Stented Tissue Valve was deployed on March 2, 2012, by his cardiothoracic surgeon. He rapidly developed postoperative AKI on CKD and required hemodialysis on the first postoperative day with worsening oliguria and associated severe volume overload (Fig. 9). He never recovered any kidney function, and he has since then remained on outpatient in-center maintenance, three times weekly, hemodialysis for ESRD, over two and half years later in November 2014. His current serum creatinine in November 2014 is 9.88 mg/dL (Fig. 9).

Late-Onset End-Stage Renal Failure from Angiotensin Blockade (LORFFAB)

We described, for the first time in 2005, the syndrome of late-onset renal failure from angiotensin blockade (LORFFAB) (Onuigbo and Onuigbo 2005, 2008). This is defined as the accelerated but potentially reversible iatrogenic renal failure from concurrent angiotensin blockade, which occurs in usually older CKD patients, despite normal renal arteries, absent traditionally acknowledged precipitating risk factors, and while remaining on the same dose of angiotensin blockade during the preceding 3 months or greater (Onuigbo and Onuigbo 2005, 2008; Onuigbo and Achebe 2013).

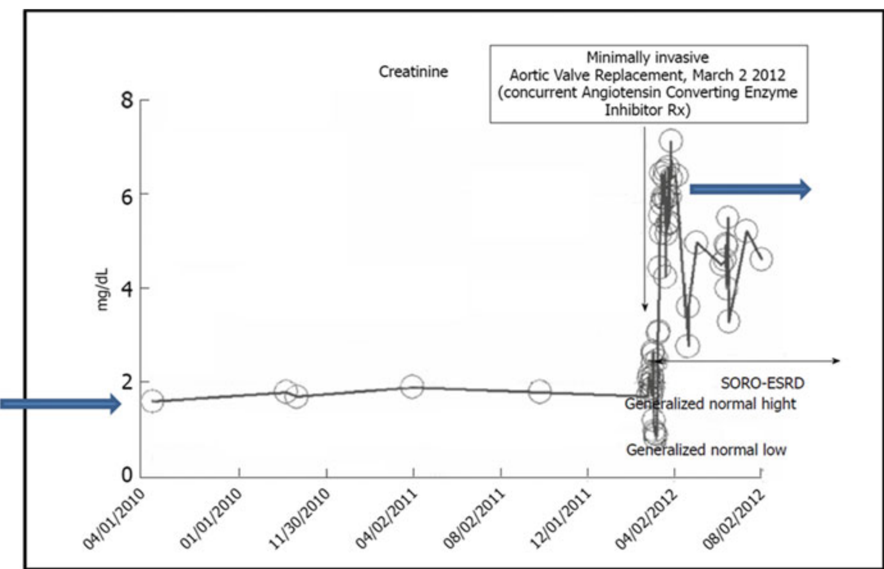

Fig. 9 Serum creatinine trajectory in a 75-year-old Caucasian obese hypertensive diabetic male who developed acute unpredictable yet irreversible AKI needing RRT consistent with the syndrome of rapid-onset ESRD (SORO-ESRD), following minimally invasive aortic valve replacement in March 2012; he has remained on maintenance hemodialysis through November 2014

The following case presentation is an illustration of the impact of LORFFAB on serum creatinine trajectories in CKD patients.

In April 2004, a 77-year-old diabetic hypertensive Caucasian male patient was admitted to our Renal Unit in Northwestern Wisconsin with symptomatic oliguric renal failure, serum creatinine of 4.6 mg/dL, eGFR of 13 ml/min/ 1.73 m^2 BSA, associated with dyspnea and volume overload (Onuigbo and Onuigbo 2008). He needed the initiation of emergent hemodialysis with ultrafiltration via a dialysis catheter. Lisinopril, 20 mg daily, which he had received for 21 months prior to presentation, was promptly discontinued. Amlodipine, 5 mg BID, was substituted for antihypertensive therapy. He continued on maintenance hemodialysis for 10.5 months and his serum creatinine improved slowly with increasing urine output (Fig. 10). By February 2005, his serum creatinine had stabilized at about 3 mg/dL and hemodialysis was discontinued (Fig. 10). He remained hemodialysis independent for almost 2 years. Unfortunately, in January 2007, at the age of 79 years, still off hemodialysis, with otherwise stable CKD IV, he suffered a heart attack and underwent a cardiac catheterization, followed by a four-vessel coronary artery bypass graft procedure. As a result of worsening postoperative AKI, he again needed hemodialysis postoperatively (Fig. 10). He subsequently was transferred out to Minneapolis, MN, for cardiac rehabilitation and was lost to our follow-up (Onuigbo and Achebe 2013).

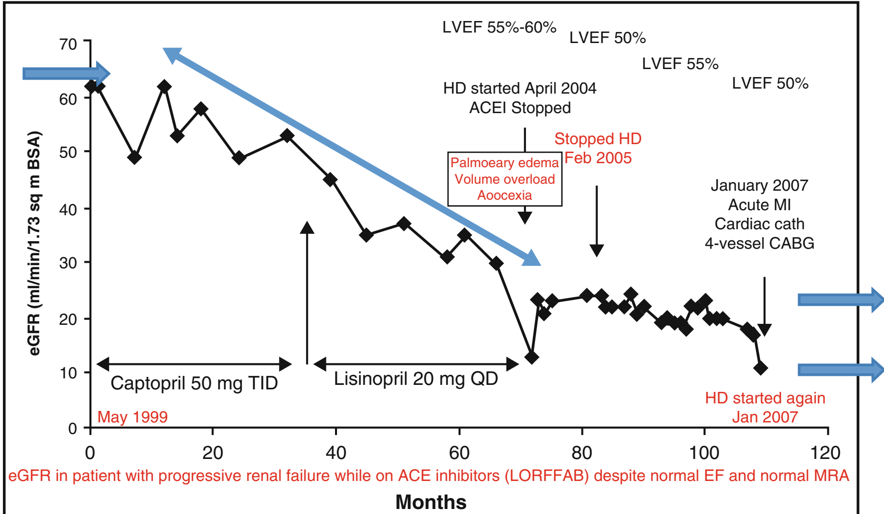

Fig. 10 Serum creatinine trajectory in the then 77-year-old diabetic hypertensive Caucasian male patient with features of LORFFAB who in 2004 required temporary hemodialysis for 11 months, was hemodialysis independent 2005–2007 following discontinuation of lisinopril, but developed postoperative AKI again requiring RRT in January 2007 following cardiac catheterization and four-vessel coronary artery bypass graft procedure

Serum Creatinine Trajectories in a Case of "Quadruple Whammy"

We recently described a new syndrome of accelerated postoperative AKI on CKD occurring in previously stable CKD patients who were concurrently at the time of the surgery on triple whammy medications of angiotensin blockade, a diuretic and an NSAID or a COX-2 inhibitor (Onuigbo and Onuigbo 2013; Onuigbo et al. 2013c; Onuigbo and Agbasi 2014). We present in the following section a representative case report.

In the Spring of 2013, our nephrology service was consulted on a 46-year-old morbidly obese (170 Kg) hypertensive Caucasian male patient, current smoker, with previously otherwise stable stage III CKD, serum creatinine of 1.21 mg/dL, eGFR of 70 ml/min/1.73 m^2 BSA. He had developed accelerated AKI on CKD following an elective right hip arthroplasty for symptomatic degenerative joint disease (Onuigbo and Onuigbo 2013; Onuigbo and Agbasi 2014) (Fig. 11). He was on lisinopril 40 mg daily and hydrochlorothiazide 25 mg daily. Incidentally, the patient also received a preoperative dose of celecoxib (Celebrex®) per orthopedic unit "operative analgesia protocol," a COX-2 inhibitor, thus completing the "triple whammy circle" (Onuigbo and Onuigbo 2013). Within 36 h, postoperatively, he quickly developed oliguric AKI on CKD, complicated by metabolic acidosis. Serum creatinine had more than doubled to 2.58 mg/dL, eGFR of 28 ml/min/1.73 m^2 BSA (Fig. 11). Notably, there was evidence of significant intraoperative as well as postoperative hypotension, despite several liters of infused normal saline (Fig. 11). Lisinopril and

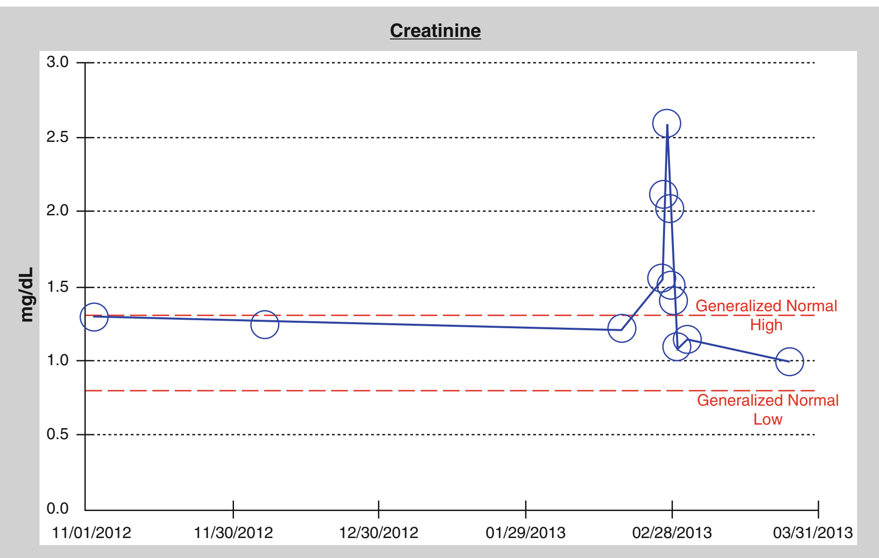

Fig. 11 Serum creatinine trajectory showing rapidly rising serum creatinine within 36 h in CKD III patient on "triple whammy" medications following elective right hip arthroplasty procedure consistent with the "quadruple whammy" syndrome

hydrochlorothiazide were promptly discontinued. He also developed postoperative anemia (hemoglobin down to 10.4 g/L from 15.8 g/L). He received more intravenous normal saline for hypotension and was started on intravenous furosemide due to persistent oliguria. Subsequently, urine output improved and serum creatinine started to fall (Fig. 11). He improved and was soon discharged from the hospital, on postoperative day 3 on amlodipine 10 mg daily and furosemide 40 mg daily for hypertension. Serum creatinine, from March 25, 2013, approximately 1 month following the hip arthroplasty, was 0.99 mg/dL, lower than his premorbid levels, with an improved eGFR of 85 ml/min/1.73 m^2 BSA (Fig. 11).

Recently, in a 2014 publication in the journal, *Nigerian Journal of Clinical Practice*, we elaborated in great detail the typical presentations of patients who experience this newly described syndrome of "quadruple whammy" (Onuigbo and Agbasi 2014). We indeed further hypothesized on a plausible role of concomitant obesity in the pathogenesis of this syndrome (Onuigbo and Agbasi 2014). For purposes of limiting AKI in CKD patients, more so in the perioperative period, we once again call on physicians, in general, and nephrologists, in particular, to pre-emptively discontinue "triple whammy" medications before any major surgical interventions (Onuigbo 2009, 2013; Onuigbo and Agbasi 2014). This is one of the cardinal principles of our "Renoprevention" mantra (Onuigbo 2009, 2013; Onuigbo and Agbasi 2014).

Perioperative AKI and Concurrent Angiotensin Inhibition Alone: A Case Presentation from Mayo Clinic Health System, Eau Claire, Wisconsin, USA

The role of concurrent angiotensin inhibition alone, without the involvement of the combination "triple whammy" medications in exacerbating postoperative AKI, continues to generate significant controversy in the nephrology literature (Onuigbo 2011, 2013, 2014; Nielson et al. 2014). However, it would appear that on the balance, accruing evidence favors the notion that more so with prevalent intraoperative hypotension, that concurrent angiotensin inhibition results in higher rates of postoperative AKI when compared to situations with its preemptive withdrawal preoperatively (Onuigbo 2011, 2013, 2014; Nielson et al. 2014). The later approach of a preoperative preemptive temporary withdrawal of angiotensin inhibition especially in the older (>65-year-old and later stage CKD) patients is indeed one of the cornerstones of our newly introduced concept of "Renoprevention" (Onuigbo 2009, 2013; Onuigbo and Agbasi 2014). We now report here a case of postoperative AKI apparently exacerbated by concurrent angiotensin inhibition.

A 57-year-old obese hypertensive type II diabetic Caucasian male patient underwent an elective pulmonary vein isolation and ablation procedure for symptomatic atrial fibrillation in early July 2014. Concurrent outpatient oral medications included chlorthalidone, metformin 1 g BID, and lisinopril 40 mg daily. Postoperative AKI triggered a nephrology consultation. Baseline serum creatinine

was 1.0 mg/dL, GFR 81 ml/min/1.73 m^2 BSA, CKD stage II. On postoperative day 1, serum creatinine had quickly increased to 1.96 mg/dL (Fig. 12a). At the time of the nephrology consultation the following day, on postoperative day 1, he was normotensive, and all available medical floor blood pressure recordings before and after the procedure were noted to be normal. We had been informed that the procedure in the operating room "went well without complications." However, our urgent and meticulous review and analysis of the operating room intraoperative anesthesia records revealed significant hypotension during the over 4-h surgical procedure (Fig. 12b). He was nonoliguric and otherwise asymptomatic except for mild lightheadedness. The patient was therefore managed conservatively. Lisinopril and metformin were promptly discontinued. Kidney function subsequently quickly improved. A few days later, with improved and stable kidney function, he was placed back on the lisinopril, at discharge. His subsequent serum creatinine in late July 2014 was 0.91 mg/dL, eGFR of 91 mL/min/1.73 m^2 BSA (Fig. 12a).

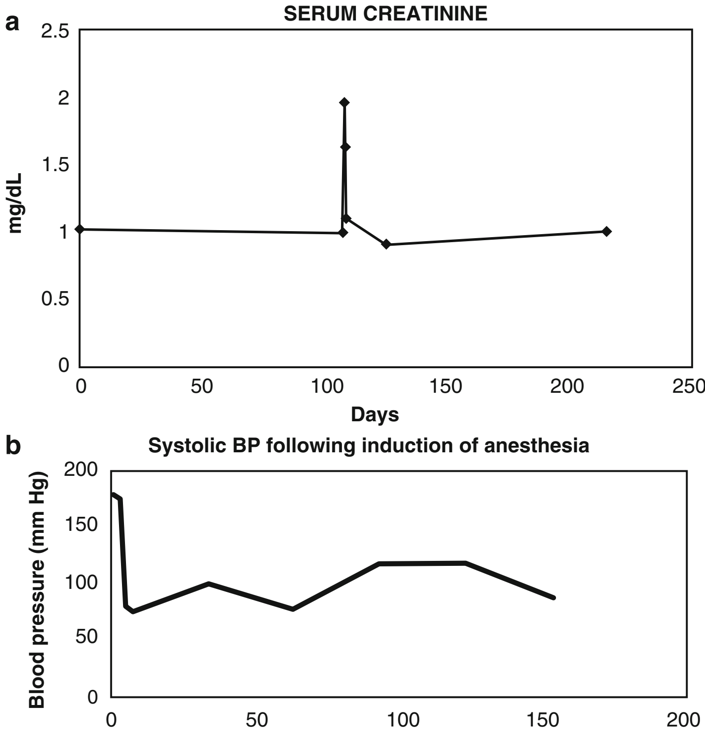

Fig. 12 (**a**) Serum creatinine trajectory 2 weeks following elective ablation procedure for symptomatic atrial fibrillation with complete recovery from AKI. (**b**) Intraoperative systolic blood pressure changes following induction of anesthesia during elective ablation procedure for symptomatic atrial fibrillation

Serum Creatinine Trajectories in HIV-Associated Nephropathy: An Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria, Renal Clinic Experience

HIV-infected individuals present with a variety of renal syndromes manifesting as acute renal failure or chronic renal failure (Weiner et al. 2003). HIV-associated nephropathy (HIVAN) is diagnosed to be present when a seropositive person presents with persistent proteinuria, progressively worsening azotemia with preservation of normal kidney size (or even enlarged kidneys), and renal histological findings of focal segmental glomerulosclerosis on renal biopsy. These subjects usually have relatively normal blood pressure and often exhibit rapid progression to renal failure and end-stage renal disease.

Among the various glomerulopathies documented in HIV or AIDS subjects, the commonest is the classic HIV-associated nephropathy with focal glomerulosclerosis (Klotman 1999). This occurs in 3–10 % of HIV-infected persons with high prevalence reported in black males with low socioeconomic status (Winston et al. 1998). It is also the third leading cause of ESRD among African Americans aged 20–64 years (United States Renal Data System USRDS, 1992–1999).

In Nigeria, the prevalence of HIV has risen exponentially from <1 % to 5.8 %, values from different communities and different states ranging from 2 % to 15 % (National AIDS/HIV/STD Control Program, Technical Reports 1997–2003), with HIVAN contributing significantly to the overall burden and magnitude of CKD and ESRD in Nigeria. Studies from Nigeria have reported high prevalence rates of renal function impairment of 53.3 % in South-South Nigeria (Okafor et al. 2011) and 52.0 % in North-Central Nigeria (Agaba et al. 2003). Another study from 2008 demonstrated the occurrence of elevated serum creatinine in a high proportion of HIV-infected Nigerian patients (Emem et al. 2008). The presentation of two HIV AIDS patients managed for HIVAN at Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria, is presented below to document the patterns of serum creatinine trajectories of such subjects in Nnewi, in Southeastern Nigeria.

Case I

A 25-year-old male Nigerian trader resident in Aba, Abia State, Southeastern Nigeria, presented to the Medical Outpatient Department (MOPD) of Nnamdi Azikiwe Teaching Hospital, Nnewi, Nigeria, in September 2013 with 2-month history of shortness of breath, intermittent leg swelling associated with facial puffiness, and diarrhea. He was recently diagnosed with HIV disease as the index illness and had been commenced on highly active antiretroviral therapy (HAART) (Abacavir, Lamivudine and Efavirenz) for about 2 weeks prior to presentation, on account of very low CD4 count of five cells/mm^3. He was chronically ill-looking and pale. Pulse was 100/min and blood pressure was 140/80 mmHg. He was dehydrated and was managed as a case of HIVAN with gastroenteritis. Pertinent test results included elevated serum creatinine, 5.9 mg/dL, metabolic acidosis with bicarbonate level of 10 mmol/L, normokalemia at 3.8 mEq/L, and albumin 26 g/dL. He demonstrated 2+ proteinuria and severe

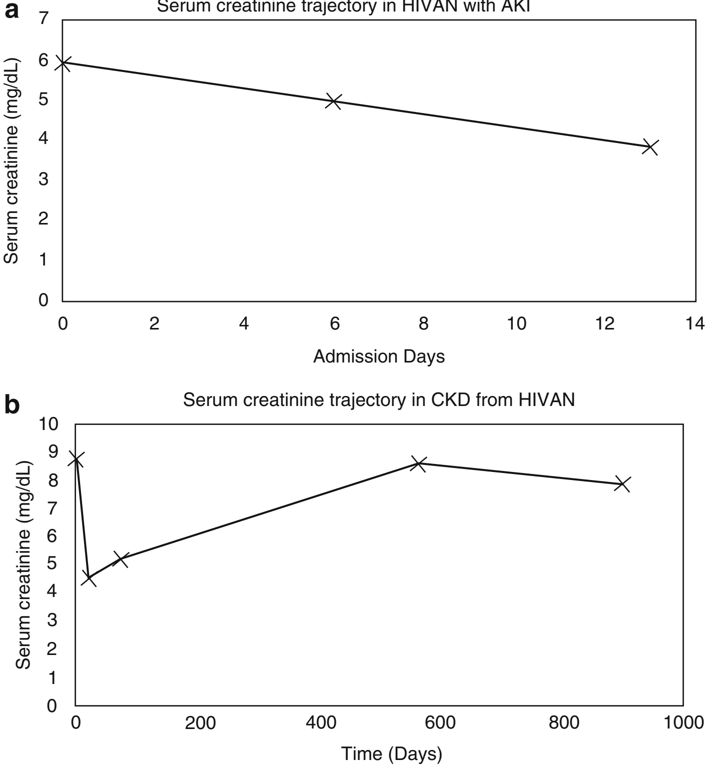

Fig. 13 (**a**) Serum creatinine trajectory in a 25-year-old Nigerian male patient who developed AKI with HIVAN and died 16 days into hospital admission. (**b**) Serum creatinine in HIV-associated CKD on conservative management with HAART

anemia, with a hematocrit of 18 %. HIV serology was positive, but serology tests for hepatitis B and C were nonreactive. He was transfused with five units of blood but developed seizures 13 days into his admission. He died on the 16th day. Kidney function tests had however started to improve prior to the patient's death on conservative management (Fig. 13a).

Case II

A 26-year-old Nigerian female student from Anambra State, Southeastern Nigeria, presented to the MOPD of Nnamdi Azikiwe Teaching Hospital, Nnewi, Nigeria, in September 2010 with 6 weeks' history of generalized body swelling and fatigue of 4 weeks' duration, with associated decrease in urine output and increased frothiness of urine. She also had dyspnea, orthopnea, and paroxysmal nocturnal dyspnea. She had been diagnosed with HIV disease 4 years previously and was commenced on HAART (zidovudine, lamivudine, and nevirapine) shortly after the diagnosis. She is not diabetic nor hypertensive. Examination showed

respiratory distress, pallor, and bilateral pitting edema. Pulse was 84/min and blood pressure was 110/60 mmHg. There was a pansystolic murmur loudest at the apex, fine bibasal crepitations, and a tender hepatomegaly, measured 4 cm below the right costal margin, together with ascites. Serum creatinine was 8.7 mg/dL, potassium 3.9 mEq/L, bicarbonate 14 mEq/L, and hematocrit of only 18 %. She was managed conservatively for chronic kidney disease secondary to HIVAN and anemic heart failure. She responded to conservative management at the MOPD after initial discharge. Her serum creatinine trajectory between August 2010 and February 2014 is shown above (Fig. 13b). She has not yet required renal replacement therapy.

The first patient with HIVAN developed symptomatic AKI while on HAART and died 13 days into the admission despite improving renal function (Fig. 13a). The second patient has features of CKD V secondary to HIVAN and on HAART (Fig. 13b). Despite the elevated serum creatinine values, she has continued to remain otherwise asymptomatic on conservative medical management without requiring renal replacement therapy (Onuigbo 2013; Onuigbo and Agbasi 2014).

Serum Creatinine Trajectories in Sickle Cell Disease Nephropathy: An Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria, Experience

A 26-year-old Nigerian male patient with sickle cell anemia (HbSS) was well until 24 months before presentation to Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria, in February 2013, when he woke up with lightheadedness and dyspnea. Prior to this, he had experienced frequent episodes of bone pain crises for which he was in and out of hospital admissions and needed regular use of opiates and other analgesics for pain control. He had earlier been placed on hydroxyurea, over a 3-month period for management of recurrent bone pain crises. Further evaluation revealed severe hypertension for which he was placed on nifedipine. Laboratory work-up revealed severe anemia, proteinuria, and azotemia, serum creatinine 4.9 mg/dL, eGFR 13 ml/min/1.73 m^2 BSA, consistent with CKD V. A diagnosis of sickle cell nephropathy was consequently made and he was then referred to the renal clinic. Following conservative management including transfusion of blood for symptomatic anemia and improved hypertension control, his CKD status improved, without a need for renal replacement therapy. The trajectory of his serum creatinine over a period of 21 months is shown in Fig. 14 above.

Sickle cell nephropathy encompasses the spectrum of morphologic, laboratory, and clinical changes due to kidney pathology associated with sickle cell disease (SCD). It specifically includes papillary necrosis, hyposthenuria, impaired renal acidification, proteinuria, hematuria, supranormal proximal tubular function, and renal failure (Stuart and Nagel 2004). It is a major risk factor for early mortality in SCD (Platt et al. 1994), hence its importance in patient evaluation and management. The magnitude of sickle cell nephropathy in Nigeria had been emphasized in

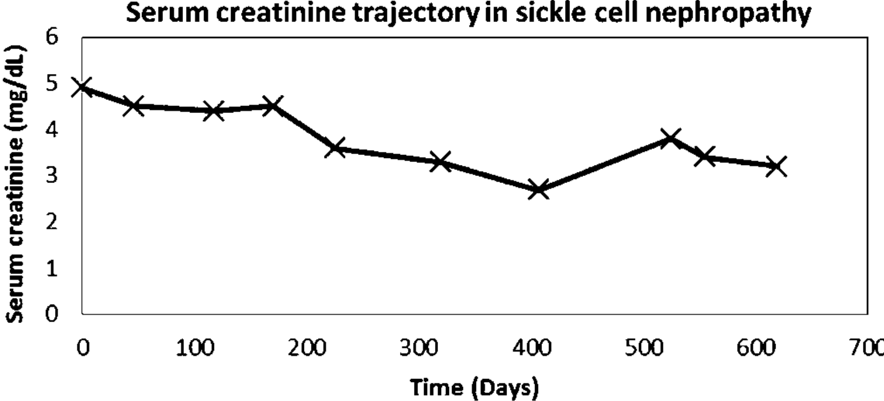

Fig. 14 Serum creatinine trajectory in a 26-year-old Nigerian male with sickle cell nephropathy and CKD IV–V, over 21 months, 2013–2014

previous reports where up to 47 %, 69.4 %, and 37.2 % of patients with SCD had chronic kidney disease (CKD), respectively (Aneke et al. 2014; Bolarinwa et al. 2012; Arogundade et al. 2011). Similarly, the median age of onset of significant renal impairment in SCD was 23.1 years, while the median age at the time of death was 27 years (Powars et al. 1991).

Serum creatinine measurement and urinalysis for proteinuria are important laboratory work-up for patients with sickle cell nephropathy; indeed they are frontline investigations in our facility for patients with this condition. Our index patient presented with biochemical evidence of nephropathy (proteinuria and deranged serum creatinine) in addition to severe anemia. Here again, a patient with SCD and sickle cell nephropathy has exhibited often unpredictable CKD stage changes during his conservative management (Onuigbo and Agbasi 2014).

Conclusions: A Need for More Preventative Renal Medicine: Renoprevention Revisited. The Introduction of the New Innovative CKD Express © IT Software

In 2002, the National Kidney Foundation established a novel chronic kidney disease (CKD) staging paradigm (NKF K/DOQI 2002; Levey et al. 2003). In 2012, the authoritative United States Preventive Task Force questioned the validity of asymptomatic CKD screening (Moyer 2012). The American Society of Nephrology and the American College of Physicians have opposite recommendations regarding this controversy (ASN 2013; ACP 2013). Moreover, CKD prediction and prognostication is clearly an inexact science (Onuigbo and Agbasi 2014, 32). CKD care must therefore be individualized. We recently developed a new IT software, the CKD Express ©, currently in US Patent Application (Onuigbo 2012; Onuigbo et al. 2013;

Onuigbo and Agbasi 2014). The incorporation of this yet to be patented IT software into existing electronic medical record (EMR) systems would go a long way in bridging this yawning gap in our knowledge and understanding of CKD initiation, propagation, and progression.

Preventative medicine is a neglected art in modern-day practice of medicine. Prevention is always better than cure. According to Yach and Calitz, the greatest increase in healthcare spending between 2000 and 2011 was attributable to drugs, medical devices, and hospital care, with the cost of treating noncommunicable diseases (NCDs) estimated to exceed 80 % of annual healthcare expenditure, whereas 3 % was spent on public health and disease prevention programs. The National Institutes of Health estimates that 20 % of its $30 billion annual budget is allocated to prevention; however, <10 % is spent on human behavioral interventions that target the major modifiable risk factors. More investment in prevention science could lead to greater health gains at lower cost (Yach and Calitz 2014). We support such reengineering of nephrology practice whereby a lot more emphasis would now be placed on preventative nephrology (Onuigbo 2009, 2013; Onuigbo and Agbasi 2014).

The role of perioperative, and more so intraoperative hypotension, in the pathogenesis of postoperative AKI has been variously described. Despite the intensive use of vasopressors including multiple infusions of intravenous phenylephrine during the ablation procedure, our patient clearly experienced significant intraoperative hypotension. Without accessing intraoperative anesthesia records to demonstrate significant hypotension, the cause of the AKI would have remained speculative. Furthermore, the fact that the patient had remained on concurrent angiotensin inhibition up until the first postoperative day could possibly have contributed to the severity of postoperative AKI in our patient (Onuigbo 2011, 2014 (2); Nielson et al. 2014). Nielson et al. (2014) in a study of 1,154 surgical patients demonstrated that surgical candidates who receive preoperative angiotensin blockade have an associated increased risk of post-induction hypotension and postoperative AKI resulting in a greater hospital length of stay. From a perspective of “Renoprevention,” we again posit here that such patients should have the angiotensin blockade preemptively withheld 3–5 days prior to the surgery and angiotensin blockade can be restarted postoperatively if kidney function remained stable (Onuigbo 2009, 2011, 2013, 2014 (2); Onuigbo and Agbasi 2014). Furthermore, more efforts must be applied in the operating room to maintain more normal blood pressures during surgical procedures. This way, we would prevent more postoperative AKI events, reduce hospital length of stay, and help save scarce US healthcare dollars (Onuigbo 2013).

Finally, we must note here that two recent large multicenter randomized clinical trials failed to demonstrate any therapeutic benefits of perioperative use of vasodilators, aspirin, clonidine, or fenoldopam to mitigate against noncardiac postoperative AKI (Bove et al. 2014; Garg et al. 2014; Winkelmayer and Finkel 2014). We strongly advocate and submit that our suggested approach, heretofore, to reduce intraoperative hypotension while at the same time limiting exposure to potential nephrotoxics including angiotensin inhibition, would be far cheaper and more

effective in limiting postoperative AKI in our CKD patients than any esoteric pharmaceutical intervention (Jo et al. 2014; Onuigbo 2009, 2011, 2013, 2014; Nielson et al. 2014; Onuigbo and Agbasi 2014).

Potential Applications of Serum Creatinine Trajectories in Kidney Disease Prognostication and Management

In this review of serum creatinine trajectories in kidney disease, we have examined and reported on the multifarious behavior of serum creatinine in acute kidney injury, in CKD to ESRD progression, in otherwise nonprogressive CKD, in late-onset renal failure from angiotensin blockade (LORFFAB), in other specifically named renal syndromes, and in postoperative acute kidney injury. Clearly, there is a broad spectrum of renal outcomes following acute kidney injury, and indeed CKD in all its stages can be "progressors" vs. "nonprogressors" or "nonimprovers" vs. "improvers" (Onuigbo and Agbasi 2014).

From this analysis of the graphical representation and analysis of real-time translations in serum creatinine trajectories at the individual patient level, the science of serum creatinine trajectories, we submit that the science of serum creatinine trajectories is a neglected paradigm of modern nephrology practice (Onuigbo and Agbasi 2014). The study of individual patient-level serum creatinine trajectories, whether real-time, concurrent, or in retrospective analysis, can indeed provide exciting and sometimes scintillating new insights in our understanding of kidney disease diagnosis and prognostication (Onuigbo and Agbasi 2014). We, at the Renal Unit of the Mayo Clinic Health System, in Northwestern Wisconsin, USA, have extensively and consistently enabled the utility of this science of individual patient-level serum creatinine trajectories in the definition of two newly described renal syndromes, the syndrome of late-onset renal failure from angiotensin blockade (LORFFAB) in 2005 and the syndrome of rapid-onset end-stage renal disease (SORO-ESRD) in 2010 (Onuigbo and Onuigbo 2005; Onuigbo 2010).

Summary Points

- The analysis of serum creatinine trajectories at the individual patient level provides additional diagnostic and prognostic insights in patient care.
- We have described the different varied patterns of renal outcomes and the resulting serum creatinine trajectories in acute kidney injury (AKI).
- We also established that CKD staging and prognostication are an inexact science and that the commonly applied 2002 NKF K/DOQI CKD staging paradigms must be seen as what they are – clinical guidelines, only.
- We further described the serum creatinine trajectory patterns in CKD to ESRD progression including the syndrome of rapid-onset end-stage renal disease (SORO-ESRD).

- Additionally, we demonstrated the patterns of serum creatinine trajectories in patients with some specific renal syndromes including autosomal dominant polycystic kidney disease (ADPKD).
- We recommend an increasing utilization of this often-neglected area of nephrology practice, especially for individualized CKD care, including CKD prognostication at the individual patient level.

References

Agaba EI, Agaba PA, Sirisena ND, Antenyi EA, Idoko JA. Renal disease in AIDS patients in North Central Nigeria. Niger J Med. 2003;12(3):120–5.

Allen PJ. Creatine metabolism and psychiatric disorders: does creatine supplementation have therapeutic value? Neurosci Biobehav Rev. 2012;36(5):1442–62. doi:10.1016/j.neubiorev.2012.03.005.PMC3340488.

American College of Physicians. Summaries for patients. Screening, monitoring, and treatment of early stage chronic kidney disease: recommendations from the American College of Physicians. Ann Intern Med. 2013;159:I–28. [PMID: 24146038 doi:10.7326/0003-4819-159-12-201312170-00728].

American Society of Nephrology. ASN emphasizes need for early detection of kidney disease, a silent killer (Press Release, Oct 22 2013). Available from: URL: http://www.asn-online.org/news/2013/ASN_COMM_ACP_Screening_Response_102213_R12.pdf. Accessed 21 Dec 2013.

Aneke JC, Adegoke AO, Oyekunle AA, Osho PO, Sanusi AA, Okocha EC, Ibeh NC, Akinola NO, Durosinmi MA. Degrees of kidney disease in Nigerian adults with sickle cell disease. Med Princ Pract. 2014;23:271–4.

Arogundade FA, Sanusi AA, Hassan MO, Salawu L, Durosinmi MA, Akinsola A. An appraisal of kidney dysfunction and its risk factors in patients with sickle cell disease. Nephron Clin Pract. 2011;118(3):c225-31. doi:10.1159/000321138.

Baek SD, Baek CH, Kim JS, Kim SM, Kim JH, Kim SB. Does stage III chronic kidney disease always progress to end-stage renal disease? A ten-year follow-up study. Scand J Urol Nephrol. 2012;46:232–8. doi:10.3109/00365599.2011.649045. PMID: 22545920.

Ballardie FW, Gartside S, Mallick NP. Computer prediction of the need for dialysis and transplantation using calculated creatinine clearance. Br Med J (Clin Res Ed). 1983;286:1328–31. doi:10.1136/bmj.286.6374.1328. PMID: 6404450.

Bhandari S, Turney JH. Survivors of acute renal failure who do not recover renal function. QJM. 1996;89:415–21.

Bolarinwa RA, Akinlade K,S, Kuti M, Olawale OO, Akinola NO. Renal disease in adult Nigerians with sickle cell anemia: a report of prevalence, clinical features and risk factors. Saudi J Kidney Dis Transpl. 2012;12(23):171–5.

Bonomini V, Stefoni S, Vangelista A. Long-term patient and renal prognosis in acute renal failure. Nephron. 1984;36:169–72.

Bove T, Zangrillo A, Guarracino F, Alvaro G, Persi B, et al. Effect of fenoldopam on use of renal replacement therapy among patients with acute kidney injury after cardiac surgery: a randomized clinical trial. JAMA. 2014. doi:10.1001/jama.2014.13573. Epub ahead of print.

Chijioke A, Aderibigbe A, Olarenwaju TO, Makusidi AM, Oguntoyinbo AE. Prevalence and pattern of cystic kidney diseases in Ilorin, Nigeria. Saudi J Kidney Dis Transpl. 2010;21(6):1172–8.

Chiu YL, Chien KL, Lin SL, Chen YM, Tsai TJ, Wu KD. Outcomes of stage 3–5 chronic kidney disease before end-stage renal disease at a single center in Taiwan. Nephrol Clin Pract. 2008;109:c109–18. doi:10.1159/000145453. PMID: 18663322.

Emem CP, Arogundade F, Sanusi A, Adelusola K, Wokoma F, Akinsola A. Renal disease in HIV-seropositive patients in Nigeria: an assessment of prevalence, clinical features and risk factors. Nephrol Dial Transplant. 2008;23:741–6. doi:10.1093/ndt/gfm836.

Fary Ka E, Seck SM, Niang A, Cisse MM, Diouf B. Patterns of autosomal dominant polycystic kidney diseases in black Africans. Saudi J Kidney Dis Transpl. 2010;21(1):81–6.

Firth JD. Acute irreversible renal failure. QJM. 1996;89:397–9.

Garg AX, Kurz A, Sessler DI, Cuerden M, Robinson A, et al. Perioperative aspirin and clonidine and risk of acute kidney injury: a randomized clinical trial. JAMA. 2014. doi:10.1001/jama.2014.15284. Nov 15, [Epub ahead of print].

Ishani A, Xue JL, Himmelfarb J, Eggers PW, Kimmel PL, Molitoris BA, Collins AJ. Acute kidney injury increases risk of ESRD among elderly. J Am Soc Nephrol. 2009;20:223–8.

Jo SH, Lee JM, Park J, Kim HS. The impact of renin-angiotensin-aldosterone system blockade on contrast-induced nephropathy: a meta-analysis of 12 studies with 4,493 patients. Cardiology. 2014;130(1):4–14.

Johnson A, Gabow P. Identification of patients with autosomal dominant polycystic disease at highest for end stage renal disease. J Am Soc Nephrol. 1997;8:1560–7.

Kelleher CL, McFann KK, Johnson AM, Schrier RW. Characteristics of hypertension in young adults with autosomal dominant polycystic kidney disease compared with the general U.S. population. Am J Hypertens. 2004;17(11 Pt 1):1029–34.

Klahr S, Breyer JA, Beck GJ, Dennis VW, Hartman JA, Roth D, Steinman TI, Wang SR, Yamamoto ME. Dietary protein restriction, blood pressure control, and the progression of polycystic kidney disease. Modification of Diet in Renal Disease Study Group. J Am Soc Nephrol. 1995;5(12):2037–47.

Klotman PE. HIV – associated nephropathy. Kidney Int. 1999;56:1161–76.

Levey AS. Measurement of renal function in chronic renal disease. Kidney Int. 1990;38:167–84.

Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, Eknoyan G. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Ann Intern Med. 2003;139:137–47. doi:10.7326/0003-4819-139-2-200307150-00013. PMID: 12859163.

Levin A, Djurdjev O, Beaulieu M, Er L. Variability and risk factors for kidney disease progression and death following attainment of stage 4 CKD in a referred cohort. Am J Kidney Dis. 2008;52:661–71. doi:10.1053/j.ajkd.2008.06.023. PMID: 18805347.

Merino GE, Buselmeier TJ, Kjellstrand CM. Postoperative chronic renal failure: a new syndrome? Ann Surg. 1975;182:37–44.

Moyer VA. Screening for chronic kidney disease: U.S. Preventive Services Task Force recommendation statement. Ann Intern Med. 2012;157:567–70. doi:10.7326/0003-4819-157-8-201210160-00533. PMID: 22928170.

National AIDS/HIV/STD Control Programme, Federal Ministry of Health and Social Services. Technical Reports, 1997, 1999, 2000, 2002, 2003.

Nielson E, Hennrikus E, Lehman E, Mets B. Angiotensin axis blockade, hypotension, and acute kidney injury in elective major orthopedic surgery. J Hosp Med. 2014;9(5):283–8. doi:10.1002/jhm.2155. Epub 2014 Jan 24.

National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis. 2002;39:S1–266. PMID: 11904577.

Okafor UH, Unuigbe EI, Ojogwu LI, Oviasu E, Wokoma FS. Renal disease in HIV infected patients at University of Benin Teaching Hospital, Nigeria. Afr Health Sci. 2011;11(1):S28–33.

Onuigbo MA. The natural history of chronic kidney disease revisited – a 72-month Mayo Health System Hypertension Clinic practice-based research network prospective report on end-stage

renal disease and death rates in 100 high-risk chronic kidney disease patients: a call for circumspection. Adv Perit Dial. 2009a;25:85–8. PMID: 19886324.
Onuigbo MA. Reno-prevention vs. reno-protection: a critical re-appraisal of the evidence-base from the large RAAS blockade trials after ONTARGET – a call for more circumspection. QJM. 2009b;102(3):155–67.
Onuigbo MA. Syndrome of rapid-onset end-stage renal disease: a new unrecognized pattern of CKD progression to ESRD. Ren Fail. 2010;32:954–8. doi:10.3109/0886022X.2010.502608. PMID: 20722563.
Onuigbo MA. Can ACE, inhibitors and angiotensin receptor blockers be detrimental in CKD patients? Nephrol Clin Pract. 2011;118(4):c407–19. doi:10.1159/000324164. Epub 2011 Mar 7.
Onuigbo MA. CKD Express © – a new IT-software proposed for a paradigm change in CKD care. Open Med Inf J. 2012;6:26–7.
Onuigbo MA. The CKD enigma with misleading statistics and myths about CKD, and conflicting ESRD and death rates in the literature: results of a 2008 U.S. population-based cross-sectional CKD outcomes analysis. Ren Fail. 2013a;35:338–43. doi:10.3109/0886022X.2013.764272. PMID: 23391263.
Onuigbo MAC. Nondialytic therapy for end-stage renal disease is an underutilized care paradigm in the United States: time for a more robust reappraisal of this treatment option. Adv Perit Dial. 2013b;29:73–6.
Onuigbo MA. Evidence of the syndrome of rapid onset end-stage renal disease (SORO-ESRD) in the acute kidney injury (AKI) literature – preventable causes of AKI and SORO-ESRD – a call for re-engineering of nephrology practice paradigms. Ren Fail. 2013c;35(6):796–800. doi:10.3109/0886022X.2013.800459. Epub 2013 Jun 3.
Onuigbo MA. Syndrome of rapid-onset end-stage renal disease in two consecutive renal transplant recipients. Indian J Nephrol. 2013d;23:222–5. doi:10.4103/0971-4065.111861. PMID: 23814425.
Onuigbo MA. Renoprevention: a new concept for reengineering nephrology care – an economic impact and patient outcome analysis of two hypothetical patient management paradigms in the CCU. Ren Fail. 2013e;35(1):23–8.
Onuigbo MA. Renoprevention: a new concept for reengineering nephrology care – an economic impact and patient outcome analysis of two hypothetical patient management paradigms in the CCU. Ren Fail. 2013f;35(1):23–8. doi:10.3109/0886022X.2012.741644. Epub 2012 Nov 15.
Onuigbo MA. Renoprevention revisted – the impact of preemptive withdrawal of RAAS blockade prior to iodinated contrast exposure in older CKD patients: results of a new meta-analysis. Cardiology. 2014a;130(1):25–6.
Onuigbo MA. Preoperative angiotensin axis blockade therapy, intraoperative hypotension, and the risks of postoperative acute kidney injury. J Hosp Med. 2014b;9(9):610. doi:10.1002/jhm.2229. Epub ahead of print.
Onuigbo MA, Achebe NJ. Acute kidney injury on chronic kidney disease – the rainbow syndrome of too many colors: a Mayo Clinic Health System case series report. In: Onuigbo MAC, editor. ACE inhibitors: medical uses, mechanisms of action, potential adverse effects and related topics, vol. 1. New York: NOVA Publishers; 2013. p. 91–108.
Onuigbo M, Agbasi N. Medicare and the escalating costs of US ESRD care compared with the United Kingdom National Health Service: a case study of reengineering current US CDC hepatitis B virus testing protocols among hemodialysis patients to achieve cost savings – the use of a new IT software program, the CKD Express ©, to achieve cost-effective CKD care for the US Medicare. In: Berhardt LV, editor. Advances in medicine and biology, vol. 76. New York: Nova Publishers; 2014a. p. 21–38.
Onuigbo M, Agbasi N. "Quadruple whammy" – a preventable newly described syndrome of post-operative AKI in CKD II and CKD III patients on combination "Triple Whammy" medications: a Mayo Clinic Health System, Eau Claire, Wisconsin experience. Niger J Clin Pract. 2014b;17 (5):649–54.

Onuigbo M, Agbasi N. Chronic kidney disease prediction is an inexact science: the concept of "progressors" and "nonprogressors". World J Nephrol. 2014c;3(3):31–49.
Onuigbo M, Agbasi N. Escalating cost of pre-dialysis chronic kidney disease care in the US: the introduction of an innovative chronic kidney disease care paradigm using "The CKD Express © IT Software Program" – a new recommended tool for medicare and the affordable care act. Open Med Inf J. 2014. (In Print).
Onuigbo MA, Onuigbo NT. Late onset renal failure from angiotensin blockade (LORFFAB): a prospective thirty-month Mayo Health System clinic experience. Med Sci Monit. 2005;11: CR462–9. PMID: 16192897.
Onuigbo M, Onuigbo N, editors. Chronic kidney disease and RAAS blockade: a new view of renoprotection. London: Lambert Academic Publishing; 2011.
Onuigbo MA, Onuigbo NT. The syndrome of rapid onset end-stage renal disease (SOROESRD) – a new Mayo Clinic dialysis services experience, January 2010–February 2011. In: Di Iorio B, Heidland A, Onuigbo M, Ronco C, editors. Hemodialysis: when, how, why. New York: Nova Publishers; 2012. p. 443–85.
Onuigbo MA, Onuigbo NTC. A second case of "Quadruple Whammy" in a week in a Northwestern Wisconsin hospital. BMJ. 2013. Rapid Response, Published online 1 Mar 2013. http://www.bmj.com/content/346/bmj.f678/rr/633687
Onuigbo MA, Ozoh JO, Egbuji T, Ijeoma CK, Chukwuka C, Offodile AA, Obianigwe V. Adult polycystic kidney disease. A Nigerian family study. Niger Med J. 1991;3(4):71–3.
Onuigbo MA, Onuigbo C, Onuigbo V, Onuigbo M, Onuigbo N, Joshi M, Egbuniwe O. The CKD enigma, reengineering CKD care, narrowing asymmetric information and confronting ethicomedicinomics in CKD care: the introduction of the new 'CKD express©' IT software program. In: Onuigbo MAC, editor. ACE inhibitors: medical uses, mechanisms of action, potential adverse effects and related topics, vol. 1. New York: NOVA Publishers; 2013a. p. 41–56.
Onuigbo MA, Onuigbo NTC. "Quadruple Whammy", a new syndrome of potentially preventable yet iatrogenic acute kidney injury in the ICU. BMJ. 2013. Rapid Response, Published online 25 Feb 2013. http://www.bmj.com/content/346/bmj.f678/rr/632570
Onuigbo MA, Roman-Maradiaga BP, Achebe NJ. "Quadruple Whammy" – a newly described preventable syndrome of peri-operative AKI on CKD in patients on concurrent "Triple Whammy" medications. In: Onuigbo MAC, editor. ACE inhibitors: medical uses, mechanisms of action, potential adverse effects and related topics, vol. 1. New York: Nova Publishers; 2013c. p. 63–74.
Onuigbo MA, Achebe NJ, Musso CG. The syndrome of rapid onset ESRD in the last 100 consecutive incident Northwestern Wisconsin Mayo Clinic chronic hemodialysis patients, 2010–2011: results of the analysis of individual patient-level serum creatinine trajectories to ESRD – can there be a link with angiotensin inhibition and renal senescence in older CKD patients? In: Onuigbo MAC, editor. ACE inhibitors: medical uses, mechanisms of action, potential adverse effects and related topics, vol. 1. New York: Nova Publishers; 2013d. p. 109–25.
Onuigbo MA, Onuigbo NT, Musso CG. Syndrome of rapid onset end stage renal disease in incident Mayo Clinic chronic hemodialysis patient. Indian J Nephrol. 2014;24:75–81. doi:10.4103/0971-4065.127886. PMID:24701038.
Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, Klug PP. Mortality in sickle cell disease, life expectancy and risk factors for early death. N Engl J Med. 1994;330:1639–44.
Ponte B, Felipe C, Muriel A, Tenorio MT, Liano F. Long-term functional evolution after an acute kidney injury: a 10-year study. Nephrol Dial Transplant. 2008;23:3859–66.
Powars DR, Elliot – Mills DD, Chan L, Niland J, Hiti AL, Opas LM, Johnson C. Chronic renal failure in sickle cell disease: risk factors, clinical course and mortality. Ann Intern Med. 1991;115:614–20.
Rutherford WE, Blondin J, Miller JP, Greenwalt AS, Vavra JD. Chronic progressive renal disease: rate of change of serum creatinine concentration. Kidney Int. 1977;11:62–70. doi:10.1038/ki.1977.8. PMID: 839654.

Shah BV, Levey AS. Spontaneous changes in the rate of decline in reciprocal serum creatinine: errors in predicting the progression of renal disease from extrapolation of the slope. J Am Soc Nephrol. 1992;2:1186–91. PMID: 1591359.

Sikaneta T, Abdolell M, Taskapan H, Roscoe J, Fung J, Nagai G, Ting RH, Ng P, Wu G, Oreopoulos D, Tam PY. Variability in CKD stage in outpatients followed in two large renal clinics. Int Urol Nephrol. 2012;44:1461–6. doi:10.1007/s11255-011-9934-9. PMID: 21424573.

Stuart MJ, Nagel RL. Sickle-cell disease. Lancet. 2004;364:1343–60.

Taylor EH. Clinical chemistry. New York: Wiley; 1989. p. 58–62. 4.

United States Renal Data System (USRDS). Annual Reports. National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health. Bethesda, MD, 1992, 1994, 1995, 1996, 1997, 1998, 1999.

Wald R, Quinn RR, Luo J, Li P, Li P, Scales DC, Mamdani MM, Ray JG. University of Toronto Acute Kidney Injury Research Group: chronic dialysis and death among survivors of acute kidney injury requiring dialysis. JAMA. 2009;302:1179–85.

Walser M, Drew HH, LaFrance ND. Reciprocal creatinine slopes often give erroneous estimates of progression of chronic renal failure. Kidney Int Suppl. 1989;27:S81–5. PMID:2700001.

Weiner NJ, Goodman JW, Kimmel PL. The HIV-associated renal diseases: current insight into pathogenesis and treatment. Kidney Int. 2003;63:1618–31.

Weis L, Metzger M, Haymann JP, Thervet E, Flamant M, Vrtovsnik F, Gauci C, Houillier P, Froissart M, Letavernier E, Stengel B, Boffa JJ. Renal function can improve at any stage of chronic kidney disease. PLoS ONE. 2013;8:e81835. doi:10.1371/journal.pone.0081835. PMID:24349134.

Winkelmayer WC, Finkel KW. Prevention of acute kidney injury using vasoactive or antiplatelet treatment: three strikes and out? JAMA. 2014. doi:10.1001/jama.2014.14548. Nov 15, [Epub ahead of print].

Winston JA, Burns GC, Klotman PE. The immunodeficiency virus (HIV) epidemic and HIV-associated nephropathy. Semin Nephrol. 1998;18:373–7.

Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev. 2000;80 (3):1107–213.

Yach D, Calitz C. New opportunities in the changing landscape of prevention. JAMA. 2014;312 (8):791–2. doi:10.1001/jama.2014.8900.

Yersin C, Bovet P, Wauters JP, Schorderet DF, Pescia G, Paccaud F. Frequency and impact of autosomal dominant polycystic kidney disease in the Seychelles (Indian Ocean). Nephrol Dial Transplant. 1997;12(10):2069–74.

Random Spot Urine Markers for Kidney and Their Applications

9

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Abstract

Over the few last decades, several additional urinary biomarkers have been correlated to other organ or system abnormalities other than kidney disease. In this setting, abnormal findings on a routine urinalysis, often in an otherwise asymptomatic patient, may be the first evidence of underlying kidney disease and even other diseases or conditions like a higher cardiovascular risk set.

A 24-h urine evaluation is still considered the gold standard method for the quantification of important urinary biomarkers like proteinuria. Nevertheless, its collection is laborious and entails significant errors that could compromise the accuracy of this method. Therefore, random spot urine assessment was developed to estimate quantitative measurements of 24-h collections. Many trials have been conducted to determine which formulae (correction for other parameters) and methods (including voiding of the day or technical procedures) are better to minimize sources of false results and enhance correlation with the gold standard. Currently, random spot urine examination is already mentioned in some international guidelines as an alternative analysis to diagnose and monitor several diseases. It allows the identification of multiple markers, which can be organized into three groups, according to their laboratory method assessment: physical, chemical, and microscopic characters. However, evidence regarding its role and power is still not unanimous, at least in some diseases, and future trials to prove how best to apply it are needed.

This review outlines random spot urine biomarkers for the kidney and their applications in clinical practice.

Keywords

Urinary biomarker • Kidney disease • Cardiovascular disease • Random spot urine • Urinalysis • Proteinuria • Hematuria • Sediment

Abbreviations

AKI	Acute kidney injury
ATN	Acute tubular necrosis
Ca	Calcium
CKD	Chronic kidney disease
$CuSO_4$	Copper sulfate

DBDH	Diisopropylbenzene dihydroperoxide
DIDNTB	bis(3′,3″-Diiodo-4′,4″-dihydroxy-5′,5″-dinitrophenyl)-3,4,5,6-tetrabromo-sulfonephthalein
eAER	Estimated albumin excretion rate
ESRD	End-stage renal disease
FCU	Fractional renal clearance of urate
GFR	Glomerular filtration rate
GN	Glomerulonephritis
INTERSALT	International study of electrolyte excretion and blood pressure
IRMA-2	Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria
K	Potassium
KDIGO	Kidney Disease: Improving Global Outcomes
LE	Leukocyte esterase
LN	Lupus nephritis
MESNA	Mercaptoethane sulfonate sodium
Mg	Magnesium
Na	Sodium
P/C	Protein/creatinine ratio
Ph	Phosphate
PREVEND	Prevention of Renal and Vascular End-Stage Disease
RBCs	Red blood cells
SG	Specific gravity
SGLT2	Sodium/glucose cotransporter 2
SLC5A2	Solute carrier family 5
SLE	Systemic lupus erythematosus
SNP	Single-nucleotide polymorphisms
SSA	Sulfosalicylic acid
STENO-2	Effect of a Multifactorial Intervention on Mortality in Type 2 Diabetes
TMB	3,3′,5,5′-Tetramethylbenzidine
UACR	Urinary albumin/protein ratio
UTI	Urinary tract infection
WBCs	White blood cells
Zn	Zinc

Key Facts of Urinalysis

- Among other characteristics, an ideal biomarker should be easily accessible and noninvasive, which could be achieved with urinalysis.
- Although the 24-h urine evaluation is the gold standard for most quantitative parameters, the random spot urinalysis may give a result that correlates with this technique over a wide range of measurements.
- Random spot sample repeated measurements can be easily obtained to ascertain disease activity and response to treatment. However, the results can be seriously

affected by several variables that depend on the patient and the technique itself, so these must be regularly confirmed.

- Several formulae and methods have successfully been developed to improve acuity of measurements in a random spot urine sample.
- Currently, guidelines already recommend protein or albumin/creatinine ratio in a random spot evaluation as a first option to monitor diseases like chronic kidney disease and lupus nephritis.
- Despite these recommendations, evidence on reliability is not unanimous in all diseases (e.g., lupus nephritis), so it should be applied carefully, especially if therapeutic changes are about to be made.

Definitions

Biomarker The Oxford dictionary describes a biomarker as “a measurable substance in an organism whose presence is indicative of some phenomenon such as disease, infection, or environmental exposure.”

Chronic kidney disease Kidney damage or an estimated glomerular filtration rate below 60 mL/min/1.73 m^2 persisting for 3 months or more, irrespective of the cause.

Diabetes Group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Chronic hyperglycemia is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels.

Dipstick A thin, plastic stick with strips of chemicals on it, which is placed in urine to detect abnormalities. The chemical strips change color if certain substances are present or if their levels are above normal.

Glomerulonephritis Disease that primarily affects the glomerulus. It has clinical presentations that vary from the asymptomatic individual who is found to have hypertension, edema, hematuria, or proteinuria at a routine medical checkup to a patient who has fulminant disease, with acute kidney injury, possibly associated with life-threatening extrarenal disease.

Lupus nephritis Immune complex glomerulonephritis that is a common and serious feature of systemic lupus erythematosus.

Mercaptoethane sulfonate sodium (MESNA) A sulfhydryl compound that is used to reduce the incidence of hemorrhagic cystitis associated with certain chemotherapeutic agents, like cyclophosphamide.

Random spot urine Occasional, one-off, urine collection.

Systemic lupus erythematosus Multisystem, autoimmune disease, whose diagnosis demands a combination of clinical and laboratory criteria defined by the American College of Rheumatology.

Sodium/glucose cotransporter 2 (SLGT2) Protein encoded by the *SLC5A2* gene and member of the sodium glucose cotransporter family involved in glucose reabsorption in the kidney.

Solute carrier family 5 (SLC5A2) Gene that encodes the SGLT2 protein.

Urocrit Similarly to the hematocrit word, it has been used to define the proportion of the urine that is composed of red blood cells. It is measured by centrifugation technique and is used when erythrocyturia is too high to count by microscopy.

Introduction

Urinalysis has historically been defined as a test to evaluate kidney and urinary tract disease. However, during the last few decades, several additional urinary biomarkers have been correlated to other organ or system abnormalities other than kidney disease.

It is well known that an ideal biomarker should be easily accessible and noninvasive, among other characteristics. This profile perfectly matches a random spot urine marker because patients with nephropathy who often progress to end-stage renal disease (ESRD) and depend on hemodialysis treatment will require a vascular access (ideally, arteriovenous fistula or graft) whose correct function depends on good vessel conditions, for which venous punctures to collect blood samples are harmful and should be avoided. In this setting, abnormal findings on a urinalysis may be the first evidence of underlying kidney disease and may be a suitable test for monitoring several conditions, thereby minimizing the number of blood tests.

Twenty-four-hour urine samples used to be the only reliable samples for most of the quantitative analysis. Nevertheless, collecting them is laborious and unfeasible for many population groups, like young working adults with hard schedules and multiple workplaces, elderly people with memory problems and urinary incontinence, as well as children. In these cases, random spot urine assessment has been successfully used. Although this method may be limited in some quantitative parameters and more vulnerable to factors related to the patient's lifestyle, some correcting formulae have been developed to achieve quantitative results that are highly correlated with timed specimens. This finding supports some current guidelines that already recommend random spot urinalysis instead of the 24-h specimen

for screening, diagnosis, and monitoring of several diseases like diabetes or systemic lupus erythematosus (SLE) (Engelgau et al. 2000; Hahn et al. 2012).

Random spot urine markers can be organized into three groups, according to their laboratory method assessment: physical, chemical, and microscopic markers. Physical markers include color, odor, clarity, and specific gravity (SG). Chemical examination includes the identification of protein, blood, glucose, pH, bilirubin, urobilinogen, ketones, nitrites, and leukocyte esterase (LE). Finally, microscopic evaluation entails the detection of crystals, cells, casts, and organisms. Chemical results are the most often widely used, probably because of their (semi)quantitative presentation and possible correlation with quantitative timed collections, but physical and microscopic ones may play a key role in many diagnoses and monitoring.

This review outlines random spot urine biomarkers for the kidney and their applications in both research and clinical practice.

The Specimen for Analysis

Urine collection requires standard handling guidelines to minimize unwanted sources of variability that could compromise the acuity of test results. Good practice prevents potential sources of error; thus, patient preparation and clear information is essential for a correct collection (Strasinger and Di Lorenzo 2014). Both the suitability of a specimen and the rejection criteria must be determined. Following collection, specimens should be delivered and tested within 2 h because changes in urine composition start immediately after the voiding (Strasinger and Di Lorenzo 2014). Bacteria may proliferate and alter pH, casts may dissolve, and crystals may be lost. Refrigeration may cause precipitation of orange red crystals of uric acid, which can be redissolved by rewarming the urine (Strasinger and Di Lorenzo 2014). Additionally, urine analysis can be seriously affected by other variables that depend on the patient, the techniques, and the medical team.

Although this type of specimen is the easiest and most convenient for the patient because it can be collected at any time, some basic recommendations should be followed. Vigorous physical exercise should be avoided for at least 24 h before the collection to minimize exercise-induced proteinuria, hematuria, or cylindruria (Johnson et al. 2014). Women should avoid collection during menstruation because of blood contamination. A midstream clean-catch specimen provides a suitable specimen for routine urinalysis and bacterial culture because it is less contaminated by epithelial cells and bacteria and, therefore, is more representative of the actual urine (Strasinger and Di Lorenzo 2014).

Regarding collection schedule, the first morning void is the most ideal to prevent false-negative pregnancy tests and to evaluate orthostatic proteinuria, because it is a more concentrated specimen, thereby assuring the detection of lower levels of substances otherwise missed in a more diluted specimen (Strasinger and Di Lorenzo 2014). On the other hand, lysis of cells and casts may occur in the bladder overnight, which may lead to false-negative results. In these cases, a second morning urine is more appropriate.

Finally, before reviewing each biomarker analysis and applications, it should be remembered that urine normally contains 95 % water and 5 % solutes. As stated above, considerable variations in these solute concentrations can occur in healthy people owing to the influence of normal biological factors, but they could also be markers of pathogenic processes or pharmacological responses to a therapeutic intervention.

Physical Markers

All routine urinalysis should begin with a physical examination of the sample which includes description of color, odor, clarity, and SG.

Color

Normal urine ranges from pale to dark yellow, which is mainly due to the urochrome pigment, a product of endogenous metabolism, but also to other chemical concentrations, and pH. Changes in color can be caused by physical activity, food, drugs, or pathological conditions. The most frequent pathological conditions that can cause color changes of the urine are gross hematuria, hemoglobinuria, or myoglobinuria (pink, red, brown, or black); bilirubinuria (dark yellow to brown); and urinary infections (dark yellow, white, green, or even purple, according to germs). Less frequent causes include uric acid crystalluria (pink) and porphyrinuria and alkaptonuria (red, turning black on standing). The main drugs responsible for abnormal urine color are rifampin, phenazopyridine, and phenindione (yellow orange); desferrioxamine (pinkish); phenytoin (red); chloroquine and nitrofurantoin (brown); amitriptyline, triamterene, propofol, and blue dyes of enteral feeds (green); methylene blue (blue); and metronidazole, methyldopa, phenol derivatives, argyrol, and imipenem–cilastatin (darkening on standing). Some examples among food include beetroot (red), senna and rhubarb (yellow to brown or red), and carotene (brown) (Strasinger and Di Lorenzo 2014; Johnson et al. 2014).

Odor

A change in urine odor may be caused by the ingestion of some food, urinary tract infection (UTI), maple syrup urine disease, phenylketonuria, isovaleric academia, and hypermethioninemia.

Turbidity or Clarity

The transparency or turbidity of a urine specimen is determined by visually examining the mixed specimen in a clear container while holding it in front of a light source. Freshly voided normal urine is usually clear, particularly if it is a midstream clean-catch specimen.

Urine turbidity can have multiple causes. The most frequent non-pathological causes include squamous epithelial cells, mucous, amorphous phosphates, carbonates, urates, semen, fecal contamination, radiographical contrast media, talcum powder, and vaginal creams. On the other hand, the most common pathological causes are red blood cells (RBCs), white blood cells (WBCs), bacteria, non-squamous epithelial cells, yeast, abnormal crystals, lymph fluid, and lipids.

Clear urine is not always normal; thus, chemical analysis will increase the acuity of physical markers in detecting certain abnormalities (Strasinger and Di Lorenzo 2014).

Relative Density

The evaluation of urine concentration is made by measuring the SG, which correlates with urine osmolality, rising by approximately 0.001 for every 35–40 mosmol/kg increase in osmolality. Thus, a urine osmolality of 280 mosmol/kg (isosmotic to plasma) is usually associated with a urine SG of 1.008 or 1.009 (Wald 2014). It refers to the weight of a urine volume compared with the weight of the same volume of distilled water and depends on the mass and number of the dissolved particles. It can be evaluated by dipstick, which provides a rapid semiquantitative result, as well as by other urinary markers on a series of test pads embedded on a reagent strip (Wald 2014). This method can be influenced by urine pH and non-ionized molecules: underestimation occurs with pH above 6.5, whereas overestimation is found with urine protein concentration above 7.0 g/L (Assadi and Fornell 1986). Additionally, non-ionized molecules, such as glucose and urea, are not detected by the dipstick, so this method may not strictly correlate with the results obtained by refractometry and osmolality (Siegrist et al. 1993). Refractometry measures all solutes, rather than just ionic substances, and is therefore more accurate than dipstick. Temperature-compensated equipment eliminates the influence of temperature, and therefore it is recommended for use in everyday practice (Strasinger and Di Lorenzo 2014). Nevertheless, this method can be affected by protein, glucose, mannitol, dextrans, diuretics, radiographical contrast media, and some antibiotics (Siegrist et al. 1993). Osmolality is measured by an osmometer and depends only on the number of particles present; it is not influenced by urine temperature or molecule size.

Regarding clinical application, SG gives important insight into the patient's hydration status and the concentrating ability of the kidneys (Simerville et al. 2005). It can be used to crudely estimate how the concentration of other urine constituents may reflect total excretion of those constituents (Jung 1991) because SG correlates inversely with a 24-h urine volume (McCormack et al. 1991). An important additional function of this parameter is to determine whether specimen concentration is adequate to ensure the accuracy of chemical tests because values that are extremely outside the normal range may induce false results. Most random specimens fall between 1.015 and 1.030. A SG of 1.000 to 1.003 is consistent with marked urinary dilution, as observed in patients with diabetes insipidus or water intoxication. Specific gravity of 1.010 is often called isosthenuric urine because it is similar to plasma, so it is often observed in conditions in which

urinary concentration is impaired, such as acute tubular necrosis (ATN) and chronic kidney disease (CKD). Specific gravity above 1.040 almost always indicates the presence of some extrinsic osmotic agent, such as radiocontrast. Indeed, self-monitoring this parameter may be useful for stone-forming patients, who benefit from maintaining dilute urine (Siegrist et al. 1993).

In conclusion, dipstick is the least accurate method, so it should be replaced by refractometry if a precise measurement is needed or error interference factors are suspected. Osmolality is more reliable than both methods, and most clinical decisions should be based on this determination when evaluating pathological urine.

Chemical Markers

Chemical urinary markers are probably most useful in daily practice, because their value in the diagnosis, monitoring, and prognosis of glomerular diseases has long been proven. This subgroup also contains multiple methods, discussed below, to measure each type of marker.

Reagent strips currently provide a simple, affordable, and rapid evaluation. As written above, they consist of absorbent pads impregnated with chemicals and attached to a plastic strip. A color-producing chemical reaction takes place when the absorbent pad comes in contact with urine (Fig. 1). The timing for reactions to take place varies between tests and manufacturers and ranges from an immediate reaction for pH to 120 s for LE. The results are interpreted by comparing the color within a chart supplied by the manufacturer. A semiquantitative value of trace, 1+, 2+, 3+, or 4+, can be reported, and a corresponding estimation of the milligrams per deciliter is available for appropriate testing areas (Strasinger and Di Lorenzo 2014). Improved specificity and sensitivity of the most recent dipsticks, as well as the use of automated strip readers, have reduced the need for routine use of confirmatory tests of this method.

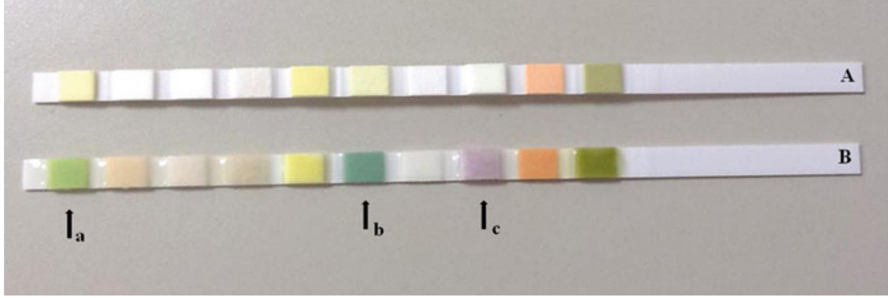

Fig. 1 Dipstick image. The first strip (**A**) shows a regular pad before being dipped in urine. The second strip (**B**) has been dipped in a random spot urine sample of a patient with glomerulonephritis, detecting trace hematuria (*a*), 3+ proteinuria (*b*), and 2+ leukocyturia (*c*)

Blood

Blood may be present in the urine, either in the form of intact RBCs (hematuria) or as the product of RBC destruction, hemoglobin (hemoglobinuria). Therefore, chemical tests for hemoglobin provide the most accurate means of determining the presence of blood.

Hematuria can be measured quantitatively by any of the following: indirect examination by dipstick, determination of RBC number through chamber count, and direct examination of urinary sediment.

The dipstick method is based on the pseudoperoxidase activity of the heme, which catalyzes the reaction of peroxide and a chromogen to form a colored product (green to blue). Red blood cells, hemoglobin, and myoglobin will catalyze this reaction, so a positive test result may indicate hematuria, hemoglobinuria, or myoglobinuria, respectively (Brenner et al. 2011). This latest condition may be suspected when a red-brown urine is seen. Dipsticks can detect concentrations as low as five RBCs per microliter. The terms trace, small, moderate, and large or trace, 1+, 2+, and 3+ are used for reporting (Strasinger and Di Lorenzo 2014). A positive test with green spots mostly results from intact erythrocytes. On the other hand, a homogeneous, diffuse green pattern can result from three main situations: marked hematuria with a high number of erythrocytes that cover the whole pad surface; lysis of erythrocytes favored by delayed examination, alkaline urine pH, or low SG, as well as hemoglobinuria secondary to intravascular hemolysis. False-negative results are mainly caused by ascorbic acid (reducing agent) (Rauta et al. 2002) and high SG. On the other hand, false-positive results can occur due to myoglobinuria and to a high concentration of bacteria with pseudoperoxidase activity (*Enterobacteriaceae*, *Staphylococci*, *Streptococci*) (Lam 1995). Given the limited specificity of the dipstick method (65–99 % for 2–5 RBCs per high-power microscopic field), an initial positive result should be confirmed by microscopic evaluation (Sutton 1990).

The chamber count method measures the number of RBCs per milliliter and has been suggested to have greater precision and sensitivity than the sediment count (Grossfeld et al. 2001). Although microscopic examination is only a semiquantitative method of determining the degree of hematuria, it is easier to perform, less time-consuming, and more cost-effective than the chamber count. Furthermore, it allows different elements to be distinguished by their appearance, enabling the source of the hematuria and its main etiologies to be pointed out (Johnson et al. 2014). However, both chamber and sediment counts have been shown to correlate with acceptable sensitivity (Grossfeld et al. 2001).

Regarding clinical significance, hematuria is most closely related to disorders of renal or genitourinary origin that can be caused by glomerular, non-glomerular (tubulointerstitial (Ballarin et al. 2011), toxic chemicals, anticoagulant-related nephropathy (Brodsky et al. 2011), renovascular or metabolic abnormalities), and urological (tumors, especially bladder cancer (Muto et al. 2014), trauma, pyelonephritis, or renal calculi) diseases. Glomerular hematuria is typically associated with significant proteinuria (Simerville et al. 2005). However, 20 % of patients with biopsy-proven glomerulonephritis (GN) present hematuria alone

(Fassett et al. 1982), among which IgA nephropathy is the most common (Simerville et al. 2005). Hematuria of non-pathological significance is observed following strenuous exercise and during menstruation. In both cases, results of repeated urinalysis after 48–72 h should be negative. On the other hand, hemoglobinuria may result from RBC lysis after urination or intravascular hemolysis (hemolytic anemia, transfusion reactions, severe burns, spider bites and infections). Distinctively, myoglobinuria due to rhabdomyolysis can happen in conditions like strenuous exercise, trauma, prolonged coma, convulsions, muscle-wasting diseases, alcoholism, as well as from statin medication and several kinds of drug abuse.

Besides the multiple conditions stated above, where hematuria has an "injury marker" value, recent information has presented its prognostic value in diseases like IgA nephropathy (Moreno et al. 2012) and acute kidney injury (AKI) (Gutierrez et al. 2007). An association with long-term incidence of ESRD has also been established ("risk factor" value) (Vivante et al. 2011). Despite this evidence, there are pitfalls in quantifying hematuria, rendering it more difficult to objectively assess the impact of therapy and to predict the outcome. Firstly, this is because it is not routinely quantified as accurately as albuminuria and proteinuria are (Moreno et al. 2012), and secondly, the assessment of glomerular hematuria may be interfered with by the presence of the non-glomerular one. Because of the absence of hard data, at the present time, it is difficult to make recommendations on the specific target values of hematuria that should influence treatment decision making.

pH

Urine pH reflects the presence of hydrogen ions, but this does not necessarily reflect the overall acid load in urine because most of the acid is excreted as ammonia (Johnson et al. 2014) and a very small amount as weak organic acids. The normal range for urine pH is 4.5–7.8.

As above, it is usually measured with a reagent test strip. Most commonly, methyl red and bromthymol blue double indicators are used to give a broad range of colors at different pH values (Brenner et al. 2011), covering a range from 5.0 to 9.0. A more precise method, pH meter, measures the concentration of hydrogen ions, whose positive charge creates an electrical potential detected by an electrode.

The importance of urinary pH is primarily as an aid in the differential diagnosis of acid–base disorders. Moreover, this marker can be used to monitor conditions that require urine to be maintained at a specific pH, to prevent drug nephrotoxicity, like that due to precipitation of methotrexate in the renal tubules, or to inhibit renal calculi formation (Sand and Jacobsen 1981). Regarding the second disorder, an alkaline pH favors the crystallization of calcium- and phosphate-containing stones, whereas an acidic pH promotes uric acid or cystine stones (Wagner and Mohebbi 2010). For that reason, pH control is essential to assess treatment response. Urine pH measurement is also mandatory if an accurate crystal microscopic evaluation is necessary.

However, pH by itself provides little useful diagnostic information, and it must be considered in conjunction with other information about the patient because many of the deviations can be explained by non-pathological conditions. A low pH is observed in metabolic acidosis, one of the main causes of which is high-protein meals (Brenner et al. 2011), and hypovolemia, in which aldosterone stimulation increases hydrogen secretion in the distal tubules. Indeed, low urine pH may help distinguish prerenal AKI from ATN, which is typically associated with higher values. Other conditions with alkaline pH are infection by urease-positive organism (Proteus), renal tubular acidosis, vomiting, and gastric suction. Non-pathological causes include vegetarian diet (minimal nitrogen and acid generation), diuretic, and alkali therapy.

Bilirubin

Bilirubin is a highly pigmented yellow compound that results from hemoglobin degradation. Its appearance in urine may provide an early indication of liver disease due to obstructive disease or hepatocellular injury, but not hemolysis, because only conjugated bilirubin is water soluble and renal excreted.

Although reagent test strips are very sensitive to bilirubin, detecting as little as 0.05 mg/dL, this method is not very sensitive for detecting hepatic abnormalities. In addition, measurement errors often occur, firstly, due to false-positive results because of urine contamination with stool and other pigments and, secondly, due to false negatives because of prolonged sample storage and exposure to light, promoting bilirubin photo-oxidation to biliverdin (Young 1990). Consequently, since the introduction of serum tests of liver enzyme function, this measurement has lost its clinical application.

Urobilinogen

Urobilinogen appears in urine because as it circulates in the blood back to the liver, it passes through the kidney and is filtered by the glomerulus. A small amount of urobilinogen (<1 mg/dL) is normally found in urine.

This strip test reaction sensitivity increases with temperature (ideally room temperature). False-positive reactions may occur due to porphobilinogen, *p*-aminosalicylic acid, sulfonamides, methyldopa, procaine, and chlorpromazine compounds, among others. False-negative results occur most frequently when specimens are improperly preserved (photo-oxidation of urobilin) or formalin is used as a preservative.

Although this parameter also has low clinical application, it can be slightly increased in chronic constipation, and main pathological causes are liver disease and hemolytic disorders (Strasinger and Di Lorenzo 2014). Some authors (Gorchynski et al. 2009) tried to prove its utility for adult blunt abdominal trauma patients, but they found it was a poor predictor for intra-abdominal injury, thus not clinically useful.

Ketones

Ketones represent intermediate products of fat metabolism, namely, acetone (2 %), acetoacetic acid (20 %), and β-hydroxybutyrate (78 %). Normally, measurable amounts of ketones do not appear in urine, because all the metabolized fat is completely broken down into carbon dioxide and water. However, when the use of carbohydrate is compromised, body fat stores are metabolized to supply energy, increasing ketones in the blood, which may lead to dehydration, electrolyte imbalance, acidosis, and coma.

With the exception of β-hydroxybutyrate, they can be detected in urine through the dipstick nitroprusside reaction (Jacobs et al. 1990), where acetoacetic acid reacts with sodium nitroprusside to produce a purple color. Results are reported qualitatively as negative, trace, small (1+), moderate (2+), or large (3+), or semiquantitatively as negative, trace (5 mg/dL), small (15 mg/dL), moderate (40 mg/dL), or large (80–160 mg/dL) (Strasinger and Di Lorenzo 2014).

Positive reactions can occur in prolonged fasting or starvation, strenuous exercise, malabsorption, vomiting, and alcoholic or diabetic ketoacidosis. Published data showed a good correlation between urinary and capillary blood ketones for lower values of glycemia, but not for higher. As a result, this marker can be used to exclude ketosis, but not to confirm ketoacidosis (Taboulet et al. 2007). Therefore, it is valuable in the management and monitoring of type 1 diabetes mellitus because ketonuria is an early indicator of insufficient insulin, indicating the need to upregulate dosage, as well as other concurrent events. False-positive reactions can be due to late readings after long sample standing and the presence of mercaptoethane sulfonate sodium (MESNA), captopril (sulfhydryl groups), levodopa, ascorbic acid, or phenazopyridine, among others. False-negative tests can happen if specimens are not preserved properly, allowing the metabolization and volatilization of ketone compounds.

Nitrites and Leukocyte Esterase

Nitrites result from the conversion of nitrates in the presence of bacteriuria. This marker is highly specific for bacteriuria, but several uropathogens other than *Enterobacteriaceae* do not reduce nitrate to nitrite (Patel et al. 2005); thus, a negative test does not necessarily mean that the urine is free of bacteria.

Nitrite is detected by the Griess reaction, in which nitrite at an acidic pH reacts with an aromatic amine to form a diazonium compound that then reacts with tetrahydrobenzoquinolin compounds to produce a pink color (positive). Different shades of pink may be produced, but they do not reflect the degree of bacteriuria. Optimal results for a urinary nitrite test are obtained by analyzing an early morning specimen that has been incubating in the bladder for 4 h or more. A clean midstream specimen is important to reduce bacterial contamination.

Apart from the first situation mentioned above, false-negative results may also occur when urine has been in the bladder for a short period, dietary nitrite is absent, nitrate reduction has gone beyond the nitrite stage to form nitrogen (Garingalo-

Molina 2000), and bacterial metabolism is inhibited by the presence of antibiotics or large amounts of ascorbic acid (Strasinger and Di Lorenzo 2014). On the other hand, false-positive tests can result from improperly preserved specimens and highly pigmented urine (Strasinger and Di Lorenzo 2014).

Although a semiquantitative culture of a urine specimen is the only method that can provide detailed documentation of a bacterial UTI, it is costly and takes at least 24 h. Thus, a screening test could be carried out using dipstick, where nitrites and LE accuracy have been correlated to culture (Lohr 1991).

The LE test detects esterase, an enzyme released by both intact and lysed WBCs (except lymphocytes), which catalyze the hydrolysis of an acid ester to produce an aromatic purple azo dye. Because it also detects lysed cells, it could be more accurate than microscopic evaluation, but should be quantified using this second method. It should be performed on a fresh specimen and reactions are reported as trace, small, moderate, and large or trace, 1+, 2+, and 3+ (1). In addition to UTI screening, it also detects infections caused by *Trichomonas*, *Chlamydia* (Rahman et al. 2014), and inflammation of renal tissues (like interstitial nephritis) that produce leukocyturia without bacteriuria.

False-positive reactions can be due to the presence of strong oxidizing agents, formalin in the container, highly pigmented urine, and nitrofurantoin. False-negative results may occur with high SG urine, antibiotics like gentamicin, cephalexin, cephalothin, and tetracycline, as well as high concentrations of protein (>500 mg/dL), glucose (>3 g/dL), oxalic, and ascorbic acid (Strasinger and Di Lorenzo 2014).

Although several studies have confirmed the low sensitivity and specificity of the dipstick test, these two positive reactions suggestive of UTI can be used to determine empirical treatment, as well as in follow-up (Demilie et al. 2014), because their combination improves each other's performance (Semeniuk and Church 1999). In contrast, a negative reaction should be an indication for culture if clinical features are present. Even though they are not intended to replace urine culture, they can be cost-effective in reducing the necessity to perform these cultures (Wise et al. 1984). Currently, they are often ordered to screen high-risk patients who are frequently asymptomatic, like pregnant women, children, the elderly, and patients with recurrent UTI, CKD, and diabetes.

Glucose

Glycosuria is the most frequently performed chemical analysis on urine.

One of the oldest methods available is based on copper reduction, where glucose reduces copper sulfate to cuprous oxide in the presence of alkali and heat (Benedict reaction). Color ranges from a negative blue through a positive green to orange/red and should be compared with the manufacturer's chart to estimate quantitative measurement of glucose. Its sensitivity is reduced to a minimum of 200 mg/dL, but at high glucose levels, a "pass through" phenomenon may occur and a return to a negative color can happen without reporting the previous temporary positive result (Strasinger and Di Lorenzo 2014). In contrast to the causes of false-negative results,

this test is not specific for glucose, so other reducing substances, such as sugars including galactose, lactose, fructose, maltose, and pentose, ascorbic acid, certain drug metabolites, containers oxidizing detergents, and antibiotics, like cephalosporins, may induce false-positive reactions (Brigden et al. 1992). Although it has a low sensitivity and specificity to detect glycosuria, this method detects urine galactose; thus, it can be used to screen newborns for "error of metabolism," in which a lack of the enzyme galactose-1-phosphate uridyl transferase prevents breakdown of ingested galactose (Strasinger and Di Lorenzo 2014).

A more recent method is performed through a dipstick impregnated with a mixture of components, including glucose oxidase, which promotes a double sequential enzyme reaction, since glucose triggers the production of gluconic acid and peroxide, which in turn catalyzes the oxidation of a chromogen to form a colored compound that will vary according to the chromogen used by each manufacturer. Under ideal conditions, the intensity of the color should be directly proportional to glucose concentration, thereby allowing a quantitative estimation (Gray and Millar 1953). Here, interference by other reducing agents does not occur thanks to the additional chemicals in the strip. Results are reported in terms of negative, trace, 1+, 2+, 3+, and 4+, which correspond to quantitative measurements ranging from 100 mg/dL to 2 g/dL, or 0.1 % to 2 %, provided by the manufacturers (Strasinger and Di Lorenzo 2014). Although its sensitivity decreases with high SG and low temperature, the greatest source of false-negative results is the long-standing samples subjecting glucose to bacterial degradation. High ketones also may interfere with the results but only in the presence of low glycosuria levels, which rarely happens.

Glycosuria clinical application occurs when kidney proximal tubules are unable to reabsorb all the filtered glucose, despite normal plasma glucose levels, or urinary leak occurs due to hyperglycemia above 180 mg/dL (10 mmol/L), when tubular reabsorption of glucose has reached its threshold. In the first setting, glycosuria can occur in an isolated setting in ESRD and cystinosis, but usually occurs with Fanconi syndrome (phosphaturia, uricosuria, and aminoaciduria). Possible causes are multiple myeloma, heavy metal exposure, and treatment with certain medications including tenofovir, lamivudine, cisplatin, valproic acid, and aminoglycosides (Haque et al. 2012). In rarer situations, it may also be an isolated defect associated with genetic mutations, as in solute carrier family 5 (SLC5A2) gene encoding sodium/glucose cotransporter 2 (SGLT2), which affects renal glucose transport (Magen et al. 2005). Hyperglycemic scenarios are mostly caused by diabetes, but a minority can be due to pancreatitis, acromegaly, Cushing syndrome, hyperthyroidism, pheochromocytoma, and thyrotoxicosis. Nevertheless, healthy people can have glycosuria caused by a temporary lowering of the renal threshold during pregnancy and after a high glucose content meal. Therefore, collection conditions should be controlled (previous 12-h fasting recommended) and results interpreted according to settings. The use of currently available reagent strip methods for both blood and urine glucose testing has greatly increased early diagnosis of diabetes, allowed patients to monitor themselves at home, and improved outcomes. For purposes of diabetes monitoring, specimens are usually tested 2 h after meals. Additionally, a

first morning specimen does not always represent a fasting specimen because glucose from an evening meal may remain in the bladder overnight, so patients should be advised to empty the bladder and collect the second specimen. Overall, fasting glycosuria testing has a specificity of 98 % and a sensitivity of 17 % (Singer et al. 1989) as a screening test for diabetes.

Albuminuria

Albuminuria has been the hallmark used to develop clinical practice and research in the area of diabetic nephropathy, CKD, and cardiovascular disease, among others.

The normal rate of albumin excretion is less than 30 mg/day (20 mcg/min), about 4–7 mg/day (3–5 mcg/min) in healthy young adults, and increases with age and with body weight. Microalbuminuria is currently defined as a persistent albumin excretion of between 30 and 300 mg/day (20–200 mcg/min) or a urinary albumin/creatinine ratio (UACR) of 2.5–35 mg/mmol in male subjects and 3.5–35 mg/mmol in female subjects (KDIGO 2013; Stevens and Levin 2013). Albumin excretion above 300 mg/day (200 mcg/min) is considered macroalbuminuria. Previous reviews and clinical practice guidelines have called for the abandonment of the term "microalbuminuria" (Ruggenenti and Remuzzi 2006). The Kidney Disease: Improving Global Outcomes (KDIGO) 2012 clinical practice guideline for the evaluation and management of CKD discourages the use of the term when classifying patients to a CKD stage according to their level of albuminuria. The suggested approach is to slot albuminuria into three categories: A1, normal to mildly increased (instead of normoalbuminuria); A2, moderately increased (instead of microalbuminuria); and A3, severely increased (instead of macroalbuminuria or proteinuria) (KDIGO 2013).

Transient elevations in the excretion of albumin can be seen in fever, infection, exercise, heart failure, nonspecific joint inflammation, poor glycemic control (hemoglobin A1c >8 %), obesity, and hyperlipidemia (low-density lipoprotein cholesterol >120 mg/dL) (Wald 2014). Posture, smoking, and diet may also influence albumin excretion rate (MacIsaac et al. 2014). However, persistent microalbuminuria has not only been consistently associated with diabetic nephropathy, but also with hypertension (>160/100 mmHg), coronary heart disease, and overall cardiovascular disease, in both diabetic and nondiabetic patients (Newman et al. 2005). As illustrated in the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study (Abdelmalek et al. 2014), it also enhances the predictive value of ST and T wave changes for cardiovascular disease. Concerning kidney disease, it is predictive of developing clinical proteinuria, faster decline in glomerular filtration rate (GFR), and higher risk of ESRD (Newman et al. 2005). Microalbuminuria has generally been accepted as the first injury marker of diabetic nephropathy. One other study showed that cardiovascular risk for patients with type 2 diabetes starts to increase with urinary albumin excretion levels above 1 mcg/min, even before the upper limit of normoalbuminuria is reached (Ruggenenti et al. 2012). In addition to its "injury marker" value, it may also be useful in monitoring treatment. Additionally to this

evidence, both Effect of a Multifactorial Intervention on Mortality in Type 2 Diabetes (STENO-2) and Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria (IRMA-2 trials) trials concluded that early intervention with anti-albuminuric drugs and monitoring is cost-effective, leading to amelioration of GFR loss, decrease in ESRD incidence, and prolongation of life. A similar association between those drug effects and other well-accepted surrogate cardiovascular end points, such as blood pressure and cholesterol, has been reported (Roscioni et al. 2014). However, it should be noticed that there is no test capable of distinguishing between microalbuminuria linked to cardiovascular disease and that linked to renal disease (MacIsaac et al. 2014).

Regarding its measurement, although a 24-h urine collection is the gold standard for the detection of microalbuminuria (Bennett et al. 1995), it has been suggested that screening can be more simply achieved through a random spot specimen.

Albumin dipsticks are based on binding antibody to the strip to form a conjugated enzyme, which reacts with the substrate to produce a color range according to pH changes. Colors range from pale green to aqua blue; intensity is proportional to the concentration of albumin itself and is graded on a scale from 0 to 4+ (Lamb et al. 2009), which can be correlated to a quantitative scale supplied by the manufacturers. Although urine concentration may strongly influence this marker result, limited data suggest that correcting this value to urine SG improves ability to identify abnormal results (Constantiner et al. 2005). Other false positives may occur after the use of iodinated radiocontrast agents (Morcos et al. 1992), with a highly alkaline urine (pH >8) (Simerville et al. 2005), with gross hematuria or a urocrit >1 % (Tapp and Copley 1988), and when specific antiseptics (chlorhexidine, benzalkonium) are used before collection (Magen et al. 2005). Abnormally high values should always be confirmed by repeated measurements (KDIGO 2013), and if a true positive is confirmed, accurate quantification by another method is needed. On the other hand, other factors unrelated to low SG may be associated with false-negative rates, as some albumin components are not immunologically reactive with the strip component (Busby and Bakris 2004).

Overall, albumin dipstick test is a very specific but not sensitive method, because many patients with microalbuminuria may be missed unless their urine is highly concentrated. Still, more reliable strips have been designed and been successful in detecting moderately increased albuminuria with a sensitivity and specificity of 80–97 % and 33–80 %, respectively (Comper and Osicka 2005).

In addition to this method, the high-performance liquid chromatography technique is able to assess all intact urinary albumin, even non-immunoreactive albumin, thereby increasing sensitivity and specificity (Busby and Bakris 2004) and proving itself useful for earlier detection. Other types of reagent strip provide simultaneous measurement of albumin (or protein) and creatinine, thereby allowing the comparison between their excretion and producing a semiquantitative UACR, which corresponds to a more accurate estimation of the 24-h excretion.

Conventional reagent pads detect a minimum of 30 mg/dL and may include other proteins besides albumin. Strips using dye bis(3′,3″-diiodo-4′,4″-dihydroxy-5′,5″-dinitrophenyl)-3,4,5,6-tetrabromo-sulfonephthalein (DIDNTB) can measure albumin as low as 8 mg/dL, without the inclusion of other proteins, increasing overall sensitivity and specificity. Reaction interference by highly alkaline urine

can be also controlled with bis-(heptapropylene glycol) carbonate reagent (Strasinger and Di Lorenzo 2014).

As stated before, limitations regarding the 24-h collection adequacy are not negligible, so this vital step can be checked by quantifying the 24-h urine creatinine and comparing this value to the expected one. As a general rule, in adults under the age of 50, daily creatinine excretion should be 20–25 mg/kg (177–221 micromol/kg) of lean body weight in men and 15–20 mg/kg (133–177 micromol/kg) of lean body weight in women, figures which progressively decline to 50 % in their 90s. Due to these limitations and the need for laborious control, some alternatives have been proposed. Most commonly, random spot UACR is used to estimate a 24-h proteinuria and to follow the effects of treatment in patients with cardiovascular and albuminuric kidney diseases.

Reagent strips containing copper sulfate (CuSO4), 3,3′,5,5′-tetramethylbenzidine (TMB), and diisopropylbenzene dihydroperoxide (DBDH) allow for the detection of creatinine based on the pseudoperoxidase activity of copper–creatinine complexes, producing a color change from orange through green blue. Quantitative results may be estimated according to charts as 10, 50, 100, 200, or 300 mg/dL and 0.9, 4.4, 8.8, 17.7, or 26.5 mmol/L. Falsely elevated results can be caused by bloody or colored urine and cimetidine. The absence of creatinine in readings is considered abnormal, as it is usually found in concentrations of 10–300 mg/dL (Strasinger and Di Lorenzo 2014).

According to several studies, UACR measured in a morning spot urine correlates well with the timed excretion rate (Ginsberg et al. 1983; Heerspink et al. 2010). One trial reported that a random UACR above 30 mg/g had a sensitivity of 100 % for the detection of moderately increased albuminuria (Nathan et al. 1987). Usually, albumin concentration is measured in mg/dL and is divided by the creatinine concentration also in mg/dL, yielding a dimensionless number that estimates the 24-h albumin excretion in grams per day (Shidham and Hebert 2006). If SI units (mg/mmol) are used, the value is divided by 8.8.

The quantification of UACR is mandatory in patients with AKI and CKD and all patients with a previously elevated semiquantitative result. On the one hand, it is imperative to exclude a false positive; on the other hand, the quantification of excretion is essential in establishing the diagnosis, prognosis, and monitoring response to treatment.

The optimal time to measure the UACR remains uncertain (Wald 2014). In an initial study, the best correlation with a 24-h collection occurred with first morning and before bedtime samples (Ginsberg et al. 1983). A larger study also found the best correlation for the first morning void, although the difference regarding other timings was not significant (Witte et al. 2009). There is some evidence that random untimed samples may be more prone to error because they generally show higher intra- and interindividual coefficients of variation than those found for the first or second morning samples (Witte et al. 2009; Naresh et al. 2012). In this setting, one report (Saydah et al. 2013) showed that random urine samples appear to overestimate the prevalence of albuminuria compared to first morning collection. Additionally, creatinine excretion variance may also influence both random spot and 24-h sample results. According to the rates reported before, the average 24-h urine creatinine excretion is approximately 1000 mg/day (11.4 mmol/day) per 1.73 m^2, so this ratio

accuracy is diminished if creatinine excretion is either markedly higher or lower than the average population. The groups more prone to error are individuals with large muscle mass, male gender, black/hispanic race, and younger in age, who may exhibit a much higher creatinine excretion rate than 1000 mg/day, and spot UACR will underestimate a 24-h albuminuria, while among patients who have small muscle mass like cachectic, older, and females, creatinine excretion may be much lower, and 24-h results overestimated. Given these UACR limitations, several investigators have developed an "estimated albumin excretion rate" (eAER) to more accurately predict the 24-h albumin excretion (Abdelmalek et al. 2014; Fotheringham et al. 2014). The eAER can be calculated by multiplying the spot UACR by the expected 24-h creatinine generation. This method may be particularly important in the groups mentioned above.

In conclusion, KDIGO Guidelines recommend screening for moderately increased albuminuria among diabetic patients for early detection of nephropathy and nondiabetic patients at increased risk for CKD or cardiovascular disease, such as those with hypertension and metabolic syndrome (KDIGO 2013).

Protein

Proteinuria is the hallmark of renal disease because it often implies an increase in glomerular permeability, which allows the filtration of normally non-filtered macro-molecules. According to the previous evidence described for albuminuria, protein-uria of increasing severity is associated with a faster rate of decline in GFR, regardless of its baseline (Turin et al. 2013), is an independent risk predictor for ESRD (Iseki 2013), and is associated with the risk of myocardial infarction and mortality (Hemmelgarn et al. 2010).

Proteinuria is defined as urinary protein excretion of more than 150 mg/day (10–20 mg/dL). Normal urinary proteins include albumin, serum globulins, and Tamm–Horsfall protein (uromodulin) secreted by the renal distal tubular epithelial cells. Proteinuria can be organized into different categories according to the source and pathogenesis of the defect, as well as to its clinical significance. It may be divided into glomerular, tubular, overflow, and post-renal proteinuria. Patients may have more than one type of proteinuria. Glomerular proteinuria is due to increased filtration through the glomerular capillary wall. It is a sensitive marker of glomerular disease and hyper-tension, where increased pressure from the blood entering the glomerulus overrides the selective filtration of the glomerulus, as well as all types of GN where different kinds of membrane lesions occur. More benign causes, such as orthostatic, high fever, or exercise-induced proteinuria, usually cause an isolated, transient, and asymptomatic proteinuria, less than 1–2 g/day (Wald 2014). Differential diagnosis of orthostatic proteinuria is carried out when a patient is requested to empty the bladder before going to bed, to collect a specimen immediately upon rising in the morning (negative), and to collect a second one after standing for several hours (positive) (Strasinger and Di Lorenzo 2014). Tubular proteinuria includes low-molecular-weight proteins, such as beta$_2$-microglobulin, immunoglobulin light chains, retinol-binding protein, and

polypeptides derived from the breakdown of albumin, which have molecular weights under 25,000 Daltons in comparison to the 69,000 Daltons of albumin. These smaller proteins can be filtered through the glomerulus and almost completely reabsorbed in the proximal tubule. Interference with proximal tubular reabsorption due to tubulointerstitial diseases, or even some primary glomerular syndromes, can lead to increased excretion of these smaller proteins (Carter et al 2006). Tubular proteinuria often fails to be diagnosed since dipstick is not sensitive for non-albumin proteins, and they are usually found in low concentrations. Mild increased excretion of immunoglobulin light chains (Bence Jones proteins) is polyclonal (Kappa and Lambda), and not injurious to the kidney.

In contrast, the monoclonal nature of the light chains in the overflow proteinuria seen in multiple myeloma is highly nephrotoxic (Wald 2014). This type of proteinuria results from conditions affecting the plasma prior to reaching the kidney and is therefore not indicative of actual renal disease, so it may also be classified as prerenal proteinuria. The most common conditions that lead to overflow proteinuria are frequently transient. It is caused by increased levels of low-molecular-weight plasma proteins that exceed tubular reabsorptive capacity, like acute phase reactants in infection and inflammation. Common examples, other than myeloma kidney, are due to lysozyme in acute myelomonocytic leukemia, myoglobin in rhabdomyolysis, and free hemoglobin in intravascular hemolysis (Barratt and Topham 2007). Patients with myeloma kidney may also develop a component of tubular proteinuria since the excreted light chains may be toxic to the tubules, leading to diminished reabsorption (Wald 2014). Based on false-negative dipstick results, suspected cases of multiple myeloma must be diagnosed by performing serum and urinary immunoelectrophoresis.

Post-renal proteinuria may be caused by inflammation in the urinary tract (often in UTI, but also in nephrolithiasis or tumors), although the mechanism is unclear. The excreted proteins are often non-albumin (often IgA or IgG), in small amounts, and usually accompanied by leukocyturia (Wald 2014).

Regarding measurement techniques, there are two semiquantitative methods to screen patients for proteinuria: dipstick (similar to albuminuria) and sulfosalicylic acid (SSA) precipitation.

Protein dipstick principles and main interference conditions are similar to those described above in the section on "Albuminuria." Although very specific, the dipstick test is not sensitive to low levels of proteinuria (<10–20 mg/dL) or low concentrations of γ-globulins and Bence Jones proteins. A result of 1+ corresponds to approximately 30 mg/dL, 2+ to 100 mg/dL, 3+ to 300 mg/dL, and 4+ to 1,000 mg/dL (House and Cattran 2002). Dipstick urinalysis can reliably predict proteinuria with sensitivities and specificities of greater than 99 % (Woolhandler et al. 1989).

In contrast to the dipstick test, which primarily detects albumin, SSA is a cold precipitation test that detects all proteins in urine at a sensitivity of 5–10 mg/dL (Rose 1987). A significantly positive SSA test in conjunction with a negative dipstick often indicates immunoglobulin light chain excretion caused by dysproteinemias. Various concentrations and amounts of SSA can be used to precipitate protein, and methods vary greatly among laboratories. It is mostly performed by mixing one part of urine supernatant (2.5 mL) with three parts of 3 % SSA and grading the resultant turbidity according to one scheme given by the manufacturer: 0 or no turbidity corresponds to

0 mg/dL; trace or slight turbidity to 1–10 mg/dL; 1+ or mild turbidity to 15–30 mg/dL; 2+ or white cloud without precipitate to 40–100 mg/dL; 3+ or white cloud with fine precipitate to 150–350 mg/dL; and 4+ or flocculent precipitate to >500 mg/dL (Strasinger and Di Lorenzo 2014). The SSA test will be overestimated by as much as 1.5–2 g/L in the presence of iodinated radiocontrast agents (Morcos et al. 1992), penicillins, sulfisoxazole, and with gross hematuria (Simerville et al. 2005).

Both the SSA and dipstick test can detect urinary lysozyme. Total lysozyme excretion is usually below 1 g/day but can exceed 4.5 g/day in some patients with acute monocytic or myelocytic leukemia (Mok et al. 1994). Thus, lysozyme excretion should be measured in patients who have a persistently positive urine dipstick for proteinuria in the absence of albuminuria, particularly if other signs of the nephrotic syndrome are absent.

Once again, patients with persistent proteinuria should undergo a quantitative measurement. As for albuminuria, the 24-h sample is the gold standard method. Nevertheless, based on the assumption that the potential error in determining proteins in a spot urine sample does not exceed the 24-h sample error (Morales et al. 2004), the protein/creatinine ratio (P/C) in spot urine was developed as an alternative (Methven et al. 2010). The same sample can even be used for microscopic investigation. Nevertheless, although the correlation between P/C ratio and 24-h proteinuria has been established, authors are not unanimous (Birmingham et al. 2007), because the results may be influenced by factors like a creatinine excretion deviation from the average. Other authors suggest that this correlation varies in accordance with different levels of proteinuria (Methven et al. 2011), with the majority finding acceptable agreement in the range 0.5–2 g/day (Leung et al. 2007), but not for nephrotic range (Antunes et al. 2008). Moreover, some data also revealed that this correlation is not reliable for some diseases. One example is lupus nephritis (LN), where the accurate evaluation of proteinuria is critical to clinical management, because it is currently the most important available biomarker of disease activity and renal prognosis. Furthermore, proteinuria is often a primary end point in clinical trials of new therapies and therefore must be measured with precision. Based on the possible lack of correlation in this disease, short-interval timed urine collections have been studied as a surrogate for 24-h collections to increase patient compliance and improve accuracy of the results. One study found that a 12-h overnight collection provides a more accurate result than shorter ones (Fine et al. 2009). Although a random spot P/C measurement may not be reliable to make decisions for patients with LN, it may be useful as a screening and monitoring test justified by its advantages concerning facility, reliability, accuracy, and diagnostic speed (Guedes-Marques et al. 2013). It could be used as the preferential marker in subgroups of subjects who find it more difficult to properly collect 24-h urine, such as children, elderly people, patients with intellectual disabilities, incompatible professional activities, or lack of adherence. Another factor to be considered is the timing of the sample, which is influenced by the daily circadian fluctuation of both protein and creatinine excretion. According to some data, the best estimation is probably obtained with morning samples, but not the first void (Saydah et al. 2013).

The technical and methodological details about spot P/C ratio are described in the section on “Albuminuria.”

Proteinuria selectivity can be assessed in nephrotic patients through the ratio of IgG clearance (160,000 D) to transferrin clearance (88,000 D). Although infrequently used, highly selective proteinuria (ratio <0.1) in nephrotic children suggests the diagnosis of minimal change disease and predicts corticosteroid responsiveness (Johnson et al. 2014).

In conclusion, some authors consider that a normal spot P/C ratio is sufficient to rule out pathological proteinuria, but that an elevated value should be confirmed and quantified with a 24-h collection (Price et al. 2005). Other investigators have reported a poor correlation at high levels of proteinuria, as well as in some diseases like LN, where this ratio is useful to monitor so a 24-h ratio is recommended before treatment decisions are made.

Uric Acid

Hyperuricemia and gout correlate with risk factors for cardiovascular disease. They are caused by an overproduction and/or inefficient renal clearance of urate.

The fractional renal clearance of urate (FCU, renal clearance of urate/renal clearance of creatinine) expresses urate clearance as a fraction of creatinine clearance. It provides information about the efficiency of the renal tubular mechanisms of urate clearance by correcting for the effect of the glomerular filtration rate (Kannangara et al. 2012). This spot-FCU has been demonstrated to be a convenient, valid, and reliable indicator of the efficiency of the kidney in removing urate from the system. Spot-FCU has been used in studies investigating molecular mechanisms of kidney clearing urate from the blood and so identifying people at increased risk for cardiovascular disease.

Furthermore, urinary uric acid assessment is necessary for the early diagnosis and adequate treatment of urolithiasis. A recent paper (Sáez-Torres et al. 2014) proposed the analysis of late-afternoon spot urine collection as an appropriate sample to evaluate patient-specific urinary risk factors.

Uric acid excretion is more favorable in alkaline urine. Based on this evidence, dietary intervention in metabolic syndrome, focused on hyperuricemia, recommends taking alkaline-rich fruit and vegetables which could be monitored through urinary pH and uric acid excretion in a random spot sample.

Electrolytes

Urinary excretion of electrolytes can be very useful for diagnosing and monitoring several diseases, as it mainly distinguishes between systemic and kidney-limited disorders. The first condition is caused by abnormal levels of serum electrolytes, to which the kidney tries to adapt its function to maintain homeostasis. The second condition is found with renal tubular disorders with abnormal electrolyte excretion.

Once more, the electrolyte/creatinine ratio in a random spot urine was found to be an alternative to 24-h measurements. Furthermore, as described for uric acid, a

random spot urine sample may also be used to measure the fractional renal clearance of an electrolyte, which expresses its excretion corrected for creatinine clearance. The main practical disadvantage of this method is that a blood sample is required, which is invasive and inconvenient to the patient.

Sodium

In steady-state conditions, the kidneys handle most of the sodium (Na) consumed in a day, and the majority (up to 95 %) is excreted in urine within 24 h. A systematic review of studies comparing 24-h and spot urine collections for estimating population salt intake (Ji et al. 2012) found that most available studies are heterogeneous and there is no uniform pool of data to assess the suitability of alternative random spot samples for measuring 24-h Na excretion (salt intake estimation). However, most studies found acceptable reproducibility. In this setting, most authors believe that using random spot urine samples requires a greater number of collections, but it would still be more convenient and feasible for monitoring. So, regarding programs of population salt reduction, this method is less desirable to provide an initial absolute measure of salt intake, but it may be useful in following repeated assessments.

According to one study (Mann and Gerber 2010), late-afternoon or early-evening samples are more reliable in predicting 24-h Na excretion and may be a cost-effective alternative in salt intake assessment in clinical practice and epidemiological studies.

Potassium

A study with the INTERSALT trial population (Tanaka et al. 2002) demonstrated that the ratio of Na or potassium (K) to creatinine concentration in a random spot specimen is directly proportional to the 24-h ratio. Furthermore, a Na:K ratio may be more reliable in estimating increased risk in cardiovascular disorders such as arterial hypertension.

Phosphate

Calcium (Ca)/creatinine and phosphate (Ph)/creatinine ratios in random spot urine specimens were also found to be a reliable method for estimating daily urinary Ca and Ph excretion (Gokçe et al. 1991) useful in evaluating mineral bone disease in CKD, diagnosis and monitoring of urolithiasis and in the differential diagnosis of hereditary diseases of renal tubular transport (Bartter, Gitelman and Fancony syndrom).

Others

One study (Ilich et al. 2009) compared the concentrations of essential elements magnesium (Mg) and zinc (Zn) in 24-h and spot urine samples and found that

although spot urine sampling might not replace 24-h measurements in all cases, it could be a reliable alternative in those evaluated. Urinay chloride measured in a spot sample is quite useful to evaluate volume and acid-base status and establish the differential diagnosis between extra-renal losses of Na (very low chloride like 1 mmol/L) and renal tubular disorders (> 20 mmol/L).

A significant number of other substances may be detected from random spot urinalysis. This is useful for detecting drug consumption or toxicity (alcohol, benzodiazepines, others), fibroblast growth factor 23 (FGF23), and a phosphaturic hormone to assess bone mineral disease in CKD, among many other approaches less widely used and explored.

Microscopic Markers

Microscopic evaluation is an essential part of spot urine analysis because it provides confirmation of urine dipstick findings and also allows the identification of formed elements that are not evaluated through other methods. Their quantification is mostly semiquantitative, and there is a general interobserver variability, which fails to make them ideal markers. As an example, one study shows only fair to moderate agreement among nephrologists in identifying important structures (Wald et al. 2009). Nevertheless, their presence in urine can be evaluated through a random spot sample and is often crucial to point toward a specific diagnosis.

Regarding specimen handling, it should be examined while fresh or adequately preserved, because elements like RBCs or WBCs and hyaline casts disintegrate rapidly, particularly in dilute alkaline urine. Refrigeration may cause precipitation of amorphous urates, phosphates, and other non-pathological crystals that can obscure other elements in the urine sediment.

The second morning voiding is the most appropriate to obtain because it avoids the lysis of particles that can occur in the bladder overnight. The midstream clean-catch specimen minimizes external contamination and bacterial growth without pathological significance. As for other techniques, diluted specimens may cause false-negative readings.

Concerning sample preparation, after centrifugation, the supernatant is removed. After this, the sediment is resuspended, then transferred to the slide, and prepared using a coverslip. Phase contrast microscopy is recommended because it improves the identification of almost all particles, whereas polarized light is mandatory for the correct identification of some lipids and crystals (Fogazzi 2010). For correct examination, both pH and SG of the sample should be known, because both alkaline pH ($\geq$7.0) and low SG (especially $<$1.010) favor the lysis of cells, which causes discrepancies between dipstick and microscopic examination. The various elements observed are quantified as number per microscopic field, and if counting chambers are used, the elements are quantified as number per milliliter (very precise, but rarely used in everyday practice) (Strasinger and Di Lorenzo 2014).

The main elements formed are cells (erythrocytes, leukocytes, and epithelial), lipids, casts and crystals.

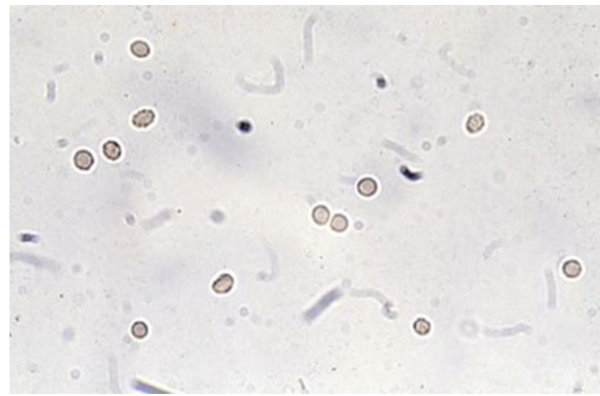

Fig. 2 Microscopic observation of dysmorphic erythrocytes. Microscopic observation (×400) of dysmorphic erythrocytes in a random spot urine sample of a patient with glomerulonephritis (Kindly provided by Dr[a]. Fernanda Carvalho from Curry Cabral Hospital)

Erythrocytes

The assessment of these elements may play a key role in the isolated hematuria diagnostic approach. Although there is no agreement on hematuria definition criteria, it is commonly defined as the presence of two or more RBCs per high-powered field (Cohen and Brown 2003). Additionally, microscopic evaluation may distinguish two main types of erythrocytes: isomorphic, with regular shapes and contours, derived from the urinary excretory system, and dysmorphic, with irregular shapes and contours, which have a glomerular origin (Fig. 2). Thus, according to their relative proportion, hematuria may be defined as non-glomerular or glomerular, respectively. However, this proportion threshold is not unanimous between authors, either.

As was already described above (see chemical parameters – blood, above), among the most common pathological causes of microscopic hematuria are kidney stones, malignancy, and glomerular disease.

Leukocytes

More than simply identifying leukocytes by chemical analysis, it is valuable to distinguish the different types that exist because they may point to specific diseases. Neutrophils usually mean bacterial UTI, but they may also result from urine contamination caused by genital secretions, especially in young women. Nevertheless, they can also be found in interstitial nephritis and proliferative GN. In both conditions they are lower in number, and in GN they are mostly accompanied by dysmorphic erythrocytes and proteinuria. Eosinophils can be detected by applying Wright's or Hansel's stain to the urine sediment (Nolan et al. 1986) and are currently seen as a nonspecific finding because they may be present in various types of GN, prostatitis, chronic pyelonephritis, urinary schistosomiasis, and cholesterol embolism. Lymphocytes may indicate acute cellular rejection in renal allograft recipients. Macrophages may be engorged with lipid droplets, appearing as "oval fat bodies," usually seen in nephrotic syndrome, but may also appear in other settings like Fabry disease.

Cells

Different kinds of cells (renal tubular epithelial, transitional epithelial, and squamous) can be found in normal or pathological conditions according to their proportion and presentation. Epithelial cells may appear in the urine after being shed from anywhere within the genitourinary tract and are mostly common in ATN.

Lipids

Lipids are found in urine as drops of different sizes that can be isolated or in clusters, as oval fat bodies (Hotta et al. 2000). All these particles mainly contain cholesterol esters and free cholesterol, showing a Maltese cross appearance with symmetric arms, under polarized light. These lipids are typical of glomerular diseases associated with nephrotic range proteinuria and Fabry disease.

Casts

Casts are cylindrical structures formed in the lumen of distal renal tubules and collecting ducts (Fig. 3). Their matrix is made of Tamm–Horsfall glycoprotein (uromodulin), and they have different appearances, each of which has different features and a specific clinical significance summarized in Table 1.

Crystals

Correct identification of urine crystals requires a wide knowledge of crystal morphology and appearance under polarizing light. Whether crystals form in the urine depends upon a variety of factors, including relative concentration of constituent molecules,

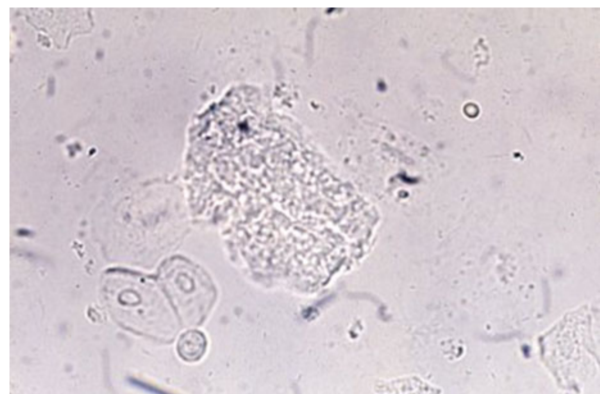

Fig. 3 Microscopic observation of a large granulous cast. Microscopic observation (×400) of a large granulous cast in a random spot urine sample of a patient with chronic kidney disease (Kindly provided by Dr[a]. Fernanda Carvalho from Curry Cabral Hospital)

Table 1 Individual features and clinical significance of urinary casts

Cast	Appearance	Associated conditions
Hyaline	Uniform pale pink or purple	High concentrated urine (normal conditions) Diuretic therapy Nonspecific
Hyaline–granular	Fine purple granules in pale pink or purple matrix	Normal
Granular	Dark purple granules in dark purple matrix	ATN
Waxy	Homogeneous pale pink or faint yellow (darker than hyaline) with sharp indentations and darker edges	Nephrotic syndrome CKD Nonspecific (acute and chronic conditions)
Erythrocyte	Pink to orange red	Glomerulonephritis (glomerular hematuria)
Hemoglobin	Uniform orange red	Intravascular hemolysis
Leukocyte	Mostly granular aspect	Interstitial nephritis Pyelonephritis Glomerulonephritis
Epithelial	Cellular bodies in a hyaline matrix	ATN Acute interstitial nephritis Glomerulonephritis
Broad	Larger casts	CKD
Myoglobin	Dark pigments	Rhabdomyolysis
Bilirubin	Greenish yellow	Jaundice (liver disease)
Microorganisms	Pure or mixed	Bacterial or fungal urinary tract infection

ATN acute tubular necrosis, *CKD* chronic kidney disease

urine pH, and the presence of crystallization inhibitors. Examination of urine for crystals is informative in the assessment of patients with stone disease, some rare inherited metabolic disorders (like cystinuria, oxalosis, phosphoribosyltransferase deficiency), and suspected drug nephrotoxicity (Fogazzi 2010). The main individual features and clinical significance of crystals are summarized in Table 2.

Organisms

Bacteria are often seen in urine, but their clinical significance is generally guided by patient symptoms and other urinary features like the presence of neutrophils (Fig. 4). Fungi are also frequent (Fig. 4). Examiners should be aware that specimen handling is vital to this examination because a sample that is not fresh can be contaminated.

Table 2 Individual features and clinical significance of urinary crystals

Crystal	Composition	Appearance	Urine conditions	Associated disorders
Uric acid	Amorphous urates	Rhomboids and barrels (polychromatic polarize)	pH 5.0–5.8 (acid)	Transient supersaturation of urine (no significance) Mild dehydration Urate nephropathy/tumor lysis syndrome (AKI)
Calcium oxalate	Bihydrated Monohydrated	Bipyramidal (no polarize) Ovoid or biconcave (polarize)	pH 5.4–6.7 (acid)	Transient supersaturation of urine (no significance) Mild dehydration Calcium lithiasis Ethylene glycol intoxication (AKI)
Brushite (calcium phosphate)	Amorphous phosphates	Pleomorphic: prism, stars, needles (polarize), granular plates (no polarize))	pH $\geq$7 (alkaline)	Transient supersaturation of urine (no significance) Mild dehydration
Struvite (triple phosphate)	Magnesium ammonium phosphate and calcium carbonate apatite	Coffin lids (polarize)	pH $\geq$7 (alkaline)	Urinary tract infection (*Ureaplasma urealyticum* and *Corynebacterium urealyticum)*
Cholesterol	Cholesterol and other lipids	Thin, transparent plates		
Cystine	Cystine	Hexagonal irregular plates	Acid pH	Cystinuria
2,8-Dihydroxyadenine	2,8-Dihydroxyadenine	Spherical, brownish, central umbilicus, birefringent cross-like appearance (polarize)		Homozygotic deficiency of adenine phosphoribosyltransferase
Tyrosine	Tyrosine			Acute liver disease Tyrosinemia
Leucine	Leucine			Acute liver disease
Drugs	Drug Calcium oxalate (naftidrofuryl oxalate, orlistat, and vitamin C)	Unusual morphology		Drug overdose, dehydration, or hypoalbuminemia (sulfadiazine, amoxicillin, ciprofloxacin, acyclovir, indinavir, pyridoxylate, naftidrofuryl oxalate, primidone, felbamate, orlistat, intravenous vitamin C)

AKI acute kidney injury

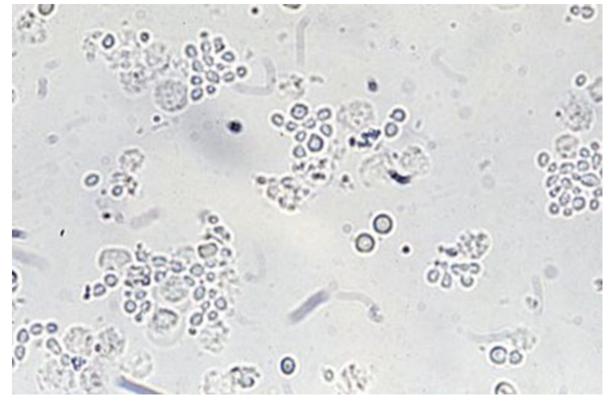

Fig. 4 Microscopic observation of fungus, bacteria, and leukocytes. Microscopic observation (×400) of fungus, bacteria, and leukocytes in a random spot urine of a patient with sepsis and acute kidney injury (Kindly provided by Dr[a]. Fernanda Carvalho from Curry Cabral Hospital)

Conclusions

It is unusual to only focus on a single risk marker for the development and progression of a disease. In this setting, random spot urine analysis may provide the identification of several markers at just one moment, without an invasive procedure. There is a need to develop broader models of progressive kidney diseases and the relationship they have with the cardiovascular background, which include novel pathways and risk markers apart from those related to the traditional proteinuric pathway. In addition, more trials are needed to find out which sample is most appropriate for analysis and if this alternative method is reliable in all types of diseases.

Finally, many other biomarkers, like genetic fragments and cytokines, among others, can already be measured in a random spot urine sample, but their comparison with a 24-h sample measurement and many of their applications is still not reported.

Potential Applications to Prognosis, Other Diseases, or Conditions

Random spot urine examination is already much more than a complementary exam limited to the nephrology field. In this setting, currently identified urinary markers have been found to be associated to other diseases, other than kidney abnormalities. Moreover, new biomarkers are being identified to help in the early detection of cancer, tuberculosis, HIV, malaria, and potentially many other diseases.

In the future, random spot urinary genetic markers, as well as others, may predict chronic or acute events, even before traditional laboratorial and clinical signs become positive. Adding to this "risk" and "diagnostic" value, future biomarkers may contribute to novel disease pathogenesis models, which could be crucial in developing new therapeutic targets and activity markers.

Maybe one day, one simple occasional urine sample could be used to screen for diseases like oncological, autoimmune, metabolic, or other diseases, immediately after birth. Additionally, other markers could equally be associated to future specific therapy responses, helping to decide the best treatment for each patient according to their individual profile.

Summary Points

- This chapter focuses on the analysis of random spot urine, which can be easily obtained at any time of the day with only one urination. It allows several, otherwise asymptomatic, markers to be identified and avoids 24-h collection-related errors.
- The collection and handling of the urine sample is crucial to avoid errors and both the suitability and the rejection criteria must be determined.
- Random spot urine markers can be organized into three groups, according to their laboratory method assessment: physical, chemical, and microscopic markers.
- Physical markers include color, odor, clarity, and specific gravity.
- Chemical examination includes the identification of protein, blood, glucose, pH, bilirubin, urobilinogen, ketones, nitrites, and leukocyte esterase. They are probably the most useful in daily practice, because their value in the diagnosis, monitoring, and prognosis of glomerular diseases has long been proven.
- Microscopic evaluation entails the detection of crystals, cells, casts, and organisms.
- Chemical results are the most often widely used, because of their (semi)quantitative presentation and possible correlation with quantitative timed collections, but physical and microscopic ones may play a key role in many differential diagnoses and monitoring.

References

Abdelmalek JA, Gansevoort RT, Lambers Heerspink HJ, et al. Estimated albumin excretion rate versus urine albumin-creatinine ratio for the assessment of albuminuria: a diagnostic test study from the Prevention of Renal and Vascular Endstage Disease (PREVEND) Study. Am J Kidney Dis. 2014;63:415.

Antunes VVH, Veronese FJV, Morales JV. Diagnostic accuracy of the protein/creatinine ratio in urine samples to estimate 24-h proteinuria in patients with primary glomerulopathies: a longitudinal study. Nephrol Dial Transplant. 2008;23:2242–6.

Assadi FK, Fornell L. Estimation of urine specific gravity in neonates with a reagent strip. J Pediatr. 1986;108:995–6.

Ballarin J, Arce Y, Torra Balcells R, et al. Acute renal failure associated to paroxysmal nocturnal haemoglobinuria leads to intratubular haemosiderin accumulation and CD163 expression. Nephrol Dial Transplant. 2011;26:3408–11.

Barratt J, Topham P. Urine proteomics: the present and future of measuring urinary protein components in disease. CMAJ. 2007;177:361.
Bennett PH, Haffner S, Kasiske BL, et al. Screening and management of microalbuminuria in patients with diabetes mellitus: recommendations to the Scientific Advisory Board of the National Kidney Foundation from an ad hoc committee of the Council on Diabetes Mellitus of the National Kidney Foundation. Am J Kidney Dis. 1995;25:107–12.
Birmingham DJ, Rovin BH, Shidham G, et al. Spot urine protein/creatinine ratios are unreliable estimates of 24 h proteinuria in most systemic lupus erythematosus nephritis flares. Kidney Int. 2007;72:865.
Brenner B, Taal M, Chertow G, et al. Brenner & Rector's the kidney, 9th ed. Boston: Elsevier Health Sciences; 2011.
Brigden ML, Edgell D, McPherson M, et al. High incidence of significant urinary ascorbic acid concentrations in a west coast population – implications for routine urinalysis. Clin Chem. 1992;38:426.
Brodsky SV, Nadasdy T, Rovin BH, et al. Warfarin-related nephropathy occurs in patients with and without chronic kidney disease and is associated with an increased mortality rate. Kidney Int. 2011;80:181–9.
Busby DE, Bakris GL. Comparison of commonly used assays for the detection of microalbuminuria. J Clin Hypertens (Greenwich). 2004;6:8.
Carter JL, Tomson CR, Stevens PE, et al. Does urinary tract infection cause proteinuria or microalbuminuria? A systematic review. Nephrol Dial Transplant. 2006;21:3031.
Cohen RA, Brown RS. Clinical practice. Microscopic hematuria. N Engl J Med. 2003;348:2330.
Comper WD, Osicka TM. Detection of urinary albumin. Adv Chron Kidney Dis. 2005;12:170.
Constantiner M, Sehgal AR, Humbert L, et al. A dipstick protein and specific gravity algorithm accurately predicts pathological proteinuria. Am J Kidney Dis. 2005;45:833.
Demilie T, Beyene G, Melaku S, et al. Diagnostic accuracy of rapid urine dipstick test to predict urinary tract infection among pregnant women in Felege Hiwot Referral Hospital, Bahir Dar. North West Ethiopia. BMC Res Notes. 2014;7:481.
Engelgau MM, Narayan KM, Herman WH. Screening for type 2 diabetes. Diabetes Care. 2000;23 (12):1868–9.
Fassett RG, Horgan BA, Mathew TH. Detection of glomerular bleeding by phase-contrast microscopy. Lancet. 1982;1:1432–4.
Fine DM, Ziegenbein M, Petri M, et al. A prospective study of protein excretion using short-interval timed urine collections in patients with lupus nephritis. Kidney Int. 2009;76(12): 1284–8.
Fogazzi GB. The urinary sediment: an integrated view. 3rd ed. Milan: Elsevier; 2010.
Fotheringham J, Campbell MJ, Fogarty DG, et al. Estimated albumin excretion rate versus urine albumin-creatinine ratio for the estimation of measured albumin excretion rate: derivation and validation of an estimated albumin excretion rate equation. Am J Kidney Dis. 2014;63:405.
Garingalo-Molina FD. Asymptomatic bacteriuria among pregnant women: overview of diagnostic approaches. Phil J Microbiol Infect Dis. 2000;29:177–86.
Ginsberg JM, Chang BS, Matarese RA, et al. Use of single voided urine samples to estimate quantitative proteinuria. N Engl J Med. 1983;309:1543.
Gökçe C, Gökçe O, Baydinç C, et al. Use of random urine samples to estimate total urinary calcium and phosphate excretion. Arch Intern Med. 1991;151(8):1587–8.
Gorchynski J, Dean K, Anderson CL. Analysis of urobilinogen and urine bilirubin for intra-abdominal injury in blunt trauma patients. West J Emerg Med. 2009;10(2):85–8.
Gray CH, Millar HR. Tests for glycosuria. A comparison of BenediCrS test, Clinitest and Glucotest. Br Med J. 1953; 1:1361–3.
Grossfeld G, Litwin M, Wolf J, et al. Evaluation of asymptomatic microscopic hematuria in adults: the American Urological Association best practice policy – part I: definition, detection, prevalence and etiology. Urology. 2001;57:599–603.

Guedes-Marques M, Cotovio P, Ferrer F, et al. Random spot urine protein/creatinine ratio: a reliable method for monitoring lupus nephritis? Clin Kidney J. 2013;6:590–4.

Gutierrez E, Gonzalez E, Hernandez E, et al. Factors that determine an incomplete recovery of renal function in macrohematuria-induced acute renal failure of IgA nephropathy. Clin J Am Soc Nephrol. 2007;2:51–7.

Hahn B, Mcmahon M, Wilkinson A, et al. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. Arthritis Care Res. 2012;64(6):797–808.

Haque SK, Ariceta G, Batlle D. Proximal renal tubular acidosis: a not so rare disorder of multiple etiologies. Nephrol Dial Transplant. 2012;27:4273.

Heerspink HJL, Gansevoort RT, Brenner BM, et al. Comparison of different measures of urinary protein excretion for the prediction of renal events. J Am Soc Nephrol. 2010;21:1355–60.

Hemmelgarn BR, Zhang J, Manns BJ, et al. Nephrology visits and health care resource use before and after reporting estimated glomerular filtration rate. JAMA. 2010;303:1151–8.

Hotta O, Yusa N, Kitamura H, et al. Urinary macrophages as activity markers of renal injury. Clin Chim Acta. 2000;297:123–33.

House AA, Cattran DC. Nephrology: 2. Evaluation of asymptomatic hematuria and proteinuria in adult primary care. CMAJ. 2002;166:348–53.

Ilich JZ, Blanusa M, Orlić ZC, et al. Comparison of calcium, magnesium, sodium, potassium, zinc, and creatinine concentration in 24-h and spot urine samples in women. Clin Chem Lab Med. 2009;47(2):216–21.

Iseki K. Proteinuria as a predictor of rapid eGFR decline. Nat Rev Nephrol. 2013;9:570–1.

Jacobs DS, De Mott WR, Willie GR. Urinalysis and clinical microscopy. In: Jacobs DS, Kasten BL, De Mott WR, Wolfson WL, editors. Laboratory test handbook. Baltimore: Williams & Wilkins; 1990. p. 912.

Ji C, Sykes L, Paul C, Dary O, et al. Systematic review of studies comparing 24-hour and spot urine collections for estimating population salt intake. Rev Panam Salud Publica. 2012;32(4):307–15.

Johnson R, Feehally J, Floege J. Comprehensive clinical nephrology, 5th ed. Philadelphia: Elsevier Health Sciences; 2014.

Jung K. Enzyme activities in urine: how should we express their excretion? A critical literature review. Eur J Clin Chem Clin Biochem. 1991;29:725–9.

Kannangara D, Ramasamy S, Indraratna S, et al. Fractional clearance of urate: validation of measurement in spot-urine samples in healthy subjects and gouty patients. Arthritis Res Ther. 2012;14:R189. Kannangara et al002E.

KDIGO. Chapter 1: definition and classification of CKD. Kidney Int Suppl. 2013;3:19. http://www.kdigo.org/clinical_practice_guidelines/pdf/CKD/KDIGO_2012_CKD_GL.pdf. Accessed on Oct 2014.

Lam MO. False hematuria due to bacteriuria. Arch Pathol. 1995;119:717–21.

Lamb EJ, MacKenzie F, Stevens PE. How should proteinuria be detected and measured? Ann Clin Biochem. 2009;46:205–17.

Leung YY, Czeto CC, Tam LS, et al. Urine protein-to-creatinine ratio in an untimed urine collection is a reliable measure of proteinuria in lupus nephritis. Rheumatology (Oxford). 2007;46:649–52.

Lohr JA. Use of routine urinalysis in making a presumptive diagnosis of urinary tract infection in children. Pediatr Infect Dis J. 1991;10:646–50.

MacIsaac R, Ekinci E, Jerums G. Progressive diabetic nephropathy. How useful is microalbuminuria?: Contra. Kidney Int. 2014;86:50–7.

Magen D, Sprecher E, Zelikovic I, et al. A novel missense mutation in SLC5A2 encoding SGLT2 underlies autosomal-recessive renal glucosuria and aminoaciduria. Kidney Int. 2005;67:34–41.

Mann SJ, Gerber LM. Estimation of 24-hour sodium excretion from spot urine samples. J Clin Hypertens (Greenwich). 2010;12:174–80.

McCormack M, Dessureault J, Guitard M. The urine specific gravity dipstick: a useful tool to increase fluid intake in stone forming patients. J Urol. 1991;146:1475–7.

Methven S, MacGregor MS, Traynor JP, et al. Assessing proteinuria in chronic kidney disease: protein–creatinine ratio versus albumin–creatinine ratio. Nephrol Dial Transplant. 2010;25 (9):2991–6.

Methven S, MacGregor MS, Traynor JP, Hair M, O'Reilly DJ, Deighan CJ. Comparison of urinary albumin and urinary total protein as predictors of patient outcomes in CKD. Am J Kidney Dis. 2011;57(1):21–8.

Mok CC, Tam SC, Kwong YL. Pseudonephrotic syndrome caused by lysozymuria. Ann Intern Med. 1994;121:818.

Morales JV, Weber R, Wagner MB, et al. Is morning urinary protein/creatinine ratio a reliable estimator of 24-hour proteinuria in patients with glomerulonephritis and different levels of renal function? J Nephrol. 2004;17(5):666–72.

Morcos SK, El-Nahas AM, Brown P, et al. Effect of iodinated water soluble contrast media on urinary protein assays. BMJ. 1992;305:29.

Moreno JA, Martı'n-Cleary C, Gutiérrez E. Editorial reviews haematuria: the forgotten CKD factor? Nephrol Dial Transplant. 2012;27:28–34.

Muto S, Sugiura S, Nakajima A, et al. Isomorphic red blood cells using automated urine flow cytometry is a reliable method in diagnosis of bladder cancer. Int J Clin Oncol. 2014;19(5):928–34.

Naresh CN, Hayen A, Craig JC, et al. Day-to-day variability in spot urine protein-creatinine ratio measurements. Am J Kidney Dis. 2012;60:561.

Nathan DM, Rosenbaum C, Protasowicki VD. Single-void urine samples can be used to estimate quantitative microalbuminuria. Diabetes Care. 1987;10:414.

Newman DJ, Mattock MB, Dawnay BS, et al. Systematic review on urine albumin testing for early detection of diabetic complications. Health Technol Assess. 2005;9:30.

Nolan 3rd CR, Anger MS, Kelleher SP. Eosinophiluria – a new method of detection and definition of the clinical spectrum. N Engl J Med. 1986;315:1516.

Patel HD, Livsey SA, Swann RA, et al. Can urine dipstick testing for urinary tract infection at point of care reduce laboratory workload? J Clin Pathol. 2005;58:951–4.

Price CP, Newall R, Boyd JC. Use of protein/creatinine ratio measurements on random urine samples for prediction of significant proteinuria: a systematic review. Clin Chem. 2005;51:1577–86.

Rahman MS, Beever W, Skov S, et al. Using urinary leucocyte esterase tests as an indicator of infection with gonorrhoea or chlamydia in asymptomatic males in a primary health care setting. Int J STD AIDS. 2014;25(2):138–44.

Rauta V, Finne P, Fagerudd J, et al. Factors associated with progression of IgA nephropathy are related to renal function – a model for estimating risk of progression in mild disease. Clin Nephrol. 2002;58:85–94.

Roscioni SS, Heerspink HJ, Zeeuw D. Microalbuminuria: target for renoprotective therapy PRO. Kidney Int. 2014;86:40–9.

Rose BD. Pathophysiology of renal disease. 2nd ed. New York: McGraw-Hill; 1987. p. 11.

Ruggenenti P, Remuzzi G. Time to abandon microalbuminuria? Kidney Int. 2006;70:1214–22.

Ruggenenti P, Porrini E, Motterlini N, et al. Measurable urinary albumin predicts cardiovascular risk among normoalbuminuric patients with type 2 diabetes. J Am Soc Nephrol. 2012;23:1717–24.

Sáez-Torres C, Rodrigo D, Grases F, et al. Urinary excretion of calcium, magnesium, phosphate, citrate, oxalate, and uric acid by healthy schoolchildren using a 12-h collection protocol. Pediatr Nephrol. 2014;29:1201–8.

Sand TE, Jacobsen S. Effect of urine pH and flow on renal clearance of methotrexate. Eur J Clin Pharmacol. 1981;19(6):453–6.

Saydah SH, Pavkov ME, Zhang C, et al. Albuminuria prevalence in first morning void compared with previous random urine from adults in the National Health and Nutrition Examination Survey, 2009–2010. Clin Chem. 2013;59(4):675–83.

Semeniuk H, Church D. Evaluation of the leukocyte esterase and nitrite urine dipstick screening tests for detection of bacteriuria in women with suspected uncomplicated urinary tract infections. J Clin Microbiol. 1999;37(9):3051–2.

Shidham G, Hebert LA. Timed urine collections are not needed to measure urine protein excretion in clinical practice. Am J Kidney Dis. 2006;47:8.

Siegrist D, Hess B, Montandon M, et al. Spezifi sches Gewicht des Urins-vergleichende Messungen mit Teststreifen und Refraktometer bei 340 Morgenurinproben. Schweiz Rundsch Med Prax. 1993;82:112–6.
Simerville JA, Maxted WC, Pahira JJ. Urinalysis: a comprehensive review. Am Fam Physician. 2005;71:1153.
Singer DE, Coley CM, Samet JH, et al. Tests of glycemia in diabetes mellitus: their use in establishing a diagnosis and in treatment. Ann Intern Med. 1989;110:125–37.
Stevens PE, Levin A, Kidney Disease: Improving Global Outcomes Chronic Kidney Disease Guideline Development Work Group Members. Evaluation and management of chronic kidney disease: synopsis of the kidney disease: improving global outcomes 2012 clinical practice guideline. Ann Intern Med. 2013;158:825.
Strasinger S, Di Lorenzo M. Urinalysis and body fluids, 6th ed. Philadelphia: F. A. Davis Company; 2014.
Sutton JM. Evaluation of hematuria in adults. JAMA. 1990;263:2475–80.
Taboulet P, Deconinck N, Thurel A, et al. Correlation between urine ketones (acetoacetate) and capillary blood ketones (3-beta-hydroxybutyrate) in hyperglycaemic patients. Diabetes Metab. 2007;33(2):135–9.
Tanaka T, Okamura T, Miura K, et al. A simple method to estimate populational 24-h urinary sodium and potassium excretion using a casual urine specimen. J Hum Hypertens. 2002;16:97–103.
Tapp DC, Copley JB. Effect of red blood cell lysis on protein quantitation in hematuric states. Am J Nephrol. 1988;8:190.
Turin TC, James M, Ravani P, et al. Proteinuria and rate of change in kidney function in a community-based population. J Am Soc Nephrol. 2013;24(10):1661–7.
Vivante A, Afek A, Frenkel-Nir Y, et al. Persistent asymptomatic isolated microscopic hematuria in Israeli adolescents and young adults and risk for end-stage renal disease. JAMA. 2011;306:729–36.
Wagner CA, Mohebbi N. Urinary pH and stone formation. J Nephrol. 2010;23(16):S165–9.
Wald R. Urinalysis in the diagnosis of kidney disease. In Uptodate. http://www.uptodate.com/contents/search. Accessed Oct 2014.
Wald R, Bell CM, Nisenbaum R, et al. Interobserver reliability of urine sediment interpretation. Clin J Am Soc Nephrol. 2009;4:567.
Wise KA, Sagert LA, Grammens GL. Urine leukocyte esterase and nitrite tests as an aid to predict urine culture results. Lab Med. 1984;15(3):186–7.
Witte EC, Lambers Heerspink HJ, de Zeeuw D, et al. First morning voids are more reliable than spot urine samples to assess microalbuminuria. J Am Soc Nephrol. 2009;20:436–43.
Woolhandler S, Pels RJ, Bor DH, Himmelstein DU, Lawrence RS. Dipstick urinalysis screening of asymptomatic adults for urinary tract disorders. I. Hematuria and proteinuria. JAMA. 1989;262:1214–9.
Young DS. Effects of drugs on clinical laboratory tests, vol. 3. 3rd ed. Washington, DC: American Association for Clinical Chemistry Press; 1990. p. 356–7.

Overview of Neutrophil Gelatinase-Associated Lipocalin (NGAL) as a Biomarker in Nephrology

10

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Abstract

Neutrophil gelatinase-associated lipocalin (NGAL) is a small 25-kD peptide belonging to the lipocalin superfamily. It mainly binds to siderophores, iron-carrying molecules which are essential to bacterial and eukaryotic cells to meet their needs of this mineral and then ensure survival. Indeed, NGAL primarily acts as an innate nonspecific antibacterial factor, since it prevents bacteria to internalize iron thus inhibiting their growth. However, serum NGAL levels rise not only in the course of infective processes but also in other pathological conditions, as the result of an

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increased production and release from several tissues after an injury. Numerous experimental and clinical data support a role for NGAL as a biomarker in nephrology. It has been demonstrated that NGAL predicts the onset of acute kidney injury (AKI), closely correlates with the extent of renal impairment in patients with chronic kidney disease (CKD) irrespective of the underlying renal disease; it is higher in kidney transplant patients who will develop a delayed graft function (DGF) compared to those who will not experience this complication. Furthermore, NGAL predicts CKD progression and may represent a precocious marker of therapeutic response in different clinical situations. A number of studies also established the involvement of this molecule in carcinogenesis and progression of several human tumors, in the regulation of erythropoiesis and in the pathophysiology of cardiovascular diseases. The aim of the present review has been therefore to summarize all the most recent findings concerning the potential use of NGAL as a diagnostic and prognostic marker as well as a marker of therapeutic response in patients with renal diseases.

Keywords

Acute kidney injury • Biomarker • Chronic kidney disease • Neutrophil gelatinase-associated lipocalin • Renal transplantation

Abbreviations

ADPKD	Autosomal-dominant polycystic kidney disease
AKI	Acute kidney injury
CIN	Contrast-induced nephropathy
CKD	Chronic kidney disease
CPB	Cardiopulmonary bypass
CT	Computed tomography
DGF	Delayed graft function
ESRD	End-stage renal disease
FABP	Fatty acid-binding protein
FeNGAL	Fractional excretion of NGAL
GFR	Glomerular filtration rate
KIM-1	Kidney injury molecule-1
NAG	*N*-acetyl-β-D-glucosaminidase
NGAL	Neutrophil gelatinase-associated lipocalin
PCI	Percutaneous coronary intervention
shRNA	Short hairpin RNA
sNGAL	Serum NGAL
uNGAL	Urinary NGAL

Key Facts of Creatinine

- Creatinine is an endogenous substance produced by the muscle metabolism of creatine that, phosphorylated by creatine kinase to creatine phosphate, acts as a reserve of energy for muscle contraction.

- Creatinine production is poorly correlated to diet and physical exercise, whereas it depends on the development of the muscular masses.
- In adults, serum creatinine values range between 0.8 and 1.3 mg/dl in men and between 0.6 and 1.1 mg/dl in women.
- The glomerular function is evaluated primarily by the determination of creatinine clearance.
- The diagnosis of acute kidney injury (AKI) is mainly based on increased serum creatinine, which is not an ideal indicator of renal impairment. Serum creatinine is produced endogenously, excreted by the kidney, easily measured in body fluids, and inexpensive. However, its levels vary slowly and are influenced by several factors including sex, age, nutritional status, muscle mass, blood volume, and some drugs. Besides, during AKI, the tubular secretion of creatinine is increased; in this condition, it cannot therefore be regarded as a reliable marker of the actual glomerular filtration rate.
- Serum creatinine proves not to be an ideal marker even in the course of chronic kidney disease: indeed, it is kept within the normal range until the glomerular filtration rate is not reduced up to about 50 ml/min. Therefore, its serum concentration may remain unchanged as long as a substantial percentage of renal function does not become compromised.
- Since creatinine is not an ideal marker, researchers are trying to identify new markers of renal function, which have the characteristic to change early on and to predict the evolution of renal disease with sufficient accuracy. Among them, one of the most promising is neutrophil gelatinase-associated lipocalin (NGAL).

Definitions

Acute kidney injury (AKI) Sudden decline, from 1 to 7 days, of renal function with retention of nitrogen products (urea, creatinine), metabolic acidosis, hyperkalemia, salt and water retention, decreased urine flow, and anuria.

Autosomal-dominant polycystic kidney disease (ADPKD) The most common hereditary renal disease; it is characterized by the progressive cystic dilatation of renal tubules with volume increase of the kidneys and gradual progression to end-stage renal disease.

Biomarker Molecular substance or characteristic that can be used as an indicator in a living organism. Its presence, absence, or modification, detectable and quantifiable using appropriate tests, are the expression of a normal or pathological biological process or the response to a certain pharmacological treatment.

Cold ischemia time Time elapsed between the start of perfusion with cold solutions in the explantation operating room and the extraction of the organ from the ice in which it is preserved (or from other refrigerated systems of preservation) at the moment of transplantation.

Contrast-induced nephropathy (CIN) Absolute (≥0.5 mg/dl) or relative increase (≥25 %) in serum creatinine level at 48–72 h after exposure to a contrast agent compared to serum creatinine at baseline.

Delayed graft function Dialysis requirement during the first week after kidney transplantation.

Siderophores Iron-carrying molecules which are essential to bacterial and eukaryotic cells to meet their needs of this mineral and then ensure survival.

Introduction

NGAL (neutrophil gelatinase-associated lipocalin), also referred to as lipocalin-2, siderocalin, uterocalin, and 24p3, is a small 25-kD peptide belonging to the lipocalin superfamily. The latter includes molecules with a three-dimensional structure characterized by an eight-stranded antiparallel b-barrel surrounding a central pocket; through this central calyx, they interact with different kinds of low-molecular-weight ligands (Flower 1996; Kjeldsen et al. 2000; Bolignano et al. 2008a).

NGAL mainly binds to siderophores (Goetz et al. 2000), small iron-carrying molecules which are essential to bacterial and eukaryotic cells to meet their needs of this mineral and then ensure survival.

NGAL name originates from the fact that this protein was firstly discovered in activated neutrophils where it is covalently bound to the enzyme gelatinase present in the cytoplasmic azurophilic granules, and serum NGAL mainly derives from these cells under normal conditions. It is also produced by other elements of the immune system, such as macrophages, in response to the addition of lipopolysaccharide and other bacterial products. Indeed, NGAL primarily acts as an innate nonspecific antibacterial factor, since it prevents bacteria to internalize iron thus inhibiting their growth (Meheus et al. 1993; Malyszko et al. 2010; Helanova et al. 2014).

The interesting observation that viral infections were able to stimulate NGAL production also in cells not belonging to the immune system, as demonstrated in in vitro models of murine kidney cells infected with strains of SV-40 virus (Hraba-Renevey et al. 1989), has for the first time raised the hypothesis that this protein could be much more than just a factor involved in nonspecific mechanisms of cellular immunity. This theory was further supported by the demonstration that NGAL was able to interact with many ligands other than siderophores such as hepatocyte growth factor, gelatinase-B, different kinases, and proteins of intra- and extracellular matrix (Kjeldsen et al. 1993; Yan et al. 2001). Moreover, later studies revealed that the promoter region of the NGAL gene is subject to transcriptional regulation by various factors including, for example, NF-kB (Cowland and Borregaard 1997; Cowland et al. 2003), a key element involved in tissue proliferation, in tumor growth, in the inflammatory acute phase mechanisms, and, most importantly, in the cellular response to and defense against various types of acute and chronic injuries such as ischemia and oxidative stress.

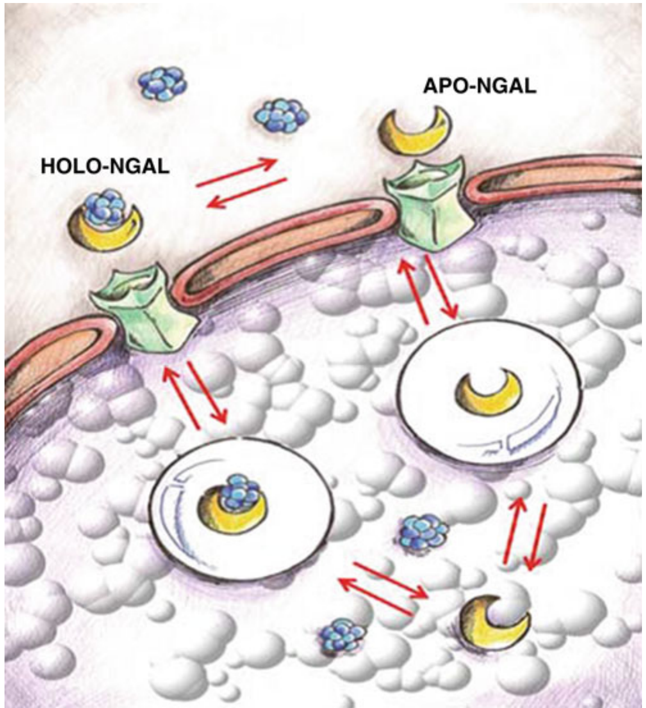

Fig. 1 Molecular mechanisms of action of NGAL at the cellular level. NGAL can be internalized into the cell either alone (*Apo-NGAL*) or as a complex with the iron-binding siderophores (*Holo-NGAL*) (Bolignano and Buemi 2009; with permission from Publisher Nuova Editoriale Bios)

NGAL cellular activities are closely influenced by the interaction with specific membrane receptors present on almost all human cells; among them are 24p3R, which belongs to the family of brain-type organic cation transporters, and the multi-scavenger complex of megalin that is mainly found on the brush border of renal tubular cells (Devireddy et al. 2005; Hvidberg et al. 2005).

Following the interaction with these receptors, NGAL can be internalized into the cell either alone (Apo-NGAL) or as a complex with the iron-binding siderophores (Holo-NGAL). The different configuration with which NGAL enters the cell is crucial because this may lead to diametrically opposite effects. Holo-NGAL is captured within endosomal vesicles and transported into the cytoplasm where it can release its iron-siderophore complexes, so enhancing specific iron-mediated intracellular activation pathways such as cell growth or mitigation of oxidative stress. The protein core can at this stage be degraded or expelled outside the cell as Apo-NGAL. Conversely, Apo-NGAL, once internalized, is able to attract the intracellular iron and release it outside the cell with resulting depletion of iron reserves; this condition can sometimes stimulate gene pathways producing exactly opposite effects compared to the previous ones including the induction of apoptosis and subsequent programmed cell death (Devireddy et al. 2005; Schmidt-Ott et al. 2007; Fig. 1).

Most likely, this dual cellular action ascribed to NGAL is at the base of the numerous inconsistencies reported by various studies with the consequent impossibility, sometimes, to assign a univocal role to this protein in the pathophysiology of some diseases.

According to what just said above, serum NGAL levels raise not only in the course of infective processes but also in other pathological conditions, as the result of an increased production and release from several tissues after an injury. For instance, NGAL is significantly high during inflammatory diseases involving intestinal epithelium and endothelium (Playford et al. 2006), the skin, and distal and proximal airways (Cowland et al. 2003); it is also hyper-expressed in atherosclerotic plaques and infarcted myocardium (Hemdahl et al. 2006).

A special attention should be paid to the numerous experimental and clinical data supporting a role for NGAL as a biomarker in nephrology. Indeed, several studies have been performed to evaluate the usefulness of NGAL in the clinical settings of acute kidney injury (AKI), chronic kidney disease (CKD), autosomal-dominant polycystic kidney disease (ADPKD), and renal transplantation.

The aim of the present review has been therefore to summarize all the most recent findings concerning the potential use of NGAL as a diagnostic and prognostic marker as well as a marker of therapeutic response in patients with renal diseases.

NGAL and Acute Kidney Injury

In current clinical practice, AKI is typically diagnosed by measuring the concentrations of serum creatinine. This parameter, however, is not very reliable in detecting acute changes in renal function for several reasons: it can significantly vary with age, sex, muscle mass, and hydration status and can be distorted by particular drug treatments, and especially it may remain unchanged as long as a substantial percentage of renal function does not become compromised. In addition, it is always necessary to attain a steady state in glomerular filtration before the levels of creatinine rise, and this, in most cases, may take several days.

Unfortunately, also other parameters such as the amount of diuresis, the urinary levels of certain high-molecular-weight proteins (e.g., beta-2-microglobulin, fatty acid-binding protein [FABP], etc.), and the calculation of the excreted sodium fraction or that of the cylinders in the sediment are not very sensitive or specific for the early detection of AKI; hence, the need of finding new biomarkers able to overcome such limitations. To date, the ideal biomarker of AKI still does not exist. It should first be precocious, that is, detectable long before the positivization of the most common parameters of renal impairment (urine output, creatinine, etc.), sensitive, able of minimizing the possibility of false negatives, as specific as possible for AKI, easily available and analyzable, and of course inexpensive.

The surprising predictive properties of NGAL for the onset of AKI have been for the first time revealed by Mishra et al. (2005) in a famous study published in Lancet. The authors analyzed NGAL serum (sNGAL) and urinary (uNGAL) levels in a cohort of 71 children undergoing cardiopulmonary bypass (CPB) surgery, a procedure known to be burdened with high risk of postoperative AKI. The 20 subjects

who developed this complication showed a significant increase of NGAL already 2 h after the end of the surgical intervention (whereas creatinine increased only after 1–3 days), unlike the others whose NGAL levels remained almost unchanged throughout the entire length of hospital stay. Multivariate analyses confirmed the validity of NGAL assay in serum and (especially) in the urine as an independent predictor of AKI, and the use of ROC curves showed an excellent diagnostic power of uNGAL considering a cutoff of 50 ng/ml (specificity 98 %, sensitivity 100 %). Two subsequent studies, extended to more numerous cohorts of pediatric patients subjected to the same type of surgery (CPB), have clearly validated these preliminary results. In the first case, Dent et al. (2007) confirmed the predictive power of sNGAL through its measurement in 120 subjects, of which 45 (37 %) developed AKI. In these patients, an increase of sNGAL levels up to three times was observed already 2 h after surgery; in multivariate analysis, sNGAL value measured after 2 h also turned out to be the most significant independent predictor of AKI (beta = 0.004, $p < 0.0001$), furthermore showing a great diagnostic power with an AUC of 0.96, a sensitivity of 0.84, and a specificity of 0.94 for a cutoff of 150 ng/ml. Similar results were reported by Bennett et al. (2008), this time taking into consideration uNGAL measurement in 196 children undergoing CPB. In those who developed AKI (51 % of patients), uNGAL levels increased by approximately 15 times 4 h after surgery and even up to 25 times after 6 h, while creatinine began to increase only in the second day. The ROC analysis showed the excellent diagnostic power of uNGAL at 2 h in predicting the future onset of AKI (cutoff 100 ng/ml, AUC 0.95, sensitivity 82 %, specificity 90 %). This parameter also appeared to be directly and independently correlated with the severity and duration of AKI, length of hospitalization, need for replacement therapy, and even mortality.

Later studies have attempted to extend these findings also to populations of adult patients; in an interesting work, Haase-Fielitz et al. (2009a) have indeed confirmed that NGAL plasma levels are independent predictors of AKI development (established as a 50 % increase in serum creatinine), as well as of a composite endpoint represented by the need of dialysis treatment and the inhospital mortality, in patients undergoing major heart surgery. The predictive ability of NGAL also increased with the severity of AKI and was directly related to the stage of renal function impairment according to the RIFLE or AKIN criteria, the two main scales of AKI severity used by the intensive care nephrologists (Haase-Fielitz et al. 2009b).

Wagener et al. (2008) have instead analyzed uNGAL levels in a much larger cohort of patients (n. 426) undergoing major heart surgery. These values increased immediately after the procedure, remained significantly elevated up to 24 h after the operation, and were also related to the duration of the intervention and that of aortic clamping. uNGAL showed however a poor diagnostic power at each measurement time (3, 18, and 24 h) in predicting the future onset of AKI (AUC from 0.573 to 0.584), defined by an increase in serum creatinine >50 % or >0.3 mg/dl within 48 h. On the contrary, the diagnostic performance of uNGAL significantly improved if the measurement of this parameter was supplemented by that of other similar biomarkers, such as kidney injury molecule-1 (KIM-1) and *N*-acetyl-β-D-glucosaminidase (NAG) (AUC 0.780) (Han et al. 2009).

The interesting NGAL predictive abilities toward the onset of AKI have recently led to the launch of specific automated "point-of-care" systems able to provide a rapid quantification of NGAL in biological samples obtained from patients (for the most critical) at high risk of developing AKI. Similarly to what already happens long since for troponin (as a marker of myocardial infarction) and, more recently, for BNP (whose levels seem able to discriminate a cardiogenic dyspnea from those of other causes), the purpose of these tools would be to provide the intensivist taking charge of a patient at risk of AKI with a single datum in real time that indicates the presence or absence of such a complication in an immediate way. The fundamental premise for this is of course to have two important validated parameters: the best timing of measurement and the exact threshold value which discriminates the impending AKI from its sure absence. If for the first parameter "pathophysiological" prerequisites exist that address to NGAL measurement from 2 to 6 h after the alleged traumatic event for the kidney (there is a strong clinical and experimental agreement on the peak of release by the tubular cells), probably it has been instead tried to establish the elusive discriminating threshold value with too much "haste." In the study of Haase-Fielitz et al. (2009a), for instance, a value of 150 ng/ml (sNGAL) is proposed as a cutoff capable of distinguishing, through a single measurement, the imminent onset of AKI in the patient just arrived in the intensive care unit. This would allow the "preventive" implementation of therapeutic supports (vasotonic drugs, volume expansion, or even dialysis) able to avoid the complication in question.

Since the critical patient often presents different conditions or comorbidities (infections, malignancies, anemia, chronic inflammations, atherosclerosis, diabetes, vascular disease, chronic kidney diseases, or recent treatment with steroids) that are causes per se of increased NGAL levels well beyond the recently proposed threshold values, the idea to report the presence of looming AKI to a single measurement (e.g., <150 ng/ml $=$ no AKI, >150 ng/ml $=$ ongoing AKI) has been recently criticized, as potentially affected by too many conditions (Bolignano et al. 2009a). Conversely, the evaluation of a "delta" value (i.e., the analysis of the pre- and postoperative change in NGAL levels) might be most useful and reliable: the presence of a documentable sharp increase of NGAL values would in fact reflect more realistically an underway kidney suffering.

Anyway, the predictive abilities of NGAL toward the onset of AKI have recently been extended also to other types of risk patients with similar interesting results. Makris et al. (2009) analyzed uNGAL levels in 31 patients with multiple trauma, respectively, at the admission to the intensive care unit, 24 and 48 h after. uNGAL had a great diagnostic power (AUC 0.977) in predicting the future onset of AKI defined according to the RIFLE criteria, with an optimal cutoff of 25 ng/ml (sensitivity 0.91, specificity 0.95). In agreement with the previously mentioned concerns, the authors excluded from the study patients with preexisting heart or chronic kidney disease.

Contrast-induced nephropathy (CIN) is another important cause of AKI. The identification of predisposing conditions such as the use of iodinated hyperosmolar contrast media, the presence of diabetes or preexisting renal impairment, hypovolemia, hematological disorders, and concomitant nephrotoxic drugs intake proved not to be sufficient alone to drastically reduce the incidence of this complication. Moreover, to date there is not even a consensus on possible preventive and/or

precocious therapeutic strategies (volume expansion, intravenous, and/or oral bicarbonates, *N*-acetylcysteine, etc.) to be put in place to counteract the evolution of this threat, with the result that the risk of CIN still remains, especially in the renal patient, one of the most serious contraindications to the execution of important diagnostic-therapeutic procedures such as coronary arteriography and computed tomography (CT).

It has been shown how, in a cohort of mostly diabetic patients undergoing percutaneous coronary intervention (PCI), sNGAL and uNGAL levels increased, respectively, from 2 to 4 h and from 4 to 12 h after the end of this procedure (Bachorzewska-Gajewska et al. 2006). Despite not considering the relationship between NGAL values and outcome (CIN or non-CIN), nevertheless this study has the merit to have demonstrated for the first time that the contrastographic procedure induces an important alteration in NGAL levels, suggesting that this reflects an early renal suffering still not detectable by the increase in serum creatinine.

Similar observations were afterward reported by another work that has taken in consideration not only the urinary values of NGAL but also those of IL-18, another potential biomarker of AKI (Ling et al. 2008). Twenty-four hours after the execution of a coronary angiography, the levels of both biomarkers were significantly increased from baseline in subjects who would have later developed CIN and then AKI. NGAL also showed a diagnostic ability toward the outcome significantly better than that of serum creatinine, and IL-18 resulted even independently associated with late cardiovascular events in the course of a follow-up of 17 months. In another interesting work, Hirsch et al. (2007) enrolled 91 pediatric patients with major heart malformations requiring elective cardiac catheterization and angiography with administration of iodinated contrast medium (ioversol). In the 11 subjects (12 %) who developed CIN, defined as an increase >50 % in serum creatinine from baseline, sNGAL and uNGAL levels increased significantly already 2 h after the end of the procedure, whereas the increase of creatinine appeared only after 24 h. The values of uNGAL and sNGAL at 2 h revealed to be independent predictors of CIN with an optimal cutoff of 100 ng/ml and an AUC of 0.92 (sensitivity 73 %, specificity 100 %) and 0.91 (sensitivity 73 %, specificity 100 %), respectively.

More recently, it has been demonstrated that NGAL revealed CIN within 8 h after iodinated contrast material injection and that therapy with *N*-acetylcysteine, sodium bicarbonate, or physiologic saline immediately before and after administration of the contrast agent did not influence NGAL values (Lacquaniti et al. 2013).

NGAL and Autosomal-Dominant Polycystic Kidney Disease (ADPKD)

The autosomal-dominant polycystic kidney disease (ADPKD) is a congenital disorder characterized by an alteration of the renal parenchyma which is progressively replaced by multiple bilateral cysts of tubular origin, with resulting disruption of the normal corticomedullary architecture (Santoro et al. 2015). Despite the genetic bases

of this condition are well known (mutation of the genes PKD1, PKD2, PKD3 with formation of abnormal polycystins), it is still unclear why some patients present a greater tendency to develop complications (arterial hypertension, polyglobulia, etc.) and a more rapid progression toward terminal uremia, whereas others, the cystic development being equal, maintain a perfectly preserved renal function over time.

Patients with ADPKD showed noticeably increased NGAL values compared to healthy subjects (Bolignano et al. 2007). These levels were also closely related to the amount of residual renal function, showing a direct correlation with the values of creatinine and an inverse relationship with those of estimated GFR (sNGAL/GFR, $R = -0.81$, $p = 0.006$; sNGAL/creatinine, $R = 0.90$, $p = 0.007$; uNGAL/GFR, $R = -0.49$, $p = 0.05$; uNGAL/creatinine, $R = 0.84$, $p = 0.001$). Unexpectedly, if patients were categorized into two groups based on ultrasound criteria of phenotypic expression of the disease (number of cysts $<$ or >10; renal length $<$ or >16 cm), regardless of residual renal function subjects with larger kidneys and more significant cystic development showed NGAL levels statistically higher than the others, thus suggesting a possible involvement of NGAL in the pathophysiology of the adaptations to polycystic disease. It must be remembered that in physiological conditions, NGAL is an important differentiating and structuring factor for the renal tubular epithelium, since the suppression of its gene expression in the embryonic organ leads to the formation of cystic-looking poorly organized structures instead of mature tubules (Gwira et al. 2005).

Starting from this assumption, Wei F et al. (2008) provided, through an experimental model, interesting pathophysiological explanations to the clinical observations on patients with ADPKD; the addition of recombinant NGAL in cultures of $PKD1^{-/-}$ polycystic kidney cells proved in fact to be able to significantly reduce both the area and the volume of the cystic development, whereas, on the contrary, the silencing of NGAL gene expression through the use of specific short hairpin RNAs (shRNAs) markedly increased the absolute number of these cells and their proliferative index. All this would suggest that the significant increase in the biological levels of NGAL found in patients with ADPKD could represent the exasperation of a compensatory mechanism aimed at restricting cystic growth, assuming a possible, faraway therapeutic use of this protein in the context of a disease which to date knows only symptomatic treatments.

NGAL and Chronic Kidney Disease

The potential involvement of NGAL in the pathophysiology of chronic kidney diseases does not appear to be limited to ADPKD. In particular, the close parallelism between NGAL levels and the extent of renal impairment, observed for the first time in patients with polycystic kidney disease (Bolignano et al. 2007), is evident in an unequivocal manner in any patient with CKD, irrespective of the underlying renal disease. More specifically, serum and urine levels of NGAL were evaluated in 69 patients with varying degrees of CKD secondary to different etiologies such as glomerulonephritis, nephroangiosclerosis, and obstructive nephropathy (Bolignano

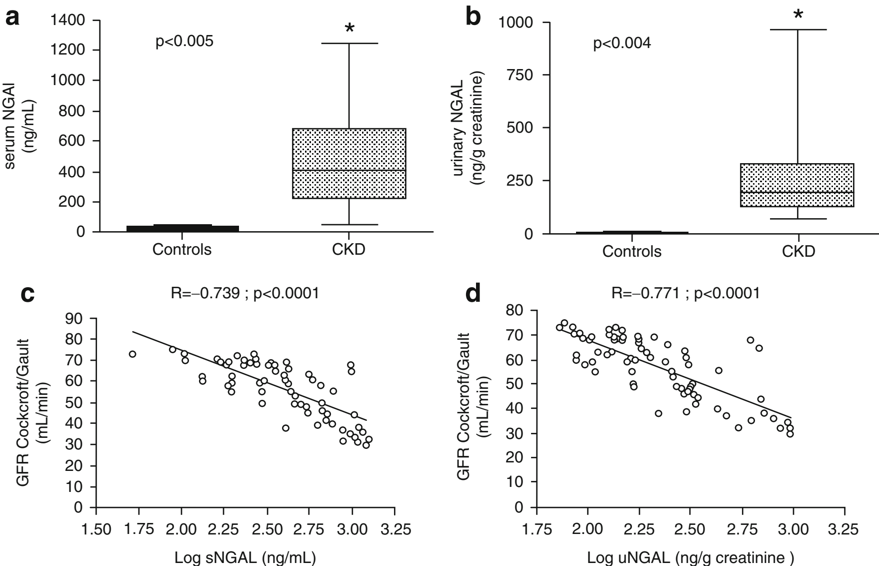

Fig. 2 Serum and urine levels of NGAL in patients with varying degrees of chronic kidney disease (*CKD*) secondary to different etiologies. Comparison of serum (**a**) and urinary (**b**) NGAL levels between controls and patients with CKD. Statistical correlations between glomerular filtration rate (*GFR*) and log-transformed sNGAL (**c**) and between GFR and log-transformed uNGAL (**d**) in CKD patients (Bolignano and Buemi 2009; with permission from Publisher Nuova Editoriale Bios)

et al. 2008b). These levels and the fractional excretion of NGAL (FeNGAL), calculated according to the formula uNGAL/sNGAL x serum creatinine/urine creatinine $\times 100$, were significantly increased compared with control subjects. Univariate correlations were also described between GFR values (Cockcroft-Gault formula/MDRD formula) and, respectively, those of sNGAL ($R = -0.739$, $p < 0.0001$/$R = -0.732$, $p < 0.0001$), uNGAL ($R = -0.771$, $p < 0.0001$/$R = -0.769$, $p < 0.0001$), and FeNGAL ($R = 0.452$, $p < 0.001$/$R = -0.450$, $p < 0.001$) as well as between the values of creatinine and those of sNGAL ($R = 0.445$, $p < 0.001$) and uNGAL ($R = 0.399$, $p < 0.001$). In addition, after multivariate adjustment, the correlations between GFR values and those of sNGAL ($= -0.645$, $p < 0.01$) and uNGAL ($= -0.688$, $p < 0.005$) remained significant, thus confirming to be independent (Fig. 2). In two other studies, the close relations between NGAL and degree of renal impairment were confirmed also in cohorts of patients much more homogeneous for etiology of CKD, e.g., membranous glomerulonephritis (Bolignano et al. 2008c) or diabetic nephropathy (Bolignano et al. 2009b): this further reinforces the idea that the relationship between NGAL and residual renal function is anything but random, representing on the contrary a constant that accompanies the progression of chronic renal failure. Considering the close correlations between NGAL and creatinine as well as those even more significant (and inverse) between NGAL and GFR, one might first assume that the increased levels of this protein are simply due

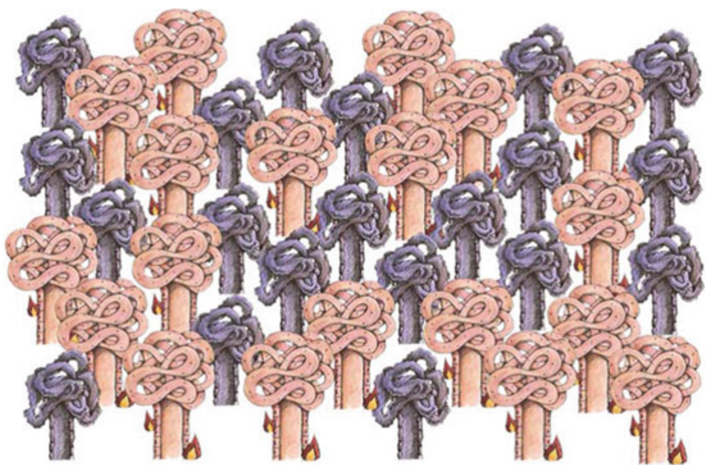

Fig. 3 Forest fire theory. According to this theory, the increased NGAL values during CKD, which is comparable to a forest fire, are the result of an active and sustained production by residual tubular cells still viable but "inflamed" by the surrounding renal disease, where instead the reduction in GFR and the consequent increase in serum creatinine are only the passive consequence of the functional loss of nephron units (and then tubules) already "burned" by the forest fire (Bolignano and Buemi 2009; with permission from Publisher Nuova Editoriale Bios)

to the reduction in the ability of renal clearance, thus representing nothing more than a mere surrogate of the function of this organ similarly to what happens for creatinine.

Mori and Nakao (2007) have instead provided an interesting alternative explanation on the meaning of the correlations between NGAL biological levels and renal function indices, developing the so-called theory of the burning forest (Forest Fire Theory; Fig. 3). According to this theory, the increase in NGAL values during CKD, comparable to a forest fire, is the result of an active and sustained production by residual tubular cells still viable but "inflamed" by the surrounding renal disease, where instead the reduction in GFR and the consequent increase in serum creatinine are only the passive consequence of the functional loss of nephron units (and then tubules) already "burned" by the forest fire. From this point of view, NGAL production by tubular cells (with a defensive meaning very similar to that described in conditions of AKI) would then represent a kind of "real-time" indicator of how much damage and active suffering are present within chronic renal impairment. Although it cannot ruled out that other sources (activated neutrophils, systemic inflammation, atherosclerosis, etc.) can contribute to the increase in NGAL levels in the course of chronic kidney diseases, there is further evidence supporting the central role of the renal tubular cells in the active production and disposal of blood and urinary NGAL. In a cohort of patients affected by childhood-onset systemic lupus erythematosus with renal involvement, Brunner et al. (2006) observed close correlations between NGAL levels and severity of the activity and chronicity scores of the disease evaluated on samples

of renal biopsies. These correlations proved to be also much stronger than those seen with any other renal and extrarenal index of disease severity. Ding et al. (2007) have instead evaluated NGAL tissue expression in a cohort of patients suffering from IgA nephropathy with varying degrees of biopsy severity (Lee classification), observing a very strong histological positivity, localized predominantly at the tubular level, in patients with high-grade kidney injury (Lee grade III). The amount of such tissue expression also strongly correlated with uNGAL levels, confirming the idea of an active production by suffering tubular cells.

As previously discussed (Bolignano et al. 2008b), CKD patients have, as well as an increase of sNGAL and uNGAL absolute values, also an elevation of FeNGAL compared to healthy subjects. Normally, the calculation of the fractional excretion is applied to sodium balance (FeNa) in subjects showing an acute diuresis contraction, in order to discriminate the renal or prerenal origin of the functional block of this organ. More in detail, a FeNa <1 % suggests a conserved ability of the kidney to reabsorb sodium aimed at preserving the hemodynamic stability threatened by various causes (cardiogenic or circulatory shock, hypovolemia, etc.): this orients toward the presence of a prerenal oliguria. Conversely, a high FeNa (>1 %) gives evidence of renal parenchymal damage with consequent active loss of this electrolyte through the tubular structures altered by different potential causes (ischemia, drugs, infections, etc.): the so-called renal or organic oliguria. By extending this formula to NGAL balance, it has been noted that not only patients with CKD present a FeNGAL significantly increased compared to healthy controls, but also this invariably results well above the value of 1 %, once again supporting the idea that the damaged kidney actively supports the increased biological levels of this protein.

As a further confirmation, even patients with diabetic nephropathy have increased values of FeNGAL compared to controls, but only those with severe renal functional impairment (macroalbuminuria and reduced GFR) show a FeNGAL >1 % (Bolignano et al. 2009b).

However, the relationships between NGAL and diabetic nephropathy go far beyond this observation, and this study designed specifically on patients with this condition has clearly demonstrated as the diagnostic potentialities (and with them, the pathophysiological significance) of NGAL in the field of chronic kidney diseases can take a particular interest precisely in those subjects who, while not having yet developed an overt CKD (then with GFR >90 ml/min/1.73 m^2, stage I according to the recent NKF-KDOQI guidelines), show however early signs of renal involvement (e.g. microalbuminuria or microproteinuria). In this regard, it was observed that in patients with type 2 diabetes mellitus treated with insulin, categorized according to the extent of albumin urinary excretion in normoalbuminuric (albuminuria/creatinuria ratio <30), microalbuminuric (ratio >30 but <300), and with overt diabetic nephropathy (ratio >300 and reduced GFR), NGAL levels increase in parallel to the extent of the glomerular damage resulting highest in subjects with reduced GFR and macroalbuminuria (Fig. 4). Very interestingly, patients with normoalbuminuria, so devoid of any clinical/laboratory sign of renal involvement

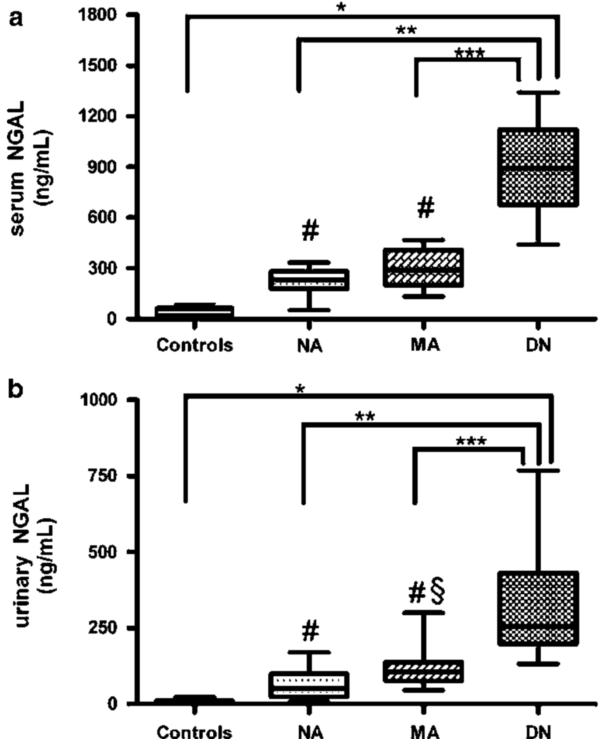

Fig. 4 In patients with type 2 diabetes mellitus, NGAL levels increase in parallel to the extent of the glomerular damage resulting highest in subjects with reduced GFR and macroalbuminuria. (**a**) sNGAL levels in healthy controls and diabetic patients with normoalbuminuria (*NA*), microalbuminuria (*MA*), and overt diabetic nephropathy (DN). #, $p < 0.01$ versus controls; *, $p < 0.001$ versus controls; **, $p < 0.01$ versus NA; ***, $p < 0.01$ versus MA. (**b**) uNGAL values in control subjects and diabetic patients with NA, MA, and DN. #, $p < 0.01$ versus controls; §, $p < 0.05$ versus NA; *, $p < 0.001$ versus controls; **, $p < 0.01$ versus NA; ***, $p < 0.05$ versus MA (Bolignano and Buemi 2009; with permission from Publisher Nuova Editoriale Bios)

from diabetes, had sNGAL and uNGAL levels already significantly increased compared to controls. NGAL also showed an excellent diagnostic ability in detecting the presence of diabetes among all subjects with normal albumin urinary excretion (controls + normoalbuminuric patients) with an AUC of 0.969 and a cutoff >88 ng/ml for sNGAL and an AUC of 0.910 and a cutoff >22 ng/ml for uNGAL. These unexpected and surprising findings indicate that NGAL may represent a biomarker of kidney (tubular) damage from diabetes even more precocious than microalbuminuria (glomerular), supporting those recent theories that sustain the existence of a phase of renal tubule suffering that temporally precedes the appearance of the best known glomerular changes responsible for microalbuminuria and proteinuria (Thomas et al. 2005).

Anyway, it is clear that even in the absence of a GFR reduction, and then of an overt renal failure, significant alterations of NGAL biological levels can already be

identified, although it is not yet possible to ascribe a precise pathophysiological significance to this phenomenon.

It is no coincidence that the most recent NKF-KDOQI US guidelines have revised the classification of CKD severity by including already in stage I those subjects with still preserved GFR (>90 ml/min) but with persistence of clinical/laboratory signs of CKD such as hematuria and especially persistent proteinuria.

Furthermore, it is known that the latter condition, regardless of the underlying disease (e.g., diabetes or primary and secondary glomerulonephritis, etc.), represents not only an expression of glomerular damage but also a cause per se of progression of nephropathy toward the establishment of an evident organ failure. The persistence of blood proteins in the tubular lumen (especially if proteinuria is in the nephrotic range, >3.5 g/24 h) is in fact harmful to the epithelial cells through activation of the complement cascade and subsequent immune-mediated damage. This leads to the appearance of tubular atrophy and interstitial fibrosis, irreversible lesions that mark the onset of a functional deficit of the nephron units and, with it, of the reduction of renal clearance ability (Abbate et al. 2006; Morita et al. 2000).

Patients with macroproteinuria due to membranous glomerulonephritis have high urinary NGAL excretion compared to healthy subjects even in the presence of normal GFR (Bolignano et al. 2008d), and, when the daily proteinuria becomes particularly severe (e.g., in the nephrotic range), uNGAL levels may increase over 500 times the norm, becoming also closely related to the values of proteinuria itself (Bolignano et al. 2008c, e).

The reasons of such an important increase, despite the absence of overt renal failure, are not as yet known; however, considering the renal turnover of NGAL in normal and pathological conditions, several hypotheses can be postulated (Fig. 5).

In the healthy adult kidney, NGAL is freely filtered through the glomerulus and physiologically almost completely reabsorbed in the proximal tubular tract (Helanova et al. 2014). This reabsorption is mediated by the coupling with the membrane transporter cubilin-megalin, particularly expressed on the surface of the cellular brush border. Afterward, NGAL is incorporated by endocytosis and removed from the tubular lumen, so that only small amounts (approximately 5 ng/ml) of this protein are found in the urine of healthy subjects (Cowland et al. 2003).

An initial hypothesis that would justify the high NGAL levels in the urine of proteinuric patients may be represented by an increased loss of circulating NGAL through the damaged glomerulus, as it happens with other plasma proteins; this concept would be partially supported by the observed correlations between NGAL levels and amount (g/24 h) of daily proteinuria as well as between sNGAL and uNGAL values (Bolignano et al. 2008c, d, e, 2009b).

Although plausible, this model does not exclude, however, that at least in part the same tubular cells may just as significantly contribute to the massive elevation of uNGAL: from this point of view, at least two mechanisms could be conceivable. Firstly, the aforementioned cubilin-megalin carrier, responsible for NGAL resorption, acts through a mechanism of nonspecific protein endocytosis that recognizes several other serum ligands such as albumin, beta2-microglobulin, and serum immunoglobulins. In confirmation of this, murine models of knockout mice for

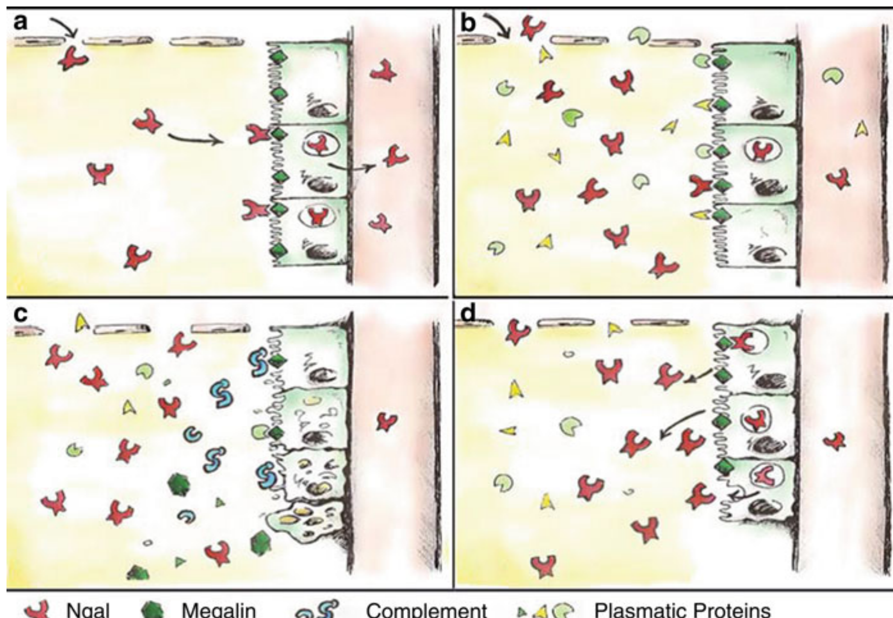

Fig. 5 Possible explanations for the increased uNGAL levels in patients with proteinuria. (**a**) Physiologically, sNGAL is filtered through the glomerulus and almost completely reabsorbed by the proximal tubule through the cubilin-megalin carrier. (**b**) In proteinuric diseases, an increased amount of NGAL could be lost through the damaged glomerulus similarly to what happens for other circulating proteins. Besides, the megalin carrier is rapidly saturated by the considerable protein tubular overload, further decreasing the ability to reabsorb NGAL. (**c**) Megalin function would be further compromised by the injured tubular brush border due to the complement cascade activation caused by persistent proteinuria. (**d**) Damaged tubular epithelial cells themselves may actively produce and release NGAL as a defense mechanism against oxidative stress and complement activation (Bolignano and Buemi 2009; with permission from Publisher Nuova Editoriale Bios)

megalin soon develop a condition of severe nonselective proteinuria, even in the absence of documentable glomerular histological lesions (Leheste et al. 1999). Under conditions of sustained proteinuria (e.g., nephrotic syndrome), this nonspecific carrier soon becomes saturated because of the massive tubular protein overload, causing further loss of plasma proteins that in part contributes to determine the extent of final proteinuria (Christensen and Gburek 2004). It is also to be considered that the main site of the damage induced by proteinuria through complement activation is precisely the brush border of the tubular cells, where the most of the cubilin-megalin complexes are located: this condition would further contribute to compromise NGAL endocytosis by its carrier. Ultimately, it cannot be excluded, however, that the same tubular cells, subjected to stress from the insult caused by the activity of complement factors, actively produce and release high amounts of NGAL with a defensive significance similar to what observed in experimental models of acute kidney damage. In accordance with this, previous studies have shown that the

tubular epithelium responds to a sustained load of plasma proteins through the release of multiple "stress proteins" including KIM-1, whose urinary levels accordingly rise in a dramatic way (van Timmeren et al. 2006).

In conclusion, waiting for specific studies doing more light on the topic, it is not to exclude that, in the end, both the passive loss and the active tubular production contribute in net measure to the increase of NGAL levels observed in the urine of proteinuric patients.

NGAL in Kidney Transplant Patients

Another interesting field of application of NGAL seems to be represented by the measurement of its biological levels in patients just subjected to kidney transplantation. Even in these patients, the risk of AKI is particularly fearful, not so much as the result of an acute rejection (which is now well prevented by the massive immunosuppressive therapy) as for a possible late organ functional recovery that leads to the need of a temporary hemodialysis support (the so-called delayed graft function, DGF); moreover, this condition is much more frequent if the transplanted kidney is from a cadaver than from a living donor, especially in relation to the occurrence of a more or less prolonged cold ischemia.

Patients waiting for a kidney, and then subjected to chronic hemodialysis treatment, have NGAL levels significantly increased compared to healthy controls (Bolignano et al. 2009c, d, 2010a; Kusaka et al. 2008). It has been shown that since the first day after transplantation, NGAL values tend to decrease progressively and in a dramatic way, preceding the recovery of normal diuresis and lowering of serum creatinine: this, however, does not happen in those subjects who will subsequently develop a DGF with the need to receive renal replacement therapy (Kusaka et al. 2008; Lebkowska et al. 2009).

In biopsy samples from just implanted organs, the levels of NGAL tissue expression are also significantly higher in cadaver than in living donor kidneys, correlating in the former with the time of cold ischemia, the postoperative creatinine peak (which occurs with a latency of several days), and the need for hemodialysis due to the appearance of a DGF (Mishra et al. 2006).

In another interesting work, Parikh et al. (2006) assessed urine NGAL and IL-18 levels in 53 kidney transplant patients immediately after surgery. In the ten patients who developed a DGF over the 2–4 following days, the basal urinary values of both biomarkers were significantly higher than those observed in patients with normal postoperative course, and both NGAL and IL-18 showed a considerable diagnostic power (AUC 0.9) in predicting the future occurrence of DGF. Nevertheless, in another study, uNGAL levels did not prove to be equally able to distinguish the histological presence of a subclinical tubular injury from a stable transplant with normal tubular histology (Schaub et al. 2007): this would slow, at least for the moment and waiting for more specific studies on the topic, the possibility of extending the measurement of NGAL in the diagnosis of chronic rejection with the same success.

Furthermore, in a very recent study (Buemi et al. 2014), plasma and urine NGAL levels were assessed in a cohort of kidney donors before organ explantation and in recipients before transplantation and then 6, 24, and 48 h after the surgical intervention, in order to evaluate the ability of this biomarker to predict DGF occurrence and posttransplant function restoration. The authors observed that recipient but not donor plasma NGAL values seem to predict DGF incidence and renal function recovery, however, too long for an interval to be able to compete with the markers of kidney function at present employed in clinical practice.

Potential Applications to Prognosis and Other Diseases or Conditions

The potential use of NGAL as a biomarker in clinical practice appears not to be limited to the early diagnosis of kidney diseases (Mishra et al. 2005; Bolignano et al. 2009b; Lacquaniti et al. 2012).

This protein has been also demonstrated to predict CKD progression (Bolignano et al. 2009e; Lin et al. 2015), in such a way becoming a reliable prognostic factor which might help nephrologists in the risk stratification and in the implementation of appropriate preventive therapeutic strategies with the ultimate purpose of improving the management of nephropathic patients.

Moreover, NGAL may represent a precocious marker of therapeutic response (Cernaro et al. 2011), as observed in patients suffering from severe proteinuria secondary to idiopathic membranous nephropathy and treated with a single high-dose bolus of intravenous immunoglobulin (Bolignano et al. 2008d) and in patients affected by Crohn's disease and receiving infliximab (Bolignano et al. 2010b). In both cases, a marked reduction was found in NGAL levels after the administration of the respective drugs. This suggests the possible application of NGAL measurement in monitoring the effectiveness of a particular treatment and predicting different clinical outcomes in the course of renal as well as extrarenal diseases characterized by an increase in the serum and urinary levels of this protein (Cernaro et al. 2011).

Indeed, recent studies propose for NGAL a role of "biomarker beyond the confines of nephrology" (Bolignano et al. 2010c). This molecule is involved, for instance, in carcinogenesis and progression of several human tumors (Bolignano et al. 2010d; Barresi et al. 2010; Lippi et al. 2014), in the regulation of red blood cell growth by inhibiting the maturation and differentiation of bone marrow erythroid precursors (Bolignano et al. 2010e), and in the pathophysiology of cardiovascular diseases (atherosclerosis, acute myocardial infarction, heart failure) (Bolignano et al. 2010c).

Although the oncological and cardiovascular settings constitute two promising horizons of application, to date, nephrology represents the field with the most imminent opportunities of NGAL transposition in daily clinical practice. In particular, the existence of a close involvement of this protein in the maturation of the embryonic kidney, as well as in defense mechanisms against acute and chronic stress in the adult kidney, has led to the real possibility in the next future of using measurement of NGAL biological levels for previously unimaginable purposes: evaluation of AKI

risk in critically ill patients with the chance of carrying out early therapeutic interventions that radically alter the incidence of adverse outcomes, stratification of the risk of progression to end-stage renal disease (ESRD) in CKD patients, monitoring of renal and systemic response to different therapeutic regimens, evaluation of iron balance, and dialysis adequacy in uremic patients on hemodialysis.

As previously described, in patients with renal disease, uNGAL levels have increasingly gained importance in addition to serum values. This has raised the problem of establishing the origin, renal or systemic, of NGAL dosed in the urine. The issue can be solved by assessing FeNGAL, similarly to what is done for sodium in order to determine the prerenal or organic etiology of AKI.

Moreover, a distinction have been made among the monomeric form of NGAL secreted by injured renal tubular epithelial cells, the homodimeric form released by activated neutrophils, and the heterodimeric form that is specific for tubular cells but seems to be produced in very low amounts even during AKI (Cai et al. 2010).

The currently available commercial assays and point-of-care devices probably measure a mixture of different forms of NGAL, having a limited ability to discriminate among them. This compromises NGAL specificity for AKI, especially in patients with systemic inflammation and several comorbidities. Such considerations have recently led some authors to express concerns about the diagnostic and clinical value of this biomarker (Mårtensson and Bellomo 2014).

In conclusion, several studies have shown the excellent ability of urinary and serum NGAL levels to predict AKI onset or CKD progression in selected populations of patients (Ronco et al. 2014). Nevertheless, the extension of these experimental results to clinical practice requires assays able to specifically measure the different forms of NGAL as well as larger trials which better define the normal range of NGAL values and the correct interpretation of their alterations, in order to confirm NGAL as the troponin of the kidney (Devarajan 2010) according to the hypothesis that has been postulated from the beginning.

Summary Points

- Neutrophil gelatinase-associated lipocalin (NGAL) is a small 25-kD peptide belonging to the lipocalin superfamily.
- Numerous experimental and clinical data support a role for NGAL as a biomarker in nephrology.
- NGAL predicts the onset of acute kidney injury (AKI) better than serum creatinine and urine output.
- Patients with autosomal-dominant polycystic kidney disease (ADPKD) show markedly increased NGAL values compared to healthy subjects.
- In chronic kidney disease (CKD) patients, NGAL closely correlates with the extent of renal impairment irrespective of the underlying kidney disease and predicts CKD progression.
- Since the first day after transplantation, NGAL values tend to decrease progressively, preceding the recovery of normal urine output and lowering of serum

creatinine; this does not happen in those patients who will subsequently develop a delayed graft function (DGF) with the need to receive renal replacement therapy.
- NGAL may represent a precocious marker of therapeutic response in different clinical situations.
- NGAL exists in three forms: the monomeric form secreted by injured renal tubular epithelial cells, the homodimeric form released by activated neutrophils, and the heterodimeric form that is specific for tubular cells but seems to be produced in very low amounts even during AKI.
- The currently available commercial assays and point-of-care systems probably measure a mixture of different forms of NGAL.
- The extension of the experimental results to clinical practice requires assays able to specifically measure the different forms of NGAL as well as larger trials which better define the normal range of NGAL values and the correct interpretation of their alterations.

References

Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? J Am Soc Nephrol. 2006;17(11):2974–84.

Bachorzewska-Gajewska H, Malyszko J, Sitniewska E, Malyszko JS, Dobrzycki S. Neutrophil-gelatinase-associated lipocalin and renal function after percutaneous coronary interventions. Am J Nephrol. 2006;26(3):287–92.

Barresi V, Ieni A, Bolignano D, Magno C, Buemi M, Barresi G. Neutrophil gelatinase-associated lipocalin immunoexpression in renal tumors: correlation with histotype and histological grade. Oncol Rep. 2010;24(2):305–10.

Bennett M, Dent CL, Ma Q, et al. Urine NGAL predicts severity of acute kidney injury after cardiac surgery: a prospective study. Clin J Am Soc Nephrol. 2008;3(3):665–73.

Bolignano D, Buemi M. Neutrophil Gelatinase-Associated Lipocalin (NGAL): un biomarker oltre i confini della nefrologia. Castrolibero: Nuova Editoriale Bios; 2009.

Bolignano D, Coppolino G, Campo S, et al. Neutrophil gelatinase-associated lipocalin in patients with autosomal-dominant polycystic kidney disease. Am J Nephrol. 2007;27(4):373–8.

Bolignano D, Donato V, Coppolino G, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a marker of kidney damage. Am J Kidney Dis. 2008a;52(3):595–605.

Bolignano D, Lacquaniti A, Coppolino G, Campo S, Arena A, Buemi M. Neutrophil gelatinase-associated lipocalin reflects the severity of renal impairment in subjects affected by chronic kidney disease. Kidney Blood Press Res. 2008b;31:255–8.

Bolignano D, Coppolino G, Lacquaniti A, Nicocia G, Buemi M. Pathological and prognostic value of Urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) in macroproteinuric patients with worsening renal function. Kidney Blood Press Res. 2008c;31:274–9.

Bolignano D, Coppolino G, Aloisi C, Romeo A, Nicocia G, Buemi M. Effect of a single intravenous immunoglobulin infusion on neutrophil gelatinase-associated lipocalin levels in proteinuric patients with normal renal function. J Investig Med. 2008d;56(8):997–1003.

Bolignano D, Coppolino G, Campo S, et al. Urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) is associated with severity of renal disease in proteinuric patients. Nephrol Dial Transplant. 2008e;23(1):414–6.

Bolignano D, Coppolino G, Lacquaniti A, Buemi M. Neutrophil gelatinase-associated lipocalin in the intensive care unit: time to look beyond a single, threshold-based measurement? Crit Care Med. 2009a;37(10):2864. author reply 2864–2865.

Bolignano D, Lacquaniti A, Coppolino G, et al. Neutrophil gelatinase-associated lipocalin as an early biomarker of nephropathy in diabetic patients. Kidney Blood Press Res. 2009b;32(2):91–8.

Bolignano D, Coppolino G, Lombardi L, Buemi M. NGAL: a new missing link between inflammation and uremic anemia? Ren Fail. 2009c;31(7):622–3.

Bolignano D, Coppolino G, Romeo A, et al. Neutrophil gelatinase-associated lipocalin (NGAL) reflects iron status in hemodialysis patients. Nephrol Dial Transplant. 2009d;24(11):3398–403.

Bolignano D, Lacquaniti A, Coppolino G, et al. Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. Clin J Am Soc Nephrol. 2009e;4(2):337–44.

Bolignano D, Coppolino G, Romeo A, Lacquaniti A, Buemi A. Neutrophil gelatinase- associated lipocalin (NGAL) levels in chronic hemodialysis patients. Nephrology (Carlton). 2010a;15(1):23–6.

Bolignano D, Della Torre A, Lacquaniti A, Costantino G, Fries W, Buemi M. Neutrophil gelatinase-associated lipocalin levels in patients with crohn disease undergoing treatment with infliximab. J Investig Med. 2010b;58(3):569–71.

Bolignano D, Coppolino G, Lacquaniti A, Buemi M. From kidney to cardiovascular diseases: NGAL as a biomarker beyond the confines of nephrology. Eur J Clin Invest. 2010c;40(3):273–6.

Bolignano D, Donato V, Lacquaniti A, et al. Neutrophil gelatinase-associated lipocalin (NGAL) in human neoplasias: a new protein enters the scene. Cancer Lett. 2010d;288(1):10–6.

Bolignano D, Coppolino G, Donato V, Lacquaniti A, Bono C, Buemi M. Neutrophil gelatinase-associated lipocalin (NGAL): a new piece of the anemia puzzle? Med Sci Monit. 2010e;16(6):RA131–5.

Brunner HI, Mueller M, Rutherford C, et al. Urinary neutrophil gelatinase-associated lipocalin as a biomarker of nephritis in childhood-onset systemic lupus erythematosus. Arthritis Rheum. 2006;54(8):2577–84.

Buemi A, Musuamba F, Frederic S, et al. Is plasma and urine neutrophil gelatinase-associated lipocalin (NGAL) determination in donors and recipients predictive of renal function after kidney transplantation? Clin Biochem. 2014;47(15):68–72.

Cai L, Rubin J, Han W, Venge P, Xu S. The origin of multiple molecular forms in urine of HNL/NGAL. Clin J Am Soc Nephrol. 2010;5(12):2229–35.

Cernaro V, Bolignano D, Donato V, et al. NGAL is a precocious marker of therapeutic response. Curr Pharm Des. 2011;17(8):844–9.

Christensen EI, Gburek J. Protein reabsorption in renal proximal tubule-function and dysfunction in kidney pathophysiology. Pediatr Nephrol. 2004;19(7):714–21.

Cowland JB, Borregaard N. Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics. 1997;45(1):17–23.

Cowland JB, Sørensen OE, Sehested M, Borregaard N. Neutrophil gelatinase-associated lipocalin is up-regulated in human epithelial cells by IL-1 beta, but not by TNF-alpha. J Immunol. 2003;171(12):6630–9.

Dent CL, Ma Q, Dastrala S, et al. Plasma neutrophil gelatinase-associated lipocalin predicts acute kidney injury, morbidity and mortality after pediatric cardiac surgery: a prospective uncontrolled cohort study. Crit Care. 2007;11(6):R127.

Devarajan P. Review: neutrophil gelatinase-associated lipocalin: a troponin-like biomarker for human acute kidney injury. Nephrology (Carlton). 2010;15(4):419–28.

Devireddy LR, Gazin C, Zhu X, Green MR. A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. Cell. 2005;123(7):1293–305.

Ding H, He Y, Li K, et al. Urinary neutrophil gelatinase-associated lipocalin (NGAL) is an early biomarker for renal tubulointerstitial injury in IgA nephropathy. Clin Immunol. 2007;123(2):227–34.

Flower DR. The lipocalin protein family: structure and function. Biochem J. 1996;318(Pt 1):1–14.

Goetz DH, Willie ST, Armen RS, Bratt T, Borregaard N, Strong RK. Ligand preference inferred from the structure of neutrophil gelatinase associated lipocalin. Biochemistry. 2000;39(8):1935–41.

Gwira JA, Wei F, Ishibe S, Ueland JM, Barasch J, Cantley LG. Expression of neutrophil gelatinase-associated lipocalin regulates epithelial morphogenesis in vitro. J Biol Chem. 2005;280 (9):7875–82.

Haase-Fielitz A, Bellomo R, Devarajan P, et al. Novel and conventional serum biomarkers predicting acute kidney injury in adult cardiac surgery – a prospective cohort study. Crit Care Med. 2009a;37(2):553–60.

Haase-Fielitz A, Bellomo R, Devarajan P, et al. The predictive performance of plasma neutrophil gelatinase-associated lipocalin (NGAL) increases with grade of acute kidney injury. Nephrol Dial Transplant. 2009b;24(11):3349–54.

Han WK, Wagener G, Zhu Y, Wang S, Lee HT. Urinary biomarkers in the early detection of acute kidney injury after cardiac surgery. Clin J Am Soc Nephrol. 2009;4(5):873–82.

Helanova K, Spinar J, Parenica J. Diagnostic and prognostic utility of Neutrophil Gelatinase-Associated Lipocalin (NGAL) in patients with cardiovascular diseases – review. Kidney Blood Press Res. 2014;39(6):623–9.

Hemdahl AL, Gabrielsen A, Zhu C, et al. Expression of neutrophil gelatinase-associated lipocalin in atherosclerosis and myocardial infarction. Arterioscler Thromb Vasc Biol. 2006;26(1):136–42.

Hirsch R, Dent C, Pfriem H, et al. NGAL is an early predictive biomarker of contrast-induced nephropathy in children. Pediatr Nephrol. 2007;22(12):2089–95.

Hraba-Renevey S, Türler H, Kress M, Salomon C, Weil R. SV40-induced expression of mouse gene 24p3 involves a post-transcriptional mechanism. Oncogene. 1989;4(5):601–8.

Hvidberg V, Jacobsen C, Strong RK, Cowland JB, Moestrup SK, Borregaard N. The endocytic receptor megalin binds the iron transporting neutrophil-gelatinase-associated lipocalin with high affinity and mediates its cellular uptake. FEBS Lett. 2005;579(3):773–7.

Kjeldsen L, Johnsen AH, Sengeløv H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem. 1993;268 (14):10425–32.

Kjeldsen L, Cowland JB, Borregaard N. Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. Biochim Biophys Acta. 2000;1482(1–2):272–83.

Kusaka M, Kuroyanagi Y, Mori T, et al. Serum neutrophil gelatinase-associated lipocalin as a predictor of organ recovery from delayed graft function after kidney transplantation from donors after cardiac death. Cell Transplant. 2008;17(1–2):129–34.

Lacquaniti A, Chirico V, Donato V, et al. NGAL as an early biomarker of kidney disease in Joubert syndrome: three brothers compared. Ren Fail. 2012;34(4):495–8.

Lacquaniti A, Buemi F, Lupica R, et al. Can neutrophil gelatinase-associated lipocalin help depict early contrast material-induced nephropathy? Radiology. 2013;267(1):86–93.

Lebkowska U, Malyszko J, Lebkowska A, et al. Neutrophil gelatinase-associated lipocalin and cystatin C could predict renal outcome in patients undergoing kidney allograft transplantation: a prospective study. Transplant Proc. 2009;41(1):154–7.

Leheste JR, Rolinski B, Vorum H, et al. Megalin knockout mice as an animal model of low molecular weight proteinuria. Am J Pathol. 1999;155(4):1361–70.

Lin HY, Hwang DY, Lee SC, et al. Urinary neutrophil gelatinase-associated lipocalin and clinical outcomes in chronic kidney disease patients. Clin Chem Lab Med. 2015;53(1):73–83.

Ling W, Zhaohui N, Ben H, et al. Urinary IL-18 and NGAL as early predictive biomarkers in contrast-induced nephropathy after coronary angiography. Nephron Clin Pract. 2008;108(3): c176–81.

Lippi G, Meschi T, Nouvenne A, Mattiuzzi C, Borghi L. Neutrophil gelatinase-associated lipocalin in cancer. Adv Clin Chem. 2014;64:179–219.

Makris K, Markou N, Evodia E, et al. Urinary neutrophil gelatinase-associated lipocalin (NGAL) as an early marker of acute kidney injury in critically ill multiple trauma patients. Clin Chem Lab Med. 2009;47(1):79–82.

Malyszko J, Tesar V, Macdougall IC. Neutrophil gelatinase-associated lipocalin and hepcidin: what do they have in common and is there a potential interaction? Kidney Blood Press Res. 2010;33 (2):157–65.

Mårtensson J, Bellomo R. The rise and fall of NGAL in acute kidney injury. Blood Purif. 2014;37(4):304–10.

Meheus LA, Fransen LM, Raymackers JG, et al. Identification by microsequencing of lipopolysaccharide-induced proteins secreted by mouse macrophages. J Immunol. 1993;151(3):1535–47.

Mishra J, Dent C, Tarabishi R, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. Lancet. 2005;365(9466):1231–8.

Mishra J, Ma Q, Kelly C, et al. Kidney NGAL is a novel early marker of acute injury following transplantation. Pediatr Nephrol. 2006;21(6):856–63.

Mori K, Nakao K. Neutrophil gelatinase-associated lipocalin as the real-time indicator of active kidney damage. Kidney Int. 2007;71(10):967–70.

Morita Y, Ikeguchi H, Nakamura J, Hotta N, Yuzawa Y, Matsuo S. Complement activation products in the urine from proteinuric patients. J Am Soc Nephrol. 2000;11(4):700–7.

Parikh CR, Jani A, Mishra J, et al. Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. Am J Transplant. 2006;6(7):1639–45.

Playford RJ, Belo A, Poulsom R, et al. Effects of mouse and human lipocalin homologues 24p3/lcn2 and neutrophil gelatinase-associated lipocalin on gastrointestinal mucosal integrity and repair. Gastroenterology. 2006;131(3):809–17.

Ronco C, Legrand M, Goldstein SL, et al. Neutrophil gelatinase-associated lipocalin: ready for routine clinical use? An international perspective. Blood Purif. 2014;37(4):271–85.

Santoro D, Pellicanò V, Visconti L, et al. An overview of experimental and early investigational therapies for the treatment of polycystic kidney disease. Expert Opin Investig Drugs. 2015;24:1199–218. Jun 30:1–20.

Schaub S, Mayr M, Hönger G, et al. Detection of subclinical tubular injury after renal transplantation: comparison of urine protein analysis with allograft histopathology. Transplantation. 2007;84(1):104–12.

Schmidt-Ott KM, Mori K, Li JY, et al. Dual action of neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol. 2007;18(2):407–13.

Thomas MC, Burns WC, Cooper ME. Tubular changes in early diabetic nephropathy. Adv Chron Kidney Dis. 2005;12(2):177–86.

van Timmeren MM, Bakker SJ, Vaidya VS, et al. Tubular kidney injury molecule-1 in protein-overload nephropathy. Am J Physiol Renal Physiol. 2006;291(2):F456–64.

Wagener G, Gubitosa G, Wang S, Borregaard N, Kim M, Lee HT. Urinary neutrophil gelatinase-associated lipocalin and acute kidney injury after cardiac surgery. Am J Kidney Dis. 2008;52(3):425–33.

Wei F, Karihaloo A, Yu Z, et al. Neutrophil gelatinase-associated lipocalin suppresses cyst growth by Pkd1 null cells in vitro and in vivo. Kidney Int. 2008;74(10):1310–8.

Yan L, Borregaard N, Kjeldsen L, Moses MA. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. J Biol Chem. 2001;276(40):37258–65.

Chemokines as Potential Markers in Pediatric Renal Diseases

11

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Abstract

Recently, biomarkers have become a focus of clinical research as potentially useful diagnostic and prognostic tools in many diseases. Among several putative biomarkers, chemokines emerge as promising molecules since they play relevant roles in the pathophysiology of chronic kidney disease. In pediatric patients, glomerular diseases and congenital anomalies of the kidney and urinary tract are the commonest cause of chronic kidney disease. The evaluation of these inflammatory mediators might help the management of diverse renal diseases in children and the detection of patients at high risk to develop chronic kidney disease. The aim of this chapter is to revise general aspects of chemokines and the potential link between chemokines and the most common causes of pediatric chronic kidney disease. The literature revision showed that the chemokines more commonly associated with pediatric renal diseases were CCL2/MCP-1 and CXCL8/IL-8. In glomerular diseases, high urinary levels of CCL2/MCP-1 have been associated to FSGS, lupus nephritis and IgA nephropathy. On the other hand, urinary levels of CXCL8/IL-8 positively correlated with proteinuria in pediatric patients with primary nephrotic syndrome, suggesting a role in glomerular permeability changes. With reference to congenital anomalies of the kidney and urinary tract, the chemokine CCL2/MCP-1 has been associated with urinary tract obstruction, whereas high urinary levels of CXCL8/IL-8 were found in patients with vesicoureteral reflux and correlated with renal scarring and renal function deterioration.

Keywords

Chemokines • Biomarker • Glomerular diseases • Congenital anomalies of the kidney and urinary tract • Chronic kidney disease

Abbreviations

CAKUT	Congenital anomalies of the kidney and urinary tract
CCL1/TCA-3	T cell activation-3
CCL11/eotaxin	Eosinophil chemotactic protein
CCL2/MCP-1	Monocyte chemotactic protein-1
CCL3/MIP-1a	Macrophage inflammatory protein-1 alpha
CCL3/MIP-1α	Macrophage inflammatory protein 1 alfa
CCL4/MIP-1b	Macrophage inflammatory protein-1 beta
CCL5/RANTES	Regulated on activation, normal T Expressed and Secreted
CKD	Chronic kidney disease
CX3CL1/fractalkine	Chemokine (C-X3-C motif) ligand 1
CXCL10/IP-10	γ-interferon-inducible protein
CXCL13/BLC	Serum B lymphocyte chemoattractant
CXCL2/MIP-2	Macrophage inflammatory protein-2
CXCL8/IL8	Interleukin 8
FSGS	Focal segmental glomerulosclerosis
GFR	Glomerular filtration rate
INS	Idiopathic nephrotic syndrome

MCNS	Minimal change nephrotic syndrome
NF-κB	Nuclear factor-kappa B
PBMC	Peripheral blood mononuclear cell
SLE	Systemic lupus erythematosus
UPJO	Ureteropelvic junction obstruction
VUR	Vesicoureteral reflux

Key Facts of Chemokines in Pediatric Renal Disease

- Urinary levels of CXCL8/IL-8 positively correlated with 24-h proteinuria in pediatric patients with idiopathic nephrotic syndrome.
- Urinary levels of CXCL8/IL-8 seem to be a marker of disease activity in pediatric patients with lupus nephritis.
- Urinary levels of CCL2/MCP-1 were increased in pediatric patients with focal segmental glomerulosclerosis and correlated with plasma cholesterol and triglycerides.
- Urinary levels of CCL2/MCP-1 from voided urine before and after surgery and from the affected pelvis of pediatric patients with ureteropelvic junction obstruction were higher than non-surgically managed cases and healthy controls.
- Urinary measurement of CXCL8/IL-8 seems to be useful for the diagnostic of vesicoureteral reflux at a cutt-off concentration of 5 pg/μmol with sensitivity of 88 % and specificity of 69 %.
- Urinary levels of CXCL8/IL-8 negatively correlated with glomerular filtration rate in pediatric patients with chronic kidney disease due to congenital anomalies of the kidney and urinary tract.

Definitions

Chemokine A large family of low molecular-weight cytokines whose main action is the recruitment of leukocyte subsets under homeostatic and pathological conditions.

Chronic kidney disease Progressive deterioration of renal function with glomerular filtration reduced for more than 3 months.

Congenital anomalies of the kidney and urinary tract This term comprises a wide *spectrum* of malformations that occur at the level of the kidney, collecting system, bladder, or urethra.

Focal segmental glomerulosclerosis This is a possible histopathology profile observed in idiopathic nephrotic syndrome, which is characterized by partial fibrosis of some of the glomerulus in renal biopsy.

Idiopathic nephrotic syndrome Primary glomerular disease characterized by proteinuria, edema, low plasma albumin levels and dyslipidemia.

IgA nephropathy Glomerular disease diagnosed by the predominance of Immunoglobulin A deposits in the glomerular mesangium.

Minimal change nephrotic syndrome This is the nephrotic syndrome due to an abnormal fusion of pedicles from podocytes.

Systemic lupus erythematosus This is a multisystemic autoimmune disease affecting predominantly women and two thirds of cases occur in the first two decades of life.

Ureteropelvic junction obstruction This is the most common cause of severe hydronephrosis in children due to a narrowing and/or obstruction of variable degrees at the ureteropelvic junction.

Vesicoureteral reflux This is a congenital anomaly of the urinary tract due to an anomalous implantation of the ureters into the bladder wall, leading to the return of urine to renal pelvis.

Chemokines: General Concepts

Chemokines constitute a large family of low molecular-weight cytokines whose main action is the recruitment of leukocyte subsets under homeostatic and pathological conditions – the word "chemokine" is the contraction of the terms "chemotactic" and "cytokine" (Charo and Ransohoff 2006). More specifically, leukocyte arrest during the rolling phase of its recruitment cascade is rapidly triggered by chemokines. After binding to specific seven transmembrane-domain G-protein-coupled receptors, chemokines regulate integrin-mediated adhesion among other effects (Ley et al. 2007). The expression of variable concentrations of different chemokines contributes to the diversity of leukocyte subsets present in inflamed tissues.

To date, approximately 50 chemokines and 20 receptors have been described in humans (Charo and Ransohoff 2006; Ransohoff 2009; Sereger and Alpers 2003). Chemokines are divided in four families based on differences in structure and function. The largest family comprises CC chemokines, so named because the first two cysteine residues are adjacent to each other, which are primarily involved in the attraction of mononuclear cells to sites of chronic inflammation. CXC family, in which the first two cysteine residues are separated by a single aminoacid, consists of two subfamilies based on the presence of a characteristic glutamic acid-leucine-arginine (ELR) motif near the N terminal of the molecule. ELR (+) CXC chemokines, of which CXCL8/IL-8 (IUPHAR nomenclature/original name) is the prototype molecule, attract polymorphonuclear leukocytes to sites of acute inflammation. Conversely ELR (-) CXC chemokines, like CXCL9/MIG, CXCL10/IP-10 and CXCL11/I-TAC, are IFN-γ inducible chemokines, being involved in the

recruitment of Th1 lymphocytes among other cell types. The remaining chemokines families comprise CX_3C (with three amino acids separating the first two cysteine residues) with a single member CX_3CL1/fractalkine, and XC (with a single cysteine residue) with XCL1/lymphotactin and XCL2 (Charo and Ransohoff 2006; Ransohoff 2009; Sereger and Alpers 2003).

In the context of leukocyte trafficking, chemokines can be functionally grouped as 'homeostatic', i.e., chemokines constitutively expressed in organs, like CXCL12/SDF-1, and 'inflammatory', i.e., chemokines induced on inflamed sites, which are bound by cognate receptors on infiltrating leukocytes (Charo and Ransohoff 2006; Ransohoff 2009). Although certain chemokines may be stored in granules of cells, such as platelets and mast cells, most chemokine expression is newly generated and released on-demand at inflammatory sites (Sereger and Alpers 2003. Indeed chemokines have been implicated in several diseases in which inflammatory mechanisms are involved, including renal diseases. Table 1 shows the subtypes of chemokines reported in humans.

With respect to renal diseases, there is much evidence that leukocyte infiltration is mediated by inflammatory chemokines released by various cell types (Sereger and Alpers 2003; Sereger et al. 2000). Infiltrating leukocytes produce chemokines that may amplify inflammatory responses in the kidney. Tubular epithelial cells can release inflammatory chemokines as CCL5/RANTES (Regulated on activation, normal T Expressed and Secreted), CCL2/MCP-1 (Monocyte chemotactic protein-1), CCL3/MIP-1α (Macrophage inflammatory protein 1 alfa), CX3CL1/fractalkine and CXCL8/IL8 (Interleukin 8) (Sereger et al. 2000). Tubular epithelial cells are also targets for chemokines since these cells respond to CCL2/MCP1 stimulation by releasing interleukin-6 and intracellular adhesion molecule-1 (Viedt et al. 2002). Messenger RNA of chemokines receptors can also be detected in podocytes and glomeruli (Sereger and Alpers 2003).

There are several techniques to measure chemokine – protein or mRNA – in tissues and body fluids. For example, chemokines could be directly measured in renal tissue by immunohistochemical or immunofluorescent techniques or by evaluating their levels in supernatants of homogenized tissues (by ELISA). In patients with renal diseases, the direct exam of tissue samples would be ideal since it may evaluate the affected organ. However, kidney biopsy is an aggressive procedure and could be harmful. On the other hand, ELISA or flow cytometry based techniques are less invasive and more useful for clinical purposes by measuring the levels of chemokines in urine or blood samples (Souto et al. 2008; Arraya et al. 2009; Vasconcelos et al. 2011; Pereira et al. 2012; Santos Jr et al. 2012; Vianna et al. 2013). Alternatively, chemokine mRNA can be measured by polymerase chain reaction or microarray in tissues or leukocytes of patients (Arraya et al. 2009; Sellares et al. 2013; Halloran et al. 2013).

Chemokines in Renal Diseases

Several studies have shown the involvement of chemokines in the inflammatory process responsible for the progression of chronic kidney disease (CKD) and renal transplant rejection (for review, see refs. Vianna et al. 2011; Pereira et al. 2009). It

Table 1 Subtypes of chemokines reported in humans (IUPHAR nomenclature/original name)

Chemokine subtype	Biochemical structure	Function	Main molecules
CC chemokines	First two cysteine residues are adjacent to each other	Recruitment of mononuclear cells to sites of chronic inflammation	CCL2/MCP-1
			CCL3/MIP-1α
			CCL5/RANTES
CXC chemokines – ELR (+)	First two cysteine residues are separated by a single aminoacid with a glutamic acid-leucine-arginine (ELR) motif near the N terminal of the molecule	Recruitment of polymorphonuclear leukocytes to sites of acute inflammation	CXCL8/IL-8
CXC chemokines – ELR (-)	First two cysteine residues are separated by a single aminoacid without ELR motif	IFN-γ inducible chemokines responsible for the recruitment of Th1 lymphocytes	CXCL9/MIG
			CXCL10/IP-10
			CXCL11/I-TAC
CX_3C chemokines	First two cysteine residues are separated by three aminoacids	Chemokines expressed on activated endothelial cells responsible for leucocyte adhesion and migration	CX_3CL1/fractalkine
XC chemokines	With a single cysteine residue	Recruitment of certain subsets of T-cells and natural killer cells	XCL1/lymphotactin-α
			XCL2/lymphotactin-β

CCL2/MCP-1 Monocyte chemotactic protein-1, *CCL3/MIP-1α* Macrophage inflammatory protein 1 alfa, *CCL5/RANTES* Regulated on activation, normal T Expressed and Secreted, *CXCL8/IL-8* interleukin-8, *CXCL9/MIG* Monokine induced by gamma interferon, *CXCL10/IP-10* Interferon gamma-induced protein 10, *CXCL11/I-TAC* interferon-inducible T-cell alpha chemoattractant

was also suggested that the chemokines might act as biomarkers of renal disease progression (Vianna et al. 2013) and as predictors of graft function in renal transplantation (Pereira et al. 2012).

Many chemokines were measured in patients with nephrotic syndrome or in animal models of the disease (for review, see refs. Pereira et al. 2014, 2015). Some of them were correlated with proteinuria and suggested as candidates for promoting glomerular permeability and, as consequence, proteinuria (for review, see ref. Moreno et al. 2014). In animal models of nephrotic syndrome, increased renal concentrations of the chemokines CCL5/RANTES, CCL11/eotaxin, CCL1/TCA-3 (T cell activation-3), CCL2/MCP-1, CCL3/MIP-1a (macrophage inflammatory protein-1 alpha) and CCL4/MIP-1b (macrophage inflammatory protein-1 beta) have been reported (Vielhauer et al. 2004). Blockade of the CCR1 chemokine receptor reduced the infiltration of macrophages, lymphocytes and fibroblasts in

renal tissue (Vielhauer et al. 2004). Wang and co-workers (1997) showed that high concentrations of albumin stimulated proximal tubular cells in culture increase the production and secretion of CCL2/MCP-1. Rats immunized with CCL2/MCP-1 and CCL5/RANTES DNA before the induction of nephrotic syndrome by doxorubicin presented low chemotaxis of monocytes/macrophages and improvement in biochemical changes and renal histopathology (Wu et al. 2005). In addition, the measurement of urinary, plasma and renal tissue levels of chemokines has been used to diagnosis and monitor various renal diseases (Souto et al. 2008; Arraya et al. 2009; Vasconcelos et al. 2011; Pereira et al. 2012; Santos Jr et al. 2012; Vianna et al. 2013). We reported below the chemokines most frequently associated to renal diseases.

CXCL8, also known IL-8, is an inflammatory chemokine that activates CXCR1 and CXCR2, and promotes neutrophil infiltration and activation in proteinuria-associated renal diseases (Souto et al. 2008; Moreno et al. 2014; Tang et al. 2003; Yokoyama et al. 1998). CXCL8/IL-8 expression was increased in tubules of patients with heavy proteinuria and non-proliferative glomerulopathy (Souto et al. 2008; Tang et al. 2003) and IgA nephropathy (Yokoyama et al. 1998). Several studies have also demonstrated high concentrations of IL-8/CXCL-8 in serum (Garin et al. 1994; Kanai et al. 2009; Garin et al. 1998) and urine (Souto et al. 2008) of patients with primary nephrotic syndrome, as well as increased levels of mRNA for CXCL-8/IL-8 in peripheral blood mononuclear cell (PBMC) culture of these patients (Garin et al. 1998). The CXCL-8/IL-8 present in PBMC culture supernatant from patients with nephrotic syndrome alters the sulfated component metabolism at the glomerular basement membrane in rats (Garin et al. 1994, 1998). In vitro studies in tubular epithelial cells have shown that albumin increases CXCL8/IL-8 through the activation of the nuclear factor-kappa B (NF-κB) transcription factor. CXCL8/IL-8 urine levels have been used as a marker of renal disease progression (Tang et al. 2003; Yokoyama et al. 1998). Increased urinary levels of CXCL8/IL-8 were observed in idiopathic nephrotic syndrome during relapses (Souto et al. 2008), IgA nephropathy (Yokoyama et al. 1998), lupus nephritis and membranoproliferative glomerulonephritis (Wada et al. 1994). In lupus nephritis, urinary levels of CXCL8/IL-8 were used as a marker of disease activity (Rovin et al. 2005). Urinary CXCL8/IL-8 levels were higher in patients with glomerular leukocyte infiltration than in those without (Rovin et al. 2005). Moreover, patients in remission showed lower CXCL8/IL-8 levels than patients with idiopathic nephrotic syndrome in relapse, and levels correlated with proteinuria values (Souto et al. 2008).

CXCL8/IL-8 is also responsible for neutrophil infiltration into the urinary tract with an important role in acute pyelonephritis (Sheu et al. 2006; Artifoni et al. 2007). In this regard, gene polymorphisms of this chemokine seem to increase the susceptibility for acute pyelonephritis (Sheu et al. 2006). For instance, the presence of the IL-8-251A allele in the genotype of children with urinary tract infection without vesicoureteral reflux has increased the risk of pyelonephritis (Artifoni et al. 2007). Table 2 summarizes the studies about CXCL-8/Il-8 in renal diseases.

Chemokine C–C motif ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP-1), is one of the most widely studied chemokines. CCL2/MCP-1 is involved in the recruitment of monocytes and lymphocytes to sites of inflammation through the activation of several receptors, mainly CCR2

Table 2 Studies reporting potential role for Interleukin-8 (CXCL8/IL-8) in renal diseases

Author	Year	Main findings
Garin et al.	1994	High levels of mRNA for CXCL-8/IL-8 in PBMC culture of patients with nephrotic syndrome
Garin et al.	1998	CXCL-8/IL-8 present in the PBMC culture supernatant from patients with nephrotic syndrome alters the sulfated component metabolism at the glomerular basement membrane in rats
Yokoyama et al.	1998	Urinary levels of chemokines, including CXCL8/IL-8, reflect distinct disease activities and phases of human IgA nephropathy
Tang et al.	2003	Albumin increases CXCL8/IL-8 through the activation of the NF-κB transcription factor in proximal tubular cells in vivo and in vitro
Rovin et al.	2005	Urine chemokine measurements have a potential role as biomarkers of lupus nephritis activity
Sheu et al.	2006	Gene polymorphisms of CXCL8/IL-8 seem to increase the susceptibility for acute pyelonephritis
Artifoni et al.	2007	Presence of the IL-8-251A allele in the genotype of children with urinary tract infection without vesicoureteral reflux increases the risk of pyelonephritis
Souto et al.	2008	Urinary levels of CXCL8/IL-8 are positively correlated with 24-h proteinuria in pediatric patients with primary nephrotic syndrome
Kanai et al.	2009	Urinary levels of CXCL8/IL-8 are associated with idiopathic steroid sensitive nephrotic syndrome
Vianna et al.	2013	Urinary levels of CXCL8/IL-8 inversely correlate with GFR in pediatric CKD patients due to CAKUT

PBMC peripheral blood mononuclear cell, *NF-κB* nuclear factor-kappaB, *GFR* glomerular filtration rate, *CKD* chronic kidney disease, *CAKUT* congenital anomalies of the kidney and urinary tract

(Viedt et al. 2002; Pereira et al. 2012; Vianna et al. 2013; Yadav et al. 2010; Kim and Tam 2011; Marks et al. 2010; Wada et al. 2000; Bobkova et al. 2006; Eddy and Warren 1996; Stangou et al. 2009; Giunti et al. 2006; Kolattukudy and Niu 2012; Dubinski et al. 2008; Ho et al. 2013). Many studies have associated MCP-1/CCL2 to glomerulopathies (Viedt et al. 2002; Vianna et al. 2013; Marks et al. 2010; Wada et al. 2000; Bobkova et al. 2006; Eddy and Warren 1996; Stangou et al. 2009) and to renal transplantation (Pereira et al. 2012; Dubinski et al. 2008; Ho et al. 2013). Wada and co-workers found significantly elevated urinary levels of this chemokine in adults with diabetic nephropathy, whereas serum levels remained similar to those of healthy volunteers (Wada et al. 2000). Patients with active proteinuric forms of chronic glomerulonephritis have higher urine excretion of CCL2/MCP-1 than healthy controls (Bobkova et al. 2006). In pediatric lupus nephritis, it was recently shown that increased urinary, but not plasma, CCL2/MCP-1 levels correlated with disease activity (Marks et al. 2010). Taken together, these studies indicate a potential role for CCL2/MCP-1 in glomerular inflammation. Concerning renal transplantation, urinary levels of CCL2/MCP-1 were significantly higher in patients with acute rejection and a significant reduction of this chemokine was found in patients who responded to anti-rejection treatment (Dubinski et al. 2008). In addition, increased urinary levels CCL2/MCP-1 at 6 months after renal transplantation might predict

Table 3 Studies reporting potential role for monocyte chemoattractant protein 1 (CCL2/MCP-1) in renal diseases

Author	Year	Main findings
Eddy et al.	1996	MCP-1 gene and protein expression are increased in the kidneys of rats with aminonucleoside nephrosis
Wada et al.	2000	Adults with diabetic nephropathy have elevated urinary levels of CCL2/MCP-1
Viedt et al.	2002	CCL2/MCP-1 induces inflammatory activation of human tubular epithelial cells
Bobkova et al.	2006	Patients with active proteinuric forms of chronic glomerulonephritis have higher urine excretion of CCL2/MCP-1 than healthy controls
Giunt et al.	2006	The MCP-1/CCR2 system has direct proinflammatory effects in human mesangial cells
Dubinski et al.	2008	Urinary levels of CCL2/MCP-1 are higher in patients with acute rejection and the reduction of this chemokine occurs in response to anti-rejection treatment
Stangou et al.	2009	Up-regulation of urinary CCL2/MCP-1 predicts outcome in IgA nephropathy
Marks et al.	2010	Increased urinary levels of CCL2/MCP-1 correlate with lupus nephritis activity in pediatric patients
Pereira et al.	2012	Urinary levels of CCL2/MCP-1 significantly reduce from 30 to 300 days after renal transplantation in live donors subgroup
Ho et al.	2013	Increased urinary CCL2:Cr ratio at 6 months is associated with late renal allograft loss
Vianna et al.	2013	Urinary CCL2/MCP-1 levels are increased in FSGS patients and positively correlated with plasma cholesterol

CCR2 receptor for the monocyte chemoattractant protein 1, *Cr* creatinin, *FSGS* focal segmental glomerulosclerosis

renal allograft loss (Ho et al. 2013). Urinary MCP-1/CCL2 measurements may be an early marker of therapy responsiveness in patients with acute rejection. Table 3 displays the main findings of the studies of CCL2/MCP-1.

Chemokine C–C motif ligand 5 (CCL5), also known as RANTES, and its specific receptors CCR1, CCR3 and CCR5 have also been associated with renal diseases (Krensky and Ahn 2007). CCL5/RANTES is a chemokine produced by human T lymphocytes at a 'late' stage (3–5 days) after activation through their T-cell receptors. It is broadly chemoattractive for T lymphocytes, monocytes, natural killer cells, basophils and eosinophils, and can also activate immune cells. This chemokine is involved in AIDS, cancer, atherosclerosis, asthma, organ transplantation, and autoimmune diseases such as arthritis, diabetes and glomerulonephritis (Krensky and Ahn 2007). Albumin and other filtered urinary proteins, such as IgA and IgG, can induce up-regulation of CCL5/RANTES. As compared with CCL2/MCP-1, CCL5/RANTES induction is delayed, but remains increased for days. CCL5/RANTES was expressed by tubular epithelial cells during proteinuria, and its expression was associated with interstitial CCR5-positive mononuclear cells (predominantly CD3-positive T lymphocytes) and fibrosis in patients with acute interstitial nephritis

(Sereger et al. 1999). CCL5/RANTES levels are found increased in kidneys of autoimmune nephritis that is characterized by proteinuria and monocyte recruitment (Xie et al. 2011).

Chemokines in Glomerular Diseases

In the last 5 years, several studies measuring chemokines in different forms of glomerular diseases such as IgA nephropathy, lupus nephritis and idiopathic nephrotic syndrome (INS) including minimal change lesion (MCNS) and focal segmental glomerulosclerosis (FSGS) have been performed.

IgA nephropathy is diagnosed by the predominance of IgA deposits in the glomerular mesangium and is present in around 13.8 % of renal biopsies of children, being the second more frequent renal disease, and the mesangioproliferative glomerulonephritis is the most common histological presentation (Bazina et al. 2007). Chemokines have been evaluated in IgA patients and some of them seemed to predict the outcome of the disease (Stangou et al. 2013).

Systemic lupus erythematosus (SLE) is a multisystemic autoimmune disease affecting predominantly women and two thirds of cases occur in the first two decades of life. Lupus nephritis is a very common condition in 50–67 % of children with SLE (Mina and Brunner 2010). A wide range of chemokines (CXCL10/IP-10, CCL3/MIP-1α, CCL5/RANTES, CXCL8/IL-8, CCL2/MCP-1 and CCL4/MIP-1β) was tested for correlation with lupus activity as assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-1) (Barbado et al. 2012). The chemokine CCL2/MCP-1 was the best biomarker of SLE activity (Barbado et al. 2012). Regarding lupus nephritis, the serum B lymphocyte chemoattractant (CXCL13/BLC) was increased in 31 adult patients in comparison with 60 SLE patients without renal involvement and might be a surrogate marker as well (Schiffer et al. 2009).

MCNS is the most common cause of nephrotic syndrome in children. This disease reflects a disorder of T lymphocytes and some patients can progress to FSGS (Cho et al. 2007). Our research group measured plasma and urinary chemokines in 32 children with INS divided according to steroid responsiveness and 12 healthy controls (Souto et al. 2008). We found increased levels of urinary CXCL8/IL-8 in relapsed steroid resistant children when compared to steroid sensitive patients in remission, with a positive correlation with urinary protein levels (Souto et al. 2008). These findings suggest that the renal release of the chemokine CXCL8/IL-8 might be associated with changes in glomerular permeability (Souto et al. 2008). More recently, by studying a group of pediatric patients at stages 2–4 of CKD, we detected higher levels of urinary CCL2/MCP-1 in patients with FSGS than in cases of uropathies at the same stage of CKD (Vianna et al. 2013). In addition, urinary levels of CCL2/MCP-1 positively correlated with serum total cholesterol and triglycerides concentrations (Vianna et al. 2013). This study supports the concept that differences in chemokine profile may be related to CKD etiology and disease-associated changes.

Figure 1 shows inflammatory mechanisms that linked chemokines and the emergence of CKD in glomerular diseases.

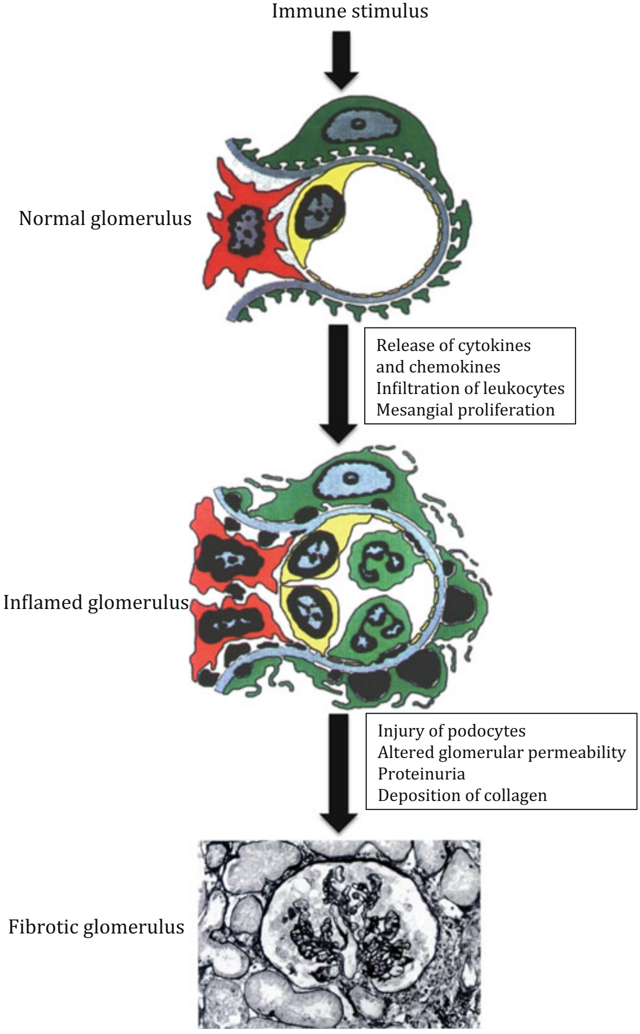

Fig. 1 Inflammatory mechanisms related to the emergence of chronic kidney disease in glomerulopathies

Chemokines in Congenital Uropathies

Congenital anomalies of the kidney and urinary tract (CAKUT) comprise a *spectrum* of malformations that occur at the level of the kidney (e.g., hypoplasia and dysplasia), collecting system (e.g., idiopathic hydronephrosis, ureteropelvic junction obstruction, and megaureter), bladder (e.g., ureterocele and vesicoureteral reflux), or urethra (e.g., posterior urethral valves) (Carr and Kim 2010). A variety of

intrarenal factors lead to progressive interstitial and renal parenchyma fibrosis in patients with CAKUT, including growth factors, cytokines, chemokines and adhesion molecules (Klahr and Morrisey 2002). Altered renal expression of these factors modulates cell death by apoptosis and/or phenotypic transition of glomerular, tubular, and vascular cells. Mediators of cellular injury include hypoxia, ischemia, and reactive oxygen species, while fibroblasts undergo myofibroblast transformation with increased deposition of extracellular matrix. On the other hand, a number of endogenous antifibrotic counter-regulatory molecules has been identified, opening the possibility of enhancing the kidney's own defenses against progressive fibrosis (Klahr and Morrisey 2002; Chevalier et al. 2010).

In this regard, chemokines like CCL2/MCP-1, CCL5/RANTES, macrophage inflammatory protein-2 (CXCL2/MIP-2) and ɣ-interferon-inducible protein (CXCL10/IP-10) have been evaluated in experimental hydronephrosis (Stephan et al. 2002; Vielhauer et al. 2001; Crisman et al. 2001; Madsen 2013). Stephan and co-workers produced partial or complete ureteral obstruction in 28-day-old Wistar rats (Stephan et al. 2002). CCL2/MCP-1 mRNA expression was moderately increased in partial ureteral obstruction, whereas kidneys without significant damage did not show any up-regulation of this chemokine. The study qualified CCL2/MCP-1 mRNA expression as a prognostic marker of partial ureteral obstruction (Stephan et al. 2002). In addition, Vielhauer and co-workers found an increased expression of the CC chemokines, CCL2/MCP-1 and CCL5/RANTES, at sites of progressive tubulointerstitial damage in murine obstructive nephropathy model (Vielhauer et al. 2001). It was also observed an interstitial infiltration of macrophages and T lymphocytes, which differentially expressed CCR2 receptors. These data suggest that CCR2- and CCR5-positive monocytes and CCR5-positive lymphocytes are attracted by locally released CCL2/MCP-1 and CCL5/RANTES, resulting in chronic interstitial inflammation (Vielhauer et al. 2001). Crisman and co-workers detected the expression of CCL2/MCP-1, CCL5/RANTES and CXCL10/IP-10 at 1 day of unilateral ureteral obstruction in mice (Crisman et al. 2001). At 7 days, CCL5/RANTES became the most abundant chemokine in the obstructed kidney and the cortical tubular cells significantly contributed to this elevation (Crisman et al. 2001). The study of chemokines in hydronephrosis might provide new insights for treatment or novel ways to blunt renal damage in obstructive nephropathy (Madsen 2013). Figure 2 proposes a link between chemokine release and CKD in patients with congenital uropathies.

Very few data about the role of chemokines in CAKUT have been provided by clinical studies and the majority of them evaluated ureteropelvic junction obstruction (UPJO) and vesicoureteral reflux (VUR).

Ureteropelvic Junction Obstruction

UPJO is the most common cause of severe hydronephrosis in children (Quirino et al. 2012). Postnatal differentiation between obstructive and non-obstructive hydronephrosis is quite difficult (Dias et al. 2013). Several studies have been made

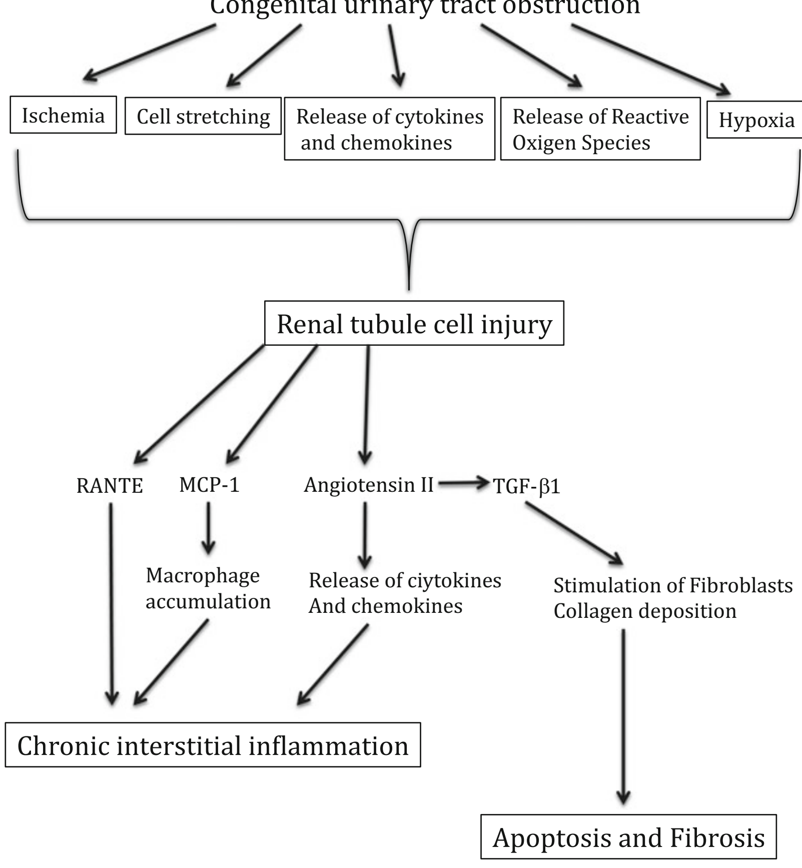

Fig. 2 Schematic view of chemokine and fibrogenic factors release at renal tissue in congenital uropathies

in patients with UPJO in order to find out noninvasive biomarkers to allow the diagnosis and treatment of these patients (Decramer et al. 2007; Lee 2009). Specifically for chemokines, the most promising results were obtained with CCL2/MCP-1 (Grandaliano et al. 2000; Bartoli et al. 2011; Madsen et al. 2013).

Healthy children presented high expression of epidermal growth factor (EGF) mRNA in renal tissue, whereas CCL2/MCP-1 mRNA was normally undetectable. In UPJO patients, CCL2/MCP-1 gene expression was strikingly increased at the tubulointerstitial level, while the EGF gene expression was markedly reduced. Moreover, the interstitial mononuclear cell infiltrate in UPJO patients correlated with the degree of tubulointerstitial damage (Grandaliano et al. 2000). Accordingly, urinary concentrations of EGF were reduced in UPJO patients, whereas the CCL2/MCP-1 levels were increased (Grandaliano et al. 2000; Madsen et al. 2013). After

Table 4 Studies reporting potential role for monocyte chemoattractant protein 1 (CCL2/MCP-1) in ureteropelvic junction obstruction (UPJO)

Author	Year	Main findings
Grandaliano et al.	2000	Urinary levels of CCL2/MCP-1 are increased in UPJO patients before surgery and the levels reduce after the surgical procedure
Bartoli et al.	2011	Urinary levels of CCL2/MCP-1 increase in patients with UPJO compared with healthy controls and significantly reduce after surgical treatment
Madsen et al.	2012	Urinary levels of CCL2/MCP-1 are increased in preoperative samples collected in UPJO patients before surgical procedure in comparison to urine from healthy children
Taranta-Janusz et al.	2012	Urinary levels of CCL2/MCP-1 from voided urine before and after surgery and from the affected pelvis are higher than non-surgically managed cases of UPJO and control group

surgical correction of UPJO, there was a significant reduction in urinary levels of CCL2/MCP-1 accompanied by a marked increase in EGF concentration. Therefore, these two biomarkers could be useful for the follow-up of obstructed patients (Grandaliano et al. 2000). In a prospective study, Madsen and co-workers reported that urinary concentrations of EGF and CCL2/MCP-1 were significantly increased in preoperative samples collected in UPJO patients before surgical procedure in comparison to urine from healthy children (Madsen et al. 2013). At the same study, the concentrations of CCL2/MCP-1, CCL3/MIP-1α, CXCL10/IP-10 and CCL5/RANTES were increased in the urine from the obstructed kidney compared to the urine from the contralateral non-obstructed kidney (Madsen et al. 2013). These urine samples were collected during surgical procedure. One year after surgery, the concentrations of these chemokines decreased to levels comparable to healthy controls (Madsen et al. 2013).

Taranta-Janusz and co-workers compared obstructed prenatal hydronephrosis cases (who underwent surgery) with non-surgically managed cases and healthy subjects (control group) (Taranta-Janusz et al. 2012). Urinary levels of CCL2/MCP-1 from voided urine before and after surgery and from the affected pelvis were significantly higher than non-surgically managed cases and control group (Taranta-Janusz et al. 2012). The authors also studied the level of CCL5/RANTES in urine samples, which were significantly higher in urine samples from affected pelvis collected during surgery than in voided urine before pyeloplasty (Taranta-Janusz et al. 2012). Three months after surgery, urinary levels of these biomarkers did not return to control values (Taranta-Janusz et al. 2012). Table 4 shows clinical studies of chemokines in UPJO.

Vesicoureteral Reflux

VUR is a congenital anomaly that increases the risk of repeated pyelonephritis and, consequently, can result in renal scarring, renin-mediated hypertension, and, in some

Table 5 Studies reporting potential role for Interleukin-8 (CXCL8/IL-8) in vesicoureteral reflux (VUR)

Author	Year	Main findings
Haraoka et al.	1996	Urinary levels of CXCL8/IL-8 are higher in children with renal scarring than without and in patients with VUR than without
Galanakis et al.	2006	Urinary measurement of CXCL8/IL-8 is used for the diagnostic of VUR at a cutt-off concentration of 5 pg/μmol with sensitivity of 88 % and specificity of 69 %
Merrikhi et al.	2012	Urinary levels of CXCL8/IL-8 are higher in children with VUR than without and this chemokine could be a marker of renal scarring due to VUR
Vianna et al.	2013	Urinary levels of CXCL8/IL-8 negatively correlate with GFR in CAKUT patients, suggesting that this chemokine might be associated to renal scarring

GFR glomerular filtration rate, *CAKUT* congenital anomalies of the kidney and urinary tract

cases, renal insufficiency (Silva et al. 2006, 2009; Simões e Silva et al. 2007). There is persistent inflammatory reaction in VUR despite the occurrence or not of urinary tract infection (UTI). The elevated urinary level of CXCL8/IL-8 in children with reflux and without UTI might contribute to reflux nephropathy (Haraoka et al. 1996; Galanakis et al. 2006; Merrikhi et al. 2012). Haraoka and co-workers found a significant difference between urinary levels of CXCL8/IL-8 in children with and without renal scarring and in patients with and without VUR (Haraoka et al. 1996). Merrikhi and co-workers also showed significantly higher levels of CXCL8/IL-8 in patients with VUR than in those without VUR (Merrikhi et al. 2012). This finding suggests that urinary CXCL8/IL-8 measurements could be useful to detect VUR patients with more pronounced renal damage and who need strict follow-up (Merrikhi et al. 2012). Galanakis and co-workers proposed the use of CXCL8/IL-8 as a biomarker for the diagnostic of VUR (Galanakis et al. 2006). A cutt-off concentration of 5 pg/μmol has a sensitivity of 88 % and a specificity of 69 % (Galanakis et al. 2006). Our research group has recently reported a correlation between high urinary levels of CXCL8/IL-8 and reduced glomerular filtration rate in CAKUT patients, suggesting that this chemokine might be associated to renal scarring and CKD (Vianna et al. 2013). Table 5 shows clinical studies of chemokines in VUR.

Potential Applications to Prognosis, Other Diseases or Conditions

Clinical and experimental evidence leaves no doubt about the role of inflammation in renal diseases. Understanding the effects of chemokines on the onset and progression of renal injury is of great interest as new diagnostic and prognostic markers, and maybe as alternative therapeutic targets.

CCL2/MCP-1 and CXCL8/IL-8 are the chemokines more commonly associated with pediatric renal diseases. In glomerular diseases, high urinary levels of CCL2/

MCP-1 have been associated to FSGS, lupus nephritis and IgA nephropathy. There is also evidence of a potential link between monocyte recruitment and dyslipidemia in pediatric patients with CKD due to FSGS. On the other hand, urinary levels of CXCL8/IL-8 positively correlated with proteinuria in pediatric patients with primary nephrotic syndrome, suggesting a role in glomerular permeability changes. With reference to CAKUT, the chemokine CCL2/MCP-1 has been associated with urinary tract obstruction in patients with UPJO, whereas high urinary levels of CXCL8/IL-8 were found in patients with VUR and correlated with renal scarring and renal function deterioration.

What is more, new roles are emerging for chemokines outside the renal disease field: for example, in Parkinson disease, high plasma levels of the chemokine CXCL10/IP-10 seem to be associated with worse performance on cognitive tests (Rocha et al. 2014) and, in bipolar disorder, plasma levels of CXCL8/IL-8 was decreased, whereas CCL11, CCL24 and CXCL10/IP-10 concentrations were increased in comparison to healthy controls (Barbosa et al. 2013).

Finally, despite great advances in our knowledge on the pathways linking chemokines to renal diseases, much still remains to be elucidated.

Summary Points

- Chemokines are responsible for recruitment of leukocytes.
- There are several techniques to measure chemokines.
- Chemokines seem to have a role in the pathogenesis of renal diseases.
- CCL2/MCP-1 and CXCL8/IL-8 are the chemokines more commonly associated with pediatric renal diseases.
- High urinary levels of CCL2/MCP-1 is found in glomerular diseases.
- CXCL8/IL-8 seems to have a role in glomerular permeability changes and in renal scarring.

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References

Araya C, Diaz L, Wasserfall C, Atkinson M, Mu W, Johnson R, Garin E. T regulatory cell function in idiopathic minimal lesion nephrotic syndrome. Pediatr Nephrol. 2009;24:1691–8.

Artifoni L, Negrisolo S, Montini G, Zuchetta P, Molinari PP, Cassar W, Destro R, Anglani F, Rigamonti W, Zacchello G, Murer L. Interleukin-8 and CXCR1 receptor functional polymorphisms and susceptibility to acute pyelonephritis. J Urol. 2007;177:1102–6.

Barbado J, Martin D, Vega L, Almansa R, Gonçalves L, Nocito M, Jimeno A, Ortiz de Lejarazu R, Bermejo-Martin JF. MCP-1 in urine as biomarker of disease activity in Systemic Lupus Erythematosus. Cytokine. 2012;60:583–6.
Barbosa IG, Rocha NP, Bauer ME, de Miranda AS, Huguet RB, Reis HJ, Zunszain PA, Horowitz MA, Pariante CM, Teixeira AL. Chemokines in bipolar disorder: trait or state? Eur Arch Psychiatry Clin Neurosci. 2013;263(2):159–65.
Bartoli F, Penza R, Aceto G, Niglio F, D'Addato O, Pastore V, Campanella V, Magaldi S, Lasalandra C, Di Bitonto G, Gesualdo L. Urinary epidermal growth factor, monocyte chemotactic protein-1, and β2-microglobulin in children with ureteropelvic junction obstruction. J Pediatr Surg. 2011;46:530–6.
Bazina M, Glavina-Durdov M, Sćukanec-Spoljar M, Bazina A, Vukojević K, Ljutić D, Saraga M. Epidemiology of renal disease in children in the region of southern Croatia: a 10-year review of regional renal biopsy databases. Med Sci Monit. 2007;13:172–6.
Bobkova IN, Chebotareva NV, Kozlovskaia LV, Varshavskii VA, Golitsyna EP. Urine excretion of a monocytic chemotaxic protein-1 and a transforming growth factor beta1 as an indicator of chronic glomerulonephritis progression. Ter Arkh. 2006;78:9–14.
Carr MC, Kim SS. Prenatal management of urogenital disorders. Urol Clin N Am. 2010;37:149–58.
Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med. 2006;354:610–21.
Chevalier RL, Thornhill BA, Forbes MS, Kiley SC. Mechanisms of renal injury and progression of renal disease in congenital obstructive nephropathy. Pediatr Nephrol. 2010;25(4):687–97.
Cho MH, Hong EH, Lee TH, Ko CW. Pathophysiology of minimal change nephrotic syndrome and focal segmental glomerulosclerosis. Nephrology (Carlton). 2007;12 Suppl 3:S11–4.
Crisman JM, Richards LL, Valach DP, Valach DF, Franzoni DF, Diamond JR. Chemokine expression in the obstructed kidney. Exp Nephrol. 2001;9:241–8.
Decramer S, Bascands J, Schanstra JP. Non-invasive markers of ureteropelvic junction obstruction. World J Urol. 2007;25:457–65.
Dias CS, Silva JM, Pereira AK, Marino VS, Silva LA, Coelho AM, Costa FP, Quirino IG, Simoes e Silva AC, Oliveira EA. Diagnostic accuracy of renal pelvic dilatation for detecting surgically managed ureteropelvic junction obstruction. J Urol. 2013;190:661–6.
Dubinski B, Boratynska M, Kopec W, Szyber P, Patrzalek D, Klinger M. Activated cells in urine and monocyte chemotactic peptide-1 (MCP-1)- sensitive rejection markers in renal graft recipients. Transpl Immunol. 2008;18:203–7.
Eddy AA, Warren JS. Expression and function of monocyte chemoattractant protein-1 in experimental nephrotic syndrome. Clin Immunol Immunopathol. 1996;78:140–51.
Galanakis E, Bitsori M, Dimitriou H, Giannakopoulou C, Karkavitsas NS, Kalmanti M. Urine interleukin-8 as a marker of vesicoureteral reflux in infants. Pediatrics. 2006;117:e863–7.
Garin EH, Blanchard DK, Matsushima K, Djeu JY. IL-8 production by peripheral blood mononuclear cells in nephrotic patients. Kidney Int. 1994;45:1311–7.
Garin EH, Laflam P, Chandler L. Anti-interleukin 8 antibody abolishes effects of lipoid nephrosis cytokine. Pediatr Nephrol. 1998;12:381–5.
Giunti S, Pinach S, Arnaldi L, Viberti G, Perin PC, Camussi G, Gruden G. The MCP-1/CCR2 system has direct proinflammatory effects in human mesangial cells. Kidney Int. 2006;69:856–63.
Grandaliano G, Gesualdo L, Bartoli F, Ranieri E, Monno R, Leggio A, Paradies G, Caldarulo E, Infante B, Schena FP. MCP-1 and EGF renal expression and urine excretion in human congenital obstructive nephropathy. Kidney Int. 2000;58:182–92.
Halloran PF, Pereira AB, Chang J, Matas A, Picton M, De Freitas D, Bromberg J, Seron D, Sellares J, Einecke G, Reeve J. Potential impact of microarray diagnosis of T cell-mediated rejection in kidney transplants: the INTERCOM study. Am J Transplant. 2013;13:2865–74.
Haraoka M, Senoh K, Ogata N, Furukawa M, Matsumoto T, Kumazawa J. Elevated interleukin-8 levels in the urine of children with renal scarring and/or vesicoureteral reflux. J Urol. 1996;155:678–80.

Ho J, Wiebe C, Rush DN, Rigatto C, Storsley L, Karpinski M, Gao A, Gibson IW, Nickerson PW. Increased urinary CCL2: Cr ratio at 6 months is associated with late renal allograft loss. Transplantation. 2013;95:595–602.
Kanai T, Yamagata T, Momoi MY. Macrophage inflammatory protein-1b and interleukin-8-associated with idiopathic steroid sensitive nephrotic syndrome. Pediatr Int. 2009;51:443–7.
Kim MJ, Tam FW. Urinary monocyte chemoattractant protein-1 in renal disease. Clin Chim Acta. 2011;412:2022–30.
Klahr S, Morrisey J. Obstructive nephropathy and renal fibrosis. Am J Renal Physiol. 2002;283: F861–75.
Kolattukudy PE, Niu J. Inflammation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant protein-1/CCR2 pathway. Circ Res. 2012;110:174–89.
Krensky AM, Ahn YT. Mechanisms of disease: regulation of RANTES (CCL5) in renal disease. Nat Clin Pract Nephrol. 2007;3:164–70.
Lee RS. Biomarkers for pediatric urological disease. Curr Opin Urol. 2009;19:397–401.
Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 2007;7:678–89.
Madsen MG. Urinary biomarkers in hydronephrosis. Dan Med J. 2013;60:B4582.
Madsen MG, Nørregaard R, Palmfeldt J, Olsen LH, Frøkiær J, Jørgensen TM. Epidermal growth factor and monocyte chemotactic peptide-1: potential biomarkers of urinary tract obstruction in children with hydronephrosis. J Pediatr Urol. 2013;9:838–45.
Marks SD, Shah V, Pilkington C, Tullus K. Urinary monocyte chemoattractant protein-1 correlates with disease activity in lupus nephritis. Pediatr Nephrol. 2010;25:2283–8.
Merrikhi AR, Keivanfar M, Gheissari A, Mousavinasab F. Urine interlukein-8 as a diagnostic test for vesicoureteral reflux in children. J Pak Med Assoc. 2012;62(3 Suppl 2):S52–4.
Mina R, Brunner HI. Pediatric lupus-are there differences in presentation, genetics, response to therapy, and damage accrual compared with adult lupus? Rheum Dis Clin N Am. 2010;36:53–80.
Moreno JA, Moreno S, Rubio-Navarro A, Gómez-Guerrero C, Ortiz A, Egido J. Role of chemokines in proteinuric kidney disosrders. Expert Rev Mol Med. 2014;16:e3.
Pereira AB, Rezende NA, Teixeira Jr AL, Teixeira MM, Simoes e Silva AC. Cytokines and chemokines in renal transplantation. Braz J Nephrol. 2009;31:286–96.
Pereira AB, Teixeira AL, Rezende NA, Pereira RM, Miranda DM, Araújo EA, Teixeira MM, Simões e Silva AC. Urinary chemokines and anti-inflammatory molecules in renal transplanted patients as potential biomarkers of graft function: a prospective study. Int Urol Nephrol. 2012;44:1539–48.
Pereira WF, Brito-Melo GE, Guimaraes FT, Carvalho TG, Mateo EC, Simoes e Silva AC. The role of the immune system in idiopathic nephrotic syndrome: a review of clinical and experimental studies. Inflamm Res. 2014;63:1–12.
Pereira WF, Brito-Melo GE, Almeida CA, Moreira LL, Cordeiro CW, Carvalho TG, Mateo EC, Simões e Silva AC. The experimental model of nephrotic syndrome induced by Doxorubicin in rodents: an update. Inflamm Res. 2015;64:287–301.
Quirino IG, Diniz JS, Bouzada MC, Pereira AK, Lopes TJ, Paixão GM, Barros NN, Figueiredo LC, Cabral AC, Simões e Silva AC, Oliveira EA. Clinical course of 822 children with prenatally detected nephrouropathies. Clin J Am Soc Nephrol. 2012;7:444–51.
Ransohoff RM. Chemokines and chemokine receptors: standing at the crossroads of immunobiology and neurobiology. Immunity. 2009;31:711–21.
Rocha NP, Scalzo PL, Brabosa IG, Souza MS, Morato IB, Vieira EL, Christo PP, Teixeira AL, Reis HJ. Cognitive status correlates with CXCL10/ IP-10 levels in Parkinson's Disease. Park Dis. 2014;2014:903796.
Rovin BH, Song H, Birmingham DJ, Hebert LA, Yu CY, Nagaraja HN. Urine chemokines as biomarkers of human systemic lupus erythematosus activity. J Am Soc Nephrol. 2005;16:467–73.

Santos Jr ACS, Lima EM, Penido MGMG, Silveira KD, Teixeira MM, Oliveira EA, Simões e Silva AC. Plasma and urinary levels of cytokines in patients with idiopathic hypercalciuria. Pediatr Nephrol. 2012;27:941–8.

Schiffer L, Kümpers P, Davalos-Misslitz AM, Haubitz M, Haller H, Anders HJ, Witte T, Schiffer M. B-cell-attracting chemokine CXCL13 as a marker of disease activity and renal involvement in systemic lupus erythematosus (SLE). Nephrol Dial Transplant. 2009;24:3708–12.

Sellares J, Reeve J, Loupy A, Mengel M, Sis B, Skene A, de Freitas DG, Kreepala C, Hidalgo LG, Famulski KS, Halloran PF. Molecular diagnosis of antibody-mediated rejection in human kidney transplants. Am J Transplant. 2013;13:971–83.

Sereger S, Alpers CE. Chemokines and chemokine receptors in renal pathology. Curr Opin Nephrol Hypertens. 2003;12:243–9.

Sereger S, Mack M, Regele H, Kerjaschki D, Schlöndorff D. Expression of the C-C chemokine receptor 5 in human kidney diseases. Kidney Int. 1999;56:52–64.

Sereger S, Nelson PJ, Schlondorff D. Chemokines, chemokine receptors, and renal disease: from basic science to pathophysiologic and therapeutic studies. J Am Soc Nephrol. 2000;11:152–76.

Sheu JN, Chen MC, Lue KH, Cheng SL, Lee IC, Chen SM, Tsay GJ. Serum and urine of interleukin-6 and interleukin-8 in children with acute pyelonephritis. Cytokine. 2006;36:276–82.

Silva JM, Diniz JS, Simões e Silva AC, Azevedo MV, Pimenta MR, Oliveira EA. Predictive factors of chronic kidney disease in severe vesicoureteral reflux. Pediatr Nephrol. 2006;21:1285–92.

Silva JM, Diniz JS, Lima EM, Pinheiro SV, Marino VP, Cardoso LS, Colosimo EA, Simões e Silva AC, Oliveira EA. Independent risk factors for renal damage in a series of primary vesicoureteral reflux: a multivariate analysis. Nephrol (Carlton). 2009;14:198–204.

Simões e Silva AC, Silva JM, Diniz JS, Pinheiro SV, Lima EM, Vasconcelos MA, Pimenta MR, Oliveira EA. Risk of hypertension in primary vesicoureteral reflux. Pediatr Nephrol. 2007;22:459–62.

Souto MFO, Teixeira AL, Russo RC, Penido MGMG, Silveira KD, Teixeira MM, Simões e Silva AC. Immune mediators in idiopathic nephrotic syndrome: evidence for a relationship between interleukin 8 and proteinuria. Pediatr Res. 2008;64:637–42.

Stangou M, Alexopoulos E, Papagianni A, Pantzaki A, Bantis C, Dovas S, Economidou D, Leontsini M, Memmos D. Urinary levels of epidermal growth factor, interleukin-6 and monocyte chemoattractant protein-1 may act as predictor markers of renal function outcome in immunoglobulin A nephropathy. Nephrol (Carlton). 2009;14:613–20.

Stangou M, Papagianni A, Bantis C, Moisiadis D, Kasimatis S, Spartalis M, Pantzaki A, Efstratiadis G, Memmos D. Up-regulation of urinary markers predict outcome in IgA nephropathy but their predictive value is influenced by treatment with steroids and azathioprine. Clin Nephrol. 2013;80:203–10.

Stephan M, Conrad S, Eggert T, Heuer R, Fernandez S, Huland H. Urinary concentration and tissue messenger RNA expression of monocyte chemoattractant protein as an indicator of the degree of hydronephrotic atrophy in partial ureteral obstruction. J Urol. 2002;167:1497–502.

Tang S, Leung JC, Abe K, Chan KW, Chan LY, Chan TM, Lai KN. Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells in vitro and in vivo. J Clin Invest. 2003;111:515–27.

Taranta-Janusz K, Wasilewska A, Dębek W, Waszkiewicz-Stojda M. Urinary cytokine profiles in unilateral congenital hydronephrosis. Pediatr Nephrol. 2012;27:2107–13.

Vasconcelos MA, Bouzada MC, Silveira KD, Moura LR, Santos FF, Oliveira JM, Carvalho FF, Teixeira MM, E Silva AC, Oliveira EA. Urinary levels of TGF β-1 and of cytokines in patients with prenatally detected nephrouropathies. Pediatr Nephrol. 2011;26(5):739–7.

Vianna HR, Bouissou CMMS, Tavares MS, Teixeira MM, Simoes e Silva AC. Inflammation in chronic kidney disease: role of cytokines. Braz J Nephrol. 2011;33:351–64.

Vianna HR, Soares CM, Silveira KD, Elmiro GS, Mendes PM, de Sousa Tavares M, Teixeira MM, Miranda DM, Simões e Silva AC. Cytokines in chronic kidney disease: potential link of MCP-1 and dyslipidemia in glomerular diseases. Pediatr Nephrol. 2013;28:463–9.

Viedt C, Dechend R, Fei J, et al. MCP-1 induces inflammatory activation of human tubular epithelial cells: involvement on transcription factors, nuclear factor-kappaB and activating protein-1. J Am Soc Nephrol. 2002;13:1534–47.

Vielhauer V, Anders HJ, Mack M, Cihak J, Strutz F, Sangasswinger M, Luckow B, Gröne HJ, Schlöndorff D. Obstructive nephropathy in the mouse: progressive fibrosis correlates with tubulointerstitial chemokine expression and accumulation of CC chemokine receptor 2- and 5-positive leukocytes. J Am Soc Nephrol. 2001;12:1173–87.

Vielhauer V, Berning E, Eis V, Kretzler M, Segerer S, Strutz F, Horuk R, Gröne HJ, Schlöndorff D, Anders HJ. CCR1 blockade reduces interstitial inflammation and fibrosis in mice with glomerulosclerosis and nephrotic syndrome. Kidney Int. 2004;66:2264–78.

Wada T, Tomosugi N, Naito T, Yokoyama H, Kobayashi K, Harada A, Mukaida N, Matsushima K. Prevention of proteinuria by the administration of anti-interleukin 8 antibody in experimental acute immune complex-induced glomerulonephritis. J Exp Med. 1994;180:1135–40.

Wada T, Furuichi K, Sakai N, Iwata Y, Yoshimoto K, Shimizu M, Takeda SI, Takasawa K, Yoshimura M, Kida H, Kobayashi KI, Mukaida N, Naito T, Matsushima K, Yokoyama H. Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions of human diabetic nephropathy. Kidney Int. 2000;58:1492–9.

Wang Y, Chen J, Chen L, Tay Y-C, Rangan GK, Harris DCH. Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. J Am Soc Nephrol. 1997;8:1537–45.

Wu H, Wang Y, Tay Y-C, Zheng G, Shang C, Alexander S, et al. DNA vaccination with naked DNA encoding MCP-1 and RANTES protects against renal injury in Adriamycin nephropathy. Kidney Int. 2005;67:2178–86.

Xie C, Liu K, Fu Y, Qin X, Jonnala G, Wang T, Wang HW, Maldonado M, Zhou XJ, Mohan C. RANTES deficiency attenuates autoantibody-induced glomerulonephritis. J Clin Immunol. 2011;31:128–35.

Yadav A, Saini V, Arora S. MCP-1: chemoattractant with a role beyond immunity: a review. Clin Chim Acta. 2010;411:1570–9.

Yokoyama H, Wada T, Furuichi K, Segawa C, Shimizu M, Kobayashi K, Su S, Mukaida N, Matsushima K. Urinary levels of chemokines (MCAF/MCP-1, IL-8) reflect distinct disease activities and phases of human IgA nephropathy. J Leukoc Biol. 1998;63:493–9.

Kidney and Neoplastic Disease: Overview with a Particular Interest to Interpretation of Cancer Biomarkers

12

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Abstract

Tumor markers represent useful tools in diagnosis and clinical management of patients with cancer, because they are easy to use, minimally invasive, and easily measured either by blood or urine. Unfortunately, such an ideal marker, as yet, does not exist. Different pathological states may increase the level of a tumor marker in the absence of any neoplasia, or alternatively during these conditions, not every subject with cancer has abnormally high levels of the tumor marker usually associated with that neoplasia. We aimed at reviewing study literature examining the association between tumor markers and different renal impairment conditions. Each tumor marker was found to be differently influenced by these criteria; additionally, we revealed in many cases a lacking of available published data.

Keywords

Tumor markers • Chronic renal failure • Renal impairment • Hemodialysis • Transplant

Abbreviations

ADPKD	Autosomal dominant polycystic kidney disease
AFP	Alpha-fetoprotein
ARF	Acute renal failure
Beta-hCG	Beta-human chorionic gonadotropin
CAPD	Continuous ambulatory peritoneal dialysis
CEA	Carcinoembryonic antigen
CKD	Chronic kidney disease
CRF	Chronic renal failure
ESRD	End-stage renal disease
NSAIDs	Nonsteroidal anti-inflammatory drugs
NSE	Neuron-specific enolase
PSA	Prostate-specific antigen
PTH	Parathyroid hormone

Key Facts of Renal Disease Subsequent to Neoplasia

- Renal impairment of different origin and severity occurs in patients affected by a neoplasia.
- It could be caused by a prerenal volume depletion, for example, due to the vomiting and diarrhea associated with chemotherapy.
- It may be originated by a postrenal compression to excretory system but also by multiple intrarenal causes of kidney function impairment, including glomerular, tubulointerstitial, and vascular diseases.

Key Facts of Neoplasia Risk in Renal Patients

- Patients in different stages of chronic kidney disease, but in particular in renal failure requiring substitutive therapy with hemodialysis or peritoneal dialysis, have an increased risk.
- Probably as a result of the accumulation of uremic toxins, patients in chronic renal failure are in immunosuppressed state and more prone to translate into an increased incidence of cancer.
- In renal transplant patients, the risk is furtherly increased by immunosuppression therapy.

Key Facts of Neoplasia Risk in Renal Patients

- Elevated levels of several tumor markers can be frequently detected in patients with impaired kidney function because their renal elimination is retarded.
- In other cases, neoplasia is really present, given the higher risk of developing malignancies in these patients.
- During renal replacement therapy by hemodialysis or peritoneal dialysis, tumor markers could result even lower because of removal by the dialysis procedure.
- Consequently, while cancer screening and surveillance are important in this population, the possibility of false-positive results notably reduces the diagnostic value of those markers that are mainly eliminated by renal excretion.

Definitions

Chronic kidney disease (CKD) A pathological condition characterized by a reduced renal function with consequent alterations in fluid regulation, blood pressure control, waste product elimination, and metabolic alterations. CKD may progress, more or less rapidly, to end stages.

End-stage renal disease (ESRD) The final stage of CKD, also known as terminal uremia. Residual renal function is not anymore sufficient to control the body homeostasis so that patients need to start in due course chronic hemodialysis treatment or be transplanted.

Hemodialysis (HD) Chronic or acute therapy for replacing renal function. ESKD patients usually undergo chronic hemodialysis thrice a week. Each HD session lasts 3.5–4 h. During the HD session, fluid and electrolyte excess and waste products are removed from the blood circulation.

Peritoneal dialysis (PD) Alternative technique to hemodialysis consisting in using the patient's own peritoneal membrane as an exchange surface for balancing fluid, electrolyte, and waste homeostasis. The peritoneal cavity is accessed via a

permanent catheter placed in the patient's abdomen that is linked to an external machine. Exchanges are induced and regulated according to diffusion and convection principles by fluid bags with established content.

Introduction

Cancer biomarkers are useful tools for cancer diagnosis and clinical management of neoplastic patients. Additionally, their determination involves mini-invasivity for the patient and simple lab procedures. Although reference ranges have been developed for the correct interpretation of cancer biomarkers, kidney function often represent a confounder, due to several different aspects influencing renal load and clearance in the course of neoplasia. In fact, renal involvement is a frequent event associated with cancer. By contrast, a preexisting renal impairment represents an independent-risk factor for cancer development. Finally, in the presence of all cause of renal function decline, cancer markers raise without a clinical meaning (Coppolino et al. 2014). In the following paragraphs discussed in detail are all these issues, with particular attention to the interpretation of cancer markers as diagnostic and prognostic lab parameters influenced by the renal function.

Renal Disease Associated with Malignancies

The occurrence of renal failure is an additional morbidity and mortality risk factor in the course of neoplasia (Lameire et al. 2005). Renal involvement occurs as consequence of the neoplasia or due to the nephrotoxicity of antitumoral drugs (Table 1). Acute renal failure (ARF) is the most frequent renal complication induced by chemotherapeutic treatment occurring in 12–49 % of terminal cancer patients. Although a preexisting renal impairment might influence the outcome, it is estimated that 9–32 % of ARF cancer patients need hemodialysis showing a high mortality rate (72–85 %) (Darmon et al. 2006). Chronic renal failure (CRF) accompanied with clinical appearance of nephrotic syndrome or isolated proteinuria or tubulopathy is another frequent renal complication associated with cancer and its treatment (Humphreys et al. 2005). More often than in noncancer-associated CRF, electrolyte disorders occur. Hypercalcemia is mainly due to local osteolysis but can also depend on the ectopic production of PTH-like peptides and/or calcitriol (Stewart 2005). Sodium homeostasis is often altered for several reasons such as paraneoplastic antidiuretic hormone inappropriate secretion, gastrointestinal losses, and/or diabetes insipidus. Kalemia and magnesemia abnormalities might occur due to associated ARF or to electrolyte loss in the renal tubule or in the gastrointestinal tract. A milder presentation of renal involvement is common in oncohematologic diseases, showing trend to reversibility after treatment suspension and/or disease remission (Manning et al. 1996).

The tumor lysis syndrome is another remarkable cause of renal impairment. The syndrome is determined by metabolic abnormalities such as hyperuricemia,

Table 1 Causes of renal failure in cancer patients

Prerenal
Extracellular fluid depletion (vomiting, diarrhea, and hyperkalemia)
Hepatorenal syndrome (VOD and hepatic resection)
Drugs (calcineurin inhibitors and NSAIDs)
Renal
Glomerular membranous nephropathy amyloidosis (MM)
Pamidronate-associated glomerulopathy (incidence unknown) LCDD
Acute tubulointerstitial necrosis (toxic/ischemic)
Lymphomatous renal infiltration
LCDD
Drug (cisplatin and ifosfamide) endovenous contrast media
Cast nephropathy (MM)
Vascular thrombotic thrombocytopenic purpura/hemolytic uremic syndrome
Tumor infiltration (renal cell carcinoma with renal vein thrombosis)
Postrenal
Intratubular obstruction uric acid nephropathy Methotrexate
Cast nephropathy (MM) extrarenal obstruction
Ureteral diseases (primary diseases and retroperitoneal lymphadenopathies)
Retroperitoneal fibrosis

LCDD light-chain deposition disease, *MM* multiple myeloma

hyperkalemia, hypocalcemia, hyperphosphatemia, and renal failure related to rapid tumoral cell lysis due to apoptosis or chemotherapy.

With regard to pathophysiological mechanisms determining renal involvement in the course of neoplastic disease, it is practical and useful to look for obstructive, parenchymal, or prerenal *primum movens* for a correct diagnosis and treatment modulation. In more than half cases, vomiting or/and diarrhea related to antineoplastic treatment, especially if not balanced by appropriate liquid intake due to ureteral obstruction or to renal hypoperfusion, induce renal damage. The evidence of acute postrenal failure due to bilateral obstruction in patients not diagnosed for cancer and in the absence of lithiasis has to be highly suspected for a neoplastic obstructive cause (Rosad et al. 2014). The obstruction can affect any urinary segment, even in the absence of hydronephrosis, and can depend on extrinsic mechanical obstructive causes (retroperitoneal lymphadenopathy, metastases of urogynecological cancers) (Wong et al. 2007) or on *ab intrinseco* blockage to urinary flow. As for intrinsic obstruction, regardless of the cause (tubular deposit of urate or calcium phosphate crystals in high-turnover cancers; direct crystallization of methotrexate), the general indication is to maintain an adequate urine output, evaluating the administration of urate-lowering therapy and urinary alkalizing agents, aimed at implementing renal damage prophylaxis (Kjellstrand et al. 1974; Thomas and Chisholm 1973; Buemi et al. 2009).

Renal parenchymal damage occurs with a variable frequency (6–60 %) in cancer patients. Microangiopathy, disseminated intravascular coagulation, tumoral infiltration of the renal tissue, and tumor lysis are typical causes of parenchymal kidney

impairment strictly dependent on tumoral disease. Also, severe forms of acute leukemia or lymphoma induce parenchymal impairment when able to infiltrate both kidneys causing a nephromegaly without hydronephrosis, renal function loss, and/or urinary abnormalities (Simsek et al. 2003). Solid tumors infrequently infiltrate or metastasize the kidney. However, primary lung, breast, and stomach tumors are described for metastasizing the kidney (Wagle et al. 1975). Another interesting modality for renal damage classification in neoplastic patients is based on determining the nephron portion primarily affected. Glomerular paraneoplastic syndromes are generally due to the intraglomerular deposition of amyloid or neoplastic antigens. In most cases, a secondary membranous glomerulonephritis can be detected, representing 9 % of all biopsies diagnostic for glomerulonephritis. Therefore, the finding of a membranous glomerulonephritis could be a "spy" for occult malignancy in adults and should be followed by neoplastic screening, especially looking for lung and gastrointestinal cancer (Birkeland and Storm 2003; Burstein et al. 1993). Beside cancer-related parenchymal causes, the chemotherapeutic agents' nephrotoxicity is a major factor for renal involvement in the course of cancer. In fact, antitumoral drugs, such as bisphosphonates and mitomycin C, can induce a specific glomerular damage. Pamidronate at high-dose infusion commonly induces collapsing focal and segmental glomerulosclerosis (Perazella and Markowitz 2008), while treatment of solid tumors with mitomycin C significantly correlates with thrombotic microangiopathy occurrence (Antman et al. 1979). Tubulointerstitial nephropathy secondary to cancer is a common example of tubular involvement mainly depending on cisplatin and ifosfamide effects on proximal tubule and being potentially age dependent (Kintzel 2001). A well-established correlation between neoplasia and tubulointerstitial damage is also described for multiple myeloma with remarkable prevalence (20 %). Moreover, renal involvement in the course of multiple myeloma has to be considered as a marker of disease severity and predictor of mortality (Blade et al. 1998). Although multiple myeloma represents 1 % of all-type cancers, end-stage renal disease (ESRD) secondary to multiple myeloma has accounted for 58 % of all cases of cancer-related kidney damage between 1997 and 2001. The renal deposition of monoclonal light chains in the course of multiple myeloma induces three different mechanisms of renal damage, depending on amyloidosis, light-chain deposition disease, or cast nephropathy (2003; Tang et al. 1989).

Malignancy Associated with Renal Disease

Development of cardiovascular and infectious complications is the main contributor for high mortality in patients with renal failure. However, the occurrence of cancer remains a major concerning comorbidity factor increasing mortality rate in both ARF and CRF. Several data suggest a role for CRF as an independent-risk factor for cancer development, with high incidence rates (Vajdic et al. 2006; Wong et al. 2009). According to the Michigan Kidney Registry collected between 1973 and 1984, prostate cancer, renal cell carcinoma, and cervical carcinoma are the most common tumoral diseases occurring in CRF patients (Arican et al. 1999). Even neoplasms arising in other organs such as the liver, thyroid, and tongue resulted more frequently

in CRF patients than in the general population. Among oncohematologic diseases, multiple myeloma and non-Hodgkin lymphoma are the most commonly associated with CRF, especially in patients with glomerulonephritis. Bladder transitional cancer has also being found associated with nonsteroidal anti-inflammatory drug (NSAID) nephropathy (Maisonneuve et al. 1999). Not surprisingly, a different geographical distribution has been found for kidney and urinary tract tumors. Kidney cancers are more common in Europe, Australia, and New Zealand, while urinary tract cancers show prevalence peaks in Taiwan and in the Balkan nephropathy-endemic area, mostly affecting females and localizing in the upper urinary tract (Wang et al. 2014). ESRD patients undergoing renal replacement treatment are considered at high-risk population for developing cancer with an overall standardized cancer incidence of 1:18. Moreover, cancer incidence in ESRD patients appears age correlated showing prevalence three times higher in over 65-year-old patients compared to younger patients. Dialysis age is an additional risk factor for cancer-related mortality with highest incidence of neoplasm occurrence after 3 years of dialytic treatment (Collins et al. 2003). A specific tumoral disease associated with kidney transplant is Kaposi sarcoma. Kidney transplant together with glomerulonephritis treated with immunosuppressants is a condition at higher risk than renal insufficiency alone for cancer development. Latrogenic influence by immunosuppressant agents in fact adds the impairment of DNA repair mechanisms and alterations of immunosurveillance processes, to the accumulation of carcinogenic agents and the reduced antioxidant response due to the renal impairment (Kooman et al. 2014; Heidland et al. 2000; Wang et al. 2014). Another renal disease showing an increased risk for neoplastic transformation is the autosomal dominant polycystic kidney disease (ADPKD) due to the tendence to malignant evolution of renal cysts. An additional factor predisposing to cancer in ADPKD is represented by long-term abuse of NSAIDs as painkillers, which contributes to metaplasia occurrence in the bladder (Stewart et al. 2003).

Breast Cancer

Breast cancer is the most common cancer in the world, although females are prevalently affected. In 2008, more than one million of cases of breast cancer were diagnosed, with a higher incidence in industrialized areas (North America, Australia, New Zealand, Northwest Europe, South Asia, and sub-Saharan Africa), confirming the influence of environmental risk factors in cancer pathogenesis (Parkin et al. 2005; Kajbaf et al. 2002). The prevalence of breast cancer in women with CRF is comparable to the general population. However, life expectancy is reduced due to the comorbidity influence on death rate. It remains valid the recommendation for annual mammography screening after menopause with anticipation in women over 40 years old on hemodialysis treatment and waiting for kidney transplantation (Holley and Von Roenn 2010). However, mammography is more difficult to interpret in these patients because of the interference of vascular calcifications (Castellanos et al. 2006; Siegel et al. 2013). Breast cancer biomarkers, CA 15-3, CA 27.29, and CEA, cannot be used for diagnostic purpose in renal patients due to the tendence to

accumulation. However, they could find space as recurrence markers in the follow-up. Other tissutal markers, particularly estrogen and progesterone receptors, were identified as prognostic indices for predicting tumor aggressiveness and response to treatment.

Colorectal Cancer

Colorectal cancer is the second tumor most diagnosed in women and the third in men. However, men are more affected than women in absolute. In recent years, a trend to reduction of prevalence and incidence has been registered, probably due to screening programs and improved therapy efficacy. However, in 2008 over one million of incident cases and over 600,000 deaths for colorectal cancer were reported with variable geographical distribution (Jemal et al. 2011, 2013). According to general data, colorectal cancer has a higher incidence in kidney transplant recipients, overlapping that of 60-year-old subjects that are reported to have the highest risk for colorectal cancer. Hence, the current recommendation is for starting antineoplastic screening in all 40-year-old kidney transplant recipients or, regardless of anagraphic age, at 5-year after transplant (Park et al. 2010). Patients on chronic hemodialysis have also a greater risk for developing colorectal cancer. However, reliability of fecal occult blood for determining eventual indication for colonoscopy is limited in this population due to the high frequency of gastritis and gastrointestinal telangiectasias (Ajam et al. 1990). On the other hand, not even cancer markers are reliable tools for diagnosing colorectal cancer in both general population and patients with renal failure. CEA and CA 19-9, in fact, result altered in the advanced tumoral disease, limiting their usefulness to the follow-up. Moreover, CA 19-9 specificity is low, being shared as a marker also by pancreatic cancer.

Women-Specific Tumoral Diseases: Cervical Cancer and Ovary Cancer

The uterine cervical cancer is the third most common cancer in sexually active women, because it is closely related to HPV infection. Being considered a sexually transmitted disease, about 85 % of new cases occur in developing countries. The squamous cell variant has a higher prevalence compared to adenocarcinoma. In the United States, 12,360 new cases were reported in 2014, accounting for an estimation of 4,020 cancer-related deaths, the equivalent of about 1.5 % of cancer deaths in women (Siegel et al. 2014; Jemal et al. 2011). Screening for uterine cervical cancer is entrusted to cervical cytology and to the HPV DNA individuation. Controversial is the utility of the HPV vaccination aimed at protecting women from the high-risk viral genotypes responsible for 70 % of uterine cervical cancer (Ault 2006). In CKD patients and in hemodialysis patients waiting for a renal transplant, HPV vaccination has not yet a strong indication. However, in over 21-year-old renal transplant candidates, a strong recommendation applies to annual Pap test and HPV DNA assay (Kajbaf et al. 2002). The second most common gynecological neoplasia is

ovarian cancer, although it belongs to the group of rare cancers. In industrialized countries, it is estimated that ovarian cancer incidence is equivalent to 9,4 new cases per 100,000, and mortality rate reaches 5.1 related deaths per 100,000. Furthermore, it is consistent that the risk for ovarian cancer development increases with age. The epithelial variant of ovarian cancer is the most common accounting for 95 % of cases. The most specific marker for epithelial ovarian cancer is CA 125, although CA 72-4, CEA, and LASA-P are other less-specific but useful markers for such variant. The germ cell variant of ovarian cancer is more sporadic and correlates with the increase of human chorionic gonadotropin (hCG) and AFP. However, regardless of the variant, the diagnosis of ovarian cancer is often tardive due to its radiological escape, and not so rarely, the diagnosis is based on the exploratory surgery (Siegel et al. 2013). However, the use of the high-specific markers hCG and AFP appears useful not only in the follow-up but also in the diagnosis of the germ cell variant, while CA 125 is validated only for the follow-up of the epithelial variant of ovarian cancer.

Lung Cancer

The lung cancer is the leading cause of death for cancer in men and the second in women with a variable incidence not only by gender but also with respect to the geographical area and the smoking cigarette habit (Parsons et al. 2010). It is remarkable that most data on lung cancer come from industrialized countries, where industrial fumes and environmental pollution could play a major role. A previous renal impairment does not appear as a significant risk factor for lung cancer, while renal complications due to chemotherapy are very common. Apart from carcinoembryonic antigen (CEA) in ongoing non-small cell lung cancer and neuron-specific enolase (NSE) in small-cell lung cancer in patients with intact renal function, lab markers show low specificity and sensibility in early detecting lung cancer. On the contrary, tomography screening is recognized to be effective and reliable and, thus, is recommended in all cigarette smokers with more than 30 years of exposure (Boiselle 2013).

Liver Cancer

Liver cancer is another leading form of neoplastic disease with different incidence within men and women. Liver cancer is fifth neoplastic disease diagnosed in men and the second leading cause of death for cancer. Instead, in women, it represents the seventh carcinoma for prevalence and the sixth causing death for cancer. The estimated incidence in the United States in 2010 was six cases per 100,000 in adults and 0.05 cases per 100,000 in children (El-Serag and Kanwal 2014). Because of his aggressiveness, the incidence of liver cancer virtually coincides with the associated mortality rate. Chronic HBV or HCV infection is the major causal determinant of hepatocellular carcinoma (Perz et al. 2006; Allan et al. 2014). The cornerstone of

liver cancer screening and follow-up, especially in patients with cirrhosis and/or hepatitis, is alpha-fetoprotein (AFP). However, the routinary use of AFP as screening has been found more useful in Asia, where there is a greater spread of liver cancer.

Prostate Cancer

Prostate cancer is the most common neoplasm in men and in patients with CKD (Port et al. 1989). Overall, prostate cancer is the most diagnosed within visceral cancers with a report in the United States of 200,000 new cases and 30,000 related deaths in 2014. On this basis, all American men show an estimated risk for developing prostate cancer of 16 % and a risk for prostate cancer-related mortality of 2.9 % (Siegel et al. 2014). Consequently, prostate cancer is the leading cause of cancer-related death in men after nonmelanoma skin cancer and lung cancer. Furthermore, epidemiological data tend to underestimate the magnitude of the problem. In fact, slow-progressor patients can remain clinically silent, and prostate cancer is often found incidentally at autopsy (Dorr et al. 1993). The 5-year prognosis for prostate cancer is strongly influenced by the timing of the diagnosis, being very favorable in the case of local extension of the cancer and extremely poor for metastatic tumors. In the past, the obsolete prostatic acid phosphatase was the only available prostate cancer marker but showed very low sensitivity. Since 1990, prostate-specific antigen (PSA) has been validated as the cornerstone marker in the diagnosis and follow-up of prostate cancer. In 1992, in fact the ability of PSA in early detecting the tumor resulted in a peak of prostate cancer diagnosis mainly at a localized stage. However, PSA in rare variants of prostate cancer (e.g., small-cell neuroendocrine tumor) is not affected and could represent a confounder delaying the diagnosis. Instead, the most reliable cancer marker for these atypical prostate tumors is chromogranin A. These are tumors responsive to hormone therapy, which profit instead of chemotherapy. Their diagnosis may be delayed only by the observation of the PSA or, on the contrary, confirmed by an alteration in the levels of chromogranin A. PSA appears also as a very useful tool in the assessment of the response to treatment or relapse. An increase of PSA after prostatectomy is a sign of relapse of the disease. A different behavior is observed after radiotherapy. PSA values, in fact, undergo a drastic reduction after treatment with a gradual return to normal levels within a few years, and the recovery is identified only with a further PSA increase exceeding the standard range. Unfortunately, PSA levels are influenced by the renal function; therefore, its use as routinary screening is controversial in CKD patients. A cost-benefit balance should be assessed between the trend to overdiagnosis leading to improper prostate biopsies and the recognized advantage deriving from an early detection of prostate cancer (Smith et al. 2001; Khairullah et al. 2004). However, the utility of PSA as routinary screening is more consolidated in kidney transplant and in young patients undergoing dialysis, although in these patients cannot be considered as a marker of tumor aggressiveness and extension (Joseph et al. 2010).

Tumors of the Urinary Tract: Kidney, Ureters, and Bladder

Renal cell carcinoma affects preferentially male subjects in old age and has a high incidence in the Czech Republic and North America (Siegel et al. 2014). The kidney cancer tends to develop from a renal cyst especially in the context of a hereditary renal cystic disease with a risk about 30 times higher than the general population. In contrast, patients with acquired renal cysts seem to have a risk of neoplastic transformation approximately comparable to the general population (6 %) (Brennan et al. 1991; Truong et al. 1995). Also, in patients with a cystic hereditary disease, the tumor assumes clinical characteristics that differ from sporadic renal tumors, having a multicentric and bilateral localization multicentric with a sarcomatoid aspect (Keith et al. 1994). Upper urinary tract cancer and CKD appear bidirectionally related probably due to the action of some oncogenic nephrotoxins, such as aristolochic acids and analgesics. These cancerogenic agents not only increase the risk for cancer development but also are responsible for chronic interstitial nephritis, accounting for renal failure occurrence in 80 % of cases with progression to ESRD in 10 % of patients. Hematuria is the main clinical symptom of urinary tract cancer; however, its onset in patients on hemodialysis cannot be considered as highly significant due to additional causes of hematuria such as intradialytic heparinization. Furthermore, urinary cytology and cystoscopy, especially in anuric patients, show low sensitivity and require the integration with imaging for the diagnosis definition. In specific geographical areas in Asia (China and Taiwan), the prevalence of CKD in patients with upper urinary tract carcinoma exceeds 55 %, and a strong correlation has been reported with all age, aristolochic acid nephropathy and previous nephroureterectomy. Moreover, Hung et al. demonstrated a linear correlation between the prevalence of cancer and CKD severity, being prevalence increased up to 55 % and 71 % in CKD stages 4 and 5, respectively. Such correlation was found stronger in female patients being on chronic hemodialysis treatment or undergone renal transplantation (Wang et al. 2014). Bladder cancer is the fifth most common of cancer in the United States, and 13,000 related deaths are reported annually. Standard detection methods for bladder cancer diagnosis include urine cytology and cystoscopy. Only an early detection of bladder cancer leads to a favorable prognosis, while cytology and cystoscopy are generally performed at advanced stages of the disease. Specific urinary cancer markers such as bladder tumor antigen (BTA) and nuclear matrix protein-22 (NMP22) increase the accuracy of detection of bladder cancer when coupled with cystoscopy. In addition, BTA and NMP22, used alone, are useful for addressing instrumental control timing during the follow-up (Grossman et al. 2005).

Markers of Cancer and Renal Function

Among different hormones, metabolites, immunoglobulins, and antigens recognized as markers of neoplasia, the majority consists of relatively high molecular weight glycoproteins (3,400–5,000 kD) undergoing renal and/or liver metabolism. Their use

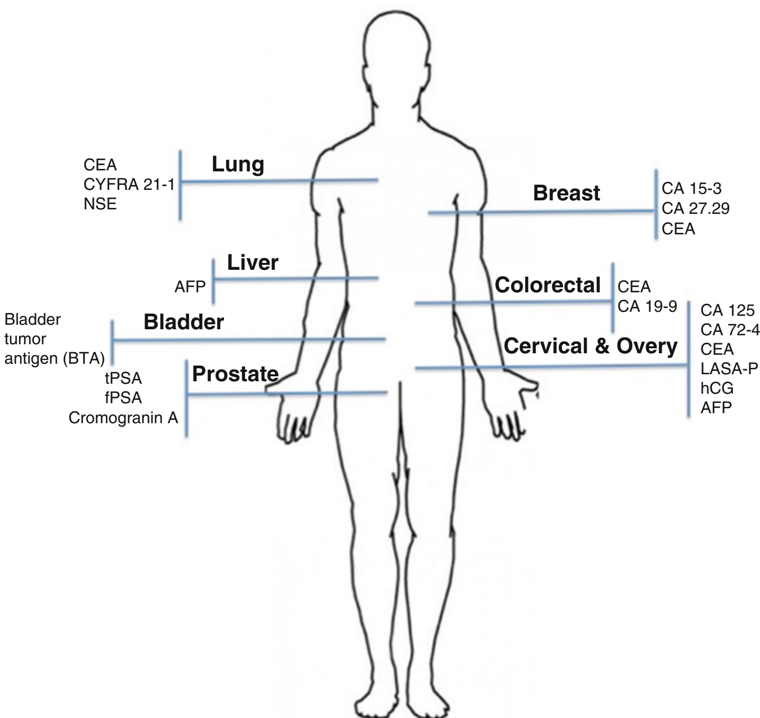

Fig. 1 Representation of main tumor markers on body

in screening for cancer the general population is progressively decreasing due to a poor sensitivity and specificity (Fig. 1). Furthermore, if a renal impairment is present at cancer biomarker determination, their predictive validity and specificity are even more limited due to kinetics alterations (Tzitzikos et al. 2010). Therefore, CRF should be considered as a nonneoplastic disease responsible for altering tumor marker blood concentration. Although the span of error in cancer biomarker determination according to various stages of renal failure has not been quantified, it is notorious that the specificity of some markers and renal failure stage are inversely correlated (Xiaofang et al. 2007). Consequently, cancer biomarker use could result in misleading the diagnostic process or the follow-up if a renal impairment coexists. As consequence, tumor markers should be classified as stable or unstable with respect to renal function. However, many unstable tumor markers tend to restabilize with dialysis, and a relative reduction of their concentration compared to pre-dialysis values should not be interpreted as an improvement of the neoplastic disease (Arik et al. 1996). Among the different variables influencing cancer, biomarker dosing should be considered the pro-inflammatory state associated with CRF, which could influence many biomarker levels. Furthermore, other than CFR, renal diseases can alter the interpretation of tumor markers: proteinuria affects metabolism and excretion of those protein structured; nephrolithiasis can trigger the production of certain

Table 2 Chemical structure and biological function of main tumor markers

Tumor marker	Chemical structure	Biological function
CEA	Glycoprotein	Membrane carcinoembryonic antigen
AFP	Glycoprotein	Alpha-fetoprotein
PSA	Protease	Prostate-specific antigen
CA125	Glycoprotein	Carbohydratic antigen (mucins)
CA19.9	Glycoprotein	Carbohydratic antigen (mucins)
CA15.3	Glycoprotein	Carbohydratic antigen (mucins)
CYFRA	Cytokeratin	Tissue polypeptide antigen
β- hCG	Glycoprotein	Hormonal function

ones already produced in small concentrations from normal tissues. Moreover, an analytical variability intrinsic to the dialysis process should be considered when determining cancer biomarkers. In fact, certain markers tend to accumulate or rapidly decrease based on several treatment parameters such as length, type of dialysis, and hemodialysis membrane characteristics (Lye et al. 1994). Cancer biomarkers have different molecular weights, and this represents an additional reason for the variable behavior of certain biomarkers in hemodialysis patients. In deep, cancer markers having a small molecular weight (<5–50 kD) easily undergo dialysis clearance, while those with a higher molecular weight are only partially removed by dialysis processes (Xiaofang et al. 2007; Table 2). The variability further increases with interindividual, demographic characteristics and comorbidity of the population undergoing treatment (Soletormos et al. 1993). It is particularly useful to evaluate for each patient on dialysis the different comorbidities able to cause an increased tumor marker concentration: inflammatory states of bowel or interesting coelomic epithelium tissues (pleura, pericardium, and peritoneum) and liver disease secondary to viral hepatitis B and C (Arican et al. 1999). This may result in false positive and false negative, with a significant reduction of the specificity of each marker. A remarkable example is CEA, a 180-kDa glycoprotein produced exclusively during fetal development, used as a marker of relapse in several malignancies. Because of its molecular weight, CEA is not removed by any dialysis modality (Arican et al. 1999). On the contrary, despite its hepatic metabolism, CEA level is affected by renal clearance (Lye et al. 1994). Moreover, smoking habit, chronic obstructive pulmonary disease, and chronic inflammatory bowel disease tend to increase CEA levels with high possibility of false-positive results. Biomarkers with independent metabolism are also available. AFP, a 65-kDa-oncofetal protein produced by the fetal liver, yolk sac, and adult liver, is considered as a “historical” marker, which is altered in 80 % of hepatomas and in 60 % of germ cell tumors. AFP is used for the management of hepatocarcinoma, liver metastasis, or non-seminoma testicular cancer. Nonetheless, as expressed by multiple tumors, it shows reduced specificity and can rise even in the course of ovarian cancer. A further example of stable cancer biomarker is PSA, a serine protease regulated by androgens and having a molecular weight of 33-kD. Differentiated cells of ductal and alveolar prostatic epithelium provide to PSA physiological secretion. However, PSA level increases at prostate cancer development resulting the main lab parameter useful for screening, diagnosis, risk

stratification, and follow-up of one of the most incident tumors, even in the dialysis population (Lindblom and Liljegren 2000). Unfortunately, several confounding variables can influence PSA, and recent prostatitis, digital prostatic examination, transrectal ultrasound, and colonoscopy should be excluded before PSA dosing for preserving specificity and lowering risk of false-positive results. A recent optimization in PSA lab determination has been introduced. Circulating PSA exists as free or complexed antigen, and both forms are detectable separately with specific laboratory techniques. This advancement allows to distinguish increases in PSA due to benign causes from prostate cancer. In the absence of the malignancy, an increase in total PSA due to diagnostic procedures or benign prostatic hypertrophy can be individualized in the isolated increase of free PSA (fPSA) levels (Robitaille et al. 2006). Thereby, current indication is for referring to fPSA in general population screening. However, in patients with reduced renal clearance, even fPSA has low specificity. Regardless of race and age, in fact, fPSA tends to accumulate with glomerular filtration rate (GFR) reduction. Similarly, in hemodialysis, patient fPSA accuracy is not optimal due to its low molecular weight and high permeability to dialysis filtration membranes (Joseph et al. 2010). A different class of cancer markers is that of carbohydratic antigens CA 19-9, CA 15.3, and CA 125, for which renal function influence on specificity is not clearly determined. CA19-9 belongs to Lewis blood type carbohydratic antigens and has low specificity (70 %) and high sensitivity (90 %) for malignancies of the gastrointestinal tract and pancreas. CA 15.3 is mainly useful in the surveillance of metastatic breast cancer. However, CA 15.3 specificity is affected by the coexistence of HCV infection and pregnancy, although with less interference from renal function (Han et al. 2012). CA 125 is a 90-kD membrane protein ubiquitary expressed in the eye, respiratory tract, and female reproductive epithelium. CA 125 is widely validated for ovarian cancer screening showing high sensitivity but limited specificity. CA 125 levels are in fact influenced by alterations in serous membranes, a condition frequently associated to both chronic hemodialysis and CRF. Although increased, CA 125 remains more stable during conservative phase of CRF and in hemodialysis, while in peritoneal dialysis, it has an oscillating trend. Finally, after renal transplantation, CA125 levels tend to normalization. This behavior, indeed, suggests a strict participation to CA125 metabolism and/or clearance. Moreover, CA 125 is gender specific and is higher in male CRF patients on conservative treatment (Sevinc et al. 2000). As for peritoneal dialysis, it is the particular case where a chronic stimulation on peritoneum induces a specific CA 125 production even in the absence of ovarian cancer. However, the CA 125 level increase stops when peritoneal sclerosis occurs in response to peritoneal dialysis age progression, and an underlying malignancy could be masked (Tables 3 and 4, and Fig. 2).

Conclusions

Among the wide availability of tumor markers, only a few are clinically relevant due to several reasons. Firstly, most cancer biomarkers are considered tumor associated rather than tumor specific. Their diagnostic power is often garbled, and further

Table 3 Summary of main variations of tumor markers levels in CKD, dialysis, and kidney transplantation (Coppolino et al. 2014)

	CKD	Hemodialysis	Peritoneal dialysis	Kidney transplantation
Alpha-fetoprotein (AFP)	=	=	=	=
Beta-2-microglobulin (B2M)	↑	↑	↑	↑
Beta-HCG	↑	↑	–	–
CA 15-3 and CA 27.29	↑	↑*; =*	–	=
CA 125	=	=	↑ In case of peritonitis or PD catheter placement	=
CA 19-9	=*; ↑*	–	–	–
Total tPSA	=	↓	=	–
Free fPSA	↑	↑	–	–
Chromogranin A	↑	↑	–	↑

Legend: = unvaried with respect to patients with normal renal function; ↑ increased;↓ decreased; – no sufficient data; * see text

Table 4 Hemodialysis removal of main tumor markers

Tumor marker	Molecular weight	Hemodialysis removal (cutoff: <5–50 kDa)
CEA	180 kDa	Not removed
AFP	65 kDa	Not removed
PSA	33 kDa	Removed
CA 125	90 kDa	Not removed
CA 19.9	360 kDa	Not removed
CA 15.3	300–400 kDa	Not removed
CYFRA	40 kDa	Removed
β- hCG	40 kDa (19 kDa for b subunit)	Removed

investigations are usually needed to confirm or countermand the diagnosis. Similarly, cancer biomarkers show a limited ability in monitoring the effectiveness of antineoplastic therapies or malignancy recurrence. Another important issue involves the appropriateness of antineoplastic screening in CRF. To correctly approach the problem, a distinction between CRF stages is mandatory. In young CFR patients on conservative treatment having a positive family history or clinical suspicion for malignancy, screening for cancer finds its rationale as in the general population. Conversely, patients on chronic dialysis should not be routinary screened for malignancy, unless they are a candidate for kidney transplantation. It is well established that patients undergoing chronic dialysis have a shortened life expectancy for different reasons, and the advantage in reducing neoplastic related mortality is not

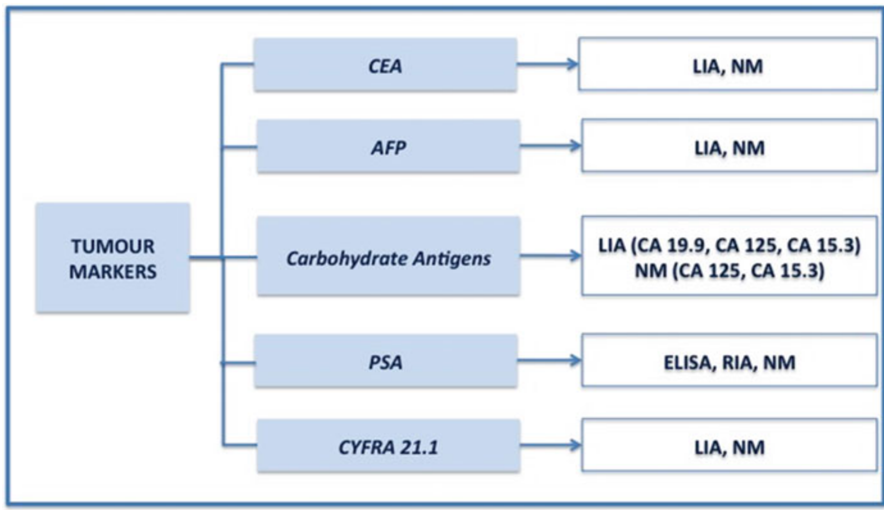

Fig. 2 Method of assay for tumor markers. *LIA* luminescence immunoenzymometric assay, *ELISA* enzyme-linked immunosorbent assay, *RIA* radioimmunoassay, *NM* microarray nanotechnology

relevant (0.2 %) compared to the costs (Kajbaf et al. 2002). Opposite is the case of dialysis patients on the waiting list for kidney transplant, in which, regardless of renal function, it is essential to discover any cancer or precancerous lesion before transplant, as the antirejection therapy may be precipitant. However, in all patients eligible for cancer biomarker screening, the following step consists in the correct interpretation of lab results in consideration of the residual renal function. Accordingly, the most reliable cancer markers are AFP, hCG, and PSA to a certain extent. In fact, compared to CEA and carbohydrates antigens, AFP, hCG, and PSA are not virtually affected by coexisting renal failure or liver infectious diseases or serous membrane diseases. An ideal cancer biomarker should be independent of renal function, stable, sensitive, and specific for achieving the goal of the reliability in the early diagnosis, staging, and monitoring of the neoplastic disease. So far, none of the available cancer biomarkers simultaneously has all these requirements. Hopefully, most advanced research in the future will allow the early detection of cancer and the management of antineoplastic therapy with a simple blood or urine test.

Summary Points

- There is a correlation between renal damage, in its various forms, and neoplastic disease.
- Chronic renal failure predisposes to a higher incidence of cancer than the general population.

- The tumor and/or its treatment can cause kidney damage, both acute and chronic.
- Tumor markers are affected in great part by renal metabolism and, therefore, by alterations of renal function whether preexisting or arisen ex novo.
- AFP is the only reliable tumor marker in case of renal damage.
- The antineoplastic screening in patients with impaired renal function should be individualized to expect life and concomitant risk factors for the development of neoplasia and then interpreted in a critical manner in relation to the behavior of each marker.

References

Ajam M, Ramanujam LS, Gandhi VC, Leehey DJ, Ing TS, Schnell TG, Daugirdas JT. Colon-cancer screening in dialysis patients. Artif Organs. 1990;14:95–7.

Allan BJ, Wang B, Davis JS, Parikh PP, Perez EA, Neville HL, Sola JE. A review of 218 pediatric cases of hepatocellular carcinoma. J Pediatr Surg. 2014;49:166–71. discussion 171.

Antman KH, Skarin AT, Mayer RJ, Hargreaves HK, Canellos GP. Microangiopathic hemolytic anemia and cancer: a review. Medicine (Baltimore). 1979;58:377–84.

Arican A, Ozdemir N, Sezer S, Erten Y, Guz G, Turan M, Inanoglu I, Haberal M. Tumor markers in hemodialysis patients. Transplant Proc. 1999;31:3367–8.

Arik N, Adam B, Akpolat T, Hasil K, Tabak S. Serum tumour markers in renal failure. Int Urol Nephrol. 1996;28:601–4.

Ault KA. Vaccines for the prevention of human papillomavirus and associated gynecologic diseases: a review. Obstet Gynecol Surv. 2006;61:S26–31.

Birkeland SA, Storm HH. Glomerulonephritis and malignancy: a population-based analysis. Kidney Int. 2003;63:716–21.

Blade J, Fernandez-Llama P, Bosch F, Montoliu J, Lens XM, Montoto S, Cases A, Darnell A, Rozman C, Montserrat E. Renal failure in multiple myeloma: presenting features and predictors of outcome in 94 patients from a single institution. Arch Intern Med. 1998;158:1889–93.

Boiselle PM. Computed tomography screening for lung cancer. JAMA. 2013;309:1163–70.

Brennan JF, Stilmant MM, Babayan RK, Siroky MB. Acquired renal cystic disease: implications for the urologist. Br J Urol. 1991;67:342–8.

Buemi M, Fazio MR, Bolignano D, Coppolino G, Donato V, Lacquaniti A, Mondello S, Buemi A, Allegra A. Renal complications in oncohematologic patients. J Invest Med. 2009;57:892–901.

Burstein DM, Korbet SM, Schwartz MM. Membranous glomerulonephritis and malignancy. Am J Kidney Dis. 1993;22:5–10.

Castellanos M, Varma S, Ahern K, Grosso SJ, Buchbinder S, D'Angelo D, Raia C, Kleiner M, Elsayegh S. Increased breast calcifications in women with ESRD on dialysis: implications for breast cancer screening. Am J Kidney Dis. 2006;48:301–6.

Collins AJ, Kasiske B, Herzog C, Chen SC, Everson S, Constantini E, Grimm R, McBean M, Xue J, Chavers B, Matas A, Manning W, Louis T, Pan W, Liu J, Li S, Roberts T, Dalleska F, Snyder J, Ebben J, Frazier E, Sheets D, Johnson R, Li S, Dunning S, Berrini D, Guo H, Solid C, Arko C, Daniels F, Wang X, Forrest B, Gilbertson D, St Peter W, Eggers P, Agodoa L. Excerpts from the United States Renal Data System 2003 Annual Data Report: atlas of end-stage renal disease in the United States. Am J Kidney Dis. 2003;42(A5-7):S1–230.

Coppolino G, Bolignano D, Rivoli L, Mazza G, Presta P, Fuiano G. Tumour markers and kidney function: a systematic review. Biomed Res Int. 2014;2014:647541.

Darmon M, Ciroldi M, Thiery G, Schlemmer B, Azoulay E. Clinical review: specific aspects of acute renal failure in cancer patients. Crit Care. 2006;10:211.

Dorr VJ, Williamson SK, Stephens RL. An evaluation of prostate-specific antigen as a screening test for prostate cancer. Arch Intern Med. 1993;153:2529–37.
El-Serag HB, Kanwal F. Epidemiology of hepatocellular carcinoma in the United States: where are we? Where do we go? Hepatology. 2014;60:1767–75.
Grossman HB, Messing E, Soloway M, Tomera K, Katz G, Berger Y, Shen Y. Detection of bladder cancer using a point-of-care proteomic assay. JAMA. 2005;293:810–6.
Han SN, Lotgerink A, Gziri MM, VAN Calsteren K, Hanssens M, Amant F. Physiologic variations of serum tumor markers in gynecological malignancies during pregnancy: a systematic review. BMC Med. 2012;10:86.
Heidland A, Bahner U, Vamvakas S. Incidence and spectrum of dialysis-associated cancer in three continents. Am J Kidney Dis. 2000;35:347–51. discussion 352–3.
Holley JL, Von Roenn J. Screening for breast cancer in women with CKD stages 4 to 5. Am J Kidney Dis. 2010;56:820–2.
Humphreys BD, Soiffer RJ, Magee CC. Renal failure associated with cancer and its treatment: an update. J Am Soc Nephrol. 2005;16:151–61.
Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69–90.
Jemal A, Simard EP, Dorell C, Noone AM, Markowitz LE, Kohler B, Eheman C, Saraiya M, Bandi P, Saslow D, Cronin KA, Watson M, Schiffman M, Henley SJ, Schymura MJ, Anderson RN, Yankey D, Edwards BK. Annual Report to the Nation on the Status of Cancer, 1975–2009, featuring the burden and trends in human papillomavirus(HPV)-associated cancers and HPV vaccination coverage levels. J Natl Cancer Inst. 2013;105:175–201.
Joseph DA, Thompson T, Saraiya M, Werny DM. Association between glomerular filtration rate, free, total, and percent free prostate-specific antigen. Urology. 2010;76:1042–6.
Kajbaf S, Nichol G, Zimmerman D. Cancer screening and life expectancy of Canadian patients with kidney failure. Nephrol Dial Transplant. 2002;17:1786–9.
Keith DS, Torres VE, King BF, Zincki H, Farrow GM. Renal cell carcinoma in autosomal dominant polycystic kidney disease. J Am Soc Nephrol. 1994;4:1661–9.
Khairullah QT, Pamatmat SD, Chatha M, Provenzano R, Telang D, Temple M. Early detection of prostate cancer in the ESRD population. Clin Nephrol. 2004;61:308–15.
Kintzel PE. Anticancer drug-induced kidney disorders. Drug Saf. 2001;24:19–38.
Kjellstrand CM, Cambell 2nd DC, Von Hartitzsch B, Buselmeier TJ. Hyperuricemic acute renal failure. Arch Intern Med. 1974;133:349–59.
Kooman JP, Kotanko P, Schols AM, Shiels PG, Stenvinkel P. Chronic kidney disease and premature ageing. Nat Rev Nephrol. 2014;10:732–42.
Lameire NH, Flombaum CD, Moreau D, Ronco C. Acute renal failure in cancer patients. Ann Med. 2005;37:13–25.
Lindblom A, Liljegren A. Regular review: tumour markers in malignancies. BMJ. 2000;320:424–7.
Lye WC, Tambyah P, Leong SO, Lee EJ. Serum tumor markers in patients on dialysis and kidney transplantation. Adv Perit Dial. 1994;10:109–11.
Maisonneuve P, Agodoa L, Gellert R, Stewart JH, Buccianti G, Lowenfels AB, Wolfe RA, Jones E, Disney AP, Briggs D, Mccredie M, Boyle P. Cancer in patients on dialysis for end-stage renal disease: an international collaborative study. Lancet. 1999;354:93–9.
Manning EC, Belenko MI, Frauenhoffer EE, Ahsan N. Acute renal failure secondary to solid tumor renal metastases: case report and review of the literature. Am J Kidney Dis. 1996;27:284–91.
Park JM, Choi MG, Kim SW, Chung IS, Yang CW, Kim YS, Jung CK, Lee KY, Kang JH. Increased incidence of colorectal malignancies in renal transplant recipients: a case control study. Am J Transplant. 2010;10:2043–50.
Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55:74–108.

Parsons A, Daley A, Begh R, Aveyard P. Influence of smoking cessation after diagnosis of early stage lung cancer on prognosis: systematic review of observational studies with meta-analysis. BMJ. 2010;340:b5569.

Perazella MA, Markowitz GS. Bisphosphonate nephrotoxicity. Kidney Int. 2008;74:1385–93.

Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol. 2006;45:529–38.

Port FK, Ragheb NE, Schwartz AG, Hawthorne VM. Neoplasms in dialysis patients: a population-based study. Am J Kidney Dis. 1989;14:119–23.

Robitaille R, Lafrance JP, Leblanc M. Altered laboratory findings associated with end-stage renal disease. Semin Dial. 2006;19:373–80.

Rosad J, Sydor A, Sulowicz W. Outcome of patients with acute kidney injury in the course of neoplastic diseases. Przegl Lek. 2014;71:72–7.

Sevinc A, Buyukberber S, Sari R, Kiroglu Y, Turk HM, Ates M. Elevated serum CA-125 levels in hemodialysis patients with peritoneal, pleural, or pericardial fluids. Gynecol Oncol. 2000;77:254–7.

Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013;63:11–30.

Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014;64:9–29.

Simsek S, Oen AL, Comans EF, V D Hoeven JJ, Zijlstra J. Acute renal failure due to non-Hodgkin lymphoma infiltration of the kidneys detected by ultrasonography and confirmed by positron emission tomography. Clin Nephrol. 2003;59:383–7.

Smith RA, Von Eschenbach AC, Wender R, Levin B, Byers T, Rothenberger D, Brooks D, Creasman W, Cohen C, Runowicz C, Saslow D, Cokkinides V, Eyre H, ACS PROSTATE CANCER ADVISORY COMMITTEE, A. C. S. C. C. A. C. A. C. S. E. C. A. C. American Cancer Society guidelines for the early detection of cancer: update of early detection guidelines for prostate, colorectal, and endometrial cancers. Also: update 2001 – testing for early lung cancer detection. CA Cancer J Clin. 2001;51:38–75. quiz 77–80.

Soletormos G, Schioler V, Nielsen D, Skovsgaard T, Dombernowsky P. Interpretation of results for tumor markers on the basis of analytical imprecision and biological variation. Clin Chem. 1993;39:2077–83.

Stewart AF. Clinical practice. Hypercalcemia associated with cancer. N Engl J Med. 2005;352:373–9.

Stewart JH, Buccianti G, Agodoa L, Gellert R, Mccredie MR, Lowenfels AB, Disney AP, Wolfe RA, Boyle P, Maisonneuve P. Cancers of the kidney and urinary tract in patients on dialysis for end-stage renal disease: analysis of data from the United States, Europe, and Australia and New Zealand. J Am Soc Nephrol. 2003;14:197–207.

Tang AL, Davies DR, Wing AJ. Remission of nephrotic syndrome in amyloidosis associated with a hypernephroma. Clin Nephrol. 1989;32:225–8.

Thomas MH, Chisholm GD. Retroperitoneal fibrosis associated with malignant disease. Br J Cancer. 1973;28:453–8.

Truong LD, Krishnan B, Cao JT, Barrios R, Suki WN. Renal neoplasm in acquired cystic kidney disease. Am J Kidney Dis. 1995;26:1–12.

Tzitzikos G, Saridi M, Filippopoulou T, Makri A, Goulioti A, Stavropoulos T, Stamatiou K. Measurement of tumor markers in chronic hemodialysis patients. Saudi J Kidney Dis Transpl. 2010;21:50–3.

USRDS: the United States Renal Data System. Am J Kidney Dis. 2003;42:1–230.

Vajdic CM, McDonald SP, Mccredie MR, VAN Leeuwen MT, Stewart JH, Law M, Chapman JR, Webster AC, Kaldor JM, Grulich AE. Cancer incidence before and after kidney transplantation. JAMA. 2006;296:2823–31.

Wagle DG, Moore RH, Murphy GP. Secondary carcinomas of the kidney. J Urol. 1975;114:30–2.

Wang LJ, Lee SY, Teh BT, Chuang CK, Nortier J. Upper tract urothelial carcinomas in patients with chronic kidney disease: relationship with diagnostic challenge. Biomed Res Int. 2014;2014:989458.
Wong LM, Cleeve LK, Milner AD, Pitman AG. Malignant ureteral obstruction: outcomes after intervention. Have things changed? J Urol. 2007;178:178–83. discussion 183.
Wong G, Hayen A, Chapman JR, Webster AC, Wang JJ, Mitchell P, Craig JC. Association of CKD and cancer risk in older people. J Am Soc Nephrol. 2009;20:1341–50.
Xiaofang Y, Yue Z, Xialian X, Zhibin Y. Serum tumour markers in patients with chronic kidney disease. Scand J Clin Lab Invest. 2007;67:661–7.

Part II

Circulating and Body Fluid Biomarkers

Creatinine Assays in Early Infancy: How to Aim for a Moving Target

13

Karel Allegaert

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Abstract

Glomerular filtration rate (GFR) in neonates is very low and can only be maintained due to a delicate balance between both vasodilatory effects at the afferent and vasoconstrictor effects at the efferent glomerular arteriole. Despite this low clearance capacity, interindividual variability is already extensive and

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can be predicted by covariates (gestational age, birth weight, postnatal age, drugs, growth restriction, or peripartal asphyxia).

We still commonly used creatinine as a proxy for renal clearance capacity. However, before creatinine values can be used to estimate renal elimination capacity, there are some issues that need to be considered related to physiology and methodology. Creatinine at birth does not yet reflect neonatal but maternal creatinine clearance, and because of passive tubular back leak instead of active secretion, creatinine clearance does not yet fully reflect GFR. Trends will be described. Moreover, absolute creatinine values also depend on the technique. The move toward harmonization through isotope dilution mass spectrometry (IDMS) traceability has helped but has not completely solved this problem. In line with recent observations in adults, more research is needed to document the potential add on the benefit of advanced biomarkers (e.g., cystatin C). In the meanwhile, IDMS-traceable creatinine observations, compared to age-dependent, assay-specific reference values, should be used to support clinical decisions.

Keywords

Assay • Clearance • Creatinine • Enzymatic • Glomerular filtration rate • Infant • Isotope dilution mass spectrometry (IDMS) • Jaffe • Maturational changes • Newborn • Preterm • Term

Abbreviations

95 % CI	95 % confidence intervals
AKI	Acute kidney injury
CALIPER	Canadian laboratory initiative on pediatric reference intervals
cGFR	Calculated glomerular filtration rate
CL_{crea}	Creatinine clearance
CLSI	Clinical and laboratory standards institute
Cr-EDTA	^{51}Chrome ethylene diamine tetra acetic acid
CysC	Cystatin C
eGFR	Estimated glomerular filtration rate
ELBW	Extreme low birth weight infants (i.e., $<$1,000 g at birth)
ELISA	Enzyme linked immunosorbent assay
GFR	Glomerular filtration rate
IDMS	Isotope dilution mass spectrometry
IgG	Immunoglobulin G
kDa	Kilodalton
NKDEP	National Kidney Disease Education Program
PENIA	Particle-enhanced nephelometric immunoassay
PETIA	Particle-enhanced turbidimetric immunoassay
pRIFLE	Pediatric risk, injury, failure, loss, end-stage (renal risk score)

S_{cr}	Serum creatinine
SD	Standard deviation
Tc-DTPA	^{99m}Technecium diethylene triamine penta acetic acid

Key Facts of Creatinine Assays and Their Clinical Relevance in Early Infancy

- The assessment of renal function is of clinical relevance. This is because it guides short-term clinical decisions (e.g., pharmacotherapy) and determines long-term outcome (e.g., cardiovascular or renal failure) and prognosis. In clinical practice, this is commonly performed by a single-point serum creatinine (S_{cr}) measurement. These values are subsequent conversion to estimated or calculated glomerular filtration rate (eGFR and cGFR). Obviously, this needs an accurate S_{cr} measurement to maintain the variability due to estimations more limited.
- Historically, serum creatinine is commonly quantified by Jaffe creatinine assays. It is a colorimetric technique method to determine creatinine, based on a proportional color change following a chemical reaction between picric acid and creatinine. However, it is well known that these techniques suffer from interferences with endogenous or exogenous compounds present in the serum. Among those interfering substances, bilirubin is the most prominent compound, but also proteins and drugs can affect the color change. More recently, efforts have been made to reduce these interferences and to standardize through isotope dilution mass spectroscopy (IDMS) traceability.
- Enzymatic methods have been developed more recently to quantify creatinine. Although theoretically more specific, these enzymatic methods can also display interferences. Compared to Jaffe, these reactions suffer less from associated bilirubin. Enzymatic methods use creatininase and creatinine hydrolase to convert creatinine to creatine, with subsequent additional reactions and a color conversion. Similar to Jaffe reactions, efforts have been made to reduce these interferences and to standardize through isotope dilution mass spectroscopy (IDMS) traceability.
- The creatinine clearance rate is the blood volume that is completely cleared of creatinine per unit time (/min or/h) and is commonly used to estimate GFR. In adults, reference values are 90–120 ml/min. These maturational trends also in part depend on the denominator (e.g., weight, body surface area) applied. In early infancy, creatinine clearance is mainly driven by weight at birth (maturation until birth) and by postnatal covariates (postnatal age, perinatal asphyxia, congenital renal malformations, nephrotoxic drugs). In the term neonate, it is 20–45 ml/min/1.73 m^2, with a subsequent fast increase to reach (when/m^2) adult reference values in the second part of the first year of life. In preterm newborns, GFR is much lower at birth (7.9 ml/kg/1.73 m^2), with a similar subsequent fast increase throughout the first weeks and months of life.

- In essence, the between- and within-patient variability of creatinine clearance in early infancy displays at least a fivefold variability. This variability is of utmost importance since this will guide clinical practices (e.g., fluid therapy, pharmacotherapy). Moreover, impaired renal function in early infancy has also been linked to long-term morbidity characteristics (e.g., persistent renal failure, cognitive outcome, retinopathy of prematurity).

Definitions

Neonatal Terminology

Newborn Any child in the first 28 days of postnatal life. This time interval is commonly further subdivided in early neonatal (<day 8) and late neonatal life (day 8–28).

Infant Any child beyond the first 28 days of postnatal life but not yet 365 days old (<1 year).

Preterm Newborn born too soon; normal pregnancy takes 40 weeks of gestational age until delivery. Preterm delivery is any delivery before 37 weeks of gestational age.

ELBW Within the preterm neonates, there is a further subdivision, either based on gestational age or on weight. Extreme low birth weight infants have a birth weight below 1,000 g at delivery.

Bio-Analysis-Related Terminology

Creatinine Creatinine is a degradation product from creatine and is produced at a fairly constant rate, reflecting muscle mass. It is commonly measured as a reflection of renal function.

Creatinine assays The *Jaffe quantification* is a colorimetric reaction method using alkaline picrate. Jaffe assays suffer from interference by endogenous (e.g., pseudocreatinines, hemoglobin F, bilirubin) and exogenous (e.g., cephalosporins). More recently, *enzymatic methods* were introduced. These assays are less prone to such interference-related errors and thus seem more suitable. Nevertheless, enzymatic assays can also be affected by interferences. It is generally accepted that uncompensated Jaffe overestimates Scr and fixed corrections (e.g., 0.2 or 0.3 mg/dl), or adaptations in the analytical procedure (e.g., rate blanking) have been suggested to adapt Jaffe assays observations.

Matrix effect This term refers to the change or the measurement bias of a given quantification method caused by (differences) in the non-analyte matrix. This may be of specific relevance in human biology since the matrix in neonates (e.g., blood)

commonly differs (e.g., albumin, bilirubin, hemoglobin F) from other populations. These differences may affect measurement results and/or accuracy.

Clearance Clearance is defined as the volume of fluid that – for a given time interval – is completely cleared of a specific compound. Creatinine clearance hereby reflects the renal elimination capacity or the glomerular filtration rate.

IDMS traceability Isotope dilution mass spectrometry traceability. This has been introduced to adapt for the differences between different creatinine assays and serves as a golden standard to all currently marketed creatinine assays. This became even more important, since estimated glomerular filtration (eGFR) values are extrapolated from single-serum creatinine (S_{cr}) measurements.

Introduction

Renal function covers glomerular filtration rate (GFR), renal tubular transport activity (excretion, absorption), and diuresis, but serum creatinine (S_{cr}) is a commonly measured and readily accessible biomarker to estimate renal (dys)function. To a certain extent, this reflects the general condition of an individual patient and supports the clinician in the decision-making process to adapt drug dosing, to formulate a prognosis, and to individualize clinical follow-up and monitoring (Levey 2014). This is also true in early infancy since renal dysfunction (reduced clearance, raised creatinine) is associated with higher mortality and morbidity (e.g., retinopathy of prematurity, neurodevelopmental impairment) and is crucial to tailor pharmacotherapy or fluid exposure to the individual newborn (Guignard and Gouyon 2008). Furthermore, an association of extreme preterm birth and renal dysfunction in later life has been reported. More adequate detection of renal dysfunction or impaired clearance in early infancy may hereby serve as an indicator for focused renal follow-up in specific patients (Walker 2011).

Besides clearance assessment based on exogenous compounds like inulin, iohexol, ^{99m}Tc-DTPA (diethylene triamine penta acetic acid), or ^{51}Cr-EDTA (ethylene diamine tetra acetic acid) with consecutive, timed sampling, creatinine clearance or estimated creatinine clearance can be used to quantify GFR. Creatinine clearance (CL_{crea}) can be calculated based on serum and urine creatinine measurements [$CL_{crea} = Crea_{ur}$ (mg/dl) x urine volume (ml/min)/S_{cr}], preferably based on 24 h urine collections (Levey 2014; Guignard and Gouyon 2008). Because of the maturational GFR changes in early life, population-specific reference values are needed. Reported reference values on creatinine clearance in neonates, developmental aspects of urine creatinine values, and related to this, the impact on assays will be covered in this chapter (2.).

Indirect calculations based on single S_{cr} values are also commonly used and are much more convenient. Estimated (eGFR) or calculated GFR (cGFR) is commonly used to screen renal function or to monitor high-risk patients (e.g., hypertension, diabetes mellitus) consecutively. The National Kidney Foundation even strongly recommends automatic calculation every time a serum creatinine measurement has been performed and has provided kidney damage stages (stage 1–5), reflecting

Table 1 Creatinine clearance (CL_{crea}) from single-serum creatinine (S_{cr}) values can be performed, based on Schwartz estimates. Besides isotope dilution mass spectrometry (*IDMS*) traceability, these Schwartz estimates further consider maturational aspects (*k* factor)

Original Schwartz estimate: serum creatinine (S_{cr}) is measured based on a non-IDMS-traceable assay, most likely Jaffe colorimetric technique	
$CL_{crea} = [k \times \text{height}/S_{cr}]$	
k , age-related factor	0.33, in low birth weight infant, <1 year
	0.45, in term infant, <1 year
	0.55, child or adolescent girl
	0.70, adolescent boy
Revised Schwartz estimate: serum creatinine (S_{cr}) is measured based on an IDMS-traceable assay, most likely enzymatic technique	
$CL_{crea} = [0.413 \times \text{height}/S_{cr}]$	

normal or near-normal eGFR (stage 1, >90 ml/min; stage 2, 60–89, both with protein and albumin in urine, cells, or casts) or decreased eGFR (stage 3, 30–59 ml/min; stage 4, 15–29 ml/min; stage 5, <15 ml/min) (US Department of Health and Human Services 2012). Obviously, once again, population-specific reference values are needed since these adult values do not yet apply to early infancy.

A major hurdle to implement the eGFR calculation concept based on single-serum creatinine measurement is the obvious need for standardization and accuracy to reduce inter-assay and intra-assay variation. This is because the assays (Jaffe, enzymatic) for creatinine quantification are commonly biased by various interfering substances, both endogenous (bilirubin, hemoglobin F) and exogenous (cephalosporins, dopamine). This standardization has been driven by the creatinine standardization program as created by the National Kidney Disease Education Program (NKDEP) laboratory working group (US Department of Health and Human Services 2012). Improvement has been made based on calibration traceability, standard reference materials, and isotope dilution mass spectrometry (IDMS) reference measurement for both Jaffe and enzymatic assays (Levey 2014). Once calibrated, creatinine clearance (CL_{crea}) from single-serum creatinine (S_{cr}) values can be performed, based on Schwartz estimates (Delanaye and Ebert 2012). These Schwartz estimates further consider maturational issues, as summarized in Table 1.

Similar to the reference values or intervals in other populations, this standardization process also altered the reference values in young infants and resulted in the need to adapt, e.g., creatinine observations driven dosing regimens and to generate assay-specific reference values (Ceriotti et al. 2008; Ceriotti 2012). The impact of covariates (gestational age, postnatal age, weight) on serum creatinine reference values for both an uncompensated Jaffe and an IDMS-traceable enzymatic assay is discussed (3.).

Because of the specific characteristics of the neonatal serum matrix, it was hypothesized that – even after IDMS standardization – the remaining S_{cr} between-assay differences (IDMS Jaffe versus IDMS enzymatic assay) is affected by population-specific aspects (e.g., bilirubin, albumin, protein) (4.).

Finally, it has been suggested that eGFR values based on cystatin C (CysC) strengthens the association between this marker and the risk of death or end-stage

renal disease across diverse adult populations (Shlipak et al. 2013). The available data on CysC in neonates were therefore discussed in the final part of this chapter (5.). Before we discuss these topics, we will first explore the development of glomerular filtration rate in early infancy, with emphasis on renal maturation (physiology) and covariates that affect this maturational pattern (*patho*physiology) (1.).

Development of Glomerular Filtration Rate in Early Infancy and Its Covariates

Glomerular filtration rate depends on the net ultrafiltration pressure across the glomerulus. The kidney of a term neonate already possesses a full set of nephrons, approximately 850,000–1,200,000 per kidney. Despite this, the GFR in neonates is still very low (2–4 ml/min or 20 ml/min/1.73 m^2) and can only be maintained due to a delicate balance between vasodilatory effects at the afferent and vasoconstrictor effects at the efferent glomerular arteriole (Guignard and Gouyon 2008). These forces recruit maximal transmembrane filtration pressure in a setting of overall low mean arterial blood pressure (30–40 mmHg) and high intrarenal vascular resistance. The postnatal changes are mainly due to changes in renal and intrarenal blood perfusion. The main drivers of renal function at birth besides age are the impressive renal and extra-renal hemodynamic changes and include increased renal blood flow, alterations of intrarenal blood flow, increased blood pressure, and raised cardiac output. This delicate balance however results in relative limited capacity to adapt to endogenous (e.g., peripartal asphyxia, hypotension, sepsis) or exogenous (e.g., nephrotoxic drugs like aminoglycosides or nonsteroidal anti-inflammatory drugs) stressors in early life (Guignard and Gouyon 2008; Smits et al. 2012).

To further put this into some perspective, the GFR is 20–45 ml/min/1.73 m^2 in the term neonate, with a subsequent progressive increase of 5–10 ml/min/1.73 m^2/week. Similar, in the preterm neonate, median GFR reference values in infants aged 27–31 weeks gestation ranged from 7.9 to 30.3 ml/min/1.73 m^2 on day 7, 10.7–33.1 ml/min/1.73 m^2 on day 14, 12.5–34.9 ml/min/1.73 m^2 on day 21, and 15.5–37.9 ml/min/1.73 m^2 on day 28 (fivefold increase) (Guignard and Gouyon 2008). When corrected for body surface area, GFR values reach adult values in the second half of infancy (8 months) (Rhodin et al. 2009). In contrast, when expressed in a weight-corrected approach (/kg), GFR relative to age is severalfold higher in toddlers. Similarly, renal tubular functions (secretion, absorption) also display maturation, but with a somewhat later onset and rate, to reach adult capacity at the end of the first year of life (Guignard and Gouyon 2008; Smits et al. 2012).

Urinary Creatinine Measurements in Early Infancy

Similar to serum, creatinine can also be quantified by Jaffe or enzymatic assays in urine. Urinary creatinine is hereby needed to calculate creatinine clearance as a reflection of GFR or as denominator to quantify urinary excretion (Shlipak et al. 2013; Junge et al. 2004).

In clinical practice, creatinine clearance (CL_{crea}) is commonly used as an indicator of the glomerular filtration rate. In adults, CL_{crea} somewhat overestimates GFR because of the additional active renal tubular secretion of creatinine. In preterm and term neonates, active renal tubular secretion still displays ontogeny, i.e., age-related activity. In addition and due to less effective tight junctions at the level of the apical renal tubular membranes, there is passive back leak of filtered creatinine across the immature leaky tubules. The need for a timed, complete urine collection, e.g., for 12 or 24 h, is another important practical hurdle and limits its use mainly to clinical research (Guignard and Gouyon 2008). Using this approach and based on a standardized Jaffe method, a CL_{crea}-based GFR nomogram for preterm neonates (27–31 weeks) in the first month of life has recently been reported (Vieux et al. 2010). These data and other CL_{crea}-based GFR estimates (all expressed by ml/min/1.73 m^2) in different cohorts as reported in the literature are provided in Table 2 (Vieux et al. 2010; Gordjani et al. 1988; Sonntag et al. 1996; Bueva and Guignard 2004; Gallini et al. 2000). In essence, these pooled cohort data nicely illustrate that GFR estimates depend on both gestational age and birth weight (reflecting renal function at birth), as well as postnatal age. Such reference values in healthy neonates can subsequently be used to quantify the impact of diseases (congenital renal malformation, peripartal asphyxia) or treatment modalities (e.g., indomethacin, ibuprofen) (Rhodin et al. 2009; Smits et al. 2012).

Urinary creatinine is also commonly used as denominator to quantify the urinary excretion or loss of electrolytes, amino acids, or proteins (Allegaert et al. 2014b). An alternative denominator is urine-specific gravity, i.e., the ratio of urine density compared to water density (1.0), with a reference range in adults between 1.005 and 1.030. However, urine density will also be affected by exogenous (e.g., drugs) and endogenous (e.g., glucosuria) compounds not routinely retrieved in urine (Jung 1991). In neonates, the urine-specific gravity reference range is 1.002–1.004 and 1.002–1.006 in infants. Beyond infancy, reference values (1.001–1.030) are similar to adult values (Guignard and Gouyon 2008). These maturational changes largely reflect the progressive increase in concentration capacity throughout infancy. The same pattern, with increasing concentration capacity and increasing renal elimination capacity throughout infancy, can be observed in reference urine creatinine values, as reflected in Table 3. This table summarizes observations on urine creatinine concentrations as reported in cohort of neonates, children, or adults (Sonntag et al. 1996; Srivastava et al. 2008; Van Lente and Suit 1989; Apple et al. 1986; Allegaert et al. 2014b). This maturational trend, with much lower creatinine (5- to 20-fold) values in neonatal urine, may also be of relevance when we consider the creatinine quantification assays (Tables 2 and 3).

Indeed – irrespective of its application (CL_{crea} estimates or denominator) – adequate standardization of creatinine quantification is of utmost importance for accurate and interchangeable test results and reference values. This is also true for the urine analysis. Historically, creatinine assays showed great variation depending on, e.g., reaction mechanism (Jaffe or enzymatic) or manufacturer, but also differences in the concentration range need to be considered (Table 2) (van Lente en Suit 1989; Apple et al. 1986). However, IDMS calibration has been more extensively verified for serum than urine matrix. Moreover, standardization itself does not eliminate reaction mechanism-related differences. Jaffe methods interfere with

Table 2 Glomerular filtration rate reference values, based on creatinine clearance estimates as reported in different neonatal cohorts

Reference	Assay	Clinical characteristics		Creatinine clearance estimates	
Vieux et al. (2010)	Jaffe	146/275 healthy neonates, 27–31 weeks			
		Day 7		19.95 (SD 9.30)	ml/min/ 1.73 m^2
		Day 14		22.11 (SD 14.90)	ml/min/ 1.73 m^2
		Day 21		24.64 (SD 10.88)	ml/min/ 1.73 m^2
		Day 28		27.66 (SD 12.66)	ml/min/ 1.73 m^2
Gordjani et al. (1988)	Jaffe	12 neonates, 28–32 weeks at birth			
		Day 4–5		10.7 (range 9.40–15.3)	ml/min/ 1.73 m^2
		Day 8–10 (7/12)		14.2 (range 12.1–28.7)	ml/min/ 1.73 m^2
		13 neonates, 33–37 weeks at birth			
		Day 4–5		20.6 (range 14.4–31.6)	ml/min/ 1.73 m^2
		Day 8–10 (4/13)		33.3 (range 23.1–43.4)	ml/min/ 1.73 m^2
Sonntag et al. (1996)	Jaffe	34 neonates, 26–34 weeks at birth			
		First week		12.5 (range 7–22)	ml/min/ 1.73 m^2
		Second week		15.7 (range 10–28)	ml/min/ 1.73 m^2
		3–4 week		19.7 (range 11–34)	ml/min/ 1.73 m^2
Bueva and Guignard (2004)	Jaffe	66 healthy neonates, 29–40 weeks at birth		Trends in median estimates	
		Term cases	Day 2–9–15	29 to 46 to 68	ml/min/ 1.73 m^2
		2,000–2,500 g	Day 2–9–15	18 to 32 to 38	ml/min/ 1.73 m^2
		1,500–2,000 g	Day 2–9–15	11 to 23 to 32	ml/min/ 1.73 m^2
		1,000–1,500 g	Day 2–9–15	8 to 17 to 19	ml/min/ 1.73 m^2
Gallini et al. (2000)	Jaffe	83 preterm neonates		Trends in median estimates	
		<27 weeks	Day 3–10–17–31	6 to 11 to 12 to 21	ml/min/ 1.73 m^2
		27–28 weeks	Day 3–10–17–31	8 to 15 to 20 to 24	ml/min/ 1.73 m^2
		29–30 weeks	Day 3–10–17–31	12 to 22 to 24 to 29	ml/min/ 1.73 m^2
		31–32 weeks	Day 3–10–17–31	13 to 23 to 26 to 32	ml/min/ 1.73 m^2

exogenous (e.g., cephalosporins) or endogenous (e.g., pseudo-creatinines, bilirubin, glucose) substances. Enzymatic methods are less prone but also suffer from interferences (e.g., dopamine, bilirubin). Consequently, maturational changes (e.g., bilirubin production, creatinine concentrations) as well as treatment modalities may have a population-specific impact (Guignard and Gouyon 2008; Ceriotti 2012; Allegaert et al. 2014b). Given the method and assay-specific interferences, urinary creatinine may still vary in a method and assay-specific way with between-assay differences related to the composition of neonatal urine and despite the use of IDMS-traceable assays. Taking these abovementioned remarks into account, the interchangeability of two IDMS-traceable assays (Jaffe and enzymatic) was verified in 84 urine samples, collected in 23 neonates (Allegaert et al. 2014b).

Samples were analyzed using two clinical IDMS-traceable assays, commercial available analytic methods [Roche Diagnostics Jaffe (urine application without compensation factor and a sample dilution factor of 1:25) and enzymatic method (dilution factor of 1:50)], and were measured on a Cobas c702 module. The total imprecision (expressed as CV) of the Jaffe assay determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines was 2.2 % at 61.5 mg/dl and 2.2 % at 138.9 mg/dl with a measuring range from 4.2 to 622 mg/dl. For the enzymatic assay, the total imprecision was 1.4 % at 64.8 mg/dl and 1.2 % at 145.8 mg/dl with a measuring range from 1.1 to 610 mg/dl. Internal quality control was performed with commercial control material (Bio-Rad unassayed) using simplified Westgard Rules for statistical process control and biological critical acceptance limits; external quality control was based on the Bio-Rad Unity urine chemistry report. The median and range in neonates for both techniques are reported in Table 3. Paired measurement of urinary creatinine in neonates based on Jaffe and enzymatic IDMS-traceable assays

Table 3 Assay specific urine creatinine concentrations as reported in neonates, children, or adults. Data reported by median and range or mean and standard deviation

Reference	Clinical characteristics	Assay	Observations		
Allegaert et al. (2014a, b)	23 neonates, 24–41 weeks gestational age	Jaffe, modified	9.25	(3.7–42.2)	mg/dl
	Postnatal age 1–26 days, paired analysis	Enzymatic	9.15	(3.8–42.9)	mg/dl
Sonntag et al. (1996)	34 preterm neonates, <1,500 g	Jaffe, modified	9.7	(3.5–17.7)	mg/dl
	First 9 weeks postnatal life				
Srivastava et al. (2008)	18 children, 40 urine samples	Jaffe, modified	69.3	(SD 66.2)	mg/dl
		Enzymatic	75.7	(SD 69.7)	mg/dl
Van Lente et al. (1989)	100 adults, 15–71 years	Jaffe	269.8	(SD 199.8)	mg/dl
	Including cases with renal impairment	Enzymatic	231	(SD 178.7)	mg/dl
Apple et al. (1986)	17 healthy adult volunteers	Jaffe	166	(SD 65)	mg/dl
		Enzymatic	138	(SD 49)	mg/dl

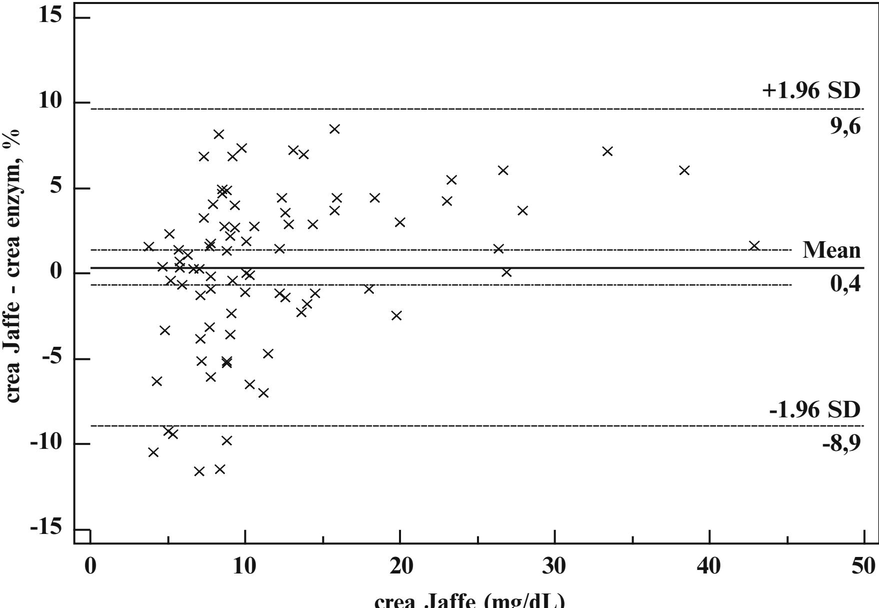

Fig. 1 Bland-Altman plot, illustrating a mean difference of 0.4 % (equal to 0.2 mg/dl) following paired measurement of urinary creatinine in neonates based on Jaffe and enzymatic IDMS-traceable assays

resulted in a limited mean difference of 0.2 mg/dl, and the regression line ($y = 0.28 + 0.95\times$, 95 % CI slope 0.93–0.98) suggests that the enzymatic assay is proportionately about 5 % higher compared to Jaffe. Figure 1 is a Bland-Altman plot, illustrating a mean difference of 0.4 % (absolute mean difference = 0.2 mg/dl).

Although the overall difference between both assays is very limited, there seems to be a urine creatinine concentration-dependent impact on the extent and the direction of the difference between both assays. At low concentration (<10 mg/dl), the Jaffe assay is higher compared to the enzymatic assay (up to 15 %), while the reverse is observed at urine creatinine concentrations above 25 mg/dl (5–10 %) (Allegaert et al. 2014b). Taking some limitations (e.g., limited number of cases with hyperbilirubin >16 mg/dl, only two specific assays) of this study into account, differences between IDMS-traceable Jaffe and enzymatic assays were limited, even in a heterogeneous group of neonates with still an assay-specific impact of bilirubin.

The Impact of the Introduction of IDMS-Traceable Assays on Creatinine Reference Values

It is generally accepted that uncompensated Jaffe overestimates S_{cr} and fixed corrections (e.g., 0.2 or 0.3 mg/dl) or adaptations in the analytical procedure (e.g.,

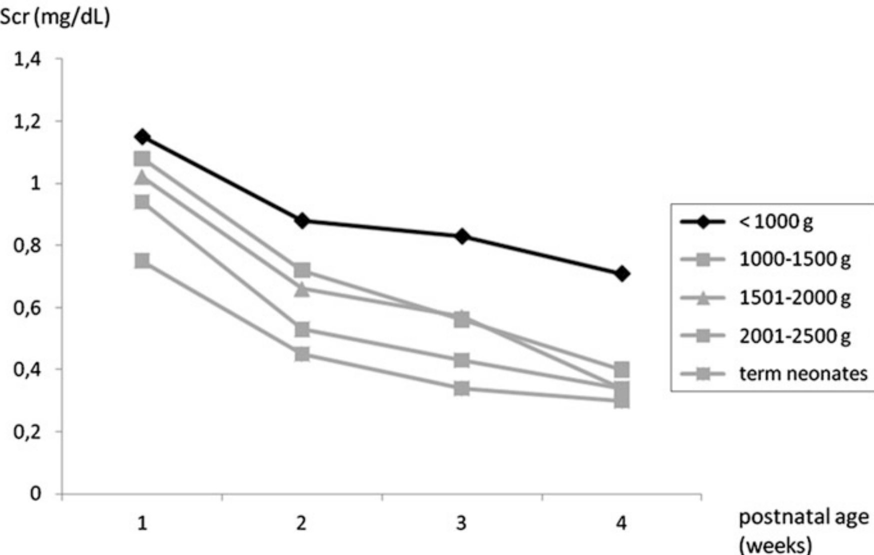

Fig. 2 Postnatal trends of serum creatinine (S_{cr}) in 151 extreme low birth weight neonates were combined with data earlier reported by Bueva and Guignard (2004)

rate blanking) have been suggested to adapt or convert Jaffe assay observations (Kaiser et al. 2014; US Department of Health and Human Services 2012; Myers et al. 2006). Because of the specific aspects of neonatal serum matrix (bilirubin, protein, albumin), such a conversion factor should not be taken for granted to apply earlier reported creatinine reference intervals or threshold to new assays. Such reference values are further complicated by the extensive inter- and intra-patient variability in early infancy (Ceriotti 2012). There is an initial increase in the first days of life, most pronounced in the most immature neonates, with a subsequent progressive decrease throughout infancy, most blunted in the most immature neonates, as illustrated in Fig. 2 (Bueva and Guignard 2004; George et al. 2011).

This extensive variability very likely also explains the absence of a universally adopted definition of acute kidney injury (AKI) in infants. Different threshold S_{cr} values (1.13–2 mg/dl) are suggested, while the modified pediatric RIFLE (pRIFLE, pediatric risk, injury, failure, loss, end-stage) recommends diagnostic criteria based on the S_{cr} increase (stage 1, + 0.3 mg/dl or 150–200 % increase from through S_{cr} value; stage 2, increase of 200–300 % from through S_{cr} value; stage 3, increase S_{cr} >300 %, or 2.5 mg/dl or dialysis) and the urine output (Akcan-Arikan et al. 2007). In essence, it remains difficult to disentangle physiology from pathophysiology. Neither an absolute value nor a proportional increase is unrelated to the maturational changes, and, e.g., stage 1 is almost “common” in extreme low birth weight infants (George et al. 2011; Allegaert et al. 2012). It seems much more reasonable to use age-dependent assay-specific reference intervals or centiles as threshold values.

To illustrate the impact of the introduction of an IDMS-traceable enzymatic assay compared to an uncompensated Jaffe assay on reference ranges, postnatal trends in serum creatinine in two cohorts of neonates before and after switch in assay (uncompensated Jaffe to IDMS-traceable enzymatic assay) were collected, with emphasis on extreme low birth weight (ELBW, i.e., <1,000 g) neonates in the first 6 weeks of postnatal life (Allegaert et al. 2012). Based on 1 883 (uncompensated Jaffe assay) and 1 295 (IDMS traceable enzymatic assay) S_{cr} observations in 151 and 116 ELBW infants, respectively, reference values for postnatal serum creatinine values in ELBW infants during the first 6 weeks of postnatal life determined by either Jaffe or enzymatic S_{cr} quantification were generated.

For both datasets, gestational age, postnatal age, ibuprofen co-administration, and other indicators of disease severity (e.g., the need for respiratory support, low Apgar score) were covariates of S_{cr} observations. To illustrate the dynamics and the impact of both postnatal age and disease characteristics, median creatinine values on day 1, 3, 14, and 28 for either the Jaffe (not IDMS traceable) or the IDMS-traceable enzymatic assay in ELBW infants either or not exposure to ibuprofen (Fig. 3) are

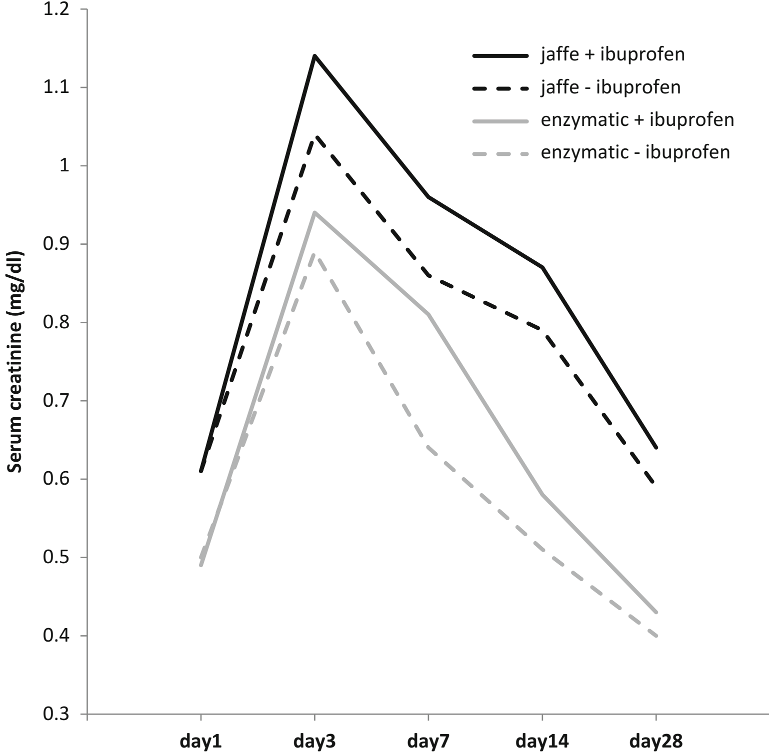

Fig. 3 Median creatinine values on day 1, 3, 14, and 28 for either the uncompensated Jaffe or isotope dilution mass spectrometry (*IDMS*)-traceable enzymatic assay in extreme low birth weight (ELBW, i.e., <1,000 g at birth) infants either or not exposure to ibuprofen

provided. In addition and based on the differences in reference values in the two consecutive cohorts, we confirm that conversion based on a single fixed value is not appropriate in early infancy. This is because such a fixed value cannot account for the variability in neonatal serum composition (e.g., albumin, IgG, bilirubin). Consequently, enzymatic quantification is the preferred method for use in early life. Unfortunately, these enzymatic reaction methods are more expensive.

To further assess the impact of the use of an IDMS-traceable enzymatic assay versus an uncompensated Jaffe quantification method, a retrospective study on S_{cr} values obtained by the Jaffe method in 1,140 neonates admitted between 2001 and 2006 was analyzed and compared to values obtained by using the enzymatic method in 1,023 neonates admitted between 2007 and 2011 in the same unit. The pooled results on postnatal trends for both cohorts (<1 kg, 1–2 kg, 2–3 kg and >3 kg) in whom either the Jaffe or enzymatic assay is provided in Table 4 (median values and difference between both values) and Fig. 4 (Allegaert et al. 2011). In essence, Jaffe is always higher compared to enzymatic techniques, but the differences in median values vary between both techniques (0.1–0.26 mg/dl).

Age- and weight-based reference values and the development of age-corrected centiles or Z scores similar to body weight or length obviously cannot be generated based on single-unit observations and re-illustrates the challenge to establish pediatric reference values for laboratory values for early infancy. Initiatives like the CALIPER (*Ca*nadian *L*aboratory *I*nitiative on *Pe*diatric *R*eference Intervals) provide online creatinine data for both Jaffe and enzymatic assays but limit this to “neonates” in the first 14 days of postnatal life (Colantonio et al. 2012). To illustrate at least the potential impact of age-corrected centiles, we refer to recently reported examples on (*i*) the use of S_{cr} centile values in early infancy as a predictor for neonates with posterior urethral valves (Lemmens et al. 2014) and on (*ii*) the use of S_{cr} centile values to further explore the variability in amikacin clearance in preterm neonates (Smits et al. 2013).

Most authors agree that the “nadir” S_{cr} (i.e., the lowest value, threshold 1 mg/dl) in infancy accurately predicts long-term renal prognosis in neonates with posterior urethral valves (Pohl et al. 2012). However, the determination of the nadir S_{cr} requires meticulous follow-up, and it is not yet available in early infancy. Using population-specific S_{cr} centiles in neonates with posterior urethral valves, peak S_{cr} but also S_{cr} values between day 9 and day 42 above the 75th centile already predicted unfavorable renal outcome (GFR <60 ml/min/1.73 m^2) at 2 years of life (Lemmens et al. 2014). Similarly, birth weight and postnatal age but not creatinine were significant covariates of amikacin clearance in neonates, despite the fact that there is claimed similarity between amikacin clearance and GFR (Smits et al. 2012). However, estimation of GFR in individual cases based on weight- and postnatal age-dependent threshold (<P25th, 25–75th or >75th centile) creatinine values for extreme low birth weight (ELBW <1,000 g) infants can be used as additional biomarker of amikacin clearance instead of an absolute value once birth weight and postnatal age have been considered (Smits et al. 2013). As illustrated in Fig. 5, an age-corrected creatinine value below the 25th centile (reflecting “improved” renal function) was associated with significantly higher

Table 4 Postnatal trends (median, mg/dl) in serum creatinine values for consecutive birth weight categories. Serum creatinine (S_{cr}) was quantified by either enzymatic analysis or Jaffe. The differences in median values are between 0.1 and 0.245 mg/dl

<1 kg	D1	D2	D3	D4	D5	D6	D7	D8	D14	D21	D28	D42
Enzyme	0,58	0,85	0,91	0,88	0,825	0,82	0,75	0,73	0,695	0,58	0,465	0,42
Jaffe	0,75	1	1,09	1,09	0,98	0,93	0,88	0,84	0,765	0,68	0,61	0,54
Difference	*0,17*	*0,15*	*0,18*	*0,21*	*0,155*	*0,11*	*0,13*	*0,11*	*0,07*	*0,1*	*0,145*	*0,12*
1–2 kg												
Enzyme	0,61	0,82	0,77	0,68	0,63	0,64	0,59	0,585	0,59	0,51	0,43	0,375
Jaffe	0,78	0,97	0,95	0,88	0,85	0,81	0,77	0,775	0,72	0,63	0,58	0,52
Difference	*0,17*	*0,15*	*0,18*	*0,2*	*0,22*	*0,17*	*0,18*	*0,19*	*0,13*	*0,12*	*0,15*	*0,145*
2–3 kg												
Enzyme	0,64	0,79	0,68	0,56	0,49	0,46	0,48	0,45	0,47	0,37	0,34	0,29
Jaffe	0,8	0,95	0,88	0,78	0,74	0,705	0,65	0,66	0,62	0,6	0,535	0,46
Difference	*0,16*	*0,16*	*0,2*	*0,22*	*0,25*	*0,245*	*0,17*	*0,21*	*0,15*	*0,23*	*0,195*	*0,17*
>3 kg												
Enzyme	0,65	0,71	0,63	0,5	0,45	0,41	0,4	0,4	0,375	0,35	0,3	0,27
Jaffe	0,88	0,935	0,81	0,71	0,66	0,67	0,65	0,63	0,59	0,55	0,505	0,51
Difference	*0,23*	*0,225*	*0,18*	*0,21*	*0,21*	*0,26*	*0,25*	*0,23*	*0,215*	*0,2*	*0,205*	*0,24*

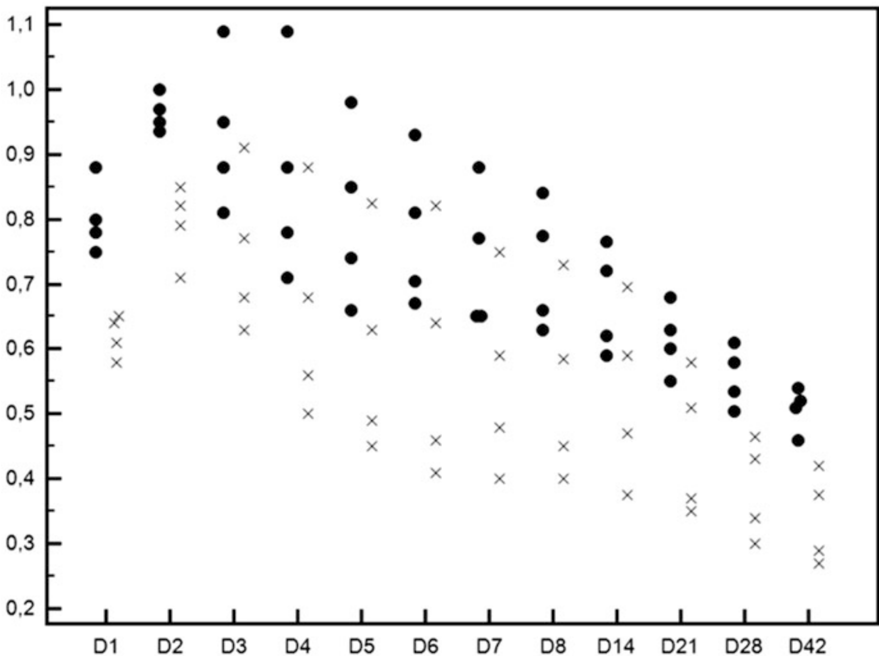

Fig. 4 Postnatal trends for both cohorts (<1 kg, 1–2 kg, 2–3 kg and >3 kg) in whom either the uncompensated Jaffe or the isotope dilution mass spectrometry (*IDMS*)-traceable enzymatic assay is provided. In essence, Jaffe is always higher compared to enzymatic techniques, but the differences in median values differ between both techniques

amikacin clearance. This remained an independent covariate of amikacin clearance, also when birth weight and postnatal age were considered. Both observations at least suggest that further exploration of such a "threshold" or centile approach is warranted, but we do need a "neonatal" CALIPER approach to further develop and validate neonatal reference values in both preterm and term neonates (Ceriotti 2012; Colantonio et al. 2012).

Between-Assay Differences of IDMS-Traceable Creatinine Assays in Early Infancy

Initially, S_{cr} reference values were based on uncompensated Jaffe quantification, a colorimetric reaction method using alkaline picrate. Jaffe assays are known to suffer from interference by multiple endogenous (e.g., hemoglobin F, IgG, bilirubin) and exogenous (e.g., cephalosporins, dopamine) substances, of which some are commonly found in the serum of neonates (Guignard and Gouyon 2008; Junge et al. 2004). Driven by the need to improve estimated GFR performance, Jaffe and enzymatic quantification methods were calibrated to IDMS (US Department of

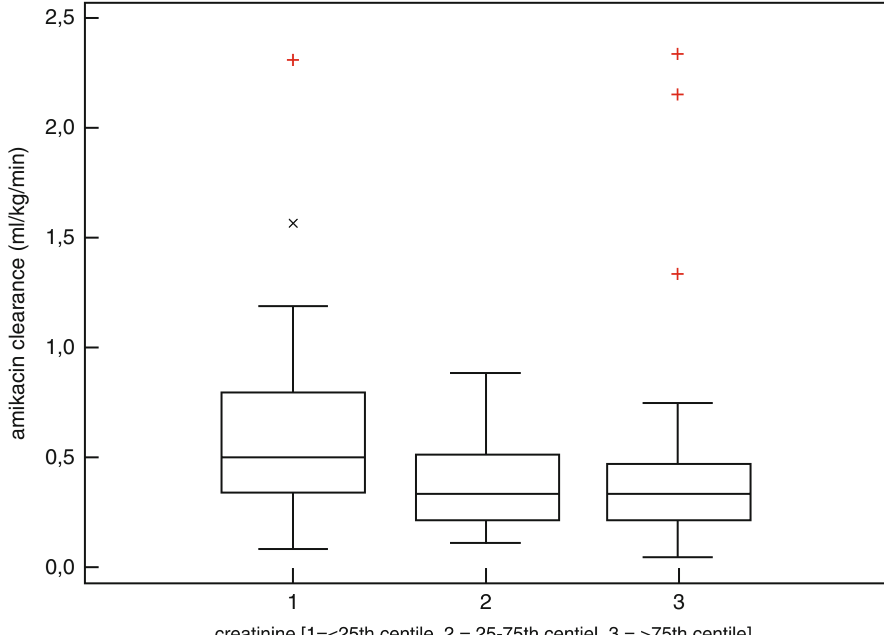

Fig. 5 In a dataset on individual amikacin clearance calculations in 175 preterm neonates, serum creatinine (S_{cr}) was a significant covariate of clearance, when creatinine age-specific reference values (<25th centile, 25–75th centile, or >75th centile) were used. Such reference values are displayed in Table 4 and Fig. 4

Health and Human Services 2012; Myers et al. 2006). To explore if IDMS-based S_{cr} standardization program affects the remaining S_{cr} between-assay differences with a population-specific impact, a study compared S_{cr} values measured by Jaffe and enzymatic methods in paired neonatal serum samples and linked this with clinical characteristics (Allegaert et al. 2014a). S_{cr} was measured in blood samples collected from 129 neonates [gestational age range 24–41 weeks, weight range 0.62–4.55 kg, albumin range 21–67 g/l, and total bilirubin range 0.2–16.1 mg/dl]. The median Jaffe S_{cr} was 0.50 (range 0.17–1.16) mg/dl, and median enzymatic S_{cr} was 0.48 (range 0.06–1.11) mg/dl, resulting in a mean difference in S_{cr} of 0.013 (range −0.2 to 0.11) mg/dl (equal to 3.9 %). Bland-Altman analysis hereby illustrates that there is no S_{cr} concentration-related impact on this difference (Fig. 6). Bilirubin (total, direct) and albumin (Fig. 7) had a significant correlation with the S_{cr} difference. Using multiple regression, only total bilirubin remained a significant covariate ($r^2 = 0.24$).

In essence, this study observed a statistical significant but clinical likely irrelevant mean difference of 0.013 mg/dl between IDMS-traceable Jaffe and enzymatic assays. The extent of this difference was in part explained by bilirubin. There are different reports that document that both Jaffe and enzymatic assays are affected by bilirubin, although interference of enzymatic methods has been minimized (Myers

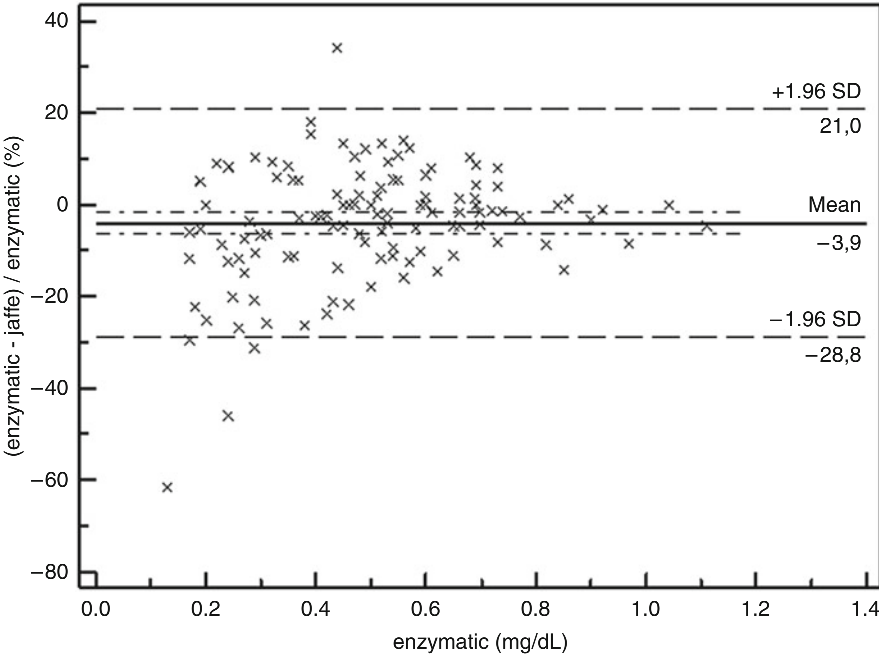

Fig. 6 Bland-Altman plot of paired serum samples from 129 (pre)term neonates. There is a mean difference in S_{cr} of 3,9 %, equal to 0.013 (range −0.2 to 0.11) mg/dl

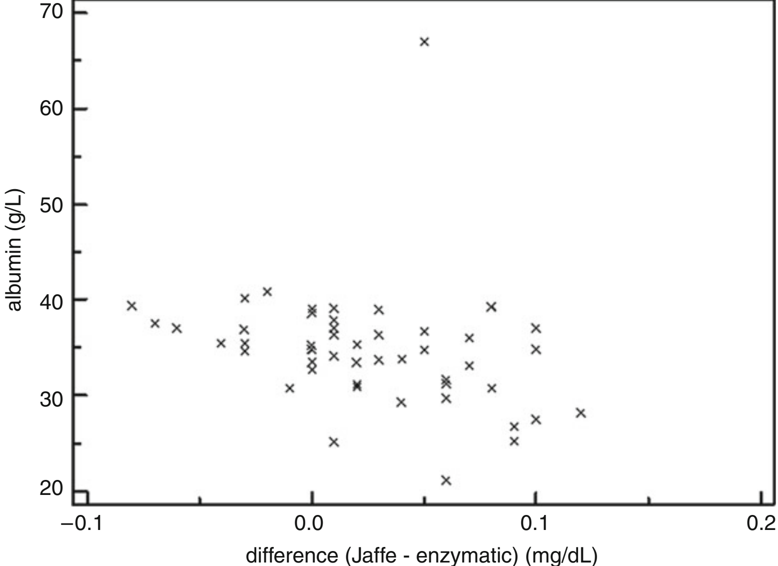

Fig. 7 The absolute mean difference in S_{cr} measurement in 129 paired serum samples in (pre)term neonates was 0.013 (range −0.2 to 0.11) mg/dl. A significant effect of albumin on the difference between both assays was documented ($p = 0.0016$)

et al. 2006). With increasing bilirubin (up to 16.1 mg/dl), Jaffe results are relatively (up to 0.1 mg/dl) lower compared to enzymatic measurements, most likely explained by overcorrection of the Jaffe assay. Bilirubin-related interference is caused by an oxidation of bilirubin to biliverdin in the alkaline Jaffe reaction medium. The Roche Jaffe assay uses rate blanking to compensate for this interference. Prior to adding picric acid (and starting the reaction), alkaline is added to the sample and the bilirubin oxidation rate is measured and later on subtracted from the measured reaction rate of creatinine with alkaline picric acid. As the oxidation of bilirubin in alkaline is curvilinear, overcorrection can occur with increasing bilirubin concentration (Apple et al. 1986; Junge et al. 2004). In clinical laboratory practice, Jaffe creatinine results from samples with high bilirubin concentrations (>10 mg/dl) should either not be reported or at least a warning should be added. In line with the earlier reports in patients with hypoalbuminemia, we confirm an albumin concentration-related effect on the difference between both assays, although the overall strength and trend of the impact is even more limited when compared to bilirubin. In conclusion and taking the limitations of this single study (only two specific assays, range of bilirubin not above 16 mg/dl, limited number of samples) into account, it seems that the differences between IDMS-traceable Jaffe and enzymatic assays are limited, even for a heterogeneous group of neonates. Only an assay-specific impact of bilirubin could be demonstrated (Allegaert et al. 2014a).

Cystatin C to Assess Glomerular Filtration Rate in Early Infancy

As discussed earlier in this chapter, there are limitations on the use of creatinine as a robust and sensitive marker for alterations in glomerular filtration rate (GFR) (Levey et al. 2014; Delanaye and Ebert 2012; Shlipak et al. 2013). Moreover, some of the creatinine-related limitations (e.g., passive tubular back leak, bilirubin, hemoglobin F, lower creatinine concentration range in urine or serum) are more relevant in perinatal life, even after IDMS traceability for the different assays has been introduced (Guignard and Gouyon 2008; Vieux et al. 2010; Allegaert et al. 2012). Low molecular weight proteins (e.g., cystatin C, beta-trace protein, beta-2 microglobulin, alpha-1 microglobulin) have been suggested as valid alternatives and biomarkers (Levey et al. 2014; Zwiers et al. 2014; Zaffanello et al. 2009). It is claimed that cystatin C (CysC) is not affected by body muscle mass, age, gender, inflammation, or nutritional conditions (Shlipak et al. 2013).

CysC is a low molecular mass (13 kDa) basic protein and belongs to the cystatin superfamily of reversible inhibitors of cysteine proteases. It is a proteinase inhibitor involved in normal intracellular protein turnover. Consequently, CysC is produced at a constant rate by any nucleated cell and is eliminated exclusively through glomerular filtration. After ultrafiltration through the basal membrane of the glomerulus, CysC is completely metabolized in the proximal renal tubular cell following megalin-mediated endocytosis at the apical renal tubular cell brush border (Levey et al. 2014; Delanaye and Mariat 2013). Consequently, serum CysC will reflect the glomerular filtration rate, while urinary CysC's presence is a reflection of renal tubular dysfunction (Levey et al. 2014; Delanaye and Mariat 2013).

There are at present at least three different registered methods for serum analysis. Besides particle-enhanced nephelometric immunoassays (PENIAs), the use of particle-enhanced turbidimetric immunoassays (PETIAs) and ELISA methods has been reported (Hossain et al. 2009). For both PENIA and PETIA, specific GFR estimators have been developed (Delanaye and Mariat 2013; Levey et al. 2014; Li et al. 2010). Unfortunately and similar to creatinine assays, the diagnostic performance at present is still suboptimal because of higher imprecision, between-assay differences or over-recovery or between laboratory differences. This resulted in the suggestion of Li et al. to apply assay-specific cystatin C-based GFR equations or estimators, until an international calibration for CysC – similar to the IDMS approach for creatinine – has been developed (Li et al. 2010).

Assay-specific reference values for healthy term (Abitbol et al. 2014; Lee et al. 2013; Finney et al. 2000; Harmoinen et al. 2000; Bahar et al. 2003; Treiber et al. 2006; Bariciak et al. 2011; Dorum et al. 2012; Cataldi et al. 1999; Randers et al. 1998), healthy preterm (Abitbol et al. 2014; Lee et al., 2013; Finney et al. 2000; Harmoinen et al. 2000; Armangil et al. 2008; Bariciak et al. 2011; Dorum et al. 2012; Demirel et al. 2013), and neonates with specific perinatal disease characteristics are summarized in Tables 5, 6, and 7, respectively. We hereby also added information on the assay used, since we are unaware of any between-assay assessment in neonatal samples. In essence (*i*) CysC concentrations are higher at birth, with a subsequent decrease in both term and preterm neonates; (*ii*) CysC concentrations are higher in preterm neonates when compared to term neonates, with the highest values in the most immature preterms; (*iii*) disease characteristics like respiratory distress (Elmas et al. 2013), bilateral kidney abnormalities (Parvex et al. 2012), peripartal asphyxia (Treiber et al. 2014), aminoglycoside exposure (Abitbol et al. 2014), renal dysfunction (Elmas et al. 2013; Montini et al. 2001), or sepsis (Maruniak-Chudek et al. 2012). Surprisingly, the CysC concentrations were lower in the septic shock newborns; (*iv*) the assay seems also to be of relevance in neonatal samples. The mean/median values for CysC measured on day 1 in term neonates are 1.64, 1.70, or 2.11 for PETIA measurements and 1.6, 1.97, 1.84, and 1.21 mg/l for PENIA quantifications. Furthermore, Fig. 8 shows all mean/median values reported in Tables 5 and 6, strongly suggesting an assay-mediated difference in mean/median values in neonatal serum samples, similar to creatinine.

Besides these analytical issues, we also have to take perinatal renal physiology into account before we consider the introduction of CysC to estimate perinatal GFR. In contrast to creatinine and because of its size, CysC does not cross the placental barrier. Consequently, there is no correlation between maternal and umbilical cord blood CysC observations (Cataldi et al. 1999; Kuppens et al. 2012). Different authors described a progressive physiological decrease in CysC values throughout infancy, with subsequent stabilization to age-independent reference values after the first year of life (0.57–1.12 mg/l) (Randers et al. 1999). This means that – similar to creatinine – age-specific reference values in early life are needed before we can consider to integrate this biomarker into neonatal practice as a sensitive and specific marker to discriminate between normal physiology and renal dysfunction (Ceriotti 2012; Colantonio et al. 2012). Finally – following ultrafiltration – CysC undergoes megalin-mediated

Table 5 Plasma cystatin C (CysC) values in healthy term neonates

Reference	Assay	Clinical characteristics	CysC values	
Abitbol et al. (2014)	PENIA	$n = 48$, term, 39 (SD 1) weeks Second part of the first week of life	1.33 (SD 0.18)	mg/l
Lee et al. (2013)	PETIA	$n = 119$ cases		
		Day 0–3	1.64 (SD 0.32)	mg/l
		Day 4–6	1.42 (SD 0.26)	mg/l
		Day 7–10	1.51 (SD 0.23)	mg/l
		Day 11–15	1.55 (SD 0.29)	mg/l
		Day 16–21	1.55 (SD 0.34)	mg/l
		Day 22–30	1.56 (SD 0.39)	mg/l
Finney et al. (2000)	PENIA	$n = 50$ cases, 0–3 months	1.37 (0.81–2.32)	mg/l
Harmoinen et al. (2000)	PETIA	$n = 50$, term cases	1.70 (SD 0.26)	mg/l
		$n = 65$, >8 days–1 year	0.75–1.87	mg/l
Bahar et al. (2003)	PENIA	$n = 112$, umbilical blood at birth	1.36 (SD 0.35)	mg/l
		$n = 98$, venous blood, day 3	1.35 (SD 0.33)	mg/l
Treiber et al. (2006)	PENIA	$n = 75$, umbilical blood at birth (34–41 weeks)	1.97 (SD 0.60)	mg/l
		$n = 75$, venous blood, day 3 (34–41 weeks)	1.93 (SD 0.33)	mg/l
Bariciak et al. (2011)	PENIA	≥36 weeks, $n = 24$, postnatal day 1	1.84 (1.32–2.63)	mg/l
		≥36 weeks, $n = 21$, postnatal day 3	1.58 (1.16–1.95)	mg/l
Dorum et al. (2012)	PENIA	At delivery, term cases ($n = 33$)	1.21 (SD 0.31)	mg/l
Cataldi et al. (1999)	PETIA	88 term cases, at delivery	2.11 (1.17–3.06)	mg/l
		Term cases, day 3	1.75 (0.75–2.7)	mg/l
		Term cases, day 5	1.63 (0.66–2.15)	mg/l
Randers et al. (1998)	PENIA	12 term cases, <1 month	1.63 (SD 0.26)	mg/l

PENIA particle-enhanced nephelometric immunoassay, *PETIA* particle-enhanced turbidimetric immunoassay, *SD* standard deviation

Table 6 Plasma cystatin C (CysC) values in healthy preterm neonates

Reference	Assay	Clinical characteristics	CysC values	
Abitbol et al. (2014)	PENIA	$n = 60$, preterm, 34 (SD 3) weeks	1.42 (SD 0.21)	mg/l
		Second part of the first week of life		
Lee et al. (2013	PETIA	$n = 72$ cases, 33–36 weeks gestational age		
		Day 0–3	1.67 (SD 0.25)	mg/l
		Day 4–6	1.68 (SD 0.27)	mg/l
		Day 7–10	1.69 (SD 0.32)	mg/l
		Day 11–15	1.72 (SD 0.24)	mg/l
		Day 16–21	1.81 (SD 0.22)	mg/l
		Day 22–30	1.64 (SD 0.23)	mg/l
		$n = 40$ cases, 29–32 weeks gestational age		
		Day 0–3	1.56 (SD 0.28)	mg/l
		Day 4–6	1.53 (SD 0.21)	mg/l
		Day 7–10	1.75 (SD 0.29)	mg/l
		Day 11–15	1.87 (SD 0.31)	mg/l
		Day 16–21	1.68 (SD 0.31)	mg/l
		Day 22–30	1.84 (SD 0.27)	mg/l
		$n = 40$ cases, ≤ 28 weeks gestational age		
		Day 0–3	1.60 (SD 0.21)	mg/l
		Day 4–6	1.55 (SD 0.28)	mg/l
		Day 7–10	1.73 (SD 0.41)	mg/l
		Day 11–15	1.87 (SD 0.26)	mg/l
		Day 16–21	1.80 (SD 0.28)	mg/l
		Day 22–30	2.02 (SD 0.42)	mg/l

(continued)

Table 6 (continued)

Reference	Assay	Clinical characteristics	CysC values	
Finney et al. (2000)	In-house immunoassay	n = 14, 29–36 weeks, day 1	1.65 (0.62–4.42)	mg/l
		n = 16, 24–28 weeks, day 1	1.48 (0.65–3.37)	mg/l
Harmoinen et al. (2000)	PETIA	n = 58, preterm cases	1.88 (SD 0.36)	mg/l
Armangil et al. (2008)	PENIA	n = 108, (32.5, SD 2.6 weeks), day 1	1.80 (SD 0.3)	mg/l
		n = 108, day 3	1.65 (SD 0.3)	mg/l
Bariciak et al. (2011)	PENIA	32–36 weeks, n = 29, postnatal day 1	1.89 (0.58–2.93)	mg/l
		32–36 weeks, n = 37, postnatal day 3	1.64 (1.17–2.19)	mg/l
		28–32 weeks, n = 33, postnatal day 1	1.79 (1.05–2.41)	mg/l
		28–32 weeks, n = 33, postnatal day 3	1.60 (1.07–2.17)	mg/l
		24–28 weeks, n = 22, postnatal day 1	1.63 (1.17–2.24)	mg/l
		24–28 weeks, n = 23, postnatal day 3	1.47 (1.14–2.08)	mg/l
Dorum et al. (2012)	PENIA	At delivery, 33–36 weeks, n = 30	1.22 (SD 0.30)	mg/l
		At delivery, 28–32 weeks, n = 25	1.41 (SD 0.27)	mg/l
Demirel et al. (2013)	PETIA	30–32 weeks, day 1	1.79 (0.68–2.31)	mg/l
		30–32 weeks, day 3	1.61 (0.92–2.21)	mg/l
		28–30 weeks, day 1	1.80 (0.65–2.48)	mg/l
		28–30 weeks, day 3	1.70 (0.56–2.31)	mg/l
		26–28 weeks, day 1	1.80 (1.51–3.19)	mg/l
		26–28 weeks, day 3	1.61 (1.10–3.41)	mg/l
		24–26 weeks, day 1	1.80 (0.80–2.20)	mg/l
		24–26 weeks, day 3	1.52 (0.54–2.0)	mg/l

PENIA particle-enhanced nephelometric immunoassay, *PETIA* particle-enhanced turbidimetric immunoassay, *SD* standard deviation

Table 7 Plasma cystatin C (*CysC*) values in neonates with specific perinatal disease characteristics

Reference	Assay	Clinical characteristics	CysC values	
Abitbol et al. (2014)	PENIA	Preterm, 34 (SD 3) weeks	1.35 (SD 0.19)–1.47 (SD 0.21)	mg/l
		Second part of first week of life		
		26/60 exposed to gentamicin *(CysC lower)*		
Treiber et al. (2014)	PENIA	50 term cases perinatal asphyxia/controls		
		At birth (umbilical cord blood)	2.12 (SD 0.53)–1.39 (SD 0.19)	mg/l
		On day 3 of life (venous blood)	1.56 (SD 0.32)–1.34 (SD 0.21)	mg/l
		28–34 weeks, birth weight 910–2,250 g		
Montini et al. (2001)	PETIA	20 preterms, postnatal 4–7 days	1.88 (1.2–2.3)	mg/l
Treiber et al. (2006)	PENIA	$n = 75$, umbilical blood at birth (34–41 weeks)	1.97 (SD 0.60)	mg/l
		Raised CysC with pH <7.2 at birth		
		Correlation ($r = 0.28$) with hemoglobin		
Parvex et al. (2012)	PENIA	100 controls (term), all umbilical cord blood	2.02 (1.54–2.64)	mg/l
		13 congenital renal anomaly cases, bilateral	2.52 (1.80–3.50) (+24.5 %)	mg/l
Elmas et al. (2013)	PENIA	34 cases without respiratory distress, day 3	1.30 (SD 0.2)	mg/l
		28 cases with respiratory distress, day 3		
		No acute kidney injury (22/28)	1.14 (SD 0.1)	mg/l
		Acute kidney injury (6/28)	1.49 (SD 0.09)	mg/l
		34 cases without respiratory distress, day 30	1.29 (0.68–1.67)	mg/l
		28 cases with respiratory distress, day 30		
		No acute kidney injury (22/28)	1.40 (1.01–1.89)	mg/l
		Acute kidney injury (6/28)	1.51 (1.16–1.70)	mg/l
Marunika-Chudek et al. (2012)	ELISA	32 neonates, 34–40 weeks, 0 and 48 h		
		Sepsis	1.47 (1.01–1.9)–1.43 (1.05–1.81)	mg/l
		Severe sepsis	1.5 (1.12–1.87)–1.31 (1.05–1.58)	mg/l
		Septic shock	1.23 (0.92–1.54)–1.21 (0.95–1.47)	mg/l

PENIA particle-enhanced nephelometric immunoassay, *PETIA* particle-enhanced turbidimetric immunoassay, *SD* standard deviation

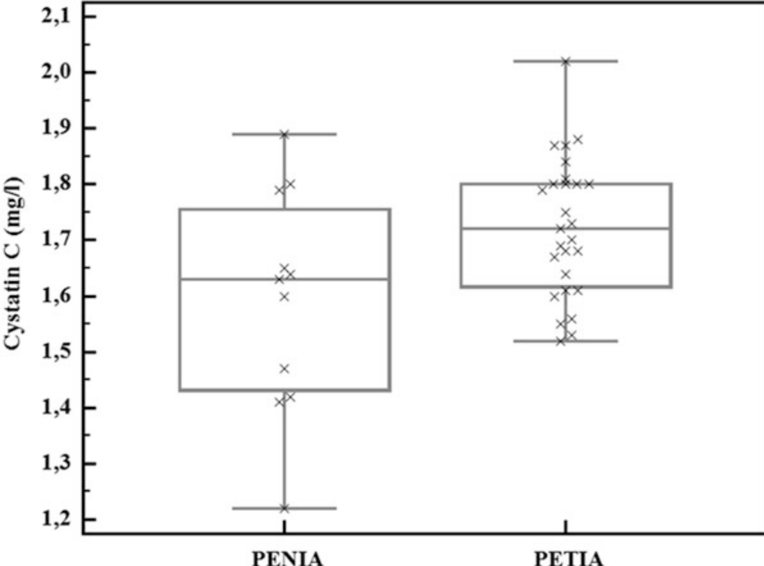

Fig. 8 Median cystatin C values as reported in different cohort of healthy (pre)term neonates. When displayed in an assay-specific way, this strongly suggests a between-assay difference, similar to what has been observed in adults. Median and range and clinical characteristics for these cohorts are provided in Tables 5 and 6 (*PENIA* particle-enhanced nephelometric immunoassay, *PETIA* particle-enhanced turbidimetric immunoassay)

endocytosis and metabolism in the proximal renal tubular cell, but little is known on the ontogeny of the megalin receptor and the maturation of CysC metabolism.

In conclusion and based on the currently available information, it seems that the range (four- to fivefold) in serum CysC observations in early infancy is extensive and only in part explained by renal (patho)physiology. This likely relates at least in part to the ontogeny of both CysC synthesis and metabolism, with an additional between-assay variability. This can be further improved by the use of assay-specific reference values, adapted to the clinical characteristics (e.g., weight, gestational age, postnatal age) and compared to inulin clearance or similar as golden standard for GFR estimation. Until such information becomes available and taking also the differences in analytical costs into account, the use of serum creatinine still remains a somewhat crude but useful biomarker of GFR (dys)function in perinatal life.

From Biochemical Quantification to Clinical Application

Neonatal care critically depends on the availability of reference intervals for any specific laboratory test or biomarker to support clinical decision-making, to tailor

therapy to the individual needs, or to support prognosis (Ceriotti 2012; Colantonio et al. 2012). However, children are not small adults and neither are newborns small children. Since maturational physiological changes are most prominent in early infancy, variability is the key feature in this population: *developmental physiology drives developmental laboratory medicine*. This is also reflected by the extensive inter- and intra-individual variability in S_{cr} and eGFR documented in early infancy and described in this chapter. This variability is in part related to physiological changes (e.g., birth weight, gestational age, postnatal age) as well as changes related to pathophysiology (e.g., peripartal asphyxia, co-medication).

The basic problem is to read and recognize the signal in this noise. Consequently, clinicians are still struggling with the translations of the (patho)physiology to the clinical setting, as, e.g., reflected in the inadequate definitions of AKI or pRIFLE (Akcan-Arikan 2007). Another interesting and emerging pattern that is not yet well explored during critical illness in adults and not yet even considered in neonates is "hyperfiltration": a disease state where renal clearance capacity is actually higher than anticipated. We commonly search for decreased renal clearance, while, e.g., ineffective pharmacotherapy may also relate to increased clearance (Grootaert 2012). Strategies to further improve the current setting either relate to novelties or to improve the use of the already available tools.

The search for novel biomarkers of renal "(dys)function" (e.g., cystatin C, beta-trace protein, beta-2 microglobulin, alpha-1 microglobulin) is valuable but will need focused studies in specific cohorts of (pre)term neonates once the within-assay issues have been considered (Levey et al. 2014; Zwiers et al. 2014; Zaffanello et al. 2009). This has been illustrated in this chapter based on the reported observations on CysC (Tables 5, 6, and 7) in neonates. Besides the search for new biomarkers, we have the strong opinion that the efforts made to standardize creatinine assays toward IDMS hold a unique opportunity to develop reference values and centiles for creatinine in (pre)term neonates. An effort similar to the CALIPER initiative but focused to early infancy is needed (Ceriotti 2012; Colantonio et al. 2012). At least, we have provided preliminary evidence that such an approach may be beneficial to improve prognosis (urethral valves and outcome) (Lemmens et al. 2014) or to tailor pharmacotherapy (S_{cr} centiles and amikacin clearance) (Smits et al. 2013). Further exploration of such a "threshold" or centile approach is warranted, but we do need a "neonatal" CALIPER approach to develop and validate neonatal reference values in both preterm and term neonates.

Potential Applications to Prognosis, Other Diseases, or Conditions

Maturational physiological changes are most prominent in early infancy. Consequently, variability is the key feature in early infancy. This is not limited to creatinine but is also reflected in age-specific reference intervals for other commonly measured biomarkers (e.g., glycemia, aminotransferases, alkaline phosphatases, hormones).

Neonatal care critically depends on the availability of reference intervals for any specific laboratory test or biomarker to support clinical decision-making, to tailor therapy to the individual needs, or to support prognosis. This is also true but not limited to creatinine measurements. A recent example is the paper on plasma aminotransferase concentrations in preterm infants (Victor et al. 2011). Extreme preterm neonates had higher plasma aminotransferase levels compared to near-term cases, again reflecting either maturational issues or disease-related differences.

For those interested in bio-analysis, we would like to make the point that this chapter also re-illustrates the relevance of the matrix in which the measurement is performed. Please take into account that the matrix matters: assays may be affected by specific composition of the matrix (serum, urine) in neonates. This is not limited to bilirubin but may also be related to differences in albumin (e.g., binding capacity), immunoglobulins, or free fatty acids.

The impact of matrix-related measurement is not limited to early infancy. We use the example of bilirubin to illustrate this. Although raised bilirubin (unconjugated) is indeed common in early infancy, it is not limited to newborns, since raised bilirubin (conjugated) is a well-known biomarker of cholestasis and liver disease, as recently reported in jaundiced adults (Kaiser 2014). In these patients, bilirubin-related interferences may also affect S_{cr} measurements. This may be of clinical relevance for both issues related to diagnosis (e.g., hepato-renal syndrome) and treatment modalities (e.g., S_{cr} is a biomarker to decide on liver transplantation) (Kaiser 2014).

Summary Points

- This chapter focuses on specific aspects of creatinine assays in early infancy. This includes both analytical aspects (between-assay differences) and clinical issues (extensive variability).
- The concept of isotope dilution mass spectrometry (IDMS) traceability improved the measurement but affected the reference values for serum and urine creatinine in early infancy. Moreover, the difference between different assays (Jaffe versus enzymatic "conversion") in neonatal matrix is not a single absolute value.
- Reference values for creatinine urine measurement are much lower in neonatal urine compared to children and adults, but the difference between IDMS-traceable enzymatic and Jaffe assays is very limited and not clinically significant.
- After the introduction of IDMS traceability of serum creatinine (S_{cr}) measurement, there is still a minor difference between IDMS-traceable enzymatic and Jaffe assays, in part explained by bilirubin.
- Cystatin C also displays assay-related differences, and the number of reference values in neonates is still very limited.
- Despite the limitations, serum creatinine (S_{cr}) therefore still remains the most commonly used biomarker for glomerular filtration rate (GFR) assessment in neonates.

- To improve the clinical application following IDMS traceability, a concerted effort is urgently needed to develop and validate neonatal reference values in both preterm and term neonates.

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References

Abitbol CL, Seeherunvong W, Galarza MG, et al. Neonatal kidney size and function in preterm infants: what is a true estimate of glomerular filtration rate? J Pediatr. 2014;164:1026–31.
Akcan-Arikan A, Zappitelli M, Loftis LL, et al. Modified RIFLE criteria in critically ill children with acute kidney injury. Kidney Int. 2007;71:1028–35.
Allegaert K, Keltemans J, Hendrickx S, et al. Creatininaemia in extreme low birth weight infants: from biochemical quantification to clinical interpretation. Sixth congress of Nephrology in Internet. 2011. http://trabajos.cin2011.uninet.edu/162/allegaert.pdf. Accessed 18 Apr 2014.
Allegaert K, Kuppens M, Mekahli D, et al. Creatinine reference values in ELBW infants: impact of quantification by Jaffe or enzymatic method. J Matern Fetal Neonatal Med. 2012;25:1678–81.
Allegaert K, Pauwels S, Smits A, et al. Enzymatic isotope dilution mass spectrometry (IDMS) traceable serum creatinine is preferable over Jaffe in neonates and young infants. Clin Chem Lab Med. 2014a;52:e107–9.
Allegaert K, Vermeersch P, Smits A, et al. Paired measurement of urinary creatinine in neonates based on a Jaffe and an enzymatic IDMS-traceable assay. BMC Nephrol. 2014b;15:62.
Apple F, Bandt C, Prosch A, et al. Creatinine clearance: enzymatic vs Jaffé determinations of creatinine in plasma and urine. Clin Chem. 1986;32:388–90.
Armangil D, Yurdakök M, Canpolat FE, et al. Determination of reference values for plasma cystatin C and comparison with creatinine in premature infants. Pediatr Nephrol. 2008;23:2081–3.
Bahar A, Yilmaz Y, Unver S, et al. Reference values of umbilical cord and third-day cystatin C levels for determining glomerular filtration rates in newborns. J Int Med Res. 2003;31:231–5.
Bariciak E, Yasin A, Harrold J, et al. Preliminary reference intervals for cystatin C and beta-trace protein in preterm and term neonates. Clin Biochem. 2011;44:1156–9.
Bueva A, Guignard JP. Renal function in preterm neonates. Pediatr Res. 2004;36:572–7.
Cataldi L, Mussap M, Bertelli L, et al. Cystatin C in healthy women at term pregnancy and in their infant newborns: relationship between maternal and neonatal serum levels and reference values. Am J Perinatol. 1999;16:287–95.
Ceriotti F. Establishing pediatric reference intervals: a challenging task. Clin Chem. 2012;58:808–10.
Ceriotti F, Boyd JC, Klein G, et al. Reference intervals for serum creatinine concentrations: assessment of available data for global application. Clin Chem. 2008;54:559–66.
Colantonio DA, Kyriakopoulou L, Chan MK, et al. Closing the gaps in pediatric laboratory reference intervals: a CALIPER database of 40 biochemical markers in a healthy and multiethnic population of children. Clin Chem. 2012;58:854–68.
Delanaye P, Ebert N. Assessment of kidney function: estimating GFR in children. Nat Rev Nephrol. 2012;8:503–4.
Delanaye P, Mariat C. The applicability of eGFR equations to different populations. Nat Rev Nephrol. 2013;9:513–22.
Demirel G, Celik IH, Canpolat FE, et al. Reference values of serum cystatin C in very low-birth weight premature infants. Acta Paediatr. 2013;102:e4–7.
Dorum S, Silfeler I, Dorum BA, et al. Reference values of serum cystatin-C for full term and preterm neonates in Istanbul. Indian J Pediatr. 2012;79:1037–42.

Elmas AT, Tabel Y, Elmas ON. Serum cystatin C predicts acute kidney injury in preterm neonates with respiratory distress syndrome. Pediatr Nephrol. 2013;28:477–84.
Finney H, Newman DJ, Thakkar H, et al. Reference ranges for plasma cystatin C and creatinine measurements in premature infants, neonates, and older children. Arch Dis Child. 2000;82:71–5.
Gallini F, Maggio L, Romagnoli C, et al. Progression of renal function in preterm neonates with gestational age < or = 32 weeks. Pediatr Nephrol. 2000;15:119–24.
George I, Mekahli D, Rayyan M, et al. Postnatal trends in creatinemia and its covariates in extremely low birth weight (ELBW) neonates. Pediatr Nephrol. 2011;26:1843–9.
Gordjani N, Burghard R, Leititis JU, et al. Serum creatinine and creatinine clearance in healthy neonates and prematures during the first 10 days of life. Eur J Pediatr. 1988;148:143–5.
Grootaert V, Willems L, Debaveye Y, et al. Augmented renal clearance in the critically ill: how to assess kidney function. Ann Pharmacother. 2012;46:952–9.
Guignard JP, Gouyon JB. Glomerular filtration rate in neonates. In: Oh W, Guignard JP, Baumgart S, editors. Nephrology and fluid/electrolyte physiology; neonatology questions and controversies. 1st ed. Philadelphia: Saunders Elsevier; 2008. p. 79–96.
Harmoinen A, Ylinen E, Ala-Houhala M, et al. Reference intervals for cystatin C in pre- and full-term infants and children. Pediatr Nephrol. 2000;15:105–8.
Hossain MA, Emara M, El Moselhi H, et al. Comparing measures of cystatin C in human sera by three methods. Am J Nephrol. 2009;29:381–91.
Lee J-H, Hahn WH, Ahn J, et al. Serum cystatin C during 30 postnatal days is dependent on the postconceptional age in neonates. Pediatr Nephrol. 2013;28:1073–8.
Jung K. Enzyme activities in urine: how should we express their excretion? A critical literature review. Eur J Clin Chem Clin Biochem. 1991;29:725–9.
Junge W, Wilke B, Halabi A, et al. Determination of reference intervals for serum creatinine, creatinine excretion and creatinine clearance with an enzymatic and a modified Jaffé method. Clin Chim Acta. 2004;344:137–48.
Kaiser T, Kinny-Köster B, Bartels M, et al. Impact of different creatinine measurement methods on liver transplant allocation. PLoS One. 2014;9:e90015.
Kuppens M, George I, Lewi L, et al. Creatinaemia at birth is equal to maternal creatinaemia at delivery: does this paradigm still hold? J Matern Fetal Neonatal Med. 2012;25:978–80.
Lemmens AS, Mekahli D, Devlieger R, et al. Population-specific serum creatinine centiles in neonates with posterior urethral valves already predict long-term renal outcome. J Matern Fetal Neonatal Med. 2014. doi:10.3109/14767058.2014.942278.
Levey AS, Inker LA, Coresh J. GFR estimation: from physiology to public health. Am J Kidney Dis. 2014;63:820–34.
Li J, Dunn W, Breaud A, et al. Analytical performance of 4 automated assays for measurement of cystatin C. Clin Chem. 2010;56:1336–9.
Maruniak-Chudek I, Owsianka-Podlesny T, Wroblewska J, et al. Is serum cystatin C a better marker of kidney function than serum creatinine in septic newborns. Postepy Hig Med Dosw. 2012;66:175–80.
Montini G, Cosmo L, Amici G, et al. Plasma cystatin C values and inulin clearance in premature neonates. Pediatr Nephrol. 2001;16:463–5.
Myers GL, Miller WG, Coresh J, et al. Recommendations for improving serum creatinine measurement: a report from the Laboratory Working Group of the National Kidney Disease Education Program. Clin Chem. 2006;52:5–18.
Parvex P, Combescure C, Rodriguez M, et al. Is cystatin C a promising marker of renal function, at birth, in neonates prenatally diagnosed with congenital kidney anomalies? Nephrol Dial Transplant. 2012;27:3477–82.
Pohl M, Mentzel HJ, Vogt S, et al. Risk factors for renal insufficiency in children with urethral valves. Pediatr Nephrol. 2012;27:443–50.
Randers E, Kristensen JH, Erlandsen EJ, et al. Serum cystatin C as a marker of the renal function. Scand J Clin Lab Invest. 1998;58:585–92

Randers E, Krue S, Erlandsen EJ, et al. Reference interval for serum cystatin C in children. Clin Chem. 1999;45:1856–8.
Rhodin MM, Anderson BJ, Peters AM, et al. Human renal function maturation: a quantitative description using weight and postmenstrual age. Pediatr Nephrol. 2009;24:67–76.
Shlipak MG, Matsushita K, Ärnlöv J, et al. Cystatin C versus creatinine in determining risk based on kidney function. N Engl J Med. 2013;369:932–43.
Smits A, Kulo A, de Hoon JN, et al. Pharmacokinetics of drugs in neonates: pattern recognition beyond compound specific observations. Curr Pharm Des. 2012;18:3119–46.
Smits A, Levtchenko E, van den Anker J, et al. Creatinine as biomarker for amikacin clearance in extreme low birth weight neonates: how to aim for a moving target ? [abstract]. Dutch Society of Clinical Pharmacology and Biopharmacy, annual meeting 2012. Br J Clin Pharmacol. 2013; 76:836.
Sonntag J, Prankel B, Waltz S. Serum creatinine concentration, urinary creatinine excretion and creatinine clearance during the first 9 weeks in preterm infants with a birth weight below 1500 g. Eur J Pediatr. 1996;155:815–9.
Srivastava T, Alon US, Althahabi R, et al. Impact of standardization of creatinine methodology on the assessment of glomerular filtration rate in children. Pediatr Res. 2008;65:113–7.
Treiber M, Gorenjak M, Pecovnik Balon B. Serum cystatin-C as a marker of acute kidney in the newborn after perinatal hypoxia/asphyxia. Ther Apher Dial. 2014;18:57–67.
Treiber M, Pecovnik-Balon B, Gorenjak M. Cystatin C versus creatinine as a marker of glomerular filtration rate in the newborn. Wien Klin Wochenschr. 2006;118 Suppl 2:66–70.
U.S. Department of Health and Human Services. About NKDEP: http://nkdep.nih.gov/about-nkdep/working-groups/laboratory-working-group.shtml (2012). Accessed 18 Aug 2014.
Van Lente F, Suit P. Assessment of renal function by serum creatinine and creatinine clearance: glomerular filtration rate estimated by four procedures. Clin Chem. 1989;35:2326–30.
Victor S, Dickinson H, Turner MA. Plasma aminotransferase concentrations in preterm infants. Arch Dis Child Fetal Neonatal Ed. 2011;96:F144–5.
Vieux R, Hascoet JM, Merdariu D, et al. Glomerular filtration rate reference values in very preterm infants. Pediatrics. 2010;125:e1186–92.
Walker MW, Clark RH, Spitzer AR. Elevation in plasma creatinine and renal failure in premature neonates without major anomalies: terminology, occurrence and factors associated with increased risk. J Perinatol. 2011;31:199–205.
Zaffanello M, Antonucci R, Cuzzolin L, et al. Early diagnosis of acute kidney injury with urinary biomarkers in the newborn. J Matern Fetal Neonatal Med. 2009;22(Suppl3):62–6.
Zwiers AJ, Cransberg K, de Rijke YB, et al. Reference ranges for serum β-trace protein in neonates and children younger than 1 year of age. Clin Chem Lab Med. 2014. doi:10.1515/cclm-2014-0371.

Urinary Angiotensinogen as a Biomarker for Renal Disease

14

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Abstract

Chronic kidney disease (CKD) is an emergent worldwide epidemic, and hypertension is a major cause of this condition. The renin-angiotensin system (RAS) is likely the most important factor in the development and maintenance of hypertension.

Recently, two distinct RASs were identified: first, the systemic or circulatory RAS and, second, the local or tissue-specific RAS. The local RAS is found in many tissues such as those of the heart, brain, kidney, and adrenal glands. The most important tissue containing the local RAS is that of the kidney because these organs contain all of the RAS components necessary to produce angiotensin II

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(Ang II), a molecule that has many direct and indirect effects on blood pressure and water homeostasis.

Animal studies have guided the definition of the RAS components in the kidney and have helped us to understand their mechanisms of action. Angiotensinogen (AGT), renin, and angiotensin-converting enzyme (ACE) are abundant in the tubular system. Most studies have shown that AGT is produced in the proximal tubule and secreted to the tubular lumen. AGT cleavage with renin results in the generation of angiotensin I (Ang I). Ang I is then transformed into Ang II with ACE, the latter of which is the active metabolite of the RAS that contributes to sodium homeostasis and the blood pressure response.

In humans, clinical studies have shown that urinary AGT (uAGT) is correlated with both systolic and diastolic blood pressure (SBP and DBP, respectively) as well as urinary protein, in patients with CKD and in patients with hypertension. uAGT is also a risk factor for the progression of CKD. Recently, uAGT was studied in patients with glomerulonephritis, amyloidosis, and autosomal-dominant polycystic kidney disease, as well as in renal transplant recipients. Limited information exists with regard to the use of uAGT for acute kidney injury (AKI).

The current chapter describes uAGT in detail. Growing evidence shows that uAGT reflects the intrarenal RAS. Some authors have also reported that uAGT is a marker of glomerular injury. Nevertheless, additional studies are needed to recognize uAGT as a biomarker.

Keywords

Angiotensin II • Angiotensinogen • Blood pressure • Chronic kidney disease • Renin-angiotensin system • Urinary angiotensinogen • Urinary protein

Abbreviations

ACE	Angiotensin-converting enzyme
ACEi	Angiotensin-converting enzyme inhibitor
ADPKD	Autosomal-dominant polycystic kidney disease
AGT	Angiotensinogen
AGT mRNA	Angiotensinogen messenger ribonucleic acid
AKI	Acute kidney injury
Ang I	Angiotensin I
Ang II	Angiotensin II
ARB	Angiotensin II type 1 receptor blocker
AT1R	Angiotensin II type 1 receptor
BMI	Body mass index
CKD	Chronic kidney disease
DBP	Diastolic blood pressure
eGFR	Estimated glomerular filtration rate
IgA	Immunoglobulin A
MGA	Minimal glomerular abnormality
PRA	Plasma renin activity
RAS	Renin-angiotensin system

SBP	Systolic blood pressure
uAGT	Urinary angiotensinogen
uAGT/uCre	Urinary angiotensinogen/urinary creatinine ratio
uAlb/uCre	Urinary albumin/urinary creatinine ratio
uPro/uCre	Urinary protein/urinary creatinine ratio

Key Facts of Urinary Angiotensinogen

- AGT is a 60-kD glycoprotein of the alpha-2 globulin fraction of plasma proteins, which is produced mainly by the liver.
- uAGT was first described in 1972, and after the definition of local RAS, uAGT was used as a measurable parameter of intrarenal RAS activity.
- uAGT can be measured by commercially available ELISA kits.
- uAGT excretion is correlated with blood pressure levels and proteinuria in renal diseases (e.g., CKD, diabetic nephropathy, chronic glomerulonephritis).
- In the future, uAGT may be used as a prognostic biomarker in patients with renal diseases.

Definitions

LMB2 LMB2 (anti-Tac (Fv)-PE38) is an immunotoxin with specific binding to human CD25 and induces progressive nonselective proteinuria, ascites, and edema in NEP25 mice.

Megalin Megalin, a member of the low-density lipoprotein receptor gene family, is a multiligand receptor expressed in the apical membrane of proximal-tubule epithelial cells that plays a central role in the endocytic process.

RAS The renin-angiotensin system is the main pathway that regulates blood pressure and fluid and sodium homeostasis by producing Ang II from AGT.

Streptozocin Streptozocin is an antibiotic with toxic effects that induce type 1 diabetes in animals.

Introduction

The renin-angiotensin system (RAS) plays a critical role in blood pressure control, fluid and electrolyte homeostasis, and progression of renal disease (Navar et al. 1999). Knowledge of the RAS began with a study in 1898, which describes that the infusion of renal extracts obtained from the renal vein of a rabbit increased the arterial pressure of nephrectomized rabbits. These authors named this pressor compound “renin” (Tigerstedt and Bergman 1898). Subsequent experiments showed

that the blood that came from the renal veins of dogs after acute ischemia had a strong pressor effect and showed different characteristics from renin; this substance was called "hypertensin" (Braun-Menéndez et al. 1939). During the same years, another study group discovered a crystalline pressor substance, which was cleaved by renin, and they named this substance "angiotonin" (Page and Helmer 1940). Later, investigators decided that unique nomenclature was necessary for additional studies, and angiotensinogen (AGT) was defined as the only substrate of renin (ISH 1978). Favaro et al. first described the excretion of AGT in 1972 among patients with glomerulonephritis (1972); however, the source of urinary AGT (uAGT) and its pathophysiological role remained unknown.

Over the last few decades, research has shown that different tissues have elements of the RAS, and the kidney is the only organ to have all of them, primarily in the tubular system. Although the liver produces circulating angiotensinogen, studies have indicated that the kidneys produce it as well.

Recently, the focus of interest on the RAS has shifted toward the role that the local RAS plays in specific tissues. Considerable attention has been paid to the significance of the tissue RAS in the brain, heart, adrenal glands, vasculature, and kidney.

Intrarenal RAS

Studies have shown that a major source of intrarenal Ang II is AGT, which is locally produced by the proximal tubular cells. Animal studies have found AGT mRNA and protein in proximal-tubule segments (Darby et al. 1994; Kamiyama et al. 2012a). The AGT produced in proximal-tubule cells and its formed metabolites are likely secreted directly into the tubular lumen (Rohrwasser et al. 1999). Renin is secreted from the juxtaglomerular apparatus, and it might be the source of both circulating and intrarenal levels of AGT. However, Prieto-Carrasquero MC et al. (2004) also showed that Ang-II-infused rats demonstrated suppressed renin immunostaining in the juxtaglomerular apparatus cells and increased immunostaining in the distal tubules. The renal interstitium and vascular structure also contains renin and serves as a pathway in the generation of Ang I. ACE is abundant throughout the nephron, except for the late distal nephron segments. Furthermore, ACE is important in transforming Ang I into Ang II. A schematic illustration of intrarenal RAS is shown in Fig. 1. Locally formed Ang II has many direct and indirect effects in the kidney (Kobori et al. 2007a).

Ang II has direct constrictor effects on the glomerular arterioles and mesangial cells, and it indirectly enhances the tubuloglomerular feedback response (Seeliger et al. 2009). Blockage of the RAS attenuates the tubuloglomerular feedback response and increases the distal nephron perfusion rate. Ang II increases sodium and bicarbonate reabsorption at proximal and distal tubules (Hashimoto et al. 2005; Dibona and Sawin 2004). Ang II also stimulates the zona glomerulosa and indirectly increases distal tubule sodium reabsorption through aldosterone.

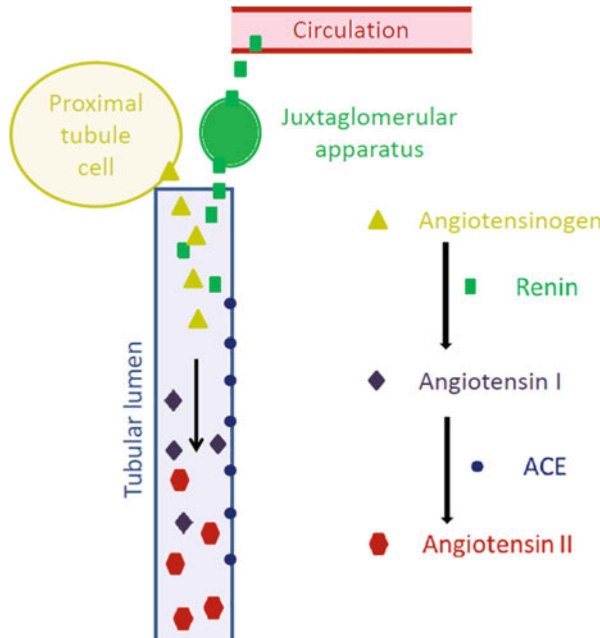

Fig. 1 Local production of RAS components in kidney. The angiotensinogen produced in proximal-tubule cell is secreted to the tubular lumen and is cleaved by renin, which is secreted from juxtaglomerular apparatus. The angiotensin I formed from the angiotensinogen is then converted to angiotensin II through the angiotensin-converting enzyme, which is abundant in the tubular system. The final product, angiotensin II, has many direct and indirect effects in the kidney and blood pressure control (*RAS* renin-angiotensin system, *ACE* angiotensin-converting enzyme)

Does uAGT Reflect Intrarenal RAS?

Multiphoton imaging of glomerular permeability has shown that under physiological and pathological conditions, systemic AGT does not cross through the glomerular filtration barrier. After glomerular damage, however, the uAGT increased, and the majority of this increase was endogenous, not the labeled AGT. These results suggest that tubules can produce AGT, and uAGT is a marker of intrarenal RAS (Nakano et al. 2012). In contrast to this study, Pohl M. et al. (2010) showed that signals of labeled AGT and AGT content in rat proximal convoluted tubule consist of the endocytic uptake from the glomerular filtrate, and this uptake is mediated through the megalin-a scavenger receptor. Mosaic megalin-deficient mice showed subapical endosomal or lysosomal accumulation of AGT in megalin-positive cells; however, megalin-deficient cells were AGT negative. These mice had similar plasma AGT levels to megalin-positive mice. Megalin-deficient mice revealed substantial uAGT excretion, but uAGT was undetectable in megalin-positive mice. AGT mRNA was detected in the proximal straight tubules, but the proximal convoluted tubules expressed weaker AGT mRNA signals. These studies supported that both early proximal convoluted tubule compartments expressed low levels of AGT mRNA, and intensive endocytosis of filtrated systemic AGT demonstrates that excreted uAGT is derived from the proximal straight tubules or more distal components of the nephron containing local RAS components.

Epidemiologic studies have shown that increased dietary salt intake is associated with increased prevalence and progression of hypertension. Salt sensitivity is variable among adults; however, patients with essential hypertension are more likely to have salt sensitivity. Salt restriction decreases plasma AGT, stimulates PRA, and increases plasma Ang I and Ang II (Ingert et al. 2000). A study regarding the effects of a low sodium diet on the intratubular RAS demonstrated that despite an increase in PRA, plasma Ang II, and kidney Ang II levels, uAGT and urinary Ang II excretion did not increase. Therefore, the physiologic stimulation of the RAS does not explain the increasing levels of uAGT. Rather, increased uAGT might merely reflect pathological stimulations of the intrarenal RAS (Shao et al. 2013).

Animal Studies

Animal studies have provided specific models to test the effects of many RAS components. These models include transgenic mice and enable the examination of the specific effect of a protein such as AGT as well as the observation of hypertensive or diabetic animals and animals with glomerulonephritis. In addition, sexual differences can be described using appropriate models. These models even allow for the description of treatment mechanisms and drug effects.

Single transgenic mice that expressed human AGT only in the kidney (group A), double transgenic mice expressing systemic human renin and human AGT in the kidney (group D) and wild-type mice (group W) all received an infusion of human AGT. Renin has a high specificity for species, and exogenously administered human AGT cannot be cleaved by mouse renin. This transgenic mouse model enabled us to test the intrarenal mouse AGT expression induced by the selective intrarenal overproduction of Ang II. Exogenous human AGT mRNA and protein was expressed in the kidneys of groups A and D. Mice liver did not express human AGT mRNA or protein in any group. Plasma Ang II levels were similar across the groups, but renal Ang II levels were higher in group D. Groups A and D had detectable human uAGT, but group W had no detectable levels of human AGT in the urine. Group D showed increased endogenous mouse AGT mRNA and protein. Group D showed kidney damage parameters with an increase in interstitial collagen, interstitial macrophage, and monocyte infiltration and afferent arteriolar hypertrophy. These findings suggest that increased selective intrarenal Ang II generation produces endogenous AGT mRNA, increases intrarenal AGT, and contributes to progressive hypertension with renal damage (Kobori et al. 2007b).

Chronic Ang II infusion enables the creation of a useful animal model of Ang-II-dependent hypertension. Chronic Ang II infusion with a high salt diet increases blood pressure and augments renal AGT mRNA and protein, plasma AGT, and liver-AGT mRNA and protein, despite suppressing plasma renin activity (Kobori et al. 2001). Ang-II-infused mice on a high salt diet showed an increase in kidney Ang II content, and this increase was correlated with uAGT; however, no correlation was found between plasma Ang II and uAGT (Kobori et al. 2002). Chronic Ang II infusion creates time- and dose-dependent increases in uAGT and protein. Although

higher urinary protein excretion rates were observed in the volume-dependent hypertension group (high salt plus deoxycorticosterone acetate [DOCA] salt group), an increase in uAGT was not observed in this group. In addition, Ang-II-infused mice and sham-operated mice received human AGT, but a urinary analysis did not show any human AGT; moreover, these mice did not show alterations in blood pressure. These data indicate that glomerular permeability prevents AGT from crossing the plasma to the tubules (Kobori et al. 2003a).

Ang-II-infused rats also showed increased urinary renin and Ang II levels. Suppressed juxtaglomerular apparatus and low levels of plasma renin activity (PRA) make filtration of the circulating renin unlikely, and urinary renin likely reflects the secreted renin from the collecting ducts and provides a pathway for augmented Ang II levels in the tubules. Therefore, renin might play a part in the maintenance of hypertension. Despite the increased PRA, the urinary renin level decreased after candesartan treatment, further supporting the tubular secretion of renin hypothesis (Liu et al. 2011).

Dahl salt-sensitive rats are genetically predisposed to hypertension, and salt loading causes the development of hypertension. A strong correlation between uAGT/renal AGT content and systolic blood pressure (SBP) was observed among Dahl salt-sensitive rats on a high salt diet. These rats have low PRA levels; thus, their systemic RAS is suppressed. Nevertheless, their intrarenal RAS seemed to be activated because of increased renal Ang II and renal AGT contents. In addition, these rats did not show Ang II type 1 receptor (AT1R) suppression, and the increase in kidney-AGT content caused inappropriate augmentation in the intrarenal RAS activity and contributed to the development and maintenance of hypertension (Kobori et al. 2003b).

Mouse AGT cDNA knock-in mice exhibiting an overexpression of AGT in the proximal tubules showed higher uAGT and urinary Ang II excretion rates and higher daytime and nighttime SBP with a higher salt diet than controls. On a normal salt diet, plasma renin content and PRA were similar between groups; with a high salt intake, however, the plasma renin content was lower in the targeted animals than controls. PRA was undetectable in both groups. This study revealed that proximal-tubule-derived AGT causes hypertension, and this effect is independent of the systemic RAS because low levels of PRA suggest that the systemic RAS is suppressed (Ying et al. 2012).

Spontaneously hypertensive rats on a high salt diet developed increased plasma Ang II, uAGT, and urinary protein excretion as well as glomerular and tubular damage. AT1R blockage with losartan did not reduce blood pressure but did reduce plasma Ang II in rats with normal salt diets. However, reductions in blood pressure and plasma Ang II were not observed in rats with high salt diets treated with losartan. Glomerular and tubulointerstitial changes decreased with AT1R blockage. Renal AGT mRNA expression was similar between groups. Despite low levels of renal AGT mRNA, the increased uAGT excretion can be explained due to the leakage from the damaged glomerulus or the increased intratubular formation of AGT. Losartan, independent of lowered blood pressure, decreased the structural and functional effects of Ang II. High blood pressure among rats with a high salt diet

might be the result of volume overload due to their diet. Furthermore, renal injury likely develops through AT1R activation (Susic et al. 2011). High salt intake also increased oxidative stress in Ang-II-infused rats, and these effects were reversed with AT1R blocker (ARB) treatment (Lara et al. 2012). These studies support the theory that a high salt intake exacerbates the renal damage in Ang-II-dependent hypertension and ARB treatment ameliorates the renal damage and its associated parameters.

In malignant hypertension, the kidneys exhibit myointimal proliferation and fibrinoid necrosis. Increased hydrostatic pressure enhances Ang II production, and this increase results in endothelial and epithelial cell apoptosis and proliferation (Efrati et al. 2007). CYP1A1-REN2 transgenic rats with malignant hypertension showed increased proteinuria and uAGT excretion, and these effects were ameliorated after candesartan treatment (Milani et al. 2010). uAGT might reflect increased intraluminal AGT, which contributes to intrarenal Ang II augmentation. Intrarenal Ang II also plays an important role in malignant hypertension.

Both systemic and intrarenal RAS have effects on the regulation of blood pressure. Ramkumar N et al. (2013) examined the effects of hepatic overexpression, kidney overexpression, or both with regard to AGT. Proximal-tubule-overexpressing mice were labeled “group K.” liver-overexpressing mice were labeled “group L.” both liver- and proximal-tubule-overexpressing mice were labeled “group KL,” and the control group was labeled “group W.” The mean arterial pressure was KL>K>L>W on a normal sodium diet. High sodium intake increased the mean arterial pressure in group L mice. Plasma AGT levels were increased in L>KL>K>W mice with normal or high sodium intake. Plasma renin concentrations were similar in the groups on a normal sodium diet. A high sodium diet decreased plasma renin concentrations similarly in all groups. The uAGT associated with a normal sodium intake was higher in groups KL and K and similar in groups L and W. uAGT increased with a high sodium intake and was higher in KL>K>L>W. The results demonstrated that uAGT levels were correlated with hypertension. Liver-AGT overexpression only caused hypertension in the presence of a high sodium intake, and proximal-tubule AGT overexpression caused hypertension, regardless of liver-AGT overexpression.

To summarize the intrarenal RAS studies regarding hypertension, Ang-II-dependent hypertension might be the result of increased intrarenal RAS activity, and Ang II likely plays a major role in the maintenance of hypertension and the progression of renal damage in its volume-dependent forms.

Although the specific pathological feature of diabetic nephropathy is nodular glomerulosclerosis, most biopsy specimens show excessive mesangial matrix depositions. Primary cultures of rat mesangial cells with high glucose concentrations showed increased intracellular renin activity as well as ACE mRNA and AGT mRNA expression, which resulted in the augmentation of Ang II. Increased Ang II might cause cell proliferation and mesangial matrix deposition with intracrine actions (Vidotti et al. 2004).

Intrarenal RAS activity plays an important role in the pathogenesis and progression of diabetic nephropathy, and urinary albumin excretion is a prognostic factor for

renal function impairment. Streptozocin-induced type 1 diabetic rats without complications were followed up after the onset of diabetes; some rats received insulin treatment. An increase in uAGT developed before an increase in urinary albumin. Insulin treatment ameliorated urinary albumin and uAGT excretion, and AGT mRNA expression was weaker in the treated group (Kamiyama et al. 2012b). Future comprehensive studies may use uAGT as an early biomarker of intrarenal RAS activation in patients with diabetes to help anticipate the development of diabetic nephropathy.

It has been shown that patients with glomerular diseases treated with Ang-converting enzyme inhibitor (ACEi) or ARB have better renal outcomes; this finding might be related to increased intrarenal RAS activity. Rats with anti-thymocyte serum nephritis developed proteinuria, and their renal histology showed mesangial proliferation. Renal AGT, Ang II expression, and uAGT excretion increased. uAGT excretion was positively correlated with glomerular damage, proteinuria, and renal AGT content. After treatment with olmesartan, uAGT, renal AGT, and Ang II expression were attenuated; however, plasma Ang II and PRA increased. These results suggest that the intrarenal RAS is regulated independent of the systemic RAS in glomerular diseases (Ohashi et al. 2008).

Another glomerulonephritis model of rats showed increased PRA, plasma Ang II, renal cortical, and medullar Ang II levels; proteinuria, microalbuminuria, and uAGT excretion; and renal AGT mRNA expression. These effects and the pathologic features of glomerulonephritis (defined as mesangial matrix expansion), except for the renal cortex Ang II content, diminished with aliskiren treatment (Miyata et al. 2014).

Renal Ang II generation in the liver and kidney or dual AGT of knockout mice was examined in the context of glomerular diseases featuring podocyte injury; the results were compared with those of control mice. LMB2-injected mice developed massive proteinuria. After podocyte injury, kidney-AGT knockout mice showed similar increases of renal AGT and Ang II content as well as uAGT excretion rates as the control group. Liver-AGT knockout mice showed decreased renal AGT and Ang II content and decreased uAGT excretion rates similar to the dual-AGT knockout mice. Liver-AGT and dual-AGT knockout mice showed more severe tubulointerstitial damage than the kidney-AGT knockout and control mice. This animal model suggests that impaired glomerular permeability allows liver-derived systemic AGT to pass through the glomerular filtration barrier. Moreover, uAGT primarily consists of liver-derived AGT (Matsusaka et al. 2014).

Premenopausal women have lower blood pressure and a reduced incidence of cardiovascular disease than age-matched men. Similar sex differences have been observed across species and in multiple animal models of hypertension. Female sex hormones, especially estrogen, modulate the renin-angiotensin-aldosterone system. Dahl salt-sensitive rats on a high salt diet exhibited sex-dependent changes in renal AGT mRNA and protein, with higher levels found in male rats. After castration, blood pressure increases, and urinary protein and albumin excretion, glomerular sclerosis, AGT mRNA, and protein levels are attenuated in male rats. Testosterone replacement after castration showed elevated blood pressure and increased renal

damage with a rise in renal AGT mRNA and protein (Yanes et al. 2009). The effects of estrogen depletion and dietary salt intake were studied with regard to AGT expression in the mRen (2) Lewis strain. The results showed that male rats on a normal salt diet exhibited a correlation between uAGT and proteinuria; however, those on a high salt diet did not show relationships among uAGT, SBP, and renal AGT expression. Female rats on a high salt diet showed the highest SBP, following ovariectomized rats and rats on a normal salt diet. Plasma AGT was not influenced by ovariectomy or salt; however, uAGT increased 180-fold among the high salt-fed female rats and 16-fold among ovariectomized rats. These rates were higher than those in the male rats on a high salt diet. Ovariectomy did not change protein excretion in female rats but did increase uAGT excretion. Although a high salt diet resulted in increased uAGT and protein excretion in both female and male rats, the expected increase in renal AGT content and AGT gene expression was not observed. These results suggest that uAGT increases because of the increase in systemic RAS activity. Male rats' higher levels of uAGT and protein excretion might reflect additional renal injury response to hypertension (Cohen et al. 2010). Another study regarding the sexual dimorphism of the intrarenal RAS showed that both salt-induced and Ang-II-dependent hypertension result in higher uAGT, protein excretion, and blood pressure responses among male rats; furthermore, they exhibit significantly more AGT gene expression in their kidneys. These results suggest that males have higher intrarenal RAS activity and are more likely to have kidney damage with hypertension (Rands et al. 2012). The increased RAS activity among males might be because of the androgen-dependent dimorphic expression of AGT mRNA in the kidney (Wang et al. 1994).

Most studies have shown that uAGT reflects intrarenal RAS activity and is generated locally in the proximal tubules; however, two studies have shown that uAGT originates from the liver (Matsusaka et al. 2012, 2014). To determine the source of uAGT, liver-AGT knockout mice, kidney-AGT knockout mice, and dual-AGT knockout mice were compared with the control group. Liver and dual-AGT knockout mice exhibited similar SBPs, which was lower than those of the kidney knockout mice and control group. The former mice groups also showed increased urine volumes, higher PRA, and renal renin activity than kidney knock-out mice and the control group. Liver-AGT mRNA and protein, plasma AGT, kidney-AGT protein, and Ang II content was lower; however, kidney-AGT mRNA was higher in the liver and dual-AGT knockout mice compared with kidney-AGT knockout and control mice. Histological examinations of the mice kidneys revealed medial hyperplasia, juxtaglomerular cell hypertrophy, and mesangial matrix expansion in the liver knockout mice as well as hypoplastic papillae, tubular dilatation, and interstitial fibrosis in the dual-AGT knockout mice. Kidney-AGT knockout mice showed normal renal morphologies. These results suggest that liver-derived AGT is the major source of uAGT (Matsusaka et al. 2012). Furthermore, the systemic RAS is likely important in maintaining normal blood pressure response and water balance. In the absence of the intrarenal RAS activity observed in the kidney-AGT knockout mice, systemic RAS activity might not lead to severe renal damage.

Rats showed an increase in uAGT, SBP, renal cortex AGT, and renal Ang II content after Ang II infusion. Treatment with olmesartan resulted in an increase of plasma Ang II; however, renal Ang II, uAGT, and urinary protein excretion was attenuated. Intrarenal AGT increases were associated with AT1R activation in Ang-II-dependent hypertension, and AT1R blockage decreased uAGT, urinary protein, and SBP (Kobori et al. 2004). When lisinopril (ACEi) was added to the treatment of Ang-II-infused rats, unlike AT1R blockage, plasma and intrarenal Ang II levels also decreased; however, this study did not explore the effects on uAGT (Gonzalez-Villalobos et al. 2009). Low-dose Ang-II-infused mice exhibited similar results with a high dose Ang II infusion, leading to remarkably increased intrarenal AGT expression, except for a slower rise in the blood pressure response. Treatment with olmesartan diminished both intrarenal AGT expression and blood pressure levels (Gonzalez-Villalobos et al. 2008). In the kidneys and adrenal glands, the intracellular uptake of Ang II appears to be mediated by AT1R. When transgenic rats exhibiting a lack of AT1R were compared with wild-type rats, 80 % of the intracellular uptake of labeled Ang II occurred through AT1R activation. The remaining 20 % of the uptake was not mediated by the receptor (Li and Zhuo 2008). All of these findings suggest that treatment with an ACEi or AT1R blocker not only ameliorates hypertension but also improves the renal histology and decreases uAGT and urinary protein excretion; these effects were not associated with blood pressure levels.

Human Studies

Twenty years after the study by Favaro et al. (1972), in the 1990s, many studies made important advances in the understanding of the pathophysiology and complications of hypertension. The chief portion of this development was with regard to the RAS. After defining the components of the RAS, their relationships and functions were examined using animal models. The results of these studies were hopeful; thereafter, many clinical studies in humans were designed to study the RAS and its components. The relationship between the intrarenal RAS and uAGT in animal models was well defined. Human genetic studies have revealed a link between the AGT gene and hypertension (Inoue et al. 1997). In addition, various clinical studies have shown that increased uAGT levels indicate the intrarenal RAS status in different populations such as patients with hypertension, type 1 and type 2 diabetes mellitus, chronic glomerulonephritis, immunoglobulin A (IgA) nephropathy, chronic kidney disease (CKD), renal amyloidosis or autosomal-dominant polycystic kidney disease (ADPKD), as well as renal transplant recipients. In 2007, Katsurada and colleagues developed a novel sandwich ELISA for human AGT. The methods and uAGT results of the discussed studies are given in Tables 1, 2, and 3.

In various studies, the relationship between uAGT and hypertension has been discussed. Kobori et al. (2009) investigated the uAGT levels in 70 patients with hypertension and 36 normotensive controls. Elevated uAGT levels were reported in patients with hypertension compared with controls. In addition, these authors demonstrated that uAGT was significantly and positively correlated with SBP, diastolic

Table 1 Human AGT results in urine

Study	Population	Method	uAGT	Comment
Katsurada et al. (2007)	Healthy volunteers	ELISA	7.1–35 ng/ml	Human AGT ELISA might be a useful disease marker
Mills et al. (2012)	Patients with CKD	ELISA	CKD: 45.4 μg/24 h	uAGT might identify patients with CKD
			Controls: 7.4 μg/24 h	

AGT angiotensinogen, *CKD* chronic kidney disease, *uAGT* urinary angiotensinogen

Table 2 Studies using log uAGT/uCre and their results

Study	Population	Method	Log uAGT/uCre	Comment
Kobori et al. (2008)	Patients with CKD	ELISA	CKD: 1.8801 ± 0.0885	uAGT might be a biomarker of CKD severity, and the ELISA method is valid for measuring uAGT
			Controls: 0.9417 ± 0.1048	
Xu et al. (2015)	Patients with CKD	ELISA	CKD: 2.02 ± 0.55 ng/mg	uAGT reflects intrarenal Ang II activity in patients with CKD
			Control 1.77 ± 0.40 ng/mg	
Kutlugun et al. (2012a)	Amyloidosis patients	ELISA	Amyloidosis: 1.88 ± 0.92 μg/g	uAGT is correlated with proteinuria in renal amyloidosis AA
			Controls: 1.25 ± 0.70 μg/g	

AGT angiotensinogen, *CKD* chronic kidney disease, *uAGT* urinary angiotensinogen

blood pressure (DBP), the urinary albumin/creatinine ratio (uAlb/uCre), and the urinary protein/creatinine ratio (uPro/uCre). uAGT was not related to race, age, gender, height, body weight, body mass index (BMI), serum sodium levels, serum potassium levels, serum creatinine levels, the urinary sodium/creatinine ratio, the urinary potassium/creatinine ratio, the fractional excretion of sodium, plasma AGT levels, or the estimated glomerular filtration rate (eGFR). They also observed that uAGT levels were significantly higher among patients with hypertension without RAS blockage compared with normotensive participants. Importantly, patients treated with RAS blockers demonstrated a prominent attenuation of this augmentation. These data suggest that the efficacy of the RAS blockage seeking to reduce intrarenal RAS activity can be assessed by measuring uAGT excretion.

Another study reported that uAGT levels were higher in 55 patients with confirmed primary hypertension than a reference group consisting of 33 participants with white-coat hypertension. This study also reported that the increased excretion of uAGT was correlated with hyperuricemia in adolescents with primary hypertension (Kuroczycka-Saniutycz et al. 2013).

Table 3 uAGT/uCre results in human studies

Study	Population	Method	uAGT/uCre	Comment
Kobori et al. (2009)	Patients with hypertension	ELISA	Hypertension with RAS blocker: 25.00 ± 4.96 μg/g	Treatment with RAS blockers suppresses uAGT
			Normotensive controls: 13.70 ± 2.33 μg/g	
Zou et al. (2012)	Patients with hypertension	ELISA	≤0.72 μg/g versus	uAGT is positively correlated with clinic and ambulatory blood pressure
			>0.72 μg/g	
Kuroczycka-Saniutycz et al. (2013)	Adolescents with hypertension	ELISA	Hypertension: 0–2.28 ng/mg Cre	uAGT is correlated with hyperuricemia
			Controls: 0–0.44 ng/mg Cre	
Saito et al. (2009)	Patients with type 1 diabetes	ELISA	Type 1 diabetes: 12.1 ± 3.2 μg/g	uAGT might be an early marker of diabetic nephropathy and the initial sign of proteinuria or microalbuminuria
			Controls: 4.2 ± 0.7 μg/g	
Terami et al. (2013)	Patients with type 2 diabetes at various stages of nephropathy	ELISA	Normoalbuminuria: 6.8 ± 11.6 μg/g Cre	uAGT is a marker of tubular injuries in the early stages of diabetic nephropathy
			Microalbuminuria: 8.5 ± 9.9 μg/g Cre	
			Macroalbuminuria: 73.3 ± 95.2 μg/g Cre	
Sawaguchi et al. (2012)	Patients with type 2 diabetes mellitus	ELISA	Patients with albuminuria: 62.0 μg/g Cre (interquartile range: 25.4–146.5)	Higher levels of uAGT among patients with type 2 diabetic and albuminuria is a strong risk factor for worsening renal and cardiovascular complications
			Patients with normoalbuminuria: 17.5 μg/g Cre (interquartile range 11.4–28.2)	
Soltysiak et al. (2014)	Normoalbuminuric children with diabetes	ELISA	Children with type 1 diabetes: 0.00 and 1.76 ng/mg	uAGT might reflect early renal involvement and precede the onset of microalbuminuria and hypertension
			Controls: 0.00 and 0.00 ng/mg	
Urushihara et al. (2010)	Patients with chronic GN	ELISA	Chronic GN: 19.79 ± 3.70 μg/g	Treatment with RAS blockers suppresses uAGT
			Chronic GN+ARB: 10.58 ± 1.23 μg/g	
			Controls: 6.22 ± 0.98 μg/g	

(*continued*)

Table 3 (continued)

Study	Population	Method	uAGT/uCre	Comment
Nishiyama et al. (2011)	Patients with IgA nephropathy	ELISA	IgA nephropathy: 39 ± 31 μg/g	uAGT is powerful marker of intrarenal RAS status and associated renal derangement
			Healthy controls: 10 ± 4 μg/g	
Kim et al. (2011)	Patients with IgA nephropathy	ELISA	IgA nephropathy: 104.96 ± 23.23 ng/mg Cre	uAGT increased and was positively correlated with uPro/uCre; patients with uAGT levels >100 ng/mg Cre might have poor renal function
			Healthy controls: 6.71 ± 1.13 ng/mg Cre	
Yamamoto et al. (2007)	Patients with CKD	RIA	<3 nmol Ang I Eq/g Cre versus	uAGT >3 nmol Ang I Eq/g Cre is a predictive marker for the deterioration of renal function
			>3 nmol Ang I Eq/g Cre	
Mills et al. (2012)	Patients with CKD	ELISA	CKD: 26.3 μg/g	uAGT might identify patients with CKD
			Controls: 4.4 μg/g	
Kocyigit et al. (2013)	Patients with ADPKD with or without hypertension	ELISA	Patients with ADPKD and hypertension: 23.7 ± 8.4 μg/g	uAGT is a potential biomarker of intrarenal RAS status among patients with ADPKD and hypertension
			Patients with ADPKD without hypertension: 16.6 ± 5.2 μg/g	
			Healthy controls: 6.9 ± 3.3 μg/g	
Kurultak et al. (2014)	Patients with ADPKD	ELISA	Patients with ADPKD: 19.35 ± 6.06 μg/g	uAGT might be a useful marker for risk classification among patients with ADPKD
			Healthy controls: 11.65 ± 1.66 μg/g	
Kutlugun et al. (2012b)	Renal transplant patients with/without hypertension	ELISA	Patients with hypertension: 8.98 ± 6.89 μg/g	uAGT can help to evaluate intrarenal RAS activation in renal transplant patients
			Patients with normotension: 5.48 ± 3.33 μg/g	
Erdogmus et al. (2013)	Renal transplant patients	ELISA	Renal transplant patients: 70.6 ± 11.3 μg/g	uAGT is associated with increased proteinuria among patients without chronic allograft injury
			Healthy controls: 9.3 ± 2.35 μg/g	

ADPKD autosomal-dominant polycystic kidney disease, *CKD* chronic kidney disease, *GN* glomerulonephritis, *uAGT* urinary angiotensinogen, *RAS* renin-angiotensin system

In the Bogalusa Heart Study, Kobori and colleagues (2010) evaluated the relationship between uAGT and traditional cardiovascular disease risk factors among asymptomatic young adults. They demonstrated that uAGT levels did not differ with regard to race or sex but were significantly correlated with SBP, DBP, uAlb/uCre, and uPro/uCre. Moreover, high correlations were found among men, especially those of African descent.

Jun Zou et al. investigated the association between uAGT excretion and ambulatory blood pressure levels (2012). They found that uAGT/uCre was significantly and positively associated with clinical and ambulatory blood pressure levels. uAGT excretion was high with a greater urinary sodium excretion as well as associated with clinical and ambulatory blood pressure measurements.

Identifying the sensitive biomarkers that can predict microalbuminuria or diabetic nephropathy during, an earlier stage of diabetes might provide meaningful information concerning early pathophysiology and an earlier clinical approach to the diagnosis and treatment of diabetic nephropathy. In this regard, a study (performed with youths with type 1 diabetes and control participants) found important results to support the hypothesis that enhanced uAGT levels are not a consequence of proteinuria. Specifically, both groups showed similar uAlb/uCre and uPro/uCre values, and these parameters were in the normal range; however, the former was significantly higher among patients with type 1 diabetes than controls. Importantly, AGT was not increased in their plasma. These data indicate that uAGT can be used as an early biomarker of diabetic nephropathy during the premicroalbuminuric phase among patients with type 1 diabetes (Saito et al. 2009). In another study conducted with children with type 1 diabetes, researchers evaluated the associations among blood pressure, uAGT, and renal sodium excretion. They showed that uAGT levels were significantly higher among patients than controls. uAGT levels were positively correlated with 24-h ambulatory SBP, DBP, and mean arterial pressure. The significant increase in uAGT was observed even in patients with prehypertension. Interestingly, significant correlations were not found between uAGT and uAlb/uCre or between uAGT and eGFR. Furthermore, uAGT, uAlb/uCre, and eGFR were negatively correlated with renal sodium excretion (Soltysiak et al. 2014). Given these results, the authors suggested that elevated levels of uAGT reflect early renal involvement and should be considered a new marker of hypertension in normoalbuminuric children with type 1 diabetes.

A study of Japanese patients with type 2 diabetes (at various stages of nephropathy) demonstrated that uAGT levels were positively correlated with uAlb/uCre and urinary alpha-1-microglobulin as well as negatively correlated with eGFR. Nevertheless, a significant and high correlation between uAGT levels and alpha-1-microglobulin was demonstrated during the normoalbuminuric stage. In addition, uAlb/uCre was higher among patients receiving an RAS inhibitor; furthermore, urinary alpha-1-microglobulin and AGT did not denote a significant increase in this group (Terami et al. 2013). This clinical study found that uAGT is a marker for tubular injuries of early-stage diabetic nephropathy in patients with type 2 diabetes.

Sawaguchi and colleagues (2012) showed that baseline uAGT levels were positively correlated with uAlb/uCre and urinary beta-2-microglobulin in patients with type 2 diabetes with normo- and microalbuminuria. However, plasma AGT levels were not correlated with these renal factors or uAGT levels. Moreover, uAGT was negatively correlated with the annual decrease of eGFR, and patients with high levels of uAGT and albuminuria demonstrated a progressive decline in eGFR and a higher incidence of renal and cardiovascular composite endpoints. These data suggest that a high level of uAGT in patients with type 2 diabetic and albuminuria is a risk factor for renal and cardiovascular complications. More recently, AGT mRNA and protein were found to be significantly higher in patients with diabetes than control participants. Moreover, the AGT mRNA levels in tubules were negatively correlated with eGFR in patients with diabetes. Kidney biopsy specimens of patients with diabetes also showed increased expression of 4-hydroxy-2-nonenal and heme oxygenase-1 than a control group (Kamiyama et al. 2013). These data suggest that intrarenal RAS activation and oxidative stress play important roles in the development of diabetic nephropathy.

Heuvel et al. (2011) studied 101 diabetic and nondiabetic patients with or without hypertension. These authors observed that RAS blockage using either ACEi or ARB increases plasma renin concentration and decreases urinary renin. The decrease in urinary renin levels after RAS blockage, which occurred independent of the plasma renin levels, reflects the activated renal RAS in patients with diabetes and the success of the RAS blockage in the kidney. Therefore, urinary renin more closely reflects renal RAS activity than uAGT. The same investigators designed a new study to confirm their findings; they measured uAGT and plasma AGT levels as well as renin and albumin in 22 patients with type 2 diabetes and hypertension accompanied by albuminuria over a 2-month treatment period compared with a placebo or RAS blocker group. They found that the urine/plasma renin ratio, not only urinary renin, reflects the renal efficacy of the RAS blockage (Persson et al. 2013). This study elucidated the origin of uAGT and renin; specifically, uAGT might be a marker of filtration barrier damage rather than intrarenal RAS activity.

CKD is a major public health problem worldwide. The investigators designed many studies to investigate the role of uAGT levels on the progression of CKD. Yamamoto et al. (2007) found that uAGT levels were higher in patients with low eGFR. Elevated urinary protein and type IV collagen excretion were also correlated with uAGT levels. These authors also reported that uAGT levels were positively correlated with renal Ang II and type I collagen immunostaining intensities. Moreover, treatment with losartan reduced uAGT, plasma AGT, urinary protein, and tip IV collagen and SBP despite concomitant increases in plasma renin and Ang II. A higher level of uAGT ($>$3.0 nmol Ang I Eq/g Cre) was a risk factor for progressive renal dysfunction in patients with CKD.

In 2008, Kobori and colleagues reported that uAGT levels were significantly higher in 80 patients with CKD than in seven healthy volunteers. They also reported that uAGT levels were significantly and positively correlated with uAlb/uCre and uPro/uCre. Furthermore, they found an inverse association between uAGT and

eGFR. In contrast, the uAGT levels in patients with minimal change disease were similar to those in control participants, although patients with minimal change disease had severe proteinuria. Furthermore, the uAGT levels were not correlated with gender, age, BMI, SBP, DBP, PRA, or plasma AGT levels.

In another study, uAGT levels were positively correlated with intrarenal immunostaining intensities of AGT, Ang II, and Ang II type 1 receptors in Chinese patients with CKD. Furthermore, a multiple regression analysis indicated that high urinary protein, urinary Ang II, and urinary collagen IV excretion were significantly correlated with high uAGT (Xu et al. 2015). Collectively, these findings indicate the potential of uAGT as a marker of intrarenal Ang II activity in patients with CKD (intrarenal Ang II activity increases in parallel with the severity of fibrotic renal damage among these patients). These data also suggest that the levels of uAGT should help to identify patients with CKD who are at increased risk for progressive renal failure.

Recently, Nakano et al. (2012) showed that the vast majority of uAGT originates from the tubules rather than via glomerular filtration using multiphoton fluorescence microscopic imaging of the glomerular permeability of AGT. These findings strongly support the hypothesis that the increase in uAGT reflects the de novo production of AGT in the kidney and marks the activation of the intrarenal RAS.

Mills et al. (2012) found that uAGT levels were positively correlated with albuminuria but negatively correlated with eGFR among 201 patients with CKD. They also reported that uAGT levels were significantly higher in patients with CKD than in controls. These associations were independent of established risk factors for CKD and the use of RAS inhibitors. These findings indicate that an association exists between uAGT excretion and CKD that is independent of albuminuria, a sensitive marker for kidney structure damage, and urinary protein leakage. Therefore, uAGT excretion might provide additional information for risk classification and prediction among patients with CKD.

In a recent clinical study, Nishijima and colleagues (2012) examined the preservation conditions of the measurements of uAGT concentrations and the ultradian rhythm of uAGT excretion in healthy individuals. They found that the preservation conditions did not change the uAGT concentration measurements. In addition, uAGT excretion in healthy volunteers did not show an ultradian change during the daytime. In a later study, Nishijima et al. (2014) investigated the relationship between circadian rhythms and plasma AGT and uAGT in healthy volunteers and patients with CKD. They did not find evidence that AGT has a circadian rhythm under any condition.

Chronic glomerulonephritis is one of the common causes of end-stage renal disease. In 2010, Urushihara and colleagues performed a study to evaluate the uAGT levels in chronic glomerulonephritis patients (26 with IgA nephropathy, 24 with purpura nephritis, 8 with lupus nephritis, 7 with focal segmental glomerulosclerosis, and 5 with non-IgA mesangial proliferative glomerulonephritis). uAGT levels were significantly increased among patients with chronic glomerulonephritis without RAS blockage, compared with control participants. Importantly, patients with glomerulonephritis treated with RAS blockers showed a prominent suppression

of uAGT. Moreover, the uAGT levels were positively correlated with uAlb/uCre and uPro/uCre.

Nishiyama et al. (2011) reported that uAGT levels were correlated with augmented intrarenal AGT gene expression and Ang II levels in normotensive patients with moderately proteinuric IgA nephropathy. They also reported that uAGT levels did not differ between healthy volunteers and patients with minimal glomerular abnormality (MGA). However, uAGT levels, renal tissue AGT expression, and Ang II immunoreactivity were significantly higher among patients with IgA nephropathy than those with MGA. Furthermore, treatment with an Ang II receptor blocker reduced uAGT levels, renal tissue AGT gene expression, and Ang II immunoreactivity in patients with IgA nephropathy.

In another study, Kim and colleagues (2011) sought to determine the role of uAGT as a predictive marker in patients with IgA nephropathy. They found that uAGT levels were significantly higher among patients with IgA nephropathy and non-IgA nephropathy than healthy participants. Using a univariate regression analysis, they found that uPro/uCre, serum creatinine, SBP, and DBP were positively correlated with uAGT. They did not find any correlations among uAGT, PRA, and aldosterone levels. In addition, a multivariate regression analysis revealed that uAGT was positively related to uPro/uCre. Few studies have addressed the cut-off value of uAGT in patients with CKD. The current authors divided patients into two groups based on uAGT levels (100 ng/ml Cr). Patients with uAGT levels >100 ng/ml Cr had higher SBP, baseline uPro/uCre, and serum creatinine levels than those who had lower uAGT levels. These results suggest that uAGT levels can assess the local activity of the RAS in the kidney and identify patients at risk for renal disease progression.

Secondary amyloidosis is a rare form of CKD in many countries; however, patients, especially those living in Mediterranean regions, are at increased risk for developing amyloidosis because of familial Mediterranean fever. Kutlugun et al. from Turkey reported that logarithmic transformation of the uAGT/uCre levels was significantly higher in 32 patients with renal AA amyloidosis than in 16 healthy controls (2012a). One of the most important findings in that study was the significant and positive correlation between uAGT and daily protein excretion among the patients with renal AA amyloidosis. However, log uAGT/UCre levels were not significantly correlated with patient age, gender, serum creatinine, eGFR, BMI, SBP, or DBP. They did not observe a significant difference in log uAGT/UCre levels between patients with and without hypertension. These findings suggest that uAGT is not a nonspecific result of proteinuria. The activated intrarenal RAS might play a role in the pathogenesis and development of proteinuria in patients with renal AA amyloidosis.

ADPKD is a multisystem disorder characterized by bilateral, multiple cysts in the kidneys and other organs such as the liver, pancreas, and arachnoid membranes. It is an inherited disease, and patients usually develop CKD during follow-up. Kurultak et al. (2014) demonstrated that uAGT/uCre levels were positively correlated with uPro/uCre and uAlb/uCre in patients with normotensive autosomal-dominant

polycystic kidney disease (ADPKD). They also found that the other parameters such as patient age, gender, serum creatinine, sodium, potassium, uric acid, eGFR, BMI, SBP, and DBP were not correlated with uAGT/uCre levels. In a similar study, Kocyigit et al. (2013) reported that uAGT/uCre levels were significantly higher among 43 patients with hypertensive ADPKD compared with 41 normotensive ADPKD patients and 40 normotensive healthy controls. In addition, uAGT/uCre levels were correlated with the uPro/uCre levels and 24-h DBP. They also observed that the uAGT/uCre and uPro/uCre levels were significantly higher in patients with hypertensive ADPKD without RAS blockage. Moreover, patients with hypertension and RAS blockage did not have this augmentation. In light of these findings, uAGT levels might indicate the risk classification of ADPKD, particularly for patients in the early stage of disease and during the early onset of hypertension.

In kidney transplantation, the activation of the intrarenal RAS might play a role in the development and progression of chronic allograft injury. Erdogmus et al. (2013) reported that uAGT levels were significantly higher in 70 renal transplant patients compared with 21 healthy volunteers. We also reported a significant positive correlation between uAGT excretion and proteinuria and a negative correlation between uAGT excretion and eGFR in renal transplant patients without overt chronic allograft injury. Moreover, uAGT levels were not associated with other patient parameters including age, sex, height, BMI, serum sodium, potassium, chloride, uric acid, creatinine or urinary sodium, potassium, or blood pressure levels in renal transplants patients. In addition, we did not observe any effect of RAS inhibitors or other antihypertensive drugs on uAGT excretion in this relatively small group of renal transplant recipients. These results suggest that uAGT excretion, which denotes the activation of the intrarenal RAS, plays a role in the development of chronic allograft injury among renal transplant recipients. Likewise, Naganuma and colleagues (2014) demonstrated that uAGT levels were higher among renal transplant recipients than renal transplant donors. They also reported that uAGT levels were significantly and positively correlated with uAlb/uCre but negatively correlated with eGFR. uAGT levels were higher in renal transplant patients with hypertension than those who were normotensive. uAGT levels were not significantly correlated with patient age, gender, serum creatinine, eGFR, BMI, SBP, DBP, dialysis modality, duration of dialysis before transplantation, or time since transplantation. These authors also showed that uAGT levels were correlated with urinary protein excretion in renal transplant recipients with hypertension; however, this correlation was not found among those who were normotensive (Kutlugun et al. 2012a).

There is limited data with respect to employing uAGT in AKI as a predictive biomarker. Alge et al. (2013a) demonstrated that uAGT could be used as a prognostic biomarker of acute kidney injury (AKI) in patients who developed this condition after cardiac surgery. In another study, these authors reported that uAGT could be used as a prognostic AKI biomarker in intensive care units. Increased uAGT was associated with adverse events in AKI patients in the intensive care unit (Alge et al. 2013b).

The Importance of uAGT

A biomarker is a characteristic that is objectively measured and evaluated to indicate normal biological processes, pathogenic processes, or pharmacologic responses to an intervention (National Research Council 2010). Urinary Ang II is unstable and therefore cannot be used as a reliable marker of intrarenal RAS activity in clinical studies. uAGT might reflect intrarenal Ang II levels, and it is the only known noninvasively measured marker for assessing the activity of intrarenal RAS (Kobori et al. 2002, 2003b).

The development of an ELISA kit for uAGT measurement would enable sensitive and specific quantifications as well as the evaluation of treatment effects; however, a cut-off level for uAGT has not yet been established. Different quantification methods for uAGT have been used in previous studies, rendering it difficult to compare past results. Future studies might reveal a cut-off value that would enable us to use uAGT in clinical practice.

In conclusion, a debate continues regarding whether uAGT indicates the impairment of glomerular permeability (like proteinuria), tubulointerstitial injury, or the activation of intrarenal RAS in various renal diseases. Therefore, additional research is needed to determine the importance of uAGT.

Potential Applications to Prognosis and Other Diseases or Conditions

The above findings suggest that uAGT can be used as an index of intrarenal RAS activation and thus may be a useful prognostic biomarker. In efforts to translate the findings from the experimental studies to human subjects, various clinical studies have demonstrated increased urinary AGT levels in hypertension, type 1 and type 2 diabetes mellitus, and several forms of chronic kidney diseases. However, no study has described any effect of uAGT other than in renal diseases. Larger clinical studies are needed in human subjects to determine if treatment with RAS inhibitors can reduce uAGT levels, thus indicating reduction in intrarenal RAS activity.

The valid measurement method of uAGT is Elisa in samples. The development of an ELISA kit for uAGT measurement would enable sensitive and specific quantifications as well as the evaluation of treatment effects; however, a cut-off level for uAGT has not yet been established. Different quantification methods for uAGT have been used in previous studies, rendering it difficult to compare past results. Future studies might reveal a cut-off value that would enable us to use uAGT in clinical practice.

Considering all the available data together, uAGT can be a potential biomarker of treatment response with an ACEi or ARB or may be defining the severity of the renal disease, but there is no evidence to use it as a screening marker for any renal disease.

Summary Points

- The kidney is a specific organ that contains and produces all of the RAS components.
- The major source of intrarenal Ang II is AGT, and it is locally produced by proximal tubular cells.
- Ang II plays an important role in maintaining hypertension and the development of renal damage.
- uAGT, rather than urinary albumin, may be an earlier marker for diabetic nephropathy.
- Treatment with an ACEi or AT1R blocker reduces blood pressure, urinary protein excretion, and uAGT in both animal and human studies.
- uAGT is correlated with urinary protein excretion and hypertension.
- uAGT can be used to detect the severity of CKD.
- Many forms of renal diseases exhibit increased uAGT excretion, but there is still a debate as to whether uAGT is an injury marker or a result of leakage from damaged renal tissue.

References

Alge JL, Karakala N, Neely BA, Janech BG, Tumlin JA, Chawla LS, Shaw AD, Arthur JM, SAKInet Investigators. Urinary angiotensinogen and risk of severe AKI. Clin J Am Soc Nephrol. 2013a;8:2043–52.

Alge JL, Karakala N, Neely BA, Janech BG, Veles JCQ, Arthur JM, SAKInet investigators. Urinary angiotensinogen predicts adverse outcomes among acute kidney injury patients in the intensive care unit. Crit Care. 2013b;17:R69.

Braun-Menéndez E, Fasciolo JC, Leloir LF, Muñoz JM. La sustancia hipertensora de la sangre del riñón isquemiado. Rev Soc Arg Biol. 1939;15:420–5.

Cohen JA, Lindsey SH, Pirro NT, Brosnihan KB, Gallagher PE, Chappell MC. Influence of estrogen depletion and salt loading on renal angiotensinogen expression in the mRen(2). Lewis strain. Am J Physiol Renal Physiol. 2010;299(1):F35–42.

Darby IA, Congiu M, Fernley RT, Sernia C, Coghlan JP. Cellular and ultrastructural location of angiotensinogen in rat and sheep kidney. Kidney Int. 1994;46(6):1557–60.

Dibona GF, Sawin LL. Effect of endogenous angiotensin II on the frequency response of the renal vasculature. Am J Physiol Renal Physiol. 2004;287(6):F1171–8.

Efrati S, Berman S, Goldfinger N, Erez N, Averbukh Z, Golik A, Rotter V, Weissgarten J. Enhanced angiotensin II production by renal mesangium is responsible for apoptosis/proliferation of endothelial and epithelial cells in a model of malignant hypertension. J Hypertens. 2007;25 (5):1041–52.

Erdogmus S, Sengul S, Kocak S, Kurultak I, Celebi ZK, Kutlay S, Keven K, Erbay B, Erturk S. Urinary angiotensinogen level is correlated with proteinuria in renal transplant recipients. Transplant Proc. 2013;45:935–9.

Favaro S, Baggio B, Castellani A, Corsini A, Antonello A, Todesco S, Borsatti A. Urinary angiotensinogen loss in chronic proteinuric glomerulonephritis. Int Urol Nephrol. 1972;4 (2):195–8.

Gonzalez-Villalobos RA, Seth DM, Satou R, Horton H, Ohashi N, Miyata K, Katsurada A, Tran DV, Kobori H, Navar LG. Intrarenal angiotensin II and angiotensinogen augmentation in chronic angiotensin II-infused mice. Am J Physiol Renal Physiol. 2008;295(3):F772–9.

Gonzalez-Villalobos RA, Satou R, Seth DM, Semprun-Prieto LC, Katsurada A, Kobori H, Navar LG. Angiotensin-converting enzyme-derived angiotensin II formation during angiotensin II-induced hypertension. Hypertension. 2009;53(2):351–5.

Hashimoto S, Adams JW, Bernstein KE, Schnermann J. Micropuncture determination of nephron function in mice without tissue angiotensin-converting enzyme. Am J Physiol Renal Physiol. 2005;288(3):F445–52.

Ingert C, Grima M, Coquard C, Barthelmebs M, Imbs JL. Effects of dietary salt changes on renal renin-angiotensin system in rats. Am J Physiol Renal Physiol. 2000;283(5):F995–1002.

Inoue I, Nakajima T, Williams CS, Quackenbush J, Puryear Y, Powers M, Cheng T, Ludwig EH, Sarma AM, Hata A, Jeunemaitre X, Lalouel JM. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. J Clin Invest. 1997;99:1786–97.

Kamiyama M, Farragut KM, Garner MK, Navar LG, Kobori H. Divergent localization of angiotensinogen mRNA and protein in proximal tubule segments of normal rat kidney. J Hypertens. 2012a;30(12):2365–72.

Kamiyama M, Zsombok A, Kobori H. Urinary angiotensinogen as a novel early biomarker of intrarenal renin-angiotensin system activation in experimental type 1 diabetes. J Pharmacol Sci. 2012b;119(4):314–23.

Kamiyama M, Urushihara M, Morikawa T, Konishi Y, Imanishi M, Nishiyama A, Kobori H. Oxidative stress/angiotensinogen/renin-angiotensin system axis in patients with diabetic nephropathy. Int J Mol Sci. 2013;14:23045–62.

Katsurada A, Hagiwara Y, Miyashita K, Satou R, Miyata K, Ohashi N, Navar LG, Kobori H. Novel sandwich ELISA for human angiotensinogen. Am J Physiol Renal Physiol. 2007;293(3): F956–60.

Kim YG, Song SB, Lee SH, Moon JY, Jeong KH, Lee TW, Ihm CG. Urinary angiotensinogen as a predictive marker in patients with immunoglobulin A nephropathy. Clin Exp Nephrol. 2011;15:720–6.

Kobori H, Harrison-Bernard LM, Navar LG. Enhancement of angiotensinogen expression in angiotensin II-dependent hypertension. Hypertension. 2001;37(5):1329–35.

Kobori H, Harrison-Bernard LM, Navar LG. Urinary excretion of angiotensinogen reflects intrarenal angiotensinogen production. Kidney Int. 2002;61(2):579–85.

Kobori H, Nishiyama A, Abe Y, Navar LG. Enhancement of intrarenal angiotensinogen in Dahl salt-sensitive rats on high salt diet. Hypertension. 2003a;41(3):592–7.

Kobori H, Nishiyama A, Harrison-Bernard LM, Navar LG. Urinary angiotensinogen as an indicator of intrarenal angiotensin status in hypertension. Hypertension. 2003b;41(1):42–9.

Kobori H, Prieto-Carrasquero MC, Ozawa Y, Navar LG. AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. Hypertension. 2004;43 (5):1126–32.

Kobori H, Nangaku M, Navar LG, Nishiyama A. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. Pharmacol Rev. 2007a;59:251–87.

Kobori H, Ozawa Y, Satou R, Katsurada A, Miyata K, Ohashi N, Hase N, Suzaki Y, Sigmund CD, Navar LG. Kidney-specific enhancement of ANG II stimulates endogenous intrarenal angiotensinogen in gene-targeted mice. Am J Physiol Renal Physiol. 2007b;293(3): F938–45.

Kobori H, Ohashi N, Katsurada A, Miyata K, Satou R, Saito T, Yamamoto T. Urinary angiotensinogen as a potential biomarker of severity of chronic kidney diseases. J Am Soc Hypertens. 2008;2:349–54.

Kobori H, Alper Jr AB, Shevava R, Katsurada A, Saito T, Ohashi N, Urushihara M, Miyata K, Satao R, Hamm LL, Navar LG. Urinary angiotensinogen as a novel biomarker of the intrarenal renin-angiotensin system status in hypertensive patients. Hypertension. 2009;53:344–50.
Kobori H, Urushihara M, Xu JH, Berenson GS, Navar G. Urinary angiotensinogen is correlated with blood pressure in men (Bogalusa Heart Study). J Hypertens. 2010;28:1422–8.
Kocyigit I, Yilmaz MI, Unal A, Ozturk F, Eroglu E, Yazici C, Orscelik O, Sipahioglu MH, Tokgoz B, Oymak O. A link between the renin angiotensin system and hypertension in autosomal dominant polycystic kidney disease. Am J Nephrol. 2013;38:218–25.
Kuroczycka-Saniutycz E, Wasilewska A, Sulik A, Milewski R. Urinary angiotensinogen as a marker of intrarenal angiotensin II activity in adolescent with primary hypertension. Pediatr Nephrol. 2013;28:1113–9.
Kurultak I, Sengul S, Kocak S, Erdogmus E, Calayoglu R, Mescigil P, Keven K, Erturk S, Erbay B, Duman N. Urinary angiotensinogen, related factors and clinical implications in normotensive autosomal dominant polycystic kidney disease patients. Ren Fail. 2014;36:717–21.
Kutlugun AA, Altun B, Aktan U, Turkmen E, Altindal M, Yildirim T, Yilmaz R, Arici M, Erdem Y, Turgan C. The relation between urinary angiotensinogen and proteinuria in renal AA amyloidosis patients. Amyloid. 2012a;19:28–32.
Kutlugun AA, Altun B, Buyukasik Y, Aki T, Turkmen T, Altindal M, Yıldırım T, Yilmaz R, Turgan C. Elevated urinary angiotensinogen a marker of intrarenal renin angiotensin system in hypertensive renal transplant recipients: does it play a role in development of proteinuria in hypertensive renal transplant patients? Transpl Int. 2012b;25:13–8.
Lara LS, McCormack M, Semprum-Prieto LC, Shenouda S, Majid DS, Kobori H, Navar LG, Prieto MC.AT1 receptor-mediated augmentation of angiotensinogen, oxidative stress, and inflammation in ANG II-salt hypertension. Am J Physiol Renal Physiol. 2012;302(1):F85–94.
Li XC, Zhuo JL. In vivo regulation of AT1a receptor-mediated intracellular uptake of [125I]Val5-ANG II in the kidneys and adrenals of AT1a receptor-deficient mice. Am J Physiol Renal Physiol. 2008;294(2):F293–302.
Liu L, Gonzalez AA, McCormack M, Seth DM, Kobori H, Navar LG, Prieto MC. Increased renin excretion is associated with augmented urinary angiotensin II levels in chronic angiotensin II-infused hypertensive rats. Am J Physiol Renal Physiol. 2011;301(6):F1195–201.
Matsusaka T, Niimura F, Shimizu A, Pastan I, Saito A, Kobori H, Nishiyama A, Ichikawa I. Liver angiotensinogen is the primary source of renal angiotensin II. J Am Soc Nephrol. 2012;23(7):1181–9.
Matsusaka T, Niimura F, Pastan I, Shintani A, Nishiyama A, Ichikawa I. Podocyte injury enhances filtration of liver-derived angiotensinogen and renal angiotensin II generation. Kidney Int. 2014;85(5):1068–77.
Milani CJ, Kobori H, Mullins JJ, Mitchell KD. Enhanced urinary angiotensinogen excretion in CYP1A1-REN2 transgenic rats with inducible Ang II dependent malignant hypertension. Am J Med Sci. 2010;340(5):389–94.
Mills KT, Kobori H, Hamm LL, Alper AB, Khan IE, Rahman M, Navar LG, Liu Y, Browne GM, Batuman V, He J, Chen J. Increased urinary excretion of angiotensinogen is associated with risk of chronic kidney disease. Nephrol Dial Transplant. 2012;27:3176–81.
Miyata K, Satou R, Inui D, Katsurada A, Seth D, Davis A, Urushihara M, Kobori H, Mitchell KD, Navar LG. Renoprotective effects of direct renin inhibition in glomerulonephritis. Am J Med Sci. 2014;348(4):306–14.
Naganuma T, Takemoto Y, Maeda S, Maeda Y, Kuwabara N, Uchida J, Nakatani T. Investigation of urinary angiotensinogen in renal transplant recipients. Transplant Proc. 2014;46:489–91.
Nakano D, Kobori H, Burford JL, Gevorgyan H, Seidel S, Hitomi H, Nishiyama A, Peti-Peterdi J. Multiphoton imaging of the glomerular permeability of angiotensinogen. J Am Soc Nephrol. 2012;23(11):1847–56.
National Research Council. Evaluation of biomarkers and surrogate endpoints in chronic disease. Washington, DC: The National Academies Press; 2010. p. 1–336.

Navar LG, Harrison-Bernard LM, Iming JD, Wang CT, Cervenka L, Mitchell KD. Intrarenal angiotensin II generation and renal effects of AT1 receptor blockade. J Am Soc Nephrol. 1999;10:266–72.

Nishijima Y, Kobori H, Sofue T, Kaifu K, Moriwaki K, Hara T, Hitomi H, Kohno M, Nishiyama A. Important aspect of urine sampling for angiotensinogen measurement: time and preservation condition in healthy individuals. Tohoko J Exp Med. 2012;228:333–9.

Nishijima Y, Kobori H, Kaifu K, Mizushige T, Hara T, Nishiyama A, Kohno M. Circadian rhythm of plasma and urinary angiotensinogen in healthy volunteers and in patients with chronic kidney disease. J Renin Angiotensin Aldosterone Syst. 2014;15(4):505–8.

Nishiyama A, Konishi Y, Ohashi N, Morikawa T, Urishihara M, Maeda I, Hamada M, Kishida M, Hitomi H, Shirahashi N, Kobori H, Imanishi M. Urinary angiotensinogen reflects the activity of intrarenal renin–angiotensin system in patients with IgA nephropathy. Nephrol Dial Transplant. 2011;26:170–7.

Nomenclature of the renin-angiotensin system. Report of the Nomenclature Committee of the International Society of Hypertension (ISH) on the occasion of the 5th meeting of the ISH in Paris, 12–14 June 1978.

Ohashi N, Yamamoto T, Huang Y, Misaki T, Fukasawa H, Suzuki H, Togawa A, Suzuki S, Fujigaki Y, Nakagawa T, Nakamura Y, Suzuki F, Kitagawa M, Hishida A. Intrarenal RAS activity and urinary angiotensinogen excretion in anti-thymocyte serum nephritis rats. Am J Physiol Renal Physiol. 2008;295:F1512–8.

Page IH, Helmer OM. A crystalline pressor substance (angiotonin) resulting from the reaction between renin and renin-activator. J Exp Med. 1940;71(1):29–42.

Persson F, Lu X, Rossing P, Garrelds IM, Danser AH, Parving HH. Urinary renin and angiotensinogen in type 2 diabetes: added valuebeyondurinary albumin? J Hypertens. 2013;31:1646–52.

Pohl M, Kaminski H, Castrop H, Bader M, Himmerkus N, Bleich M, Bachmann S, Theilig F. Intrarenal renin angiotensin system revisited: role of megalin-dependent endocytosis along the proximal nephron. J Biol Chem. 2010;285(53):41935–46.

Prieto-Carrasquero MC, Harrison-Bernard LM, Kobori H, Ozawa Y, Hering-Smith KS, Hamm LL, Navar LG. Enhancement of collecting duct renin in angiotensin II-dependent hypertensive rats. Hypertension. 2004;44(2):223–9.

Ramkumar N, Stuart D, Ying J, Kohan DE. A possible interaction between systemic and renal angiotensinogen in the control of blood pressure. Am J Hypertens. 2013;26(4):473–80.

Rands VF, Seth DM, Kobori H, Prieto MC. Sexual dimorphism in urinary angiotensinogen excretion during chronic angiotensin II-salt hypertension. Gend Med. 2012;9(4):207–18.

Rohrwasser A, Morgan T, Dillon HF, Zhao L, Callaway CW, Hillas E, Zhang S, Cheng T, Inagami T, Ward K, Terreros DA, Lalouel JM. Elements of a paracrine tubular renin-angiotensin system along the entire nephron. Hypertension. 1999;34(6):1265–74.

Saito T, Urushihara M, Kotani Y, Kagami S, Kobori H. Increased urinary angiotensinogen is precedent to increased urinary albumin in patients with type 1 diabetes. Am J Med Sci. 2009;338:478–80.

Sawaguchi M, Araki SI, Kobori H, Urushihara M, Haneda M, Koya D, Kashiwaga A, Uzu T, Maegawa H. Association between urinary angiotensinogen levels and renal and cardiovascular prognoses in patients with type 2 diabetes melitus. J Diabetes Investig. 2012;3:318–24.

Seeliger E, Wronski T, Ladwig M, Dobrowolski L, Vogel T, Godes M, Persson PB, Flemming B. The renin-angiotensin system and the third mechanism of renal blood flow autoregulation. Am J Physiol Renal Physiol. 2009;296(6):F1334–45.

Shao W, Seth DM, Prieto MC, Kobori H, Navar LG. Activation of the renin-angiotensin system by a low-salt diet does not augment intratubular angiotensinogen and angiotensin II in rats. Am J Physiol Renal Physiol. 2013;304(5):F505–14.

Soltysiak J, Skowronska B, Fichna P, Ostalska-Nowicka D, Stankiewicz W, Lewandowska-Stachowiak M, Lipkowska K, Zachwieja J. Urinary angiotensinogen and urinary

sodium are associated with blood pressure in normoalbuminuric in children with diabetes. Pediatr Nephrol. 2014;29:2373–8.

Susic D, Frohlich ED, Kobori H, Shao W, Seth D, Navar LG. Salt-induced renal injury in SHRs is mediated by AT1 receptor activation. J Hypertens. 2011;29(4):716–23.

Terami T, Wada J, Inoue K, Nakatsuka A, Ogawa D, Teshigawara S, Murakami K, Katayama A, Eguchi J, Makino H. Urinary angiotensinogen is a marker for tubular injuries in patients with type 2 diabetes. Int J Nephrol Renov Dis. 2013;22:6233–40.

Tigerstedt R, Bergman PG. Niere und Kreislauf. Skand Arch Physiol. 1898;8:223–71.

Urushihara M, Kondo S, Kagami S, Kobori H. Urinary angiotensinogen accurately reflects intrarenal renin-angiotensin system activity. Am J Nephrol. 2010;31:318–25.

Van den Heuvel M, Batenburg WW, Jainandunsing S, Garrelds IM, van Gool JM, Feelders RA, van den Meiracker AH, Danser AH. Urinary renin, but not angiotensinogen or aldosterone, reflects the renal renin-angiotensin-aldosterone system activity and the efficacy of renin-angiotensin-aldosterone system blockade in the kidney. J Hypertens. 2011;29:2147–55.

Vidotti DB, Casarini DE, Cristovam PC, Leite CA, Schor N, Boim MA. High glucose concentration stimulates intracellular renin activity and angiotensin II generation in rat mesangial cells. Am J Physiol Renal Physiol. 2004;286(6):F1039–45.

Wang E, Yayama K, Takano M, Okamoto H. Sexual dimorphism of urine angiotensinogen excretion in the rat. Jpn J Pharmacol. 1994;64(4):243–50.

Xu Z, Xu B, Xu C. Urinary angiotensinogen as a potential biomarker of intrarenal renin-angiotensin system activity in Chinese chronic kidney disease patients. Ir J Med Sci. 2015;184(2):297–304.

Yamamoto T, Nakagawa T, Suzuki H, Ohashi N, Fukasawa H, Fujigaki Y, Kato A, Nakamura Y, Suzuki F, Hishida A. Urinary angiotensinogen as a marker of intrarenal angiotensin II activity associated with deterioration of renal function in patients with chronic kidney disease. J Am Soc Nephrol. 2007;18:1558–65.

Yanes LL, Sartori-Valinotti JC, Iliescu R, Romero DG, Racusen LC, Zhang H, Reckelhoff JF. Testosterone-dependent hypertension and upregulation of intrarenal angiotensinogen in Dahl salt-sensitive rats. Am J Physiol Renal Physiol. 2009;296(4):F771–9.

Ying J, Stuart D, Hillas E, Gociman BR, Ramkumar N, Lalouel JM, Kohan DE. Overexpression of mouse angiotensinogen in renal proximal tubule causes salt-sensitive hypertension in mice. Am J Hypertens. 2012;25(6):684–9.

Zou J, Li Y, Li FH, Wei FF, Wang JG. Urinary angiotensinogen excretion and ambulatory blood pressure. J Hypertens. 2012;30:2000–6.

Flow Cytometry of Urinary Leukocytes and Lymphocytes as a Biomarker of Renal Disease

15

Philipp Enghard, Birgit Rudolph, Jan Klocke, and Gabriela Riemekasten

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Abstract

Urine is normally nearly devoid of leukocytes. In various renal diseases however, immune cells can be observed in the urine. Flow cytometry is the state-of-the-art technique for analyzing the quantity and qualities of cells in suspension, making this method an ideal approach for investigating urinary immune cells. Increased amounts of urinary T cells and macrophages are routinely observed in inflammatory renal disease. In contrast, noninflammatory renal syndromes are not associated with an increase in urinary immune cell counts. The composition and the phenotype of the urinary immune cells are reminiscent of the infiltrating cells observed in the respective kidney biopsies, indicating that the urinary immune cells mirror local kidney inflammation. Therefore, they can be used as a "window into the kidney" to investigate the local cellular pathogenesis of renal diseases. Urinary T cells and macrophages have been probed as biomarkers in IgA nephritis, lupus nephritis, ANCA-associated glomerulonephritis, and renal transplant rejection, showing promising results in all entities.

Keywords

Anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis • Flow cytometry as biomarker for renal diseases • IgA nephropathy • Lupus nephritis (LN) • Renal transplantation • Urinary leukocyte origin

Abbreviations

ANCA	Anti-neutrophil cytoplasmic antibody
Anti-HLA-DR	Antibody staining HLA-DR MHCII molecules
Anti-URO-3	Antibody specific for tubular epithelial cells
BKV	BK-virus, member of the polyomavirus family, can cause infection of renal transplants
CCR7	Chemokine receptor of the CC family among others expressed on naïve T cells
CD14	Marker to identify classical monocytes and macrophages
CD16	Surface molecule expressed by neutrophil granulocytes and a subset of monocytes/macrophages
CD3	Identifies T cells which can be subdivided in CD3+CD4+ T helper T cells and CD3+CD8+ T killer T cells
CD4+ effector/memory T cells (EM T cells)	Once naïve T cells encounter their cognate antigen, they differentiate into effector/memory T cells
CD45RO	Surface molecule expressed by memory T lymphocytes
CD54	ICAM-1 adhesion molecule
CD56	Marker expressed on NK T cells
CXCR3	Chemokine receptor of the CXC family binds CXCL9, 10, and 11. Primarily expressed on Th1 T cells and mediates recruitment of these cells

DAPI	Fluorescence stain that binds DNA visualizes cell nucleuses in immunofluorescence
Fas-Ligand (Fas-L)	Protein of the TNF family binding mediates apoptosis
GN	Glomerulonephritis
SLEDAI	Score reflecting the disease activity of SLE patients
LN	Lupus nephritis

Key Facts on Flow Cytometry of Urinary Immune Cells (For the Layperson)

- Normally, the urine is almost devoid of immune cells. In certain diseases, diverse subsets of immune cells can be detected and quantified in the urine.
- Elevated amounts of immune cells in the urine reflect inflammation of the kidney.
- Immune cells in the urine can be used as a biomarker for various inflammatory renal diseases, for diagnosis and follow-up.
- The immune cells in the urine offer the unique opportunity to directly investigate the cellular components of the renal inflammation.

Definitions

ANCA-associated glomerulonephritis ANCA autoantibody-induced systemic vasculitis affecting the kidneys and causing renal inflammation.

Flow cytometry Method for quantitative and qualitative analysis of large amounts of suspended cells. Usually, cells are stained with fluorochrome-coupled antibodies, which are detected using lasers and detectors for specific wavelengths.

Hematuria The presence of blood in the urine.

IgA nephropathy One of the most frequent forms of glomerulonephritis caused by deposition of IgA in the mesangium.

Lupus nephritis (LN) Renal inflammation associated with the systemic autoimmune disease SLE.

Proliferative nephritis Renal disease associated with the proliferation of cells in the glomeruli due to inflammation of the kidneys.

Proteinuria The presence of elevated amounts of protein in the urine.

Renal transplant rejection Immunologic reaction against the renal graft, a frequent cause of graft failure.

SLE Systemic lupus erythematosus, a systemic autoimmune disease characterized by the presence of anti-nuclear autoantibodies and inflammation of several potential target organs.

Urine sediment Microscopic analysis of the insoluble contents of urine after centrifugation.

Introduction

Simplified, the pillars of the classic laboratory workup of renal diseases consist of an evaluation of the renal glomerular filtration rate (creatinine, cystatin C), assessment of the function of the filtration barrier (proteinuria), and a microscopic analysis of the urine sediment. Analysis of the sediment in particular holds clues to whether an inflammatory kidney disease is present. However, it mainly relies on a semiquantitative evaluation of unstained cells, is observer dependent, and does not have a high sensitivity or specificity (Crop et al. 2010; Fogazzi et al. 2005).

A common observation in the pathology of inflammatory renal diseases is the recruitment of inflammatory leukocytes and lymphocytes into the inflamed renal tissue. Infiltration of immune cells can be present in the glomeruli and renal interstitium. The invading cells consist mainly of macrophages and T cells and to a lesser extent of NK cells, B cells, and plasma cells (Markovic-Lipkovski et al. 1990). In large histologic studies, the interstitial infiltration of immune cells was identified as being a main factor predicting the outcome in diverse renal diseases (Bohle et al. 1992, 1994) (Table 1).

Table 1 Main flow cytometry findings in IgA nephropathy, lupus nephritis, ANCA-associated glomerulonephritis, and renal transplantation

Summary of the main findings of flow cytometry in different renal diseases	
IgA nephropathy	Ratio of urinary CD3/CD14 distinguishes IgA nephropathy from other forms of hematuria
	Urinary CD3, CD4, and CD14 cells correlate with renal function and crescents
	Urinary macrophages correlate with disease activity
Lupus nephritis	Urinary CD4 and CD8 identify patients with active, proliferative LN
	Urinary T cells seem suitable to monitor treatment response
ANCA-associated GN	Increase in urinary EM T cells in patients with active renal involvement
	Decrease in urinary T cells correlates with remission
Renal transplant	Increased amounts of urinary tubular epithelial cells indicate acute tubular necrosis
	Increased urinary T cells in patients with acute rejection
	The presence of urinary HLA-DR+ cells and ratio of CD3+/URO+ >1 in patients with acute rejection
	Persistence of HLA-DR+ and CD3+ cells in the urine may indicate subclinical rejection

Using specific antibodies for the detection of leukocyte and lymphocyte subsets, T cells and macrophages can be detected in the urine using cytology or flow cytometry. Flow cytometry is the state-of-the-art technique for analyzing and quantifying cell suspensions in an easy and reliable form, making it a promising method for the analysis of urinary leukocytes and lymphocytes. The first report of flow cytometric analysis of urinary cells goes back to 1981 by W. Eisert et al. Flow cytometry has further evolved ever since, and while most studies in the 1990s were limited to the analysis of four parameters per analysis, modern studies were able to simultaneously assess an ever-growing number of parameters.

The present chapter will review the current data on the use of urinary flow cytometry in various kidney diseases. First, we will outline which patterns of kidney injury are associated with certain leukocyte types in the urine; then, the application of flow cytometry of urine cells will be discussed in specific renal diseases. Finally, the application of urinary flow cytometry as a "window into the kidney" to analyze and widen our understanding of renal diseases will be described. This chapter will focus on advanced flow cytometry of urine leukocytes/lymphocytes in renal diseases. Related topics, such as the automated flow cytometry of unlabelled urine cells and the usage of urine flow cytometry to detect lower urinary tract malignancies, will not be discussed.

Origin of the Urinary Leukocytes and Patterns of Associated Renal Injury

High numbers of T cells and macrophages can be observed in the urine of patients with diverse renal diseases, such as anti-neutrophil cytoplasmic antibody (ANCA)-associated rapid progressive glomerulonephritis, proliferative lupus nephritis (LN), and IgA nephropathy, and renal transplant patients with acute rejection (see Fig. 1, e. g., histology). However, the exact origin of these cells is not definitively established. The phenotype of the cells in the urine is reminiscent of the intrarenal cells observed in histology and differs from the phenotype of the cells in the peripheral blood, indicating that the urine cells originate in the kidney and do not reflect a passive leak of blood cells into the urine (Dolff et al. 2010, 2013; Enghard et al. 2009). Along that line, urinary macrophages can be used for the evaluation of patients with hematuria: Patients with glomerulonephritis (GN)-associated hematuria had high detectable numbers of macrophages and T cells in the urine. In contrast, patients with idiopathic renal hematuria, patients with hereditary nephropathy including Alport's disease, and patients with renal stone-associated hematuria had no relevant amounts of T cells or macrophages in the urine (Hotta et al. 1996; Hotta et al. 1999) (Fig. 2). Consequently it can be assumed that the leukocytes/lymphocytes observed in the urine stem from the renal tissue.

In the kidney pathology of various renal diseases, invading T cells and macrophages can be detected in various sites, such as the glomeruli, the kidney interstitium, and – in the case of tubulitis – invading the tubular epithelium itself. Whether the detectable cells in the urine originate from a certain renal compartment is presently unknown. The amount of urinary T cells and macrophages was found to correlate well with the amount of glomerular crescents and the glomerular

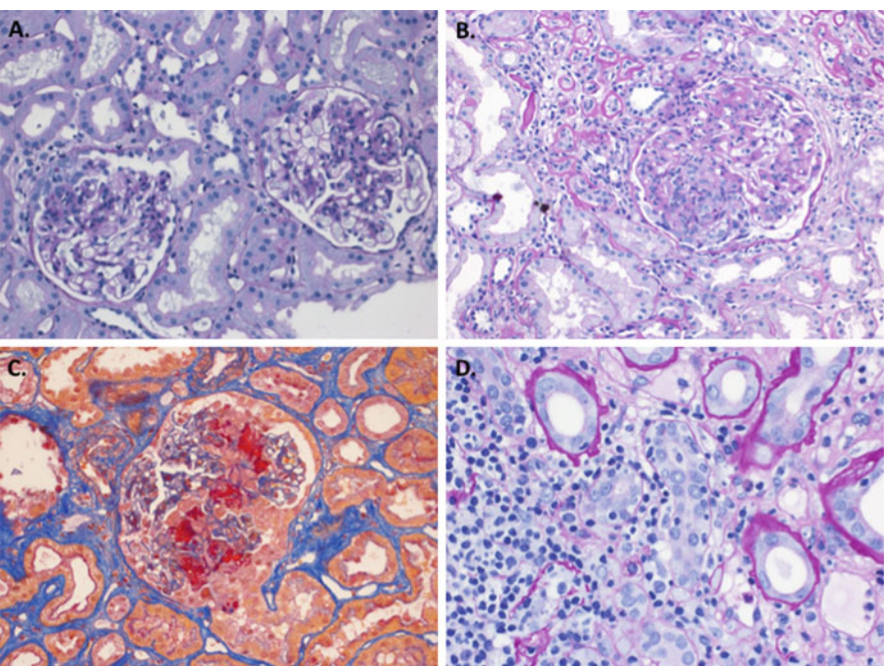


Fig. 1 Examples for the kidney pathologies associated with the presence of elevated urinary T cells and/or macrophages. (**a**) IgA nephropathy. (**b**) Proliferative lupus nephritis IV. (**c**) ANCA-associated glomerulonephritis. (**d**) Acute renal transplant rejection

injury score (Deenitchina et al. 1999; Hotta et al. 1993), suggesting a glomerular source of the urinary cells. Furthermore, the macrophages in urine and glomeruli shared certain characteristics, such as CD16 expression (Hotta et al. 1999). The vast majority of invading leukocytes/lymphocytes, however, is often observed in the renal interstitium. Urinary T cells in lupus nephritis, for example, share many features of the renal interstitial T cells, including subtype and chemokine-receptor expression, which cannot be observed in the glomeruli (Dolff et al. 2010; Enghard et al. 2009). In renal transplant patients, the amount of certain T cell subsets observed in the biopsy correlated with the urinary numbers of these cells. The intrarenal T cells in renal transplantation predominantly localize in the renal interstitium, providing further evidence that the urinary cell may mirror inflammation in this compartment.

In line with the concept of the urinary immune cells origin, two patterns of renal injury seem distinguishable according to the presence or absence of urinary T cells and macrophages. In a wide variety of inflammatory proliferative forms of renal disease, these cells can be observed in elevated numbers in the urine. In contrast, various groups reported only negligible amounts of urinary lymphocytes/leukocytes in less inflammatory renal diseases. In patients with nonproliferative nephrotic syndromes, like minimal change nephropathy, membranous glomerulonephritis, and focal segmental glomerulosclerosis, T cells and macrophages seem to be only

Fig. 2 Proposed flow chart to differentiate hematuria as proposed by Hotta et al. (Figure from Hotta et al. 1993, 2000, with permission of the publishers)

present in low numbers in the urine (Deenitchina et al. 1999; Enghard et al. 2009, 2014; Hotta et al. 1994, 1999; Sakatsume et al. 2001). In most studies, these patients have been included in small numbers as control groups. Nevertheless, the results from various groups agree in the observation of only marginal numbers of urinary inflammatory cells in nonproliferative forms of glomerulonephritis.

General Considerations

Using the immune cells in the urine as a biomarker for renal diseases has certain advantages over a renal biopsy. Obviously analyzing urine is less invasive than is a renal biopsy, and the urine analysis can be repeated as often as deemed necessary. Importantly, the urinary cells may potentially be more sensitive to detect pathological changes: Many pathological alterations are not distributed evenly throughout the renal tissue, but are patchy. Consequently, they can be missed by a random biopsy sample from the kidney tissue. In contrast, it is reasonable to assume that the urinary cells reflect the entire kidney and are therefore not at risk for sampling bias.

Given that urine is not a physiological place for leukocytes/lymphocytes, it seems natural to assume that cell survival in this fluid is limited. In a seminal paper on cell survival in urine, Stachowski et al. found that cell viability decreased with storage time of the sample and high osmolarity; in contrast, high protein content in the urine exerted a protective effect on cell survival. In line with this observation, a protocol was developed adding 30 % fetal calf serum to the urine samples, which substantially increased cell survival (Stachowski et al. 1998).

However, cell survival may not be very critical for analyzing and quantifying immune cells in the urine, as these cells remain detectable even after cell death. Only a minority of the published papers on urinary leukocytes/lymphocytes using flow cytometry did use a live gate with special dyes for staining dead cells and excluding

them from analysis. All other studies did not exclude dead cells from their analysis, which seems feasible as well.

Flow Cytometry as Biomarker for Specific Renal Diseases

IgA Nephropathy

IgA nephropathy (Morbus Berger) is among the most frequent forms of glomerulonephritis worldwide. It is characterized by the accumulation of IgA deposits in the glomerular mesangium. Hematuria is a common feature of patients with IgA nephropathy, with severity ranging from minor hematuria to phases of gross hematuria. While many patients show only minor functional impairment, some patients proceed to severe renal damage and renal insufficiency.

In the urine of patients with IgA nephropathy, CD3+ T cells and CD14+ macrophages can be observed, and the ratio of both allows for distinguishing IgA nephropathy from other forms of hematuria, such as renal stones or hereditary hematuria (Hotta et al. 1996). In IgA nephropathy, CD14+ macrophages usually outnumber the CD3 T cells. Among the T cells, a slight predominance of CD8 T cells over CD4 T cells was reported. Additionally, a considerable amount of CD56 NK cells can be found in the urine, while CD20 B cells are almost absent (Hotta et al. 1994). The amount of CD3+ T cells, CD4+ T cells, and CD14+ macrophages correlates with impaired renal function (creatinine clearance) and histopathological changes, namely, interstitial cell infiltration and glomerular crescents (Deenitchina et al. 1999; Hotta et al. 1994). The quantity of macrophages also correlates with the activity of the disease, showing increased urinary numbers during transient exacerbations, which normalize in remission. CD16, a marker for inflammatory macrophages, is also detectable on the urinary macrophages, correlating with acute exacerbations (Hotta et al. 1999) (Fig. 3).

A total of five papers from three independent research teams reported consistent data on urinary T cells and macrophages as biomarkers, with good correlation with histology and renal damage. Future studies, however, will have to prove that urinary flow cytometry also helps predict the course of the disease and guide treatment decisions.

Lupus Nephritis

Lupus nephritis (LN) is one of the most common manifestations of SLE and is associated with a poor prognosis (Mills 1994). Response to the standard therapeutic regimes is still very unsatisfactory, with 45 % of all patients showing failure to achieve remission during standard induction theraphy (Appel et al. 2009; Ginzler et al. 2005). Biomarkers could help improve the prognosis of SLE and LN in two ways: first by enabling a rapid diagnosis of LN, which is associated with better outcome (Faurschou et al. 2006; Fiehn et al. 2003), and even more importantly by providing reliable tools to monitor disease activity and treatment response, thus enabling clinicians to adjust therapy toward a patient-tailored immunosuppressive treatment regime.

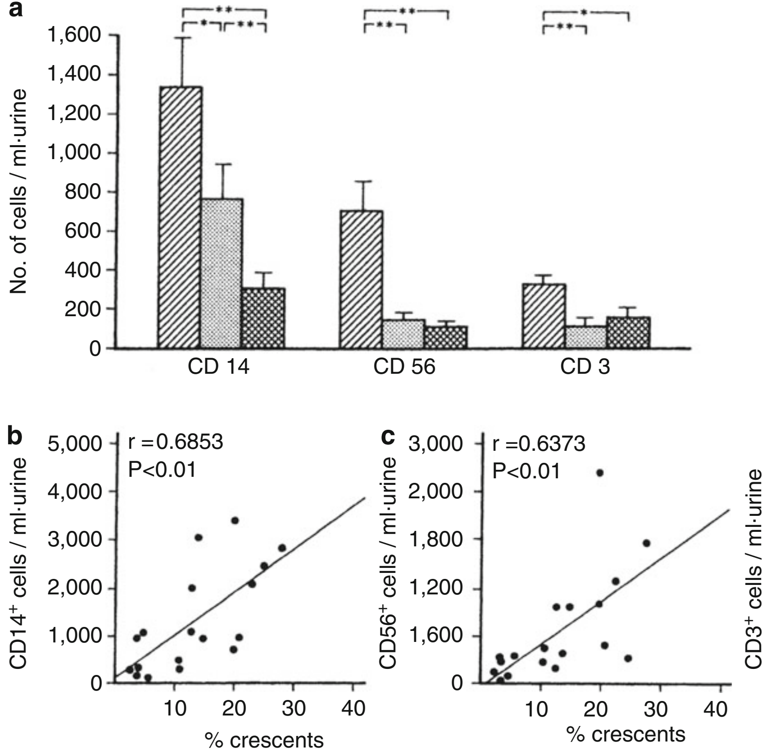


Fig. 3 Urinary mononuclear cells in IgA nephropathy. (**a**) Number of urinary CD14+, CD56+, and CD3+ cells in active crescentic (*diagonal lines*, columns on the *left*), inactive crescentic (finely checkered, columns in the *middle*), and non-crescentic IgA nephropathy (grossly checkered, columns on the right). (**b**) Correlation of the amount of CD14+ cells with the % of observed crescents on the kidney biopsy. (**c**) Correlation of the amount of CD3+ cells with the % of observed crescents (Figures from Hotta et al. 1993, with permission of the publishers)

In the course of LN, T cells and other leukocytes are recruited into the inflamed kidney tissue and are thought to play a pivotal role in the mediation of tissue damage. It was first observed in 1994 that some patients with SLE have relevant numbers of macrophages in the urine (Hotta et al. 1994). However, it was not until 2009 that the presence of urinary T cells was studied in larger cohorts of SLE patients. It was first noted that urinary CD4+ T cells could be found in high numbers in patients with active lupus nephritis. The amount of urinary CD4+ T cells correlates with the disease activity and identifies patients with active lupus nephritis (Enghard et al. 2009). Additionally, the urinary T cell counts decrease with therapy. Almost in parallel, a second group reported similar findings for urinary CD8+ T cells as a potential biomarker (Dolff et al. 2010).

In a large monocentric study, the findings of CD4+ T cells as a biomarker for lupus nephritis were further consolidated. In a cohort of 147 SLE patients, the amount of

CD4+ T cells correlated with systemic and renal disease activity in patients with past or present renal involvement (Enghard et al. 2014). In contrast, in patients without renal involvement, hardly any T cells were observed in the urine, despite high levels of disease activity. Using 800 CD4+ T cells as a cutoff, patients with active, proliferative LN were identified with a high diagnostic precision (sensitivity 100 %, specificity 98 %, area under the Receiver Operating Characteristics (ROC) curve 0.9969). Importantly, urinary T cells in terms of diagnosing proliferative LN performed better than did classical biomarkers, such as proteinuria, the urine sediment, or creatinine (Fig. 4a, b).

A group of 14 patients was monitored during the follow-up under treatment for active lupus nephritis. The majority of patients showed a rapid normalization of their urinary T cell counts as soon as 1–2 months after the initiation of treatment, indicating a response to therapy. However, several patients showed persistent or even increasing urinary CD4+ T cell counts under treatment, referred to as "non-responders." Interestingly, after the usual 6 months of induction treatment for lupus nephritis, the responders presented a lower general disease activity and better creatinine increment than did the "nonresponders" (Enghard et al. 2014) (Fig. 4c, d).

A total of 29 patients in this study were analyzed in parallel to a kidney biopsy, the current gold standard for diagnosing lupus nephritis. All patients with proliferative lupus nephritis also presented with high urinary CD4+ T cell counts. In contrast, patients with only minor abnormalities (class I) or nonproliferative forms (class V) were not associated with increased urinary CD4+ T cells counts.

A second independent research team in a cohort of 46 SLE patients observed similar results. In this study, urinary CD8 T cells (area under the ROC curve AUC 0.93) slightly outperformed urinary CD4 T cells (AUC 0.92) as a biomarker for lupus nephritis. In patients with renal involvement, urinary T cells yielded a better diagnostic precision than did creatinine or proteinuria. A subgroup of 16 SLE patients was analyzed serially in parallel to treatment. After successful induction of remission, a significant decrease in urinary CD4+ and CD8+ T cell numbers was observed (Dolff et al. 2013).

ANCA-Associated Glomerulonephritis

Anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis is one of the common manifestations of systemic ANCA vasculitis. It has an often remitting-relapsing course, and exact determination of the inflammatory activity can be challenging.

In a group of 102 patients with ANCA vasculitis, the amount of CD4+ effector/memory T cells (EM T cells, defined as CD45RO+CCR7-CD3+CD4+) was quantified in the peripheral blood and urine. In patients with active renal involvement, a notable increase in urinary EM CD4+ T cells was observed (Abdulahad et al. 2009). In parallel, the frequency of the same T cell subset decreased in the blood, indicating compartmentalization of these cells from the blood into the

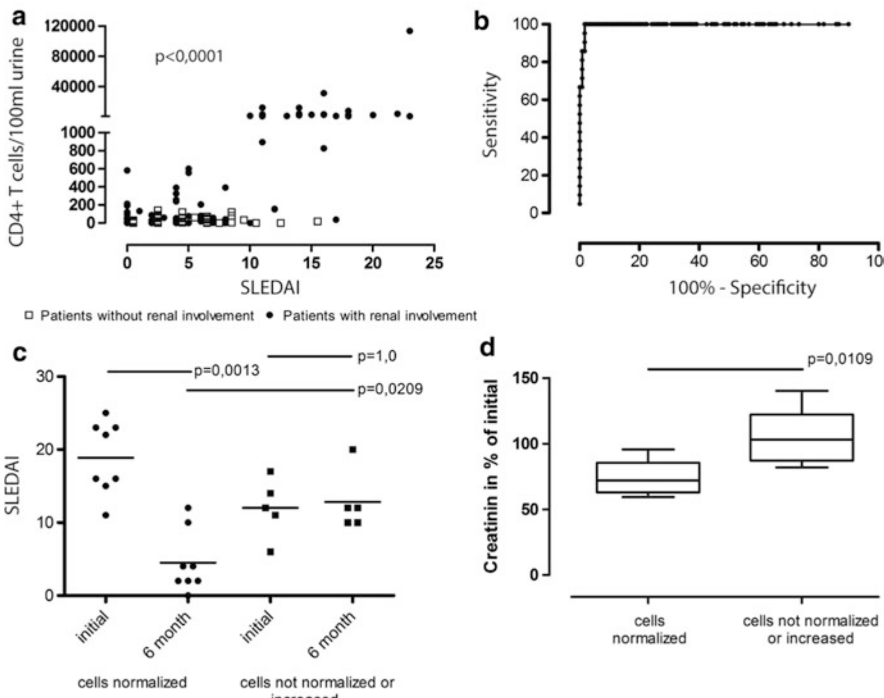


Fig. 4 Urinary T cells as a biomarker for proliferative lupus nephritis. (**a**) The amount of urinary CD4+ T cells correlates with the SLEDAI in SLE patients with renal involvement (*filled circles*). In SLE patients without renal involvement, very few urinary CD4+ T cells are detectable, regardless of disease activity (*open boxes*). (**b**) ROC curve showing the diagnostic performance of urinary CD4+ T cells for detecting proliferative/inflammatory LN in SLE patients. Using a cutoff of 800 CD4+ T cells yielded a sensitivity of 100 % and a specificity of 98.0 %. (**c**) Follow-up under therapy: Patients who normalized their urinary CD4+ T cell counts <800/100 ml within 6 months demonstrated a significant reduction of their SLEDAI. In contrast, patients with persistent or increased urinary CD4+ T cell numbers showed no treatment response regarding SLEDAI. (**d**) Patients with normalized urinary CD4+ cells have significantly decreased creatinine levels than patients with increase or persistence of these cells (6-month creatinine as % of initial creatinine) (Figures from Enghard et al. 2014, with permission of the publishers)

inflamed kidneys. The amount of urinary EM CD4+ T cells differed significantly between patients with active renal involvement and those in disease remission, and patients without past or present renal involvement had hardly any EM CD4+ T in the urine. Furthermore, the amount of urinary EM T cells decreased with successful treatment in 12 patients assessed in the follow-up (Abdulahad et al. 2009) (Fig. 5). A further case report on two patients with vasculitis-associated glomerulonephritis (one patient with ANCAs, one without) reported that besides EM CD4+ T cells, naïve CD4+ T cells and CD14+ monocytes/macrophages can also be observed in the urine (Sakatsume et al. 2001).

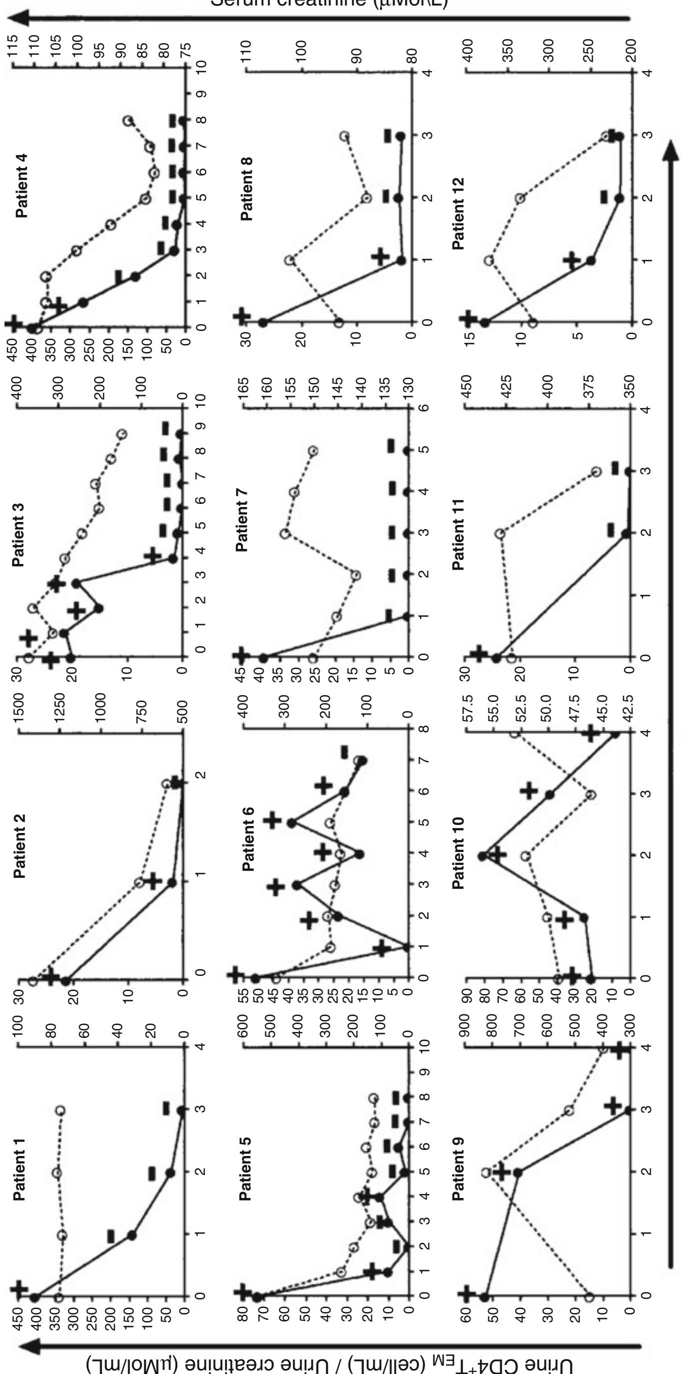


Fig. 5 Follow-up of urinary effector/memory CD4+ T cells and creatinine in patients with ANCA-associated glomerulonephritis. The *left* axis shows EM CD4+ T cells (cell/ml) divided by urine creatinine (μmol/ml) (*solid circles*). The *right* axis displays serum creatinine (μmol/l) (*open circles*) (Figure from Abdulahad et al. 2009, with permission of the publishers)

Renal Transplantation

Kidney transplantation is the preferred treatment for patients with terminal renal insufficiency. However, the need for immunosuppression and the risk of graft rejections need to be kept in balance to enable graft survival and minimize the patient's risk. Up until now, there is no established biomarker for the fine-tuning of the immunosuppression or the early, noninvasive detection of acute or chronic graft rejection (Lo et al. 2014).

Flow cytometry was used to analyze the cellular "profile" of the urine of renal transplant patients as early as 1981. The first studies relied merely on the forward and sideward scatter of the flow cytometer to identify cells according to their size and granularity (Eisert et al. 1981; Gomez Jorge et al. 1991; Lee et al. 1992; Yu et al. 1999). Using these parameters, cell debris, leukocytes, lymphocytes, and exfoliated tubular epithelial cells can be differentiated. It was recognized that different urine signatures of cell composition could be identified in patients with stable graft function, acute rejection, acute tubular necrosis, and urinary tract infection. In particular, acute tubular necrosis was identified by the presence of prominent numbers of tubular epithelial cells with a high sensitivity (100 %) in two separate studies (Lee et al. 1992; Yu et al. 1999). The diagnostic value of the cell scatter alone for identifying acute rejection was less reliable, with a sensitivity of 63 % and specificity of 79 % (Yu et al. 1999).

In a large study analyzing 223 urinary samples from 127 renal transplant patients, Nanni-Costa et al. used the scatters and specific fluorochrome-coupled antibodies for the detection of different T cell subsets and NK cells (Nanni-Costa et al. 1992). They confirmed different patterns for stable grafts, acute rejection, infection, and acute tubular necrosis. Furthermore, they reported that patients with cyclosporine toxicity might be identified by the presence of a large fraction of small cellular debris. During acute rejection, urinary lymphocytes outnumbered the monocytes and consisted predominantly of the CD8+ cytotoxic subset. Large cellular debris (presumably epithelial cells) was the dominating fraction in acute tubular necrosis, and neutrophils were frequently found in urinary tract infections. Patients with stable graft function presented only low cell numbers. Unfortunately, the study did not report receiver operator curves of sensitivities and specificities of the respective cellular profiles (Nanni-Costa et al. 1992). Another study from Brazil on 60 renal transplant patients analyzed the frequency of CD3, CD4, CD8, HLA-Dr, Fas-L, ICAM, and CD25 using flow cytometry of urine samples (Galante et al. 2006). Similar to other reports, CD3 T cells were the largest urinary leukocyte population during acute rejection. The expression of Fas-L yielded the best results for diagnosing acute rejection with an area under the ROC curve of 0.99, comparing acute rejection to patients with stable graft function or pyelonephritis. Patients with chronic allograft dysfunction also showed elevated Fas-L expression to some extent, which diminished the discriminatory power for separating acute rejection from chronic allograft dysfunction (AUC 0.91). However, the study analyzed only frequencies of cell subsets and not absolute cell numbers, and including the absolute cell count may well increase the diagnostic utility.

A major contribution to the current knowledge of flow cytometry on urine cells in renal transplantation comes from Isabel Roberti et al. over several reports. In a prospective double-blind study, 200 urine specimens from 40 renal transplant patients were

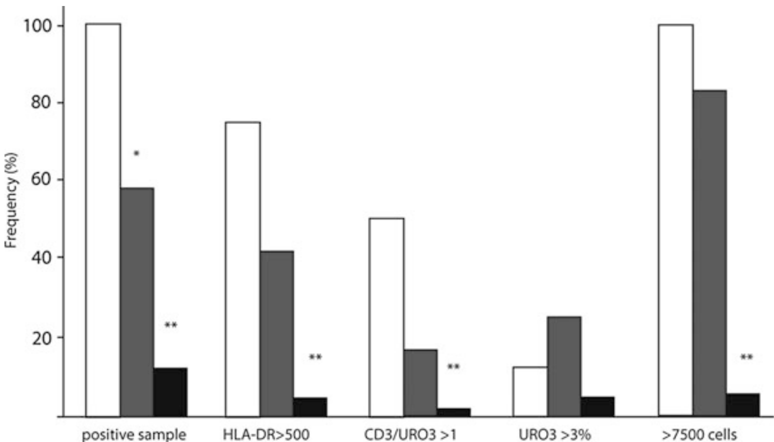


Fig. 6 Urinary flow cytometry in patients with renal transplant. Urinary cells observed in patients with acute rejection (*white columns*), ischemic injury (*gray columns*), and other pathologies (*black columns*). Samples were regarded as positive if the mean fluorescence significantly differed from the control and at least 5 % of the cells stained with anti-HLA-DR or anti-CD3. Furthermore samples with more than 500 HLA-DR positive events, samples with a CD3/URO3 ratio>1, with a proportion of URO3 cells>3 %, and samples with more than 7500 events are displayed (Figure from Roberti et al. 1995 with permission of the publishers)

analyzed. The presence of CD3+ T cells, URO+ tubular epithelial cells, and HLA-DR+ cells was analyzed using flow cytometry. These data were compared to the clinical diagnosis of the patients, which was assigned retrospectively in a double-blind manner. The presence of HLA-DR+ and the ratio of CD3+/URO+ cells differentiated patients with acute rejection from other patients. A positive flow cytometry was defined as at least 5 % of all urinary cells staining with anti-HLA-Dr or Anti-CD3. Applying these criteria, acute rejection was diagnosed with a sensitivity of 100 % and a specificity of 87.9 % using flow cytometry, which was superior to the analysis of unstained cells using urine cytology (Roberti et al. 1995) (Fig. 6). The good but non-perfect specificity was owed to the observation that some of the patients with stable graft function also presented elevated amounts of urinary HLA-DR+ and T cells. Interestingly, in a later study, the group reported the 1-year follow-up data. The outcome of three patient groups was compared: (1) patients without acute rejection and persistent low urine cells; (2) patients with early acute rejection that had normalized their cells after antirejection therapy; and (3) patients with stable function (no acute rejection), but with persistent positive urinary flow cytometry. Strikingly, group 3 had a significant worse serum creatinine increment than did group 1, indicating that detection of HLA-DR+ or T cells in the urine in the absence of acute rejection may reflect subclinical rejection (Roberti et al. 1997a). In another study, the same group reported an increase of HLA-DR, CD54, and CD3 expressing urinary cells during rejection, while the presence of CD14+ monocytes was highly specific for chronic rejection (Roberti et al. 1997b). Furthermore, the persistence of HLA-DR, CD14, and CD54 expression on urinary T cells predicted the need for intensified treatment and irreversible graft damage during acute rejection (Roberti and Reisman 2001).

A recent study of 35 renal transplant patients reported that CD4+ and CD8+ effector/memory T cells (EM T cells) and terminal differentiated T cells (TD T cells) can also be observed in the urine. Their urinary counts correlated with the respective amounts of CD4+/CD8+ EM and TD T cells in the respective renal biopsies. Urinary numbers of the CD4+ and CD8+ EM and TD T cells differentiated between the group with stable kidney function and the ones with allograft rejection. Patients with BKV infection also showed increased numbers of urinary EM T cells. However, the CD8+ EM and TD T cell numbers in the urine were significantly higher during rejection compared to BKV nephropathy (van Doesum et al. 2014).

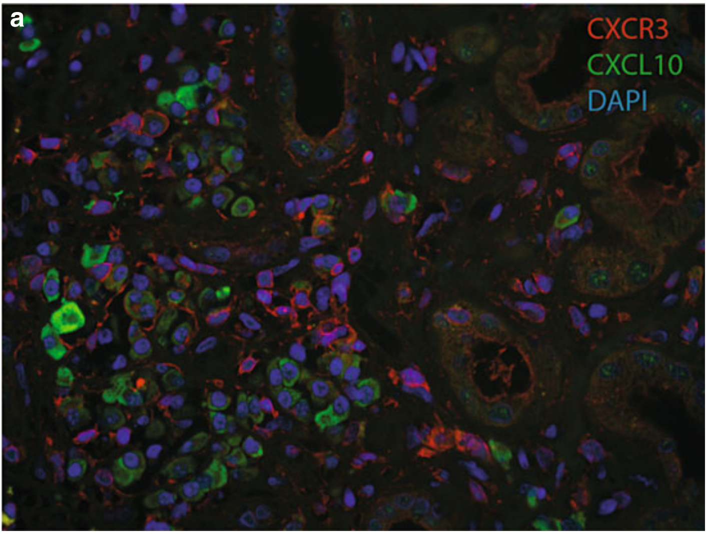
Urinary Cells as a "Window" into the Kidney

Besides their potential application as a biomarker for various renal diseases, the analysis of urinary cells offers the opportunity to directly investigate different forms of renal inflammation. As the urinary immune cells seem to mirror the inflammatory process in the kidney, they offer a "window" into the kidney, to live and noninvasively observe the cellular pathogenesis or renal diseases.

Comparing the phenotype of T cells in the peripheral blood and urine, it was demonstrated that mostly memory-effector T cells were recruited into the kidney and found in the urine of renal transplant patients and those with IgA nephropathy, ANCA-associated glomerulonephritis, and lupus nephritis (Abdulahad et al. 2009; Dolff et al. 2010; Sakatsume et al. 2001; van Doesum et al. 2014). Interestingly, in lupus nephritis and ANCA-associated glomerulonephritis, parallel to the observation of EM T cells in the urine, a reciprocal decrease of the respective T cell subsets was observed in the peripheral blood, suggesting migration of these cells from one compartment to the other (Abdulahad et al. 2009; Dolff et al. 2010).

Presently, it is not known to what extent an antigen-specific T cell response contributes to renal inflammation. A research paper on the T cell receptor repertoire reported an enrichment of T cell receptors, indicating an antigen-driven response. The same T cell receptor repertoire was detected in T cells in the urine, further supporting the notion that the cells in the urine reflect the intrarenal immune pathology (Hu et al. 2004).

Mainly using mouse models, several chemokine receptor pathways involved in the recruitment of inflammatory cells into the renal tissue were identified. Although these models have been informative, the observed results were, in part, contradictory, and whether these models reflect the recruitment patterns in human patients remains elusive. In human lupus nephritis, an enrichment of CXCR3-expressing T cells in the kidneys was reported (Enghard et al. 2009; Segerer et al. 2004). CXCR3 is also expressed by the majority of urinary CD4+ T cells in patients with active lupus nephritis. The frequency of urinary CXCR3-expressing T cells correlated with disease activity in SLE patients with renal involvement. Importantly, compared to the peripheral blood, CXCR3-expressing CD4+ T cells were significantly enriched in the urine, suggesting participation of CXCR3 in mediating the migration of these cells from the blood into the inflamed kidneys (Enghard et al. 2009) (Fig. 7).



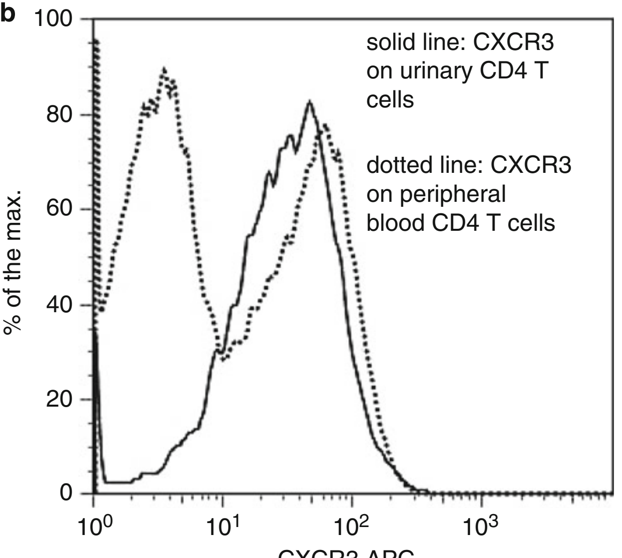


Fig. 7 CXCR3-expressing cells can be observed in the kidney and urine of patients with proliferative lupus nephritis. (**a**) Immunofluorescence staining of a kidney biopsy from patients with LN. Co-staining of CXCR3 (*red*), CXCL10 (*green*), and DAPI (*blue*) demonstrating co-localization of CXCR3+ cells next to CXCL10 producers. (**b**) Histogram showing CXCR3 expression on CD3 +CD4+ T cells in the peripheral blood (*red line*) and urine (*blue line*) analyzed by flow cytometry. While there are CXCR3 positive and negative CD4+ T cells in the blood, the urinary CD4+ T cell uniformly expresses CXCR3 (Figures from Enghard et al. 2009, with permission of the publishers)

Potential Application to Prognosis, Other Diseases, or Conditions

The analysis of urinary immune cells by flow cytometry is presumably not only applicable to the renal diseases described above, but renal diseases in general. Since the urinary immune cell signature reflects the renal inflammation, it is reasonable to assume that all kinds of renal diseases can be assessed for the extent of local kidney inflammation, to facilitate diagnosis and enable monitoring under treatment.

Summary Points

- Urinary lymphocytes/leukocytes seem to directly reflect local renal inflammation.
- The presence of urinary T cells and/or macrophages yields promising results as a biomarker for different renal diseases.
- These include IgA nephropathy, lupus nephritis, ANCA-associated glomerulonephritis, and renal transplant rejection.
- In these diseases, urinary T cells and/or macrophages can be used as a biomarker for diagnosis and, presumably, for follow-up under therapeutic intervention.
- In contrast, noninflammatory renal diseases are not associated with increased amounts of urinary immune cells.
- Additionally, flow cytometry of urinary immune cells offers an attractive tool for investigating the cellular pathology of human renal diseases.

References

Abdulahad WH, Kallenberg CG, Limburg PC, Stegeman CA. Urinary CD4+ effector memory T cells reflect renal disease activity in antineutrophil cytoplasmic antibody-associated vasculitis. Arthritis Rheum. 2009;60(9):2830–8.

Appel GB, Contreras G, Dooley MA, et al. Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis. J Am Soc Nephrol. 2009;20(5):1103–12.

Bohle A, Wehrmann M, Bogenschutz O, et al. The long-term prognosis of the primary glomerulonephritides. A morphological and clinical analysis of 1747 cases. Pathol Res Pract. 1992;188 (7):908–24.

Bohle A, Wehrmann M, Mackensen-Haen S, et al. Pathogenesis of chronic renal failure in primary glomerulopathies. Nephrol Dial Transplant. 1994;9 Suppl 3:4–12.

Crop MJ, de Rijke YB, Verhagen PC, Cransberg K, Zietse R. Diagnostic value of urinary dysmorphic erythrocytes in clinical practice. Nephron Clin Pract. 2010;115(3):c203–12.

Deenitchina SS, Shinozaki M, Hirano T, et al. Association of a T-cell receptor constant alpha chain gene polymorphism with progression of IgA nephropathy in Japanese patients. Am J Kidney Dis. 1999;34(2):279–88.

Dolff S, Abdulahad WH, van Dijk MC, Limburg PC, Kallenberg CG, Bijl M. Urinary T cells in active lupus nephritis show an effector memory phenotype. Ann Rheum Dis. 2010;69 (11):2034–41.

Dolff S, Abdulahad WH, Arends S, et al. Urinary CD8+ T-cell counts discriminate between active and inactive lupus nephritis. Arthritis Res Ther. 2013;15(1):R36.

Eisert WG, Wagner E, Beisker W. Analysis of urine sediments after kidney transplantation. Anal Quant Cytol. 1981;3(1):17–20.

Enghard P, Humrich JY, Rudolph B, et al. CXCR3+CD4+ T cells are enriched in inflamed kidneys and urine and provide a new biomarker for acute nephritis flares in systemic lupus erythematosus patients. Arthritis Rheum. 2009;60(1):199–206.

Enghard P, Rieder C, Kopetschke K, et al. Urinary CD4 T cells identify SLE patients with proliferative lupus nephritis and can be used to monitor treatment response. Ann Rheum Dis. 2014;73(1):277–83.

Faurschou M, Starklint H, Halberg P, Jacobsen S. Prognostic factors in lupus nephritis: diagnostic and therapeutic delay increases the risk of terminal renal failure. J Rheumatol. 2006;33 (8):1563–9.

Fiehn C, Hajjar Y, Mueller K, Waldherr R, Ho AD, Andrassy K. Improved clinical outcome of lupus nephritis during the past decade: importance of early diagnosis and treatment. Ann Rheum Dis. 2003;62(5):435–9.

Fogazzi GB, Saglimbeni L, Banfi G, et al. Urinary sediment features in proliferative and non-proliferative glomerular diseases. J Nephrol. 2005;18(6):703–10.

Galante NZ, Camara NO, Kallas EG, Salomao R, Pacheco-Silva A, Medina-Pestana JO. Noninvasive immune monitoring assessed by flow cytometry and real time RT-PCR in urine of renal transplantation recipients. Transpl Immunol. 2006;16(2):73–80.

Ginzler EM, Dooley MA, Aranow C, et al. Mycophenolate mofetil or intravenous cyclophosphamide for lupus nephritis. N Engl J Med. 2005;353(21):2219–28.

Gomez Jorge JT, Estrada C, Gonzalez ZA, Morales-Otero LA, Lavergne J, Santiago-Delpin EA. Flow cytometric analysis of urine sediment after kidney transplantation. Transplant Proc. 1991;23(2):1764–5.

Hotta O, Yusa N, Kitamura, Taguma Y. Urinary macrophages as activity markers of renal injury. Clin Chim Acta. 2000;297(1–2):123–33.

Hotta O, Taguma Y, Ooyama M, Yusa N, Nagura H. Analysis of CD14+ cells and CD56+ cells in urine using flow cytometry: a useful tool for monitoring disease activity of IgA nephropathy. Clin Nephrol. 1993;39(6):289–94.

Hotta O, Taguma Y, Yusa N, Ooyama M. Analysis of mononuclear cells in urine using flow cytometry in glomerular diseases. Kidney Int Suppl. 1994;47:S117–21.

Hotta O, Yusa N, Ooyama M, Taguma Y. Urinary macrophage counts and ratio to T lymphocytes: possible use in differential diagnosis and management of glomerular disease. J Clin Lab Anal. 1996;10(4):205–8.

Hotta O, Yusa N, Ooyama M, Unno K, Furuta T, Taguma Y. Detection of urinary macrophages expressing the CD16 (Fc gamma RIII) molecule: a novel marker of acute inflammatory glomerular injury. Kidney Int. 1999;55(5):1927–34.

Hu M, Zhang GY, Walters G, et al. Matching T-cell receptors identified in renal biopsies and urine at the time of acute rejection in pediatric renal transplant patients. Am J Transplant Off J Am Soc Transplant Am Soc Transplant Surg. 2004;4(11):1859–68.

Lee PH, Huang MT, Lee CS. Analysis of urine cytology by flow cytometry in renal transplantation. Transplant Proc. 1992;24(4):1543–4.

Lo DJ, Kaplan B, Kirk AD. Biomarkers for kidney transplant rejection. Nat Rev Nephrol. 2014;10 (4):215–25.

Markovic-Lipkovski J, Muller CA, Risler T, Bohle A, Muller GA. Association of glomerular and interstitial mononuclear leukocytes with different forms of glomerulonephritis. Nephrol Dial Transplant. 1990;5(1):10–7.

Mills JA. Systemic lupus erythematosus. N Engl J Med. 1994;330(26):1871–9.

Nanni-Costa A, Iannelli S, Vangelista A, et al. Flow cytometry evaluation of urinary sediment in renal transplantation. Transplant Int Off J Eur Soc Organ Transplant. 1992;5 Suppl 1:S8–12.

Roberti I, Reisman L. Serial evaluation of cell surface markers for immune activation after acute renal allograft rejection by urine flow cytometry – correlation with clinical outcome. Transplantation. 2001;71(9):1317–20.

Roberti I, Reisman L, Burrows L, Lieberman KV. Urine cytology and urine flow cytometry in renal transplantation – a prospective double blind study. Transplantation. 1995;59(4):495–500.
Roberti I, Panico M, Reisman L. Urine flow cytometry as a predictor of renal allograft function. Transplantation. 1997a;63(5):781–2.
Roberti I, Panico M, Reisman L. Urine flow cytometry as a tool to differentiate acute allograft rejection from other causes of acute renal graft dysfunction. Transplantation. 1997b;64 (5):731–4.
Sakatsume M, Xie Y, Ueno M, et al. Human glomerulonephritis accompanied by active cellular infiltrates shows effector T cells in urine. J Am Soc Nephrol. 2001;12(12):2636–44.
Segerer S, Banas B, Wornle M, et al. CXCR3 is involved in tubulointerstitial injury in human glomerulonephritis. Am J Pathol. 2004;164(2):635–49.
Stachowski J, Barth C, Lewandowska-Stachowiak M, Lammerding P, Runowski D, Baldamus CA. Flow cytometric analysis of urine lymphocytes isolated from patients with renal transplants – purification of urine lymphocytes. J Immunol Methods. 1998;213(2):145–55.
van Doesum WB, Abdulahad WH, van Dijk MC, et al. Characterization of urinary CD4(+) and CD8 (+) T cells in kidney transplantation patients with polyomavirus BK infection and allograft rejection. Transplant Infect Dis Off J Transplant Soc. 2014;16(5):733–43.
Yu DS, Sun GH, Lee SS, Wu CJ, Ma CP, Chang SY. Flow-cytometric measurement of cellular changes in urine: a simple and rapid method for perioperatively monitoring patients after kidney transplantation. Urol Int. 1999;62(3):143–6.

Proteome of Human Urinary Exosomes in Diabetic Nephropathy

16

Gloria Alvarez-Llamas and Irene Zubiri

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Abstract

Diabetic kidney disease (DKD) is the major complication in diabetic patients, the leading cause of end-stage renal disease (ESRD), and main risk factor for cardiovascular disease (CVD). Its silent development, together with the lack of specific and early accessible indicators of renal damage, often results in a late diagnosis when kidney damage is irreversible. Omics approaches (genomics, proteomics, metabolomics) account with the advantage of investigating the molecular milieu as a whole, without preselection of potential targets. The complexity and wide range of concentration levels of biological fluids as plasma, serum, or urine makes difficult

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the discovery of novel markers of kidney disease progression, other than already known high-abundance molecules (e.g., albumin). Exosomes are microvesicles derived from kidney cells in contact with the urinary space with proven roles in RNA and protein transfer and cell–cell communication. Exosomes may directly reflect pathophysiological changes taking place in the damaged kidney, constituting a feasible alternative to the invasive biopsy. Once released into urine or plasma, exosomes can be isolated and thus represent a sub-proteome where molecular messengers are enriched. This chapter overviews the current panorama in the potential use of exosomes as a novel source of biomarkers able to improve DKD current diagnosis, patients' risk stratification, and prognosis prediction.

Keywords
Kidney disease • Diabetic nephropathy • Exosomes • Proteomics • Cardiovascular disease • Urine • Plasma

Abbreviations

BP	Blood pressure
CE	Capillary electrophoresis
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DIGE	Difference gel electrophoresis
DN	Diabetic nephropathy
DKD	Diabetic kidney disease
ESRD	End-stage renal disease
GC	Gas chromatography
GFR	Glomerular filtration rate
iTRAQ	Isobaric tags for relative and absolute quantitation
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MVB	Multivesicular bodies
NTA	Nanoparticle tracking analysis
RAS	Renin–angiotensin system
SEC	Size exclusion chromatography
SELDI-TOF	Surface-enhanced laser desorption/ionization time-of-flight
TEM	Transmission electron microscopy
THP	Tamm–Horsfall protein
UAER	Urinary albumin excretion rate
UC	Ultracentrifugation
WB	Western blotting

Key Facts of Exosomes

- Exosomes are 40–100 nm vesicles with density values in the range of 1.13–1.19 g/mL.

- Exosomes derive from kidney cells in contact with the urinary space and have proven roles in intercellular communication.
- Exosomes are direct messengers of what is happening in the kidney, both in acute and chronic damage, and carry molecular markers of renal dysfunction and structural injury.
- Several methodologies have been described for isolating exosomes from urine, paying specific attention to the purity and recovery of the isolated fraction.
- In kidney disease patients, albumin can seriously interfere when being co-isolated from urine with the exosomal fraction.
- A few exosomal markers of diabetic kidney disease and other renal disorders have been found by proteomics approaches.

Definitions of Words and Terms

Albuminuria Abnormally increased amount of protein (albumin) detected in the urine.

Biomarker A characteristic (molecule) that is objectively measured and whose levels are evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention.

Chronic kidney disease Progressive and permanent kidney damage, classified in five different stages depending on severity of renal dysfunction.

Diabetic kidney disease Chronic renal disease affecting patients with type1 or type2 diabetes.

End-stage renal disease Kidney failure which requires dialysis or kidney transplantation. ESRD is the last and more severe stage in chronic kidney disease.

Exosome 40–100 nm microvesicles of endocytic origin secreted by most cell types.

Proteomics Study of the whole set of proteins present in a cell, organ, or biological fluid in a certain moment.

Introduction: Diabetic Kidney Disease (DKD) – Diagnosis, Treatment, and Challenges

Diabetes prevalence is globally increasing, and diabetes major complication is a renal disease, commonly named diabetic nephropathy (DN) and more recently referred to as diabetic kidney disease (DKD). Chronic kidney disease (CKD) is the major clinical outcome of diabetic patients with DKD being the leading cause of end-stage renal disease (ESRD) and a risk factor for cardiovascular disease (CVD), i.e., if diabetes is present, ESRD patients mainly die from CVD. Unfortunately, the disease courses silently, diagnosis is not straightforward, and kidney damage is irreversible. In many

Table 1 Clinical indicators of diabetic kidney disease (DKD) (Caramori et al. 2006; Jha et al. 2014)

Initiators
Hyperglycemia
Genetics/epigenetics
Defining parameters
Albuminuria or ACR: normo (<30 mg/g), micro (30–300 mg/g), macro (≥300 mg/g)
GFR changes
Progression factors
Albumin
GFR changes
Glucose (HbA_{1C})
↑ BP
Lipids (cholesterol, triglycerides)
Uric acid
Novel indicators of kidney injury (pending confirmation)
Glomerular (adiponectin, ceruloplasmin, laminin)
Tubular (NGAL, KIM-1, α1- and β2-microglobulin, L-FABP, cystatin C)
Fibrosis (collagen type IV, TGF-β1-to-BMP-7 ratio)
Inflammation (TNF-α, TNFR1)

ACR albumin/creatinine ratio, *GFR* glomerular filtration rate, *BP* blood pressure

cases, initial diagnosis is not made by nephrologist, to whom the patient may be often lately referred, resulting in an increase in mortality rates as no early management to prevent disease progression has been attempted. Diagnosis is based on several clinical manifestations with different interpretation depending on, e.g., patient with type 1 or type 2 diabetes (Park 2014). Urinary albumin excretion rate (UAER or AER) and rising blood pressure (BP) are the most commonly considered indicators together with histological manifestations if biopsy material is available, which mainly happens if atypical clinical course for diabetic nephropathy individuals is observed. In diabetic patients, microalbuminuria is an indicator of nephropathy and a marker of vascular damage and higher cardiovascular risk. Microalbuminuria reflects an abnormality in glomerular capillary permeability to proteins and is also dependent on the tubular capacity to reabsorb filtered albumin. Clinically, it is defined in the range 20–199 mg albumin/g creatinine in males and 30–299 mg albumin/g creatinine in females. Higher values are defined as macroalbuminuria or proteinuria and indicate a decline in the renal function. AER as predictor for DKD accounts with several limitations. It may be the case that healthy subjects with normal renal function show increased AER or that diabetic patients with high risk of developing proteinuria are normoalbuminuric in an early screening. In some cases, albuminuric patients revert to normoalbuminuria in some years without treatment, and, in the opposite case, normoalbuminuric individuals may develop DKD and progress to kidney failure (Molitch et al. 2010; Kramer et al. 2003). Table 1 summarizes most commonly used initiators, defining parameters, and progression factors of DKD.

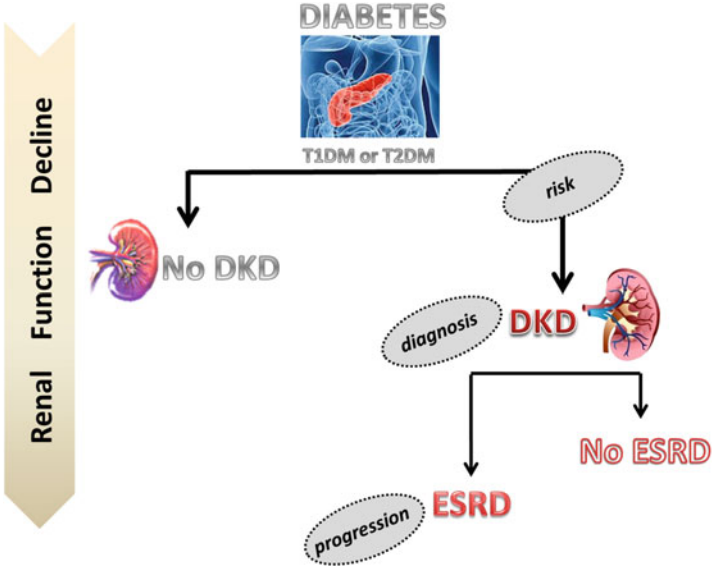

Fig. 1 Kidney disease progression in diabetic patients. Type 1 or 2 diabetic patients may or may not develop diabetic kidney disease (*DKD*). Novel markers able to predict individuals at risk for DKD are pursued. Once renal function starts declining, early diagnosis is mandatory together with the ability to predict whose patients will progress to end-stage renal disease (*ESRD*)

When facing individuals with diabetes, the main questions are as follows: Are they going to develop DKD? And if so, will they progress to ESRD (Fig. 1)? Current risk markers for DKD are albuminuria and decline in glomerular filtration rate (GFR) with cutoff stratification values depending on age, serum uric acid, and serum soluble 1 and 2 TNF receptors, among others (MacIsaac et al. 2014), together with family history, smoking habits, and ambulatory BP and lipids (Caramori et al. 2006; Gray and Cooper 2011). No cure is available. The best approach would be preventing microalbuminuria development and CVD in diabetic patients, e.g., by means of tight BP control and renin–angiotensin system (RAS) suppression (Ruilope et al. 2010) or attempting to stop progression once DKD is diagnosed (Fernandez Fernandez et al. 2012). Precise glycemic control, BP reduction, cholesterol management, and lifestyle improvement compose the current therapy which should be defined as personalized and multitargeted (Bakris 2011).

Despite of all efforts, DKD prevalence remains stable (de Boer et al. 2011), pointing to an urgent need of novel early markers of disease, markers of patient's risk, and predictors of patient's prognosis once DKD is diagnosed in early stages.

Urinary and Plasma Markers of DKD

Limitations of current clinical makers of renal damage in diabetic patients prompt further research aimed to discover novel indicators easily accessible (i.e., able to be monitored in biological fluids as urine or serum/plasma). The ultimate goal is

twofold: (1) achieving early diagnosis of diabetic kidney disease, particularly in those patients with apparent normal renal function (normoalbuminuric), and (2) successful individual stratification of CV risk and renal damage progression.

Classical approaches focus to one or various molecules for which there is evidence or proved connection with the disease under research. Preselection of potential targets implies an enormous limitation in view of the complexity of the interactions and underlying mechanisms operating in the cross talk among the different organs, tissues, and cells. The advantage of the omics strategy is that no potential marker and no key target are preselected, but all the protein and/or metabolite sets are investigated as a whole in the search for significant differences. Thus, not only particular pathways or responding molecules commonly measured in routine biochemical patient's analysis or previously discovered are being investigated but also those whose relationship with the pathophysiological processes taking place is still unknown. A validation (confirmation) phase should then follow to further investigate candidate makers discovered, to set valid conclusions for the clinical practice.

A systematic review of DKD markers is out of the scope of this chapter. Representative studies are referred in Table 2, showing main urinary and plasma/serum markers of DKD found by omics approaches mainly as responders to diabetic condition itself, diabetic nephropathy, albuminuria development or progression, and renal function decline over time or stable. Very different and complementary approaches have been used, i.e., gas chromatography (GC) or liquid chromatography (LC) or capillary electrophoresis (CE) coupled with mass spectrometry (GC-MS or LC-MS or CE-MS), differential gel electrophoresis (DIGE) analysis, MALDI-TOF-MS, SELDI-TOF-MS, and label-free or isotopic labeling (iTRAQ)-LC-MS/MS.

Exosomes: A Novel Source of Research in Kidney Disease

Urine exosomes are 40–100 nm vesicles coated with lipid bilayer membranes with density values in the range of 1.13–1.19 g/mL, derived from all types of kidney cells in contact with the urinary space, including renal tubule cells and podocytes. Exosomes have proven roles in regulating immune response, antigen presentation, RNA and protein transfer, and cell–cell interaction/signaling (Mathivanan et al. 2010; Camussi et al. 2010; Van Balkom et al. 2011). These microvesicles have an endosomal origin. They are formed by the fusion of multivesicular bodies with the plasma membrane and release of their intraluminal vesicles, which are then termed exosomes once in the extracellular space. Exosomes thus contain membrane and cytosolic cellular proteins and are considered a mechanism of nonclassical secretion of proteins, representing 3 % of the whole urine proteome. ExoCarta is a protein, lipid, and RNA exosomal database providing with the contents of exosomes which have been identified in multiple organisms, cells, and fluids.

The use of urinary exosomes as starting material for biomarker discovery has shown to be advantageous. They constitute a sub-proteome of the whole urinary proteome with minor complexity and reduced protein dynamic concentration range, which represents a better alternative for detection of low-abundance proteins that

Table 2 Representative proteomics studies to approach diabetic nephropathy and discover novel markers of disease

Clinical groups	Biological source	Technical approach	Main findings	References
T1DM ($n = 122$): Normo, micro, macro	Plasma peptidome	RPC18, wCX, MALDI-TOF	C3f, apolipoprotein C-I (markers of DN)	(Hansen et al. 2010)
T2DM ($n = 6$) Control ($n = 6$)	Plasma glycoproteins	PAGE+LC-MS/MS	Lumican, vasorin, RBP4	(Ahn et al. 2010)
T2DM ($n = 90$) Normo–micro, micro–macro HTN ($n = 150$)	Plasma and urine metabolome	LC-MS/MS	Plasma: histidine, butenoylcarnitine (T2DM vs. control) Urine: hexose, glutamine, tyrosine (risk predictors of albuminuria evolution)	(Pena et al. 2014)
DM DN ($n = 150$)	Plasma	GC-MS	NEFAs, EFAs	(Han et al. 2011)
T2DM ($n = 30$) DN ($n = 52$) Control ($n = 30$)	Plasma	UPLC-MS/MS	Phospholipids PI C18:0/22:6 SM dC18:0/20:2	(Zhu et al. 2011)
T1DM+micro (stable or declined renal function)	Plasma peptides	LC-MALDI-TOF	Kininogen	(Merchant et al. 2013)
DN ($n = 66$) T2DM ($n = 82$)	Urine peptides	CE-MS	Collagen fragments	(Alkhalaf et al. 2010)
T2DM: normo ($n = 43$) Micro ($n = 43$)	Urine	iTRAQ	Alpha-1-antitrypsin Alpha-1-acid glycoprotein 1 Prostate stem cell antigen	(Jin et al. 2012)
T1DM: normo ($n = 52$) (progressed ($n = 26$) or stable ($n = 26$))	Urine	GC-MS, LC-MS	Metabolite panel	(van der Kloet et al. 2012)
T1DM (normo and macro)	Urine	LC-MS/MS	Vanin-1	(Fugmann et al. 2011)
1. Control ($n = 20$), normo ($n = 20$), micro ($n = 18$) + T2DM 2. DN ($n = 65$), T2DM +ndCKD ($n = 10$), nDM +CKD ($n = 57$)	Urine	SELDI-TOF	Ubiquitin B2-microglobulin	(Papale et al. 2010)
T1DM+micro (normal renal function): declined renal function ($n = 21$) and stable ($n = 40$)	Urine	LC-MALDI-TOF	α1(IV) collagen α1(V) collagen Tenascin-X Inositol pentakis phosphate 2-Kinase	(Merchant et al. 2009)

(*continued*)

Table 2 (continued)

Clinical groups	Biological source	Technical approach	Main findings	References
DM+albuminuria ($n = 38$) DM w/o albuminuria ($n = 45$) noDM+albuminuria ($n = 34$) Control ($n = 45$)	Urine	SELDI-TOF	UbA52	(Dihazi et al. 2007)

DM diabetes mellitus, *T1DM* type 1 diabetes mellitus, *T2DM* type 2 diabetes mellitus, *DN* diabetic nephropathy, *CKD* chronic kidney disease, *ndCKD* nondiabetic CKD, *HTN* hypertension

otherwise could be masked by major proteins. As a consequence of their endocytic origin, urinary exosomes contain proteins characteristic of every renal tubule epithelial cell type and from the urinary collecting system, including proteins that are characteristic of the membrane and cytoplasm of the cells in which they have been generated. In particular, exosomes can be released in the kidney by cells as podocytes, pass through the renal tubule, and either be untaken by recipient epithelial cells of the collecting duct or influence them through secretion of their content (Street et al. 2011). In this sense, more than a way of exocytic cell waste elimination, exosomes should be considered as messengers, transferring information between renal and nonrenal cells and carrying molecular markers of renal dysfunction and structural injury (Salih 2014; Zhou et al. 2008). This role of exosomes as messengers between cells and tissues gains particular importance in complex scenarios where multi-organ cross talk takes place. That is the case of the cardiorenal syndrome, defined (although not fully understood) by proved evidence that an acute/chronic worsening of kidney function influences an acute/chronic cardiac dysfunction and vice versa. In cardiovascular disease, exosomes have gained increasing interest (Cosme et al. 2013) although their specific role in atherosclerosis development still constitutes an underexplored field (Gonzalez-Calero et al. 2014).

Exosomal Isolation from Urine

Independent of the methodological approach to be used in the study of exosomal molecular content, key aspects should be taken into account, which may strongly influence the purity and recovery of the exosomal isolated fraction. Collection and storage of urine samples influence in a high degree the quality of the recovered exosomal fraction, and general guidelines have been published, including the use of protease inhibitors at collection time, sample storage at −80 °C, and extensive vortexing of urine samples after thawing as mandatory steps (Zhou et al. 2006a). Exosomal isolation (purification) from urine is not straightforward. High abundant urinary proteins as Tamm–Horsfall protein (THP or uromodulin) and albumin when renal function is compromised are co-isolated together with the exosomes. This contamination source can be overcome using different methodological approaches, as detailed below.

In the last decade, different methods have been proposed for the isolation of exosomes from diverse biological fluids, and there is no consensus on the best method to obtain a pure and well-characterized exosomal fraction from urine. Despite the lack of agreement, most commonly used approaches are based on (differential) ultracentrifugation (UC) (Pisitkun et al. 2004) (density gradient- or cushion-based UC) (Raj et al. 2012) and based on the use of a nanomembrane concentrator (Cheruvanky et al. 2007), immunoaffinity (Sun et al. 2012), or microfluidic technology (He et al. 2014). There are also new commercial methods such as the Total Exosome Isolation™ precipitation solution (Invitrogen), immunobeads or immunoplates (HansaBioMed LLC), or ExoQuick™ precipitation reagent suitable for the isolation of these microvesicles from urine, serum, and plasma.

Differential centrifugation and UC isolation method is recommended by the Human Kidney and Urine Proteome Project (http://www.hkupp.org/Exosome%20Preparation.htm). Currently, this is the most frequently used methodology for the isolation of exosomes from urine (Fig. 2). In brief, urine samples are centrifuged at 17,000 × g in order to remove the whole cells, large membrane fragments, and debris and recover the supernatant, which is then ultracentrifuged (200,000 × g, 1 h, 4 °C). Exosomes are recovered in the pellet. Particular attention should be paid by

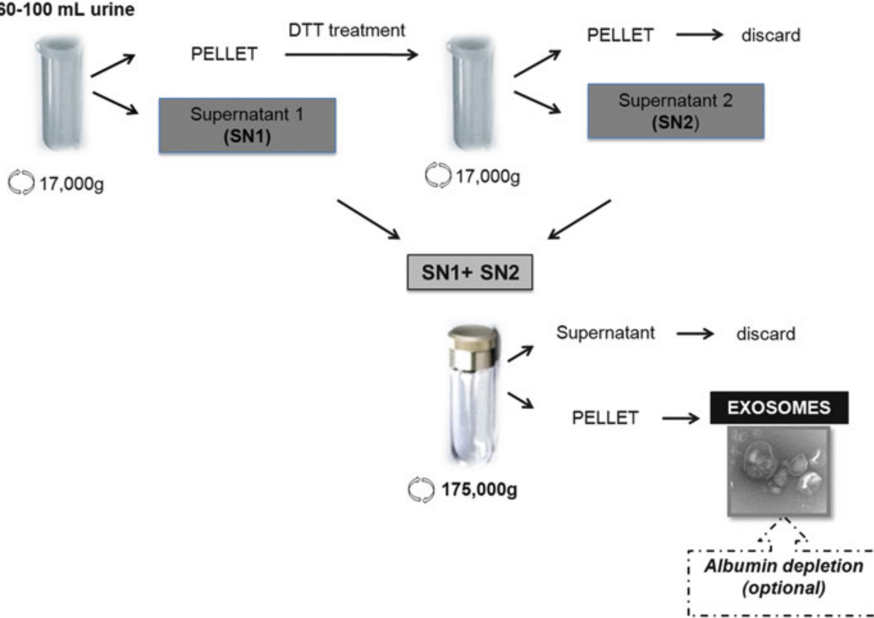

Fig. 2 Schematic view of isolation protocol of exosomes from urine. Ultracentrifugation based is one of the most commonly used methodologies for isolating urinary exosomes. Serial centrifugation steps and enrichment of the exosomal pellet by DTT treatment are applied to maximize purity and recovery of the exosomal fraction. An extra albumin depletion step is recommended when analyzing urine samples from kidney disease patients diagnosed with macroalbuminuria

the partial entrapment of exosomes by the polymerized THP network, thus reducing exosomal recovery. This drawback can be overcome by treatment of the first (low-speed centrifugation) pellet with reducing agents (e.g., DTT) and heat. Following centrifugation again, the supernatant is then collected and pulled together with the supernatant obtained in the first low-speed centrifugation to proceed with ultracentrifugation (Fernández-Llama et al. 2010). The final pellet can still contain important amounts of THP polymers coprecipitating with the exosomes which can be treated again with reducing agents and heat to dissolve the aggregates and ultracentrifugated again to obtain a final clean exosome pellet (Gonzales et al. 2010). Another option to minimize THP interference and further purify the exosomal fraction is to perform extra steps of UC using sucrose gradient or 30 % sucrose cushion.

Nanomembrane concentration is an alternative to ultracentrifugation, which is time consuming and requires instrumentation not always available. This approach is fast and simple and is based on the use of nanomembranes with a uniform pore size of 13 mm. However, protein recovery is generally not uniform nor pure, and for comparative proteomic analysis, this variation needs to be taken into account. Exoquick® is a commercial reagent designed for specific isolation of exosome by precipitation, but obtaining enough exosomal recovery from control urine samples and high purity of the isolated fraction from albuminuric samples is not guaranteed, as urine-contaminating proteins can be co-isolated in proteinuric conditions [unpublished data]. A modified Exoquick® protocol has been described with improved results (Alvarez et al. 2012). Six different protocols were compared concluding that ultracentrifugation methods result in the purest exosomal protein yield, and the fast and simple modified Exoquick® protocol proved to be the most effective alternative, particularly when analyzing exosomal mRNA and miRNA.

Exosomal isolations from plasma or secreted by B cell have been accomplished by immunoaffinity based or by microfluidic isolation technology which separates microvesicles as a function of diameter from heterogeneous populations of cancer-cell-derived extracellular shed vesicles (Santana et al. 2014). These methods will be probably adapted to the isolation of microvesicles from urine in the near future.

Characterization of the Isolated Exosomal Fraction

There are numerous techniques able to confirm the presence and purity of exosomes obtained by any of the above-described isolation methods. Transmission electron microscopy (TEM), Western blotting (WB), and most recently NanoSight technique are the approaches most commonly used. TEM requires sample fixation with 4 % paraformaldehyde to be later deposited on Formvar carbon-coated nickel grids and stained with uranyl acetate to obtain images of the exosomes that can allow the user to determine the size and shape (cup shaped) of these microvesicles under the microscope. Immuno-electron microscopy (IEM) allows the immune detection and direct imaging of exosomes. For WB characterization, specific well-known exosomal proteins are detected. ALIX, TSG101, and clathrin are involved in the

maturation of MVB and are known to be present in the human urinary exosome membrane. Exosomes are also rich in tetraspanins like CD9, CD63, and CD81 and heat shock proteins like HSP60, HSP70, and HSPA5. All these specific markers are independent from the origin of the exosomes and can be used to characterize exosomes from urine as well as from other sources. In urine, exosomes originate from podocytes and epithelial cells, and it is possible to detect the presence of proteins that are segment specific such as aquaporin 2 (AQP2, collecting duct), sodium proton exchanger 3 (NHE-3, proximal tubule), or podocalyxin (PODXL) found in podocytes. Apart from WB, enzyme-linked immunosorbent assays (ELISAs) or flow cytometry can be used for detection of specific exosomal markers. Because of their small size, exosomes can only be analyzed in a flow cytometer after linkage to larger particles of known size. Exosomes can be adsorbed to solid latex microspheres, and microspheres/exosomes can be later incubated with specific antibodies and analyzed on a flow cytometer (Benito-Martin et al. 2013). A novel tool that has proven efficiency in the characterization of exosomes is the nanoparticle tracking analysis (NTA) using the NanoSight which allows specific exosomes and microvesicles in the range of 50–1,000 nm in liquid suspension to be directly and individually visualized and counted in real time.

Albumin Potential Interference in the Study of Kidney Diseases by Proteomics

The albuminuric condition may condition the purity of the exosomal isolated fraction. Albumin overload in urine can represent an important problem when, e. g., approaching a proteomics study of kidney diseases characterized by an abnormally high content of this protein in urine (Martin-Lorenzo et al. 2014). Unspecific co-isolation of albumin in the exosomal fraction may diminish reproducibility, condition the robustness of the methodology with comparative purposes, and reduce the possibility to detect low-abundance proteins, making more challenging the comparison between healthy and disease condition. Total protein quantification of exosomal fractions can vary substantially between control and disease samples, resulting in a significantly higher total "exosomal protein content" in patient samples due to albumin and thus causing underestimation of the low-abundance exosomal proteins. Depletion of major soluble urine protein contaminants is therefore advisable to broaden our understanding of exosomal proteome changes apart from albumin content in proteinuric kidney disease. An isolation methodology by serial (ultra)centrifugation steps followed by depletion of the major proteins present in the exosome fraction was described based on ProteoPrep® Immunoaffinity Albumin & IgG Depletion Kit (Sigma-Aldrich) originally developed for plasma samples but adapted to urinary exosomal fraction. This method proved to be useful and simple, allowing an increase up to 60 % in the number of identified proteins when using LC-MS/MS techniques to investigate candidate exosomal markers of diabetic nephropathy in human samples (Zubiri et al. 2013, 2014). The efficiency of isolation methods in patients with nephrotic-range proteinuria was investigated by

comparison of three techniques: nanomembrane ultrafiltration, ultracentrifugation, and ultracentrifugation followed by size exclusion chromatography (UC-SEC). They demonstrate that highly abundant urinary proteins were still present in sufficient quantity after ultrafiltration and ultracentrifugation and were able to overcome this problem when using UC-SEC (Rood et al. 2010).

These two methods represent an improvement in the available exosomal isolation methods, particularly challenging when dealing with nephropathy urine samples.

Exosomal Markers of Diabetic Kidney Disease

Since 2004 when the presence of exosomes in urine was reported (Sun et al. 2012), a growing number of studies have been published aimed to the search of novel biomarkers of disease in these microvesicles. Protein and RNA biomarker candidates have been postulated for a variety of bladder, prostate, urinary tract diseases and kidney diseases including DKD. In this specific context, several promising biomarkers have been described in urinary exosomes from patients and animal models (Table 3). The activity of dipeptidyl peptidase IV (DPP IV) in urine microvesicles measured by ELISA positively correlated with the progression of proteinuria in type 2 diabetic nephropathy patients, being a good candidate to represent an early biomarker of renal damage before onset of albuminuria. Podocyte injury contributes to the initiation and decline of kidney function in diabetic nephropathy (Wolf et al. 2005), and podocyte apoptosis is an early mechanism leading to diabetic nephropathy (Susztak et al. 2006). Measuring podocyte protein expression changes in a noninvasive manner was possible after isolating urine exosomes. Expression of Wilms' tumor 1 (WT1) protein, a transcription factor and podocyte marker, was measured in urine exosomes from 48 type 1 diabetic patients and 25 healthy controls, showing for the first time a predominant expression of *WT1* protein in urinary exosome in type 1 diabetic patients. This protein was not present in healthy age-matched controls, and higher levels of this marker were found in exosomes from patients with proteinuria. The strong correlation found between the expression of WT1 and the increase in urine protein excretion suggests a considerable predictive value of this protein as an early biomarker of DN (Kalani et al. 2013).

Omics approaches account with the advantage of generating data which are not individual, referred to a unique molecule (protein, metabolite), but global, describing hundreds or thousands of compounds altered simultaneously in response to a certain disease or stimulus. This is possible due to the ability of a wide range of available techniques to characterize thousands of molecular species in each run, thus generating profiles or data sets which reflect the general situation of the sample (cell, tissue, biopsy, serum, urine, etc.). Different methodological approaches currently available can be applied to investigate the exosomal proteome, making the choice mainly dependent on (1) the nature of the analytes to investigate (i.e., peptides, proteins, metabolites, lipids); (2) the performance in terms of sensitivity, selectivity, specificity, and throughput; and (3) the step in the, e.g., biomarker research pipeline to approach (discovery or validation) which may require a targeted (e.g., SRM or

Table 3 Studies showing the potential of urinary exosomes in the search for biomarkers of kidney diseases

Disease	Biomarker candidate	Main technique	Isolation method	References
DKD	miR-145	RT-QPCR	UC	(Barutta et al. 2013)
DKD	AMBP	LC-MS/MS	UC	(Wolf et al. 2005)
	MLL3			
	VDAC1			
DKD	13 mitochondrial metabolite panels	GC-electron impact MS	Volume exclusion	(Sharma et al. 2013)
DKD	Xaa-Pro dipeptidase	LC-MS/MS	UC	(Raimondo et al. 2013a)
	Major urinary protein 1			
	Neprilysin			
DKD	DPP IV	ELISA	Immunoaffinity isolation	(Gonzales et al. 2010)
DKD	WT1	Immunoblotting	UC	(Zhou et al. 2013)
Podocyte injury				
CKD	OPG	Immunoblotting	UC	(Benito-Martin et al. 2013)
		ELISA		
Kidney fibrosis	mRNA of CD2AP	RT-QPCR	UC	(Lv et al. 2014)
Cystinuria	38 protein panels	IEF LC-MS/MS	UC	(Bourderioux et al. 2015)
AKI	ATF3 RNA	RT-QPCR	UC	(Chen et al. 2014)

(*continued*)

Table 3 (continued)

Disease	Biomarker candidate	Main technique	Isolation method	References
AKI	ATF3	Immunoblotting	UC	(Zhou et al. 2008)
	WT1	IHC		
AKI	Fetuin-A	2D-DIGE	UC	(Zhou et al. 2006b)
Renal carcinoma	10 protein panels	LC-MS/MS	UC	(Raimondo et al. 2013b)
Renal transplantation	NGAL	Immunoblotting	UC	(Alvarez et al. 2013)
IgA nephropathy	Aminopeptidase N vasorin	LC-MS/MS	UC	(Moon et al. 2011b)
Thin basement membrane nephropathy	α-1-Antitrypsin, ceruloplasmin			
Renal ischemia–reperfusion injury	AQP1	Immunoblotting	UC	(Sonoda et al. 2009)
Bartter syndrome type I	INKCC2	LC-MS/MS	UC	(Gonzales et al. 2009)
		Immunoblotting		
Autoimmune glomerulonephritis	miR-26a	RT-QPCR	UC	(Ichii et al. 2014)
Polycystic kidney disease	PC1/TMEM2 PC2/TMEM2	LC-MS/MS	Sucrose gradient	(Hogan et al. 2015)
Obstructive nephropathy	E-cadherin	Immunoblotting	UC	(Trnka et al. 2012)
	N-cadherin			
	TGFB			
	L1CAM			

DKD diabetic kidney disease, *AKI* acute kidney injury, *UC* ultracentrifugation

MRM) or a wider approach (e.g., label-free or (iTRAQ)-LC-MS/MS, CE-MS, SELDI-TOF-MS). Proteomics analysis has been applied in the search for potential markers of disease in isolated exosomes (Choi et al. 2013; Moon et al. 2011a; Simpson et al. 2009). By label-free LC-MS/MS quantitative analysis of exosomes isolated from urine of Zucker diabetic fatty (ZDF) rats as a model of type 2 DN, 286 proteins were identified and quantified. Confirmed by immunoblotting, increased Xaa-Pro dipeptidase and decreased urinary protein 1 were shown. In a similar study carried out in humans, spectral counting analysis revealed a total of 562 proteins identified (207 had been previously identified in urinary exosomes, 108 had been identified in exosomes from different origin, and 244 were identified as exosomal proteins for the first time). Among those, a panel of 25 proteins significantly changed in diabetic nephropathy. Confirmed by selected reaction monitoring (SRM) mass spectrometry technique, three protein candidate markers of DN in exosomes were postulated, alpha-1-microglobulin/bikunin precursor (AMBP), voltage-dependent anion-selective channel protein 1 (VDAC1), and isoform 1 of histone-lysine N-methyltransferase (MLL3), opening a new possibility to monitor DN by analyzing urinary exosomes (Wolf et al. 2005).

The metabolome represents the downstream changes in the genome, transcriptome, and proteome as a reflection of real-time processes occurring in living organisms. Compared to more than ten million proteins in the proteome, a few thousand metabolites present in an organism imply a considerable reduction in complexity. Urine metabolomics is another important field for the study of diabetic complications. By GC-MS 94 urine metabolites were quantified in cohorts of patients with diabetes mellitus with and without kidney disease and in healthy controls. Thirteen metabolites were found significantly reduced in the diabetic nephropathy cohorts, related to the mitochondrial metabolism, indicating a suppression of mitochondrial activity in diabetic kidney disease. A consequence of this dysregulation was also detectable in urine exosomes as they showed that urine exosomes from patients contain a lower amount of mitochondrial DNA. This founding was consistent with later gene expression measurements performed in the kidney tissue, where a lower expression of PGC1α, a master regulator gene of mitochondrial biogenesis, was observed.

The majority of the studies based in urine exosomes in the search of biomarkers for DN are focused on the analysis of the proteomic composition of these microvesicles in healthy and disease stages. The potential of the exosomal RNA as source of kidney disease markers has also been reported (Miranda et al. 2010). RNA present in urine tends to be easily degraded and can be originated in apoptotic or necrotic cells not being representative of the transcriptional profile (Wang and Szeto 2007). RNAs protected by the lipid membrane of the exosomes are more stable and can be recovered and analyzed through the isolation of the exosomal fraction. RNAs contained in exosomes are produced in viable cells; thus they can provide a key insight of the physiopathological processes taking place in the kidney (van Balkom et al. 2011). Exosomes contain microRNA (miRNA), a class of small nonprotein-

encoding RNAs that regulate gene expression via suppression of target mRNAs. miRNA expression was analyzed in urinary exosomes from type 1 diabetic patients with and without diabetic nephropathy. Two hundred twenty-six miRNAs were detected in the normoalbuminuric patient urinary exosomes, and 22 miRNAs showed differential expression between normoalbuminuric and microalbuminuric patients. In the validation phase, miR-145 was found enriched in urinary exosomes from microalbuminuric patients, a glomerular marker of mesangial cells (Harvey et al. 2008) induced by TGF-β1 in this cell type (Denby et al. 2011). The expression of miR-145 was explored in both streptozotocin-induced diabetic mice and cultured mesangial cells. An upregulation in miR-130a was observed in type 1 diabetic patients. On the contrary, miR-155 and miR-424 were downregulated, and this effect was observed specifically in those patients with incipient diabetic nephropathy. In conclusion, miR-145 was identified as a new potential player in diabetic glomerulopathy, and the feasibility of the study of urinary exosomal miRNA as a source for candidate biomarker discovery in diabetic and other renal diseases was demonstrated here.

The global results of these studies evidence the potential use of the urine exosomes to monitor changes occurring in the kidney, opening an interesting alternative to the invasive kidney biopsies used nowadays to diagnose patients and follow progression. Further exosomal studies will follow to expand current knowledge of underlying operating mechanisms in DKD, which ultimately end in the discovery of novel therapeutic targets, and key molecules able to (a) diagnose diabetic patients in asymptomatic stages, (b) predict who of them will or not further progress to DKD or ESRD, and (c) stratify individual cardiovascular risk.

Potential Applications to Prognosis, Other Diseases, or Conditions

This chapter shows the applicability of exosomes in the study of kidney diseases and diabetic nephropathy in particular. The silent progression, asymptomatic at early stages, and irreversible damage of kidney functionality prompt the application of novel strategies in the search for novel markers, and exosomes arise as a powerful underexplored source. Markers can be classified according to their utility in (1) “risk assessment” (markers responding to disease susceptibility), (2) “screening” (markers able to discriminate between healthy and asymptomatic diseases in large populations), (3) “prognosis” (markers able to predict probable course of disease or aggressiveness of therapy), (4) “stratification” (envisage responders and non-responders to drug), and (5) “therapy monitoring” (indicators of the efficacy of treatment once the responder status is established). Depending on the ultimate goal, the experimental study should be carefully designed and patients’ cohorts, properly matched with a healthy control group, carefully chosen (Fig. 3). Apart from these general rules, all technical and methodological improvements focused to efficiently isolate, characterize, and analyze the exosomal fraction from biological fluids can be implemented to the study of this and other diseases. This is an open

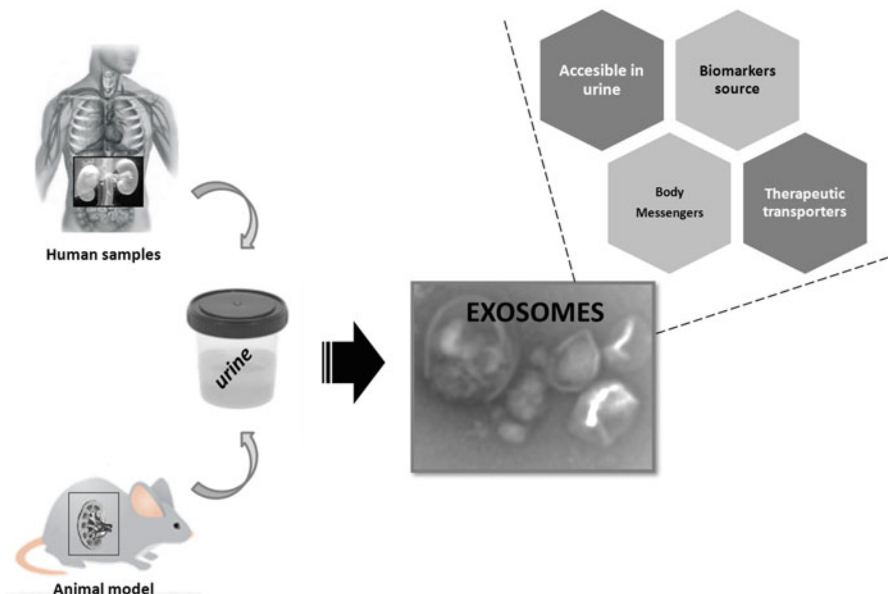

Fig. 3 Schematic workflow. Exosomal proteome is a powerful tool in the search for novel markers of disease. Isolated from urine, they constitute an enriched sub-proteome which directly reflects changes taking place in the kidney

field of research, which, although not fully mature in methodology, already accounts with proven applicability in the clinical proteomics field.

Summary Points

- Diabetic kidney disease is the major complication in diabetes and main risk factor for cardiovascular disease.
- Diabetic kidney disease develops silently, it is asymptomatic at early stages, and it is often diagnosed once the renal damage is irreversible.
- Proteomics arises as a powerful approach in the search for novel markers of diabetic nephropathy, once the challenge of the proteome dynamic range in the biological fluids is overcome.
- Exosomes are microvesicles released into urine which act as messengers of changes taking place in the kidney.
- Exosomal isolation from biological fluid is challenging, and ultracentrifugation-based method is one of the most efficient approaches. Particular care has to be taken with co-isolation of albumin from urine of renal patients.
- Exosomes constitute a novel and enriched source of biomarkers of kidney diseases and diabetic nephropathy, in particular.

References

Ahn JM, Kim BG, Yu MH, et al. Identification of diabetic nephropathy-selective proteins in human plasma by multi-lectin affinity chromatography and LC-MS/MS. Proteomics Clin Appl. 2010;4:644–53.
Alkhalaf A, Zurbig P, Bakker SJL, et al. Multicentric validation of proteomic biomarkers in urine specific for diabetic nephropathy. PLoS One. 2010;5:e13421.
Alvarez ML, Khosroheidari M, Kanchi Ravi R, et al. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. Kidney Int. 2012;82:1024–32.
Alvarez S, Suazo C, Boltansky A, et al. Urinary exosomes as a source of kidney dysfunction biomarker in renal transplantation. Transplant Proc. 2013;45:3719–23.
Bakris GL. Recognition, pathogenesis, and treatment of different stages of nephropathy in patients with type 2 diabetes mellitus. Mayo Clin Proc. 2011;86:444–56.
Barutta F, Tricarico M, Corbelli A, et al. Urinary exosomal microRNAs in incipient diabetic nephropathy. PLoS One. 2013;8:e73798.
Benito-Martin A, Ucero AC, Zubiri I, et al. Osteoprotegerin in exosome-like vesicles from human cultured tubular cells and urine. PLoS One. 2013;8:e72387.
Bourderioux M, Nguyen-Khoa T, Chhuon C, et al. A new workflow for proteomic analysis of urinary exosomes and assessment in cystinuria patients. J Proteome Res. 2015;14(1):567–77. doi:10.1021/pr501003q. Epub 2014 Nov 12.
Camussi G, Deregibus MC, Bruno S, et al. Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int. 2010;8:838–48.
Caramori ML, Fioretto P, Mauer M. Enhancing the predictive value of urinary albumin for diabetic nephropathy. J Am Soc Nephrol. 2006;17:339–52.
Chen HH, Lai PF, Lan YF, et al. Exosomal ATF3 RNA attenuates pro-inflammatory gene MCP-1 transcription in renal ischemia-reperfusion. J Cell Physiol. 2014;229:1202–11.
Cheruvanky A, Zhou H, Pisitkun T, et al. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. Am J Physiol Renal Physiol. 2007;92:F1657–61.
Choi DS, Kim DK, Kim YK, et al. Proteomics, transcriptomics and lipidomics of exosomes and ectosomes. Proteomics. 2013;13:1554–71.
Cosme J, Liu PP, Gramolini AO. The cardiovascular exosome: current perspectives and potential. Proteomics. 2013;13:1654–9.
de Boer IH, Rue TC, Hall YN, et al. Temporal trends in the prevalence of diabetic kidney disease in the United States. JAMA. 2011;305:2532–9.
Denby L, Ramdas V, McBride MW, et al. miR-21 and miR-214 are consistently modulated during renal injury in rodent models. Am J Pathol. 2011;179:661–72.
Dihazi H, Müller GA, Lindner S, et al. Characterization of diabetic nephropathy by urinary proteomic analysis: identification of a processed ubiquitin form as a differentially excreted protein in diabetic nephropathy patients. Clin Chem. 2007;53:1636–45.
Fernandez Fernandez B, Elewa U, Sanzchez-Niño MD, et al. 2012 update on diabetic kidney disease: the expanding spectrum, novel pathogenic insights and recent clinical trials. Minerva Med. 2012;103:219–34.
Fernández-Llama P, Khositseth S, Gonzales PA, et al. Tamm-Horsfall protein and urinary exosome isolation. Kidney Int. 2010;77:736–42.
Fugmann T, Borgia B, Révész C. Proteomic identification of vanin-1 as a marker of kidney damage in a rat model of type 1 diabetic nephropathy. Kidney Int. 2011;80:272–81.
Gonzales PA, Pisitkun T, Hoffert JD, et al. Large-scale proteomics and phosphoproteomics of urinary exosomes. J Am Soc Nephrol. 2009;20:363–79.
Gonzales PA, Zhou H, Pisitkun T, et al. Isolation and purification of exosomes in urine. Methods Mol Biol. 2010;641:89–99.
Gonzalez-Calero L, Martin-Lorenzo M, Alvarez-Llamas G. Exosomes: a potential key target in cardio-renal syndrome. Front Immunol. 2014;5:465.

Gray SP, Cooper ME. Alleviating the burden of diabetic nephropathy. Nat Rev Nephrol. 2011;7:71–3.
Han LD, Xia JF, Liang QL, et al. Plasma esterified and non-esterified fatty acids metabolic profiling using gas chromatography-mass spectrometry and its application in the study of diabetic mellitus and diabetic nephropathy. Anal Chim Acta. 2011;689:85–91.
Hansen HG, Overgaard J, Lajer M, et al. Finding diabetic nephropathy biomarkers in the plasma peptidome by high-throughput magnetic bead processing and MALDI-TOF-MS analysis. Proteomics Clin Appl. 2010;4:697–705.
Harvey SJ, Jarad G, Cunningham J, et al. Podocyte-specific deletion of dicer alters cytoskeletal dynamics and causes glomerular disease. J Am Soc Nephrol. 2008;19:2150–8.
He M, Crow J, Roth M, et al. Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology. Lab Chip. 2014;14(19):3773–80. doi:10.1039/c4lc00662c.
Hogan MC, Bakeberg JL, Gainullin VG, et al. Identification of biomarkers for PKD1 using urinary exosomes. J Am Soc Nephrol. 2015;26(7):1661–70. doi:10.1681/ASN.2014040354. Epub 2014 Dec 4.
Ichii O, Otsuka-Kanazawa S, Horino T, et al. Decreased miR-26a expression correlates with the progression of podocyte injury in autoimmune glomerulonephritis. PLoS One. 2014;9: e110383.
Jha JC, Jandeleit-Dahm KAM, Cooper ME. New insights into the use of biomarkers of diabetic nephropathy. Adv Chronic Kidney Dis. 2014;21:318–26.
Jin J, Ku YH, Kim Y, et al. Differential proteome profiling using iTRAQ in microalbuminuric and normoalbuminuric type 2 diabetic patients. Exp Diabetes Res. 2012;2012:168602.
Kalani A, Mohan A, Godbole MM, et al. Wilm's tumor-1 protein levels in urinary exosomes from diabetic patients with or without proteinuria. PLoS One. 2013;8:e60177.
Kramer HJ, Nguyen QD, Curhan G, et al. Renal insufficiency in the absence of albuminuria and retinopathy among adults with type 2 diabetes mellitus. JAMA. 2003;289:3273–7.
Lv LL, Cao YH, Pan MM, et al. CD2AP mRNA in urinary exosome as biomarker of kidney disease. Clin Chim Acta. 2014;428:26–31.
MacIsaac RJ, Ekinci EI, Jerums G. Markers of and risk factors for the development and progression of diabetic kidney disease. Am J Kidney Dis. 2014;63(S2):S39–62.
Martin-Lorenzo M, Gonzalez-Calero L, Zubiri I, et al. Urine 2DE proteome analysis in healthy condition and kidney disease. Electrophoresis. 2014;35:2634–41.
Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. J Proteomics. 2010;73:1907–20.
Merchant ML, Perkins BA, Boratyn GM, et al. Urinary peptidome may predict renal function decline in type 1 diabetes and microalbuminuria. J Am Soc Nephrol. 2009;20:2065–74.
Merchant ML, Niewczas M, Ficociello LH, et al. Plasma kininogen and kininogen fragments are biomarkers of progressive renal decline in type-1 diabetes. Kidney Int. 2013;83:1177–84.
Miranda KC, Bond DT, McKee M, et al. Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. Kidney Int. 2010;78:191–9.
Molitch ME, Steffes M, Sun W, et al. Development and progression of renal insufficiency with and without albuminuria in adults with type 1 diabetes in the diabetes control and complications trial and the epidemiology of diabetes interventions and complications study. Diabetes Care. 2010;33:1536–43.
Moon PG, You S, Lee JE, et al. Urinary exosomes and proteomics. Mass Spectrom Rev. 2011a;30:1185–202.
Moon PG, Lee JE, You S, et al. Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy. Proteomics. 2011b;11:2459–75.
Papale M, di Paolo S, Magistroni R, et al. Urine proteome analysis may allow noninvasive differential diagnosis of diabetic nephropathy. Diabetes Care. 2010;33:2409–15.
Park CW. Diabetic kidney disease: from epidemiology to clinical perspectives. Diabetes Metab J. 2014;38:252–60.

Pena MJ, Lambers Heerspink HJ, Hellemons ME, et al. Urine and plasma metabolites predict the development of diabetic nephropathy in individuals with Type 2 diabetes mellitus. Diabet Med. 2014;31:1138–47.
Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci U S A. 2004;101:13368–73.
Raimondo F, Corbetta S, Morosi L, et al. Urinary exosomes and diabetic nephropathy: a proteomic approach. Mol Biosyst. 2013a;9:1139–46.
Raimondo F, Morosi L, Corbetta S, et al. Differential protein profiling of renal cell carcinoma urinary exosomes. Mol Biosyst. 2013b;9:1220–33.
Raj DA, Fiume I, Capasso G, et al. A multiplex quantitative proteomics strategy for protein biomarker studies in urinary exosomes. Kidney Int. 2012;81:1263–72.
Rood IM, Deegens JK, Merchant ML, et al. Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome. Kidney Int. 2010;78:810–6.
Ruilope L, Izzo J, Haller H, et al. Prevention of microalbuminuria in patients with type 2 diabetes: what do we know? J Clin Hypertens (Greenwich). 2010;12:422–30.
Salih M. Urinary extracellular vesicles and the kidney: biomarkers and beyond. Am J Physiol Renal Physiol. 2014;306:F1251–9.
Santana SM, Antonyak MA, Cerione RA, et al. Microfluidic isolation of cancer-cell-derived microvesicles from heterogeneous extracellular shed vesicle populations. Biomed Microdevices. 2014;16:869–77.
Sharma K, Karl B, Mathew AV, et al. Metabolomics reveals signature of mitochondrial dysfunction in diabetic kidney disease. J Am Soc Nephrol. 2013;24:1901–12.
Simpson RJ, Lim JW, Moritz RL, et al. Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomics. 2009;6:267–83.
Sonoda H, Yokota-Ikeda N, Oshikawa S, et al. Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury. Am J Physiol Renal Physiol. 2009;297: F1006–16.
Street JM, Birkhoff W, Menzies RI, et al. Exosomal transmission of functional aquaporin 2 in kidney cortical collecting duct cells. J Physiol. 2011;589:6119–27.
Sun AL, Deng JT, Guan GJ, et al. Dipeptidyl peptidase-IV is a potential molecular biomarker in diabetic kidney disease. Diab Vasc Dis Res. 2012;9:301–8.
Susztak K, Raff AC, Schiffer M, et al. Glucose-Induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. Diabetes. 2006;55:225–33.
Trnka P, Ivanova L, Hiatt MJ, et al. Urinary biomarkers in obstructive nephropathy. Clin J Am Soc Nephrol. 2012;7:1567–75.
Van Balkom BW, Pisitkun T, Verhaar MC, et al. Exosomes and the kidney: prospects for diagnosis and therapy of renal diseases. Kidney Int. 2011;80:1138–45.
van der Kloet FM, Tempels FWA, Ismail N, et al. Discovery of early-stage biomarkers for diabetic kidney disease using ms-based metabolomics (FinnDiane study). Metabolomics. 2012;8:109–19.
Wang G, Szeto CC. Quantification of gene expression in urinary sediment for the study of renal diseases. Nephrology (Carlton). 2007;12:494–9.
Wolf G, Chen S, Ziyadeh FN, et al. From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. Diabetes. 2005;54:1626–34.
Zhou H, Yuen PS, Pisitkun T, et al. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int. 2006a;69:1471–6.
Zhou H, Pisitkun T, Aponte A, et al. Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int. 2006b;70:1847–57.
Zhou H, Cheruvanky A, Hu X, et al. Urinary exosomal transcription factors, a new class of biomarkers for renal disease. Kidney Int. 2008;74:613–21.

Zhou H, Kajiyama H, Tsuji T, et al. Urinary exosomal Wilms' tumor-1 as a potential biomarker for podocyte injury. Am J Physiol Renal Physiol. 2013;305:F553–9.

Zhu C, Liang QL, Hu P, et al. Phospholipidomic identification of potential plasma biomarkers associated with type 2 diabetes mellitus and diabetic nephropathy. Talanta. 2011;85:1711–20.

Zubiri I, Vivanco F, Alvarez-Llamas G. Proteomic analysis of urinary exosomes in cardiovascular and associated kidney diseases by two-dimensional electrophoresis and LC-MS/MS. Methods Mol Biol. 2013;1000:209–20.

Zubiri I, Posada-Ayala M, Sanz-Maroto A, et al. Diabetic nephropathy induces changes in the proteome of human urinary exosomes as revealed by label-free comparative analysis. J Proteomics. 2014;96:92–102.

Renal Biomarkers *N*-Acetyl-Beta-D-Glucosaminidase (NAG), Endothelin, and Their Application

17

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Abstract

The risk of acute kidney injury is rising due to aging in the world and with the increased usage of therapeutic agents and diagnostic interventions which are highly nephrotoxic. Kidneys, however, well tolerate with the injury, and with the late rise in nitrogenous waste products such as creatinine and BUN in the blood, the organ damage is underestimated. Biomarkers are being searched to find

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a more specific and sensitive one for kidney injury diagnosis like cardiac troponins (I and T) which is the gold standard in diagnosing acute coronary syndrome nowadays replaced the total creatine kinase and CK-MB. However, there is still no such a marker available to take place instead of creatinine. *N*-Acetyl-β-D-glucosaminidase is a lysosomal enzyme that leaks into urine which is mainly originated from the proximal tubular cells. This enzyme is defined as being more specific and sensitive to renal tubular injury than creatinine especially with its isoenzymes and when combined with other renal biomarkers, for example, NGAL and Kim-1. *N*-Acetyl-β-D-glucosaminidase is stable in urine, and the variation among individuals is minimal that the spot urine sample is adequate for the assay practically with colorimetric and spectrophotometric methods with its high reproducibility. Endothelins are paracrine hormones that stimulate myocardial contraction and other smooth muscle contraction such as uterus, bronchus, and stomach. They also promote vascular smooth muscle cell growth. Besides they stimulate secretion in kidney, liver, and adrenals. Endothelins are implicated in many pathophysiological conditions such as hypertension, myocardial infarctus, subarachnoidal hemorrhage, and kidney failure.

Keywords

Creatinine ratio • Endothelin 1 • Endothelin-converting enzyme • Endothelin receptor • Glomerular filtration rate • Proteinuria • Spot urine • Urinary *N*-acetyl-β-D-glucosaminidase

Abbreviations

aa	Amino acid
AKI	Acute kidney injury
ANP	Atrial natriuretic peptide
BNP	Brain natriuretic peptide
cGMP	Cyclic guanosine monophosphate
CKD	Chronic kidney disease
Cys C	Cystatin C
ECE	Endothelin-converting enzyme
ET	Endothelin
ETA	Endothelin receptor type A
ETB	Endothelin receptor type B
GFR	Glomerular filtration ratio
IL-1	Interleukin-1
IL-6	Interleukin-6
IP3	Inositol trisphosphate
LDL	Low-density lipoprotein
NAG	*N*-acetyl-β-D-glucosaminidase
NEP	Neutral endopeptidase
NGAL	Neutrophil gelatinase-associated lipocalin

NO	Nitric oxide
PKC	Protein kinase C
RBP	Retinol binding protein
SCr	Serum creatinine
SRTX	Sarafotoxin
TGF-B	Transforming growth factor beta

Key Facts of Proximal Tubular Cells

- Nephrons are the main functional units of the kidney that their function is to maintain the water, electrolyte, and acid–base balance and remove waste products from the blood.
- Glomerulus and the Bowman's capsule are the initial filtering parts of the nephron.
- Proximal tubule is the part of the nephron that lies from Bowman' s capsule to the Henle loop.
- The apical surface of the proximal tubule epithelial cells is closely packed with microvilli that gives its characteristic name brush border cell which increases the surface area.
- *N*-Acetyl-β-D-glucosaminidase is the most active of the glycosidases found in the lysosomes of the proximal renal tubule epithelial cells.

Definitions

Biomarker indicators of the normal physiological, pathological processes, or response to pharmacological compounds or drugs that can be measured in biological materials such as blood, body fluids, or tissues.

Acute kidney injury a deterioration of renal functions to maintain the fluid, electrolyte, and acid–base homeostasis in an acute manner that results in nitrogenous waste product retention in the blood.

Lysosome an intracellular cytoplasmic organelle that catabolizes the waste materials with its enzymes inside that works well at the acidic pH.

***N*-acetyl-β-D-glucosaminidase** a lysosomal enzyme that is mostly abundant in renal proximal tubular cells, and the function is to catalyze the hydrolysis of terminal glucose residues in glycoproteins.

Ischemia-reperfusion ischemic injury occurs in a tissue with an interrupted blood supply; reperfusion begins with the return of the circulation.

Hexosaminidase a term used to define both of the enzymes *N*-acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase because of their similar activities.

Endothelin-1 a localized hormone acts as a potent vasoconstrictor when stimulated or administered intravenously.

Introduction

Kidneys are vital organs that tolerate well with the injury for a period with its remaining undamaged nephrons instead which results in late response of functional tests such as BUN, creatinine, and urine output. Because kidneys are target tissues for toxic injuries by drugs such as aminoglycosides, chemotherapeutics, and chemical toxins such as cadmium, lead, and contrast media, the aging population has high rates of hospitalization, as a result of exposure to much more nephrotoxic agents (Moriguchi et al. 2009; Leung et al. 2013; Uchino et al. 2005).

The disease prevention, attenuation of the severity, and proper treatment selection require early identification. Biomarkers used for this purpose can be defined as the indicators of the normal physiological and pathological processes or response to pharmacological compounds or drugs that can be measured in biological materials such as blood, body fluids, or tissues.

Renal biomarkers aid in the early treatment choices with the informational advantage of differentiating the various types of kidney injuries such as prerenal azotemia and ischemic or nephrotoxic injuries or the duration of kidney failure such as acute, chronic, or acute-on-chronic. However, the current biomarkers are insensitive to recognize injury early enough to aid in the prevention of nephrotoxic factors in an acute manner.

The new biomarkers are being searched and studied in various patient groups to find a more specific, sensitive, and earlier marker than creatinine, the gold standard, used for decades for acute and chronic renal injury. As a valuable example, a novel biomarker troponin acts as an indicator of myocardial injury as early as in a few hours and acts as a reliable biologic parameter for diagnosis and therapeutic response which is easily measured in a single tube of venous blood. The ideal renal biomarker characteristics are listed in Table 1.

The urine itself is a useful product of the kidney which can be obtained easily than serum or plasma which is appropriate for serial measurements. It contains enzymes and protein release from injured tubular cells that can be early markers for the tissue originated. As an example urine NGAL was superior to serum NGAL for diagnosing AKI in children undergoing cardiopulmonary bypass (Mishra et al. 2005).

Table 1 Ideal renal biomarker characteristics

Ideal renal biomarker characteristics
Noninvasive (study materials are blood or urine)
Easily measured with good reproducibility
Inexpensive
Produces rapid results
Useful for early detection and monitoring
High sensitive (an early sign of organ injury)
High specific (typical of the organ damage)
Traceable among other laboratories
High sample stability
Minimum interferent factors (endogenous activators/inhibitors)
Monitors the effect of treatment

The protein or enzyme released from the segmental site of the injury, such as glomerular, tubular or cytoplasmic, lysosomal, or membranous parts gives extensive information. Urinary enzymes NAG, alanine aminopeptidase (AAP), α- and π-glutathione S-transferase (GST), and lactate dehydrogenase (LDH) are commonly used renal biomarkers. The injury to the renal tubular basal membrane sheds the brush border enzymes of alkaline phosphatase (AP), gamma-glutamyl transpeptidase (GGT), maltase, and leucine aminopeptidase into urine at increased concentrations (Geus et al. 2012).

Low molecular weight urinary proteins such as α-1 and β2-microglobulin, neutrophil gelatinase-associated lipocalin (NGAL), and retinol-binding protein (RBP) are found in urine at higher levels without kidney injury when filtration overload surpasses tubular reabsorption capacity (Câmara et al. 2009) or with tubular injury.

The incidence of acute kidney injury (AKI) is rising due to increasing age and severity of illnesses especially in critically ill patients although the annual inhospital mortality rate seems to decline (Xue et al. 2006; Waikar et al. 2006). The incidence of chronic kidney disease (CKD) is increasing primarily due to its major cause, the type 2 diabetes. Sepsis and major surgery are other common predisposing factors for acute and chronic renal diseases.

An abrupt decline in renal filtration function within 48 h is the recent definition of AKI (KDIGO 2012) where the staging laboratory criteria are mainly an increase in SCr and a reduction in urine output. Glomerular filtration rate (GFR) reduction is not considered as reliable in acute stages (Wu and Parikh 2008). However, in chronic renal failure, a small decline in the GFR is associated with a large increase in serum creatinine level.

Serum creatinine (SCr), the current gold standard, has a limited predictive performance for the early stages of acute renal injury (Waikar et al. 2009). It is produced at a constant rate with nonenzymatic dehydration of muscle creatine, freely filtered by the glomerulus, not reabsorbed by the tubules but secreted through the tubules (Ferguson and Waikar 2012). SCr varies due to the age, gender, race, dietary

intake, muscle mass, muscle metabolism, strenuous exercise, volume overload, and prerenal azotemia (Gupta et al. 2010). Because creatine is synthesized in the liver, the SCr concentration in liver disease such as cirrhosis does not correlate well with the kidney injury (Orlando et al. 2002).

The main function of the kidney is to maintain the homeostasis by glomerular filtration, tubular reabsorption, and tubular excretion. Glomerular filtration rate (GFR) is the best index to evaluate the overall kidney function (Ferguson and Waikar 2012). SCr is the best measuring method for the glomerular filtration estimation (eGFR) in clinical practice. Consequently, the creatinine-based equations such as modification of diet in renal disease equation (MDRD) or Cockcroft-Gault equation are uncertain for GFR estimation due to the defined limitations (Bagshaw 2011). Other defined gold standard for GFR assessment is inulin clearance which is impractical especially in the acute setting.

SCr and cystatin C (Cys C) are markers of glomerular filtration in renal function (Geus et al. 2012). Cys C is a cysteine protease inhibitor synthesized in all nucleated cells. Due to its low molecular weight (~13 kDa), this molecule is freely filtered by the glomerulus and nearly completely reabsorbed and catalyzed by proximal tubular cells, but different from creatinine, not secreted by the renal tubules. This particularity is an option used for GFR measurement, which is nowadays estimated as being superior to the gold method, the creatinine measurement. Consequently, it better evaluates GFR decline than SCr with a proportional rise in serum concentration (Ferguson and Waikar 2012).

At early stages of kidney function decline, the tubular secretion of creatinine is increased; hence, SCr level may be detected as unchanged that overestimates the decrease in GFR estimation (Ferguson and Waikar 2012). Significant increase is seen in 48–72 h of postinjury with a dysfunction of 30 % nephrons and more (Waikar et al. 2009). Therefore, SCr is a late marker with poor specificity and sensitivity.

N-Acetyl-β-D-Glucosaminidase

N-Acetyl-β-D-glucosaminidase (NAG: EC 3.2.1.30) was purified from pig epididymis in 1960 (Findlay and Levvy 1960) and from human spleen in 1967 (Robinson and Stirling 1968). It is found in many tissues in the body; however, it is the most active of the glycosidases found in the lysosomes of the proximal renal tubule epithelial cells (see Fig. 1a, b) (Price 1992). The enzyme NAG catalyzes the hydrolysis of terminal glucose residues in glycoproteins. The enzymes with a molecular weight higher than 70 kDa cannot filter normally into the urine. Since it is too large (140 kDa) to be filtered by the glomerulus freely, serum form of NAG is not found in urine under physiological conditions. NAG is neither absorbed nor secreted by renal tubules. Urine form of NAG exists in urine at elevated concentrations with tubular cell damage due to nephrotoxic agents (drugs, contrast media,

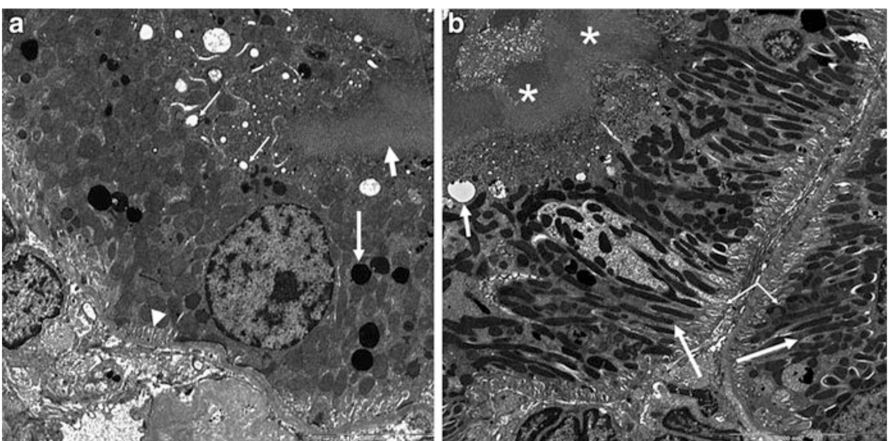

Fig. 1 (**a**) Transmission electron microscopy image of proximal renal tubule in a control rat. *Thin arrows* show vesicles of various sizes located in the apical cytoplasm. The *thick arrow* shows lysosomes, *short arrow* shows the microvilli, and head of the *arrow* shows basal interdigitating processes. (**b**) Transmission electron microscopy (*Carl Zeiss Libra 120*) image of two adjacent proximal tubules in a control rat. The basal interdigitating processes (*thin arrows*), the mitochondria among digitations (*thick arrows*), the apical surface of the cell shows the closely packed microvilli (*), and vesicles of various sizes in the apical cytoplasm (*short arrow*) (*The images are supplied kindly by Prof. Dr. Isıl Tekmen from her private archive. The images have not published anywhere. Affiliation: Prof. Dr. Isıl Tekmen: Department of Histology and Embryology, Dokuz Eylul University Medical Faculty, Izmir Turkey)

cadmium, lead) and renal illnesses such as diabetes, obstructive uropathy, allograft rejection, and hypertension being a useful early sign of tubular injury. Urinary NAG is useful for the glomerular function detection in chronic kidney disease (Hong and Lim 2012). Contrary to other urine biomarkers β2-microglobulin and α1-microglobulin which are filtered through the glomeruli, NAG (coming only from tubular cells) increase in urine reflects tubular dysfunction (Moriguchi et al. 2009). Moreover, NAG is a very sensitive indicator of renal parenchymal injury than creatinine due to its renal functional reserve.

It is important to note that in normal conditions NAG is excreted into urine at a constant rate in small quantities as a result of normal exocytosis and leakage processes. Even in the absence of renal injury, induced lysosomal activity may cause an increase in urine NAG concentration (Bosomworth et al. 1999).

Isoenzymes: NAG has two main isoenzymes: “A” (acidic) and “B” (basic) which are located mainly in human kidneys (Morita et al. 1998; Javed and Hussain 1992). The functions of NAG enzyme are hydrolytic degradation of glycoproteins, mucopolysaccharides, or glycolipids. Isoenzymes A and B have similar kinetic characteristics (Coma et al. 1992). NAG A and B were also isolated from placenta (Numata et al. 1997).

Isoenzyme A is located in the soluble part of the lysosome and normally found in urine due to exocytosis, where isoenzyme B could only be found in urine pathologically due to the tubular damage as being a part of the lysosomal membrane (Gibey et al. 1984). Normally isoenzyme A is dominant in urine in small quantities; however in state of injury, isoenzyme B increases.

The minor isoenzymes of NAG are I_1 and I_2 forms (Numata et al. 1997). Intermediate isoenzymes, I_1 and I_2, are found in normal sera, additionally I_2 in the liver. The intermediate I_1 and I_2 are described between the A and B peaks with lower activities and are extracted mainly from human brain tissue. A^S is serum and A^T is tissue form found in the liver, brain, spleen, and kidney (Ikonne and Ellis 1973).

During pregnancy, the urinary NAG activity increases due to the renal physiological adaptation that might interfere the diagnostic value of NAG during renal injury. Authors (Capodicasa et al. 2011) detected fluorometrically the prevalently increased form of NAG as isoenzyme A. The intermediate isoenzyme “I” is the placental form detected in sera (not urine) of pregnant women and is the small fraction among other NAG isoenzymes. NAG isoenzyme I is an acidic variant of isoenzyme B (Numata et al. 1997).

Isoenzymes differ in isoelectric points (pI), substrate specificity, and thermal stability. The different isoelectric points (pI) of NAG A and NAG B (5.4 and 7.9, respectively) allow the separation of these two isoenzymes by electrophoresis and ion-exchange chromatography. Isoenzyme A has an acidic and B has a basic isoelectric point. Isoenzyme isolation methods are ion-exchange chromatography, HPLC, electrophoresis methods, ELISA, fluorometric methods (Mandić et al. 2005), and a method that takes advantage of thermoinstability of NAG isoenzyme.

The heat stability of the two isoenzymes is different. The isoenzyme activities of A and B are maximum at 50 °C which abruptly decline from 50 °C to 70 °C (Nicot et al. 1987). Isoenzyme A can be inactivated after heat treatment. By heating the isoenzymes at 50–52 °C for 1–3 h reduces the activity of isoenzyme A. NAG B is more stable to heat and pH changes than isoenzyme A (Robinson and Stirling 1968).

NAG constitutes sialic acid residues which give its acidic property. Removing the sialic acid groups transfers acidic form A to basic form B. Isoenzyme A has a greater mobility toward the anode due to its acidic property (Robinson and Stirling 1968).

Isoenzyme A always exceeds isoenzyme B in normal serum and urine. Tissue form of A is different from the serum form of isoenzyme A. In diabetic patients' sera and urine, this situation does not change (Severinl et al. 1988). The intermediate form isoenzyme “I” is more evident in diabetics with microvascular complications.

Numata et al. isolated NAG isoenzymes A and B with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method showing α and β chains. Isoenzyme A dissociates into two chains of α and β and isoenzyme B into two or four β subunits that can be dissociated into β_a and β_b subgroups. Probably isoenzyme “I” can be dissociated into two β chains (Numata et al. 1997).

Detection method: NAG can be detected with enzymatic, colorimetric, fluorometric, and ELISA methods. Enzymatic colorimetric assay method is based on enzymatic cleavage of the colorimetric substrates: dichlorophenol sulfonephthalein,

p-Nitrophenyl *N*-Acetyl-*β*-D-Glucosaminide (PNP-NAG) (Horak et al. 1981), and MCP-NAG (Noto et al. 1983) (sodio *m*-cresolsulfonphthaleinyl *N*-acetyl-*β*-D-glucosaminide: MCP-NAG) where with substrate PNP-NAG detection sensitivity was 0.15 U/L (Dalva et al. 2011). Detection wavelength differs (405, 505, 580) due to the type of substrate (the formation of color) used. With the wavelength preferred, no interference is expected in case of hemolysis or hyperbilirubinemia (Dalva et al. 2011).

The first analytical method was fluorometric assay using 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as a substrate (4-methylumbelliferyl-Glc NAc). Principle: The rate of hydrolysis of nonfluorescent 4-methyllumberriferone derivative of *N*-acetylglucosamine to fluorescent 4-methylumbelliferone is quantified by spectrophotofluorometry. The fluorescent method is highly sensitive in determination of very low levels of NAG in urine diluted 20-to 50 fold to eliminate the endogenous interferent substances and has been replaced with more practical spectrophotometric and colorimetric methods (Horak et al. 1981; Skálová 2005).

NAG activity can be expressed as U/g of creatinine for spot urine specimens to minimize the dilutional or concentration effects. However, there is not a consensus about the interpretation of urinary biomarker results as a normalized ratio to urinary creatinine concentration. That might be affected by the urine creatinine excretion variability where the decreased urine creatinine level might misinterpret the urine biomarker level as lower than its exact value (Moriguchi et al. 2009; Hibi et al. 2014). This easy calculation of the ratio of biomarker level to urinary creatinine formula alternative to the gold standard excretion rate calculation of a urinary biomarker is created assuming that creatinine excretion is constant. However, it changes individually, according to the race, gender and muscle mass, etc., physiologically, and shows variations due to the tubular injury pathologically such as in state of AKI. In contrary in an experimental study by Tonomura et al., the influence of AKI on urinary NAG-to-creatinine ratio was examined. The urine creatinine correction formula to urinary flow rate correction was compared in AKI conditions for urinary enzyme NAG (Tonomura et al. 2011). According to that study, the diagnostic accuracy of urinary creatinine correction for NAG displayed a higher specificity and sensitivity (90.2 % and 72.2 %, respectively).

Assay Interferent Factors

Although urinary enzymes give information about the injury site where they are originated, the interfering factors such as activators and inhibitors have to be taken into consideration (Mueller et al. 1989). There exist low molecular weight endogenous inhibitors in urine such as urea and ascorbic acid which may cause competitive inhibition of NAG enzyme that leads to a loss in activity of nearly 6 % (Dalva et al. 2011). At high concentrations, the endogenous urea in urine inhibits NAG A and B isoenzyme activities (Bondiou et al. 1985). Gel filtration method is used to separate the enzyme from inhibitors in urine before the analysis (Horak et al. 1981); however, it is not declared as necessary (Dalva et al. 2011). NAG B is high in semen

Table 2 Advantages of *N*-acetyl-β-D-glucosaminidase assay in urine

Advantages
Noninvasive
Diurnal variation of NAG is minimal to interfere with the assay results; therefore, untimed urine specimen can be used
Detection in the site of injury
Sensitive marker for tubular damage
Excreted amount of the enzyme is correlated with the tubular injury
Allow early detection of renal injury
Identify the severity of renal injury
Stable in urine across to a range of pH and temperature
Urinary NAG is not gender dependent (Mohkam et al. 2008)
Easily detected by colorimetric or spectrophotometric assay
Reagent low cost
Reliability of measurements

(5800 μg/L), and contamination can interfere the results inevitably (Itoh et al. 1994). The advantages of NAG assay in urine are listed in Table 2.

Specimens: Serum and urine are acceptable samples.

- **Serum**: The preferred blood sample is serum. Heparin has an interferent effect on NAG activity that depresses activity by 50 % (Robinson and Stirling 1968).
- **Urine**: Untimed urine specimen can be used due to minimal diurnal variation of NAG. The second morning urine or midstream urine is recommended instead of the first morning urine. Second morning urine is the urine sample voided 2–4 h after the first morning urine (Delanghe and Speeckaert 2014). First voided urine is higher in epithelial cells, erythrocytes, leukocytes, and bacteria where gender difference is higher than midstream or second morning urine.

Reference Intervals

Serum: *Serum total NAG*: Adult: 10–30 U/L

Higher in infants <1 year: 11–59 U/L (Oláh et al. 2004)

Serum NAG A isoenzyme: 56–76 % of total activity

Urine: Adult spot urine total NAG: 3.3–4.1 U/L (Horak et al. 1981)

1.0–4.6 U/g creatinine

Isoenzyme A: 80–90 % of total NAG, 10–20 % isoenzyme B (Morita et al. 1998)

Isoenzyme B: 15.8–20 % of total NAG (Numata et al. 1997)

Sample stability: The stability of NAG isoenzymes is found as affected by the pH. Lysosome is an organelle in which the interior side is at acidic pH (4.5–5.0) where the enzymes are highly active. In cytoplasma, pH is slightly basic (7.2), and lysosomal enzymes cannot destroy the cytoplasmic organelles as they are pH sensitive and do not work well at basic pH. Therefore, NAG is stable in acidic urine with its predominant A form, where B is the active form in alkaline pH caused by urease-producing bacteria (*Proteus*,

Klebsiella, *Serratia*) or drugs (methotrexate, ranitidine, quinolone) (Morita et al. 1998; Mandić et al. 2005). Under alkaline condition of about pH 8, the enzyme activity of isoenzyme A is observed as inactivated where even at pH 8 isoenzyme B maintained its activity as much as 84 % (Morita et al. 1998).

To adjust urine to alkaline pH values leads to NAG denaturation irreversibly, so it is not recommended (Mandić et al. 2005). As an example, in an experimental study, the alkalinization decreased the NAG activity by about 30 % and later on acidification of the urine decreased by an additional 12 %, irreversibly (Mandić et al. 2005).

NAG is stable in urine for a few months stored at –80 °C or –20 °C and stable up to five times of freeze-thaw cycles (Lockwood and Bosmann 1979).

Serum NAG A and B activities are found as stable up to 1 month of storage at −20 °C. Second thaw caused a decrease in activity of ~33 % for both of the forms (Coma et al. 1992).

Clinical Significance

The term "hexosaminidase" was first used by Robinson and Stirling in 1968 to describe either the *N*-acetyl-β-D-glucosaminidase or *N*-acetyl-β-D-galactosaminidase because of their similar activities (Robinson and Stirling 1968). The two major isoenzymes liberate the terminal GlcNAc and GalNAc monomers from glycoproteins and glycosaminoglycans (so-called hexosaminidases). Isoenzyme A degrades some glycolipids such as G_{M2} and G_{A2} gangliosides. Mutations in α or β chain result in accumulation of undegraded metabolites in lysosomes and in circulation due to inactivity of hydrolytic enzymes. Tay-Sachs disease is an autosomal recessive condition, and the disease is a defect of hexosaminidase A activity which causes ganglioside accumulation (Vidgoff et al. 1973; Sandhoff 1969; Okada and O'Brien 1969). In case of Tay-Sachs disease, serum and liver hexosaminidase A are absent, but I_1, I_2, and B are present (Ikonne and Ellis 1973). Deficiency of both A and B isoenzymes is named as Sandhoff variant of G_{M2} gangliosidosis (Sandhoff 1969).

Increased urinary activity of NAG not only indicates the renal tubular damage but the increased lysosomal activity also (Bosomworth et al. 1999). In normal urine, NAG B and I isoenzymes are present with a percentage of 10–20 % which are concluded as more sensitive than total NAG (Numata et al. 1997). In pathological urine of patients with renal diseases, the NAG showed significant increases and the percentage of NAG B (Numata et al. 1997). Isoenzyme B increase was reported in patients under therapy of aminoglycoside antibiotics (Gibey et al. 1984). In premature infants exposed to aminoglycoside antibiotics, urine NAG levels were found as increased in the absence of a significant rise in SCr (Mcwilliam et al. 2012). In a study (Assal et al. 2013), the urinary NAG activity was detected as the most sensitive marker for early renal damage in type 2 diabetes than serum Cys C and urine NGAL.

NAG is an early warning of rejection of renal transplantation. Ischemia-reperfusion injury begins in the proximal tubules as an earliest occurring factor damaging the

organ. For the early identification of the damage of the transplanted kidney, NAG is used as a biomarker of the ischemia-reperfusion injury and acute rejection of the renal allograft, because early damage of the tubulus is associated with rapidly processing chronic allograft dysfunction. In their study, authors found NAG urine activity as useful in the evaluation of early proximal tubule damage and predicting the long-term function of the transplanted kidney (Kwiatkowska et al. 2014).

NAG excretion can also be found as increased due to the glomerular damage such as in diabetic nephropathy (Gonzalez and Vincent 2012). In patients with vascular complications, total NAG activity in both serum and urine was higher than in diabetics without vascular complications, and the intermediate form was more evident (Severinl et al. 1988).

In a study (Semerci et al. 2014), patients undergoing coronary angiography demonstrated increased urinary excretion of NAG and high plasma endothelin-1 in 4 h of intervention although none of these patients developed contrast-induced nephropathy. SCr increased slightly but insignificantly (from 0.71 to 0.79 mg/dL) and serum BUN unchanged. This is an example that enzymuria reflected only slight injury that is reversible and uncomplicated. In conclusion, monitoring patients at risk with these sensitive biomarkers may detect the injury earlier than other late insensitive standard clinical parameters of renal function.

Albumin is excreted into urine as a result of the changes in the glomerular permselectivity barrier and intraglomerular pressure. The glomerular disease caused proteinuria accompanied with high levels of urine NAG can be explained with abnormal traffic of proteins thus caused intrinsic tubular toxicity. In cases of massive proteinuria, the increase in tubular secretion of creatinine limits GFR estimation. NAG is considered as a reliable marker of tubular toxicity of proteinuria in early stages of nephrotic diseases (Bazzi et al. 2002). Urinary NAG is shown to be increased significantly after surgery in patients suffering from acute kidney injury, more sensitively than SCr (Jiang et al. 2013). In a study, a positive correlation of urine NAG was observed with the level of the acute kidney injury (Liangos et al. 2007).

Urinary NAG provided a high sensitivity and specificity with a AUCROC 0.97 discriminating AKI in patients with urinary tract infection (Han et al. 2008).

Enzymes leak from proximal tubular cells into the tubular lumen. The urine examination provides information about the site of the injury such as high molecular proteinuria indicates glomerular injury; low molecular weight proteinuria, urinary excretion of brush border antigens and urinary enzymes indicate proximal convulated tubule injury (Sharma 2012). It is possible to enhance the sensitivity and specificity in the diagnostic and therapeutic processes with the combination of biomarkers. In many studies, enzymes or proteins; such as NAG, Kim-1 and NGAL, combined according to the different sites of origination, glomerular or tubular, the results were observed as more reliable (Han et al. 2009; Damman et al. 2011).

Urine NAG can specifically detect clinical outcome of renal tubular damage in high-risk patients (Hiruma et al. 2014; Damman et al. 2011).

Serum NAG activity has not been investigated widely in clinical studies. However, elevated serum NAG activity was reported in HT, DM, hyperinsulinemia, and renal disease (Inoue et al. 2000) and in chronic obstructive pulmonary disease and

myocardial infarction (Iqbal et al. 2003, 2005). Serum NAG activity is increased in acute pancreatitis, as a marker of lysosomal dysfunction during the inflammatory process (Milnerowicz et al. 2014).

Endothelin

Endothelin-1 (ET-1) is the most well-known peptide form of the endothelin family. It was extracted from the endothelial cell cultures and was identified in 1988. It is particularly known for its potent vasoconstrictor property as it has a long-lasting activity for several hours when given intravenously (Yanagisawa et al. 1988a). Endothelins are 21 amino acid peptides and have two disulfide bridges at the positions of 1–15 and 3–11 cysteine amino acids. Amino acids of the N-terminal end supply binding affinity to the receptor and some amino acids on the C-terminal end cause to bind receptor (Sawamura et al. 1989).

Endothelin family consists of three members: endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3) (Inoue et al. 1989a). These members are encoded on chromosomes 6, 1, and 20, respectively, in human (Arinami et al. 1991).

ET-2 and ET-3 differ from ET-1 by two and six various amino acids, respectively (Yanagisawa et al. 1988b; Inoue et al. 1989a; Sawamura et al. 1989). ET-2 has a tryptophan residue at position 6 which makes it most hydrophobic, and ET-3 has a Lys7 residue instead of the hydrophobic Met7 which is the most polar endothelin (Fig. 2) (Inoue et al. 1989a). Porcine and human endothelins are identical but the rat endothelin has different six amino acid residues (Yanagisawa et al. 1988b).

Endothelin like toxins from the venom of the snake (*Atractaspis engaddensis*), called sarafotoxins, has four isoforms: SRTX-a, SRTX-b, SRTX-c (S6c), and SRTX-e (Yanagisawa and Masaki 1989). These 21 amino acid peptides are cardiotoxic that induce coronary vasoconstriction (Nakajima et al. 1989). The vasoconstrictor activity of ET-1 is threefold of sarafotoxin S6b (Nakajima et al. 1989). Sarafotoxin 6c (S6c) is used as a selective agonist of endothelin receptor type B (ETB) (Rubanyi and Polokoff 1994).

Among the three types of endothelins, only the ET-1 is produced by endothelial cells, but ET-2 and ET-3 cannot be obtained from endothelial cells of the vascular tissue (Inoue et al. 1989a; Bloch et al. 1989; Yanagisawa and Masaki 1989). Additionally, ET-1 mRNA is detected in vascular smooth muscle cells (VSMC) and also in nonvascular cells such as the brain, lung, placenta, fetal lung, spleen, pancreas, leukocytes and macrophages, endometrial cells, hepatocytes, Sertoli cells, breast epithelial cells, fibroblasts, cardiomyocytes, tubular epithelial cells, kidney mesangial cells and podocytes, etc. (Bloch et al. 1989; Thorin and Clozel 2010; Kohan 1997; Bagnato et al. 2011). ET-2 is found in the kidney, intestinal tissues, and heart, and ET-3 is in the brain, kidney, adrenal gland, intestinal tissues, fetal lung, spleen, and pancreas (Itoh et al. 1989; Khimji and Rockey 2010; Plumpton et al. 1993; Bloch et al. 1989; Sakurai et al. 1992; Arinami et al. 1991). Both the ET-1 and ET-3 exist in the central nervous system (Shinmi et al. 1989). Especially in the rat pituitary gland, ET-3 is higher than ET-1 (Matsumoto et al. 1989).

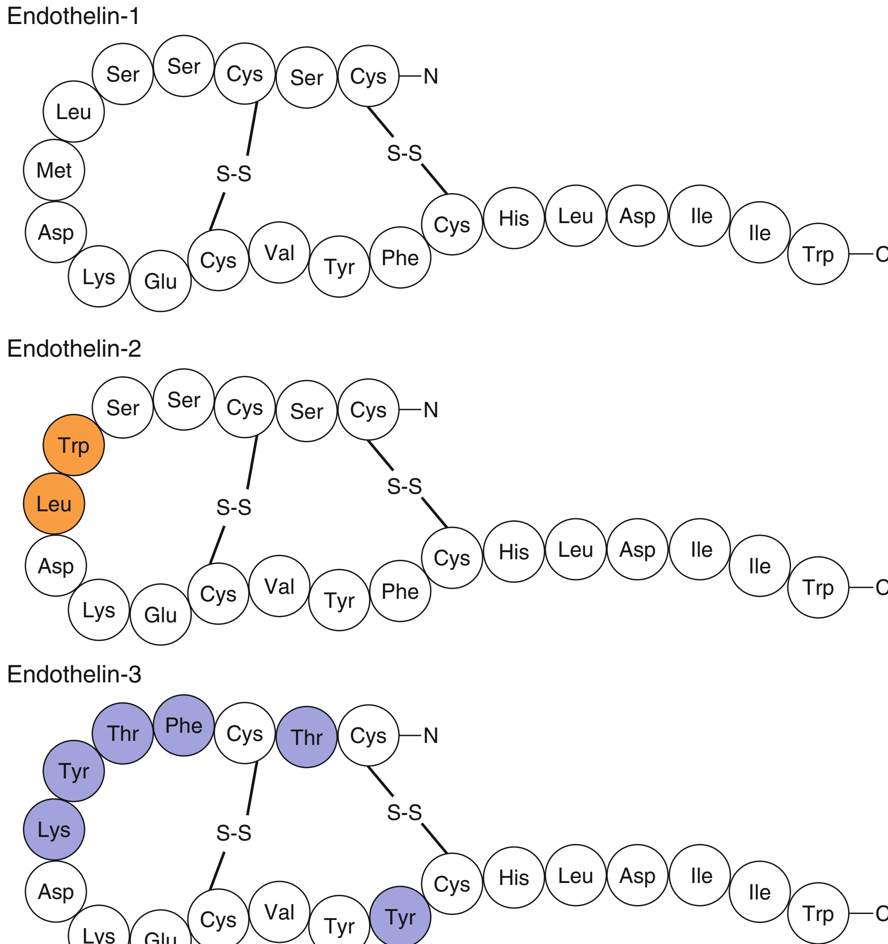

Fig. 2 Amino acid structures of endothelin-1, endothelin-2, and endothelin-3

Endothelin Synthesis

Endothelins act like local, paracrine/autocrine hormones as they are not stored but released within minutes with a stimulus such as hypoxia, ischemia, or shear stress for the regulation of the vasomotor tonus (Nakamura et al. 1990). The stimuli induce the transcription of ET-1 mRNA, synthesis, and secretion. The half-life of mRNA is ~15–20 min (Inoue et al. 1989b).

Many stimuli including vasoactive hormones, growth factors, hypoxia, shear stress, lipoproteins, free radicals, endotoxin, cyclosporine, and also endothelin increase synthesis of ET-1. Endothelium-derived nitric oxide, nitrovasodilators, natriuretic peptides, heparin, and prostaglandins which cause increases in

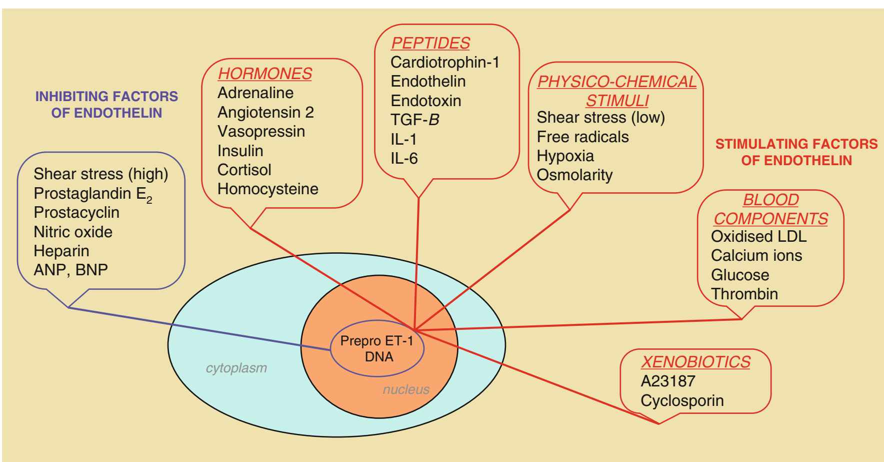

Fig. 3 Stimulator and inhibitor factors of endothelin-1 synthesis. *ANP* atrial natriuretic peptide, *BNP* brain natriuretic peptide, *IL-1* interleukin-1, *IL-6* interleukin-6, *LDL* low density lipoprotein, *TGF-B*, transforming growth factor beta

intracellular level of cyclic guanosine monophosphate (cGMP) inhibit the synthesis of endothelin (Fig. 3) (Haynes and Webb 1998; Attina et al. 2005; Kowalczyk et al. 2015; Gray and Webb 1996; Saito et al. 1995).

Endothelins are synthesized by proteolysis of preproendothelins. Human preproendothelin has 212 amino acid residues; the porcine preproendothelin has 203 amino acids (Itoh et al. 1988; Yanagisawa et al. 1988a). In the lumen of granular endoplasmic reticulum, the preproendothelin leaves its signal peptide to occur proendothelin-1 with 195 amino acids. Big endothelin-1, a 38 amino acid residue in human but 39 amino acid in porcine, is formed with cleavage of proendothelin by an enzyme named furin-like peptidase (Denault et al. 1995; Xu et al. 1994). A 21 amino acid residue ET-1 is generated from big ET by endothelin-converting enzymes 1 and 2 (ECE-1 and ECE-2) by specific proteolytic processing between Trp21 and Val22 amino acids (Sawamura et al. 1989; Inoue et al. 1989a). Big ET-2 and ET-3 have 37 and 41 amino acid residues, respectively (Arinami et al. 1991; Opgenorth et al. 1992). The synthesis pathways of ET-2 and ET-3 are similar to ET-1.

Endothelin-Converting Enzyme

In 1994 the amino acid sequence of ECE was found and called ECE-1 (Masaki 2004; Xu et al. 1994; Schmidt et al. 1994). Later on the four isoforms of ECE-1 were identified: ECE-1a, ECE-1b, ECE-1c, and ECE-1d. ECE-1a is found in intracellular secretory vesicles in the endothelial cells. ECE-1b is also located in the intracellular compartment close to the Golgi apparatus. ECE-1c and ECE-1d are located on the cell surface (Masaki 2004). ECE-1 is mainly found in smooth muscle cells which hydrolyzes bradykinin, substance P, and insulin also (Schmidt et al. 1994). ECE-2 is

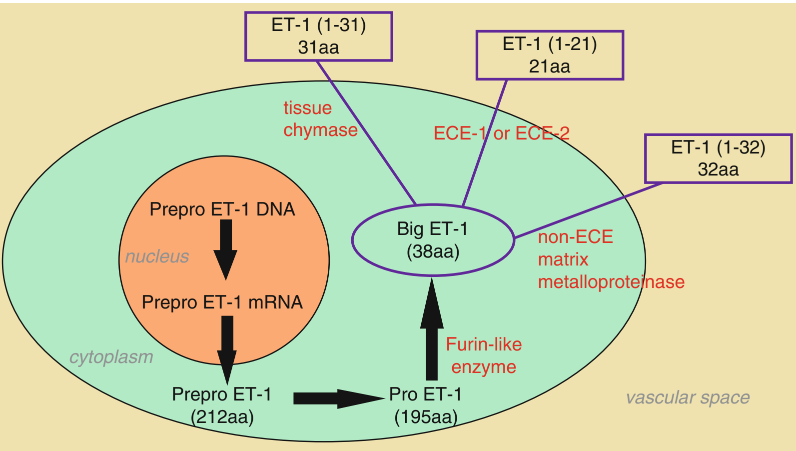

Fig. 4 Endothelin-1 synthesis pathway

an intracellular enzyme that differs from ECE-1 by its pH sensitivity and is active at acidic pH (pH 5.5) in contrast to neutral pH optimum of ECE-1. It subsists especially in endothelial and neuronal cells (Emoto and Yanagisawa 1995). ECE-1 and ECE-2 are coded by chromosomes 1 and 3, respectively.

ECE-1 is a membrane-bound metalloproteinase and is similar to neutral endopeptidase 24.11 and Kell blood group protein structurally. This enzyme is inhibited by the phosphoramidons, the metalloprotease inhibitors (Xu et al. 1994).

There exist also non-ECE pathways that convert big endothelin to various endothelin forms with tissue chymases and matrix metalloproteinases (Nakano et al. 1997; D'Orléans-Juste et al. 2003). When big endothelins are cleaved at the Tyr31-Gly33 bond by human tissue chymases (e.g., mast cell chymases), 31-aa-length endothelins (1–31) are formed. These products do not undergo any other processes and have properties like endothelins (1–21). Among the (1–31) endothelins, ET-2 (1–31) is the most potent vasoconstrictor, similar to ET-l (1–21), and stronger than ET-2 (1–21) (Nakano et al. 1997; Oka et al. 2014). Endothelins (1–32) are also formed by the matrix metalloproteinases (non-ECE metalloproteinases) (Fig. 4) (D'Orléans-Juste et al. 2003).

The vasoconstriction potency of ET-1 is 140-fold greater than big ET-1, and preproendothelin-1, which is found in circulation and has no vasoconstrictor activity (Rubanyi and Polokoff 1994). Although the contractile activity of big endothelin-1 in vascular beds is very low than ET-1, it is as potent as ET-1 in raising the blood pressure in vivo as ECE-1 converts big ET-1 to active ET-1 rapidly (Sakurai et al. 1992).

Endothelin Receptors

There are two kinds of receptors for endothelins which are called endothelin receptor type A (ETA) and type B (ETB) and are coded by chromosomes 4 and 13, respectively (Arai et al. 1990; Sakurai et al. 1992). Endothelins present their effects by these receptors (Sakurai et al. 1992). Both ETA and ETB receptors are members of the heptahelical G-protein-coupled transmembrane receptors which have seven hydrophobic membrane-spanning domains and a relatively long extracellular N- terminal (Masaki 2004). The affinity order for ETA is ET-l ≥ ET-2 >> ET-3 and ET-1 affinity is 100-fold of ET-3 (Lin et al. 1991). ETB affinity is nearly equivalent for all three endothelins and sarafotoxins (Sakamoto et al. 1991). Another receptor named ETC reported in amphibians has a greater affinity for ET-3 and S6c than ET-1 and ET-2 (Masaki et al. 1994). The binding affinity of ETA and ETB to ET-1 is equal (Davenport 2002), but the receptors' intracytoplasmic C-termini are different, so the intracellular effect of ET-1 is different (Nussdorfer et al. 1999).

ETA and ETB receptors are distinct from each other, both structurally and functionally, and are widely distributed in the kidneys, lungs, adrenal gland, brain, trigeminal nerve, fibroblasts, heart, cardiomyocytes, prostate, breast, and the female reproductive tissue in addition to their main origin of vascular system (Karet and Davenport 1996; Chichorro et al. 2010).

Type A receptors are located on vascular smooth muscle cells that cause vasoconstriction and smooth muscle proliferation (Haynes and Webb 1994; Komuro et al. 1988). Type B receptors are present on both endothelial and vascular smooth muscle cells, especially on the endothelial cells (Davenport and Maguire 1994). When ET-1 is bound to ETB receptor on smooth muscle cell, calcium ions increase and vasoconstriction occurs (Chester and Yacoub 2014; Haynes et al. 1995).

ET-1 bound to an ETB receptor on endothelial cell causes synthesis of nitric oxide (NO) and prostacyclin; consequently NO increases the cyclic guanosine monophosphate (cGMP) levels in smooth muscle cells, thus leading to vasodilatation (Chester and Yacoub 2014; Verhaar et al. 1998).

Binding of ET-1 to ETA receptor on smooth muscle cell leads to phospholipase C activation that causes diacylglycerol (DAG) and inositol triphosphate (IP3) occurrence. IP3 causes a release of calcium ions from sarcoplasmic reticulum into the cytosol. Also DAG causes an increase of protein kinase C (PKC) (Khimji and Rockey 2010). Both pathways (calcium and PKC) cause vasoconstriction.

ET-1 shows its physiological effects with cell growth induction on endothelial cells, smooth muscle cells, and astrocytes with its potent receptor ETA, whereas ETB is suggested to be involved in cell apoptosis (Davenport 2002).

ECE-1 expression in endothelial cells is inhibited by activation of ETB receptors. Pulmonary clearance of circulating ET-1 and reuptaking of ET-1 are mediated also by ETB receptors (Davenport 2002).

ET-1 shows its proliferative effects through intracellular separate pathways by increasing mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K) (Chester and Yacoub 2014).

Clearance of ET-1

The circulating ET-1 is cleared from the plasma by ETB receptor followed by lysosomal degradation for which the major site of clearance is the pulmonary circulation with a minimum contribution of renal and splanchnic circulation (Bremnes et al. 2000; Dupuis et al. 1996; Attina et al. 2005; Gasic et al. 1992). Another way of degradation is the catabolism by extracellular neutral endopeptidase 24.11 (NEP, neprilysin) mainly found in the brush border vesicles of the renal proximal tubules. NEP is a membrane-bound metallopeptidase with zinc. Although ET-1 is cleared from the circulation approximately in 1–2 min, its biological effects last longer (~60 min) (Abassi et al. 1992; de Nucci et al. 1988; Shah 2007).

Detection Method

ET-1 is at a very low concentration in plasma under physiological conditions being a paracrine/autocrine hormone. It is detected in plasma with radioimmunosorbent assay and enzyme-linked immunosorbent assay (ELISA) (Masaki 2004). In humans ET-2 has not been detected yet in plasma (Haynes and Webb 1998).

The preferred blood sample is plasma with anticoagulant EDTA (2 mg/ml). The centrifugation at $2000 \times g$ for 15 min at 4 °C is recommended. Samples can be stored at −25 °C or −30 °C until analysis (Miyauchi et al. 1991; Oka et al. 2014).

Up to date, for none of the endothelin family, no significant reference interval study was performed with a large healthy population, to our knowledge. However, there are studies performed with small groups. As an example in a study by Suzuki et al., ET-1 level was found as 1.59 ± 0.32 pg/ml ($n = 24$); however in another study by Suzaki et al., ET-1 levels were found as 15.54 ± 4.45 pg/ml ($n = 5$) (Suzuki et al. 1989; Suzaki et al. 2003). These differences might be due to the different study population, number of individuals, and different analysis methods such as the difference of the column used for the purification of the antigens (Oka et al. 2014).

Aging Difference

ET-1 showed to be increased with aging (Komatsumoto and Nara 1995) in healthy women (young, middle-aged, and older women) from 1.02 to 2.9 pg/ml (Maeda et al. 2003). However, in another study, no difference was observed with age (Oka et al. 2014).

Gender Difference

There is not a consensus whether sex-related difference exists for ET-1. In a study male ET-1 levels were found as higher than female (Miyauchi et al. 1992), but another study regretted the difference (Oka et al. 2014).

Table 3 Functions of endothelin

Functions of endothelin
Regulation of the vascular tone and blood pressure
Cell proliferation
Hormone production
Synthesis of vasoactive peptides
Bronchoconstrictor effect
Positive inotropic and chronotropic effect
Modulation of neurotransmission

Physiological Functions of Endothelin (Table 3)

Regulation of the Vascular Tone and Blood Pressure

ET-1 has a vasoconstrictor effect in vitro, and generally it increases blood pressure in vivo (Yanagisawa et al. 1988a). The vasoconstrictor activities of ET-3 and sarafotoxin S6b are about 1/60th and 1/3 of ET-1, respectively (Nakajima et al. 1989). ET-1 increases the blood pressure by activating ETA receptors on vascular smooth muscle cells and decreases the blood pressure by activating ETB receptors on endothelial and renal cells (Shah 2007; Kohan et al. 2011). ET-1 leads to vasodilatation by prostacyclin (Shah 2007). Also ET-1 has an effect on vascular tone indirectly by sympathetic nervous system (Haynes and Webb 1994).

Cell Proliferation

Endothelin-1 has a proliferative effect on smooth muscle cells, mesangial cells and fibroblasts, and cardiac myocytes. ET-1 has an increasing effect on growth-promoting proto-oncogenes (c-fos and c-myc) and on growth factors (Bloch et al. 1989; Haynes and Webb 1998).

Hormone Production

ET-1 stimulates atrial natriuretic factor secretion from atrial cardiocytes and stimulates aldosterone synthesis from cortical zona glomerulosa cells and adrenaline secretion from medullary chromaffin cells (Bloch et al. 1989; Haynes and Webb 1998).

Synthesis of Vasoactive Peptides

Endothelins activate cytokines like tumor necrosis factor (TNF), neutrophil chemotactic factor, granulocyte-macrophage colony-stimulating factor, and interleukins 1, 6, and 8 (Bloch et al. 1989; Haynes and Webb 1998).

Both ET-1 and ET-3 stimulate pulmonary bronchoconstriction by thromboxane (Levin 1995; Yanagisawa and Masaki 1989).

ET-1 has positive inotropic and chronotropic effects on myocardium. The activities of ET-1(1–31) are similar to ET-1(1–21) which are vasoconstriction, neurotransmission, cell proliferation, and production of hormone cytokine (Yanagisawa and Masaki 1989). But its effects are more weaker than ET-1(1–21) (Oka et al. 2014).

Clinical Significance

Endothelin has a role on many illnesses such as ischemic heart diseases, myocardial infarction, chronic heart failure, hypertension, atherosclerosis, pulmonary hypertension, chronic renal failure and cerebrovascular spasm, hypercholesterolemia, several malignancies (like prostate, ovarial, renal, pulmonary, colorectal, cervical, breast, bladder, and malign melanoma), sickle cell disease, Raynaud's disease, and complex regional pain syndrome, infectious diseases, and sepsis with its increased levels (Shah 2007; Masaki 2004; Levin 1995).

Endothelin and Kidney

ET-1 is the mainly potent isoform in the kidney and is produced by glomerular epithelial and mesangial cells, vasa recta, and arcuate arteries, renal tubular, and medullary collecting duct tubule cells (Eid et al. 1994; Karet and Davenport 1996). ET-1 regulates renal and intrarenal blood flow, sodium and water homeostasis, and acid–base balance. ET-1 stimulates HCO_3^- reabsorption and H^+ secretion along the collecting duct with ETA receptors.

Renal and vascular ET-1 act independently with each other. In urine, only kidney-originated ET-1 exists so the urinary ET-1 excretion is independent of its plasma form (Serneri et al. 1995; Dhaun et al. 2006; Abassi et al. 1992). ET-1 is catabolized by neutral endopeptidase that is found in the brush border vesicles of the renal proximal tubules.

The exogenously administered ET-1 mainly affects renal vasculature, as they are more sensitive than other vascular beds. The effect is seen as vasoconstriction of the afferent and efferent arterioles (esp. the afferent arteriole) and consequently a reduction in glomerular filtration rate (Rabelink et al. 1994; Loutzenhiser et al. 1990; Schildroth et al. 2011). With the increased water and sodium retention, blood pressure increases. In cases of renal function reduction, plasma ET-1 levels were found as increased (Zoccali et al. 1995).

In experimental studies, ETB receptors, both renal and extrarenal, played protective roles against hypertension (Ohkita et al. 2005). In state of hypertension, increased vascular ET system activity was observed in experimental studies performed with ET receptor antagonists (Cardillo et al. 1999). In a study, chronic blockade of the ETB receptor increased blood pressure (Pollock and Pollock 2001). It is suggested that renal ET-1 production and function might be so altered that inappropriate volume homeostasis is contributed in the pathophysiology of hypertension (Dhaun et al. 2006).

Renal cortical ET-1 causes hypertension by increasing renal vascular resistance, and glomerular ETA activation increases inflammation through enhanced production of inflammatory factors. The released factors by the inflammatory cells release a number of factors within the kidney that cause vasoconstriction and increase in sodium reabsorption resulting in increased blood pressure (Speed and Pollock 2013). Therefore, ET-1 antagonists are being searched for the arterial hypertension treatment.

ET-1 has pro-inflammatory, promitogenic, and profibrotic actions in the kidney that might take part in the hypertensive effect of ET-1. A study demonstrated that ET-1 directly increased glomerular proteinuria with increasing the permeability to albumin and renal inflammation via ETA receptor activation in vitro (Saleh et al. 2010).

ET and Chronic Kidney Disease

ET-1 is suggested to be a major factor in chronic kidney disease which contributes to hypertension, proteinuria, and renal inflammation (Dhaun et al. 2011). In chronic renal failure, both the circulating (Koyama et al. 1989) and urine (Dhaun et al. 2009) ET-1 levels were found as increased. The increased ET-1 causes the morphological and functional alterations characteristic for chronic renal failure (Richter 2006).

Summary Points

- This chapter reviews the biomarkers *N*-acetyl-β-D-glucosaminidase and endothelin and their significance for renal disorders.
- The insensitivity of creatinine leads to a new standardized biomarker research.
- *N*-Acetyl-β-D-glucosaminidase is a lysosomal enzyme mainly found in the proximal tubular cells, and the urine NAG has a diagnostic value for tubular injury.
- Endothelin-1 (ET-1) is the most well-known peptide form of the endothelin family which was extracted from the endothelial cell cultures and named in 1988.
- Serum creatinine is the gold standard used widely to estimate the glomerular filtration rate (GFR) since no biomarker was identified instead of that endogenous compound up to date.
- Serum creatinine concentrations vary among individuals with age, race, gender, muscle mass, and muscle metabolism.
- Both the physiological (children, pregnancy) and pathological (cirrhosis, muscle wasting, volume overload) conditions may underestimate the acute renal injury as the defined cutoffs.
- *N*-Acetyl-β-D-glucosaminidase is the most commonly used renal biomarker with the advantages of minimal diurnal variation, minimal variability among individuals, and easy quantification with untimed urine that gives information of the site of the injury since urine *N*-acetyl-β-D-glucosaminidase originates from the proximal tubular cells.
- Ischemia-reperfusion injury begins in the proximal tubules as an earliest occurring factor damaging the organ, and *N*-acetyl-β-D-glucosaminidase is used for early identification of the transplanted kidney injury.
- The exogenously administered ET-1 mainly affects renal vasculature. The effect is seen as vasoconstriction of afferent and efferent arterioles, thus a reduction in glomerular filtration rate.
- Endothelins act like local, paracrine/autocrine hormones and are not stored, but released within minutes with a stimulus such as hypoxia, ischemia, or shear stress for the regulation of the vasomotor tonus.

- Only endothelin-1 is produced by endothelial cells, but endothelin-2 and endothelin-3 cannot be obtained from endothelial cells of the vascular tissue.
- ETA receptor activation within the blood vessels, detected on the smooth muscle, modulates vasoconstriction. ETB receptors, mainly found on the vascular endothelium, modulate vasodilatation with prostacyclin and nitric oxide.
- ET-1 has pro-inflammatory, promitogenic, and profibrotic actions in the kidney.
- ET-1 is suggested to be a major factor in chronic kidney disease which contributes to hypertension, proteinuria, and renal inflammation.

References

Abassi ZA, Tate JE, Golomb E, et al. Role of neutral endopeptidase in the metabolism of endothelin. Hypertension. 1992;20(1):89–95.
Arai H, Hori S, Aramori I, et al. Cloning and expression of a cDNA encoding an endothelin receptor. Nature. 1990;348:730–2.
Arinami T, Ishikawa M, Inoue A, et al. Chromosomal assignments of the human endothelin family genes: the endothelin-1 gene (EDN1) to 6p23–p24, the endothelin-2 gene (EDN2) to 1p34, and the endothelin-3 gene (EDN3) to 20q13.2–q13.3. Am J Hum Genet. 1991;48(5):990–6.
Assal HS, Tawfeek S, Rasheed EA, et al. Serum cystatin C and tubular urinary enzymes as biomarkers of renal dysfunction in type 2 diabetes mellitus. Clin Med Insights Endocrinol Diabetes. 2013;6:7–13.
Attina T, Camidge R, Newby DE, et al. Endothelin antagonism in pulmonary hypertension, heart failure, and beyond. Heart. 2005;91(6):825–31.
Bagnato A, Loizidou M, Pflug BR, et al. Role of the endothelin axis and its antagonists in the treatment of cancer. Br J Pharmacol. 2011;163(2):220–33.
Bagshaw SM. Subclinical acute kidney injury : a novel biomarker-defined syndrome. Crit Care. 2011;13(3):201–3.
Bazzi C, Petrini C, Rizza V, et al. Urinary *N*-acetyl-β-glucosaminidase excretion is a marker of tubular cell dysfunction and a predictor of outcome in primary glomerulonephritis. Nephrol Dial Transplant. 2002;7:1890–6.
Bloch KD, Eddy RL, Shows TB, et al. cDNA cloning and chromosomal assignment of the gene encoding endothelin 3. J Biol Chem. 1989;264:18156–61.
Bondiou MT, Bourbouze R, Bernard M, et al. Inhibition of A and B *N*-acetyl-beta-D-glucosaminidase urinary isoenzymes by urea. Clin Chim Acta. 1985;149(1):67–73.
Bosomworth MP, Aparicio SR, Hay AWM. Nephrology dialysis transplantation urine *N*-acetyl-β-D-glucosaminidase – a marker of tubular damage? Nephrol Dial Transplant. 1999;14:620–6.
Bremnes T, Paasche JD, Mehlum A, et al. Regulation and intracellular trafficking pathways of the endothelin receptors. J Biol Chem. 2000;275:17596–604.
Câmara NO, Williams Jr WW, Pacheco-Silva A. Proximal tubular dysfunction as an indicator of chronic graft dysfunction. Braz J Med Biol Res. 2009;42(3):229–36.
Capodicasa E, Angelini A, Tassi C. Isoenzyme A and urinary *N*-acetyl-β-D-glucosaminidase activity in normal pregnancy. Ren Fail. 2011;33(6):650–3.
Cardillo C, Kilcoyne CM, Waclawiw M, et al. Role of endothelin in the increased vascular tone of patients with essential hypertension. Hypertension. 1999;33(2):753–8.
Chester AH, Yacoub MH. The role of endothelin-1 in pulmonary arterial hypertension. Glob Cardiol Sci Pract. 2014;2014(2):62–78.
Chichorro JG, Fiuza CR, Bressan E, et al. Endothelins as pronociceptive mediators of the rat trigeminal system: role of ETA and ETB receptors. Brain Res. 2010;1345:73–83.
Coma P, Gomez-Chacon L, Garcia-Serrano B, et al. α-Glucosidase and *N*-acetyl-beta-D-glucosaminidase isoenzymes in serum. Clin Chem. 1992;38(2):223–6.

D'Orléans-Juste P, Plante M, Honoré JC, et al. Synthesis and degradation of endothelin-1. Can J Physiol Pharmacol. 2003;81:503–10.
Dalva K, Balk M, Saydam GS, et al. İdrar *N*-Asetil-Beta-D-Glukosaminidaz aktivitesi : iki farklı tayin yönteminin karşılaştırılması. J Ank Uni. 2011;64(1):001–005.
Damman K, Masson S, Hillege HL, et al. Clinical outcome of renal tubular damage in chronic heart failure. Eur Heart J. 2011;32(21):2705–12.
Davenport AP. International union of pharmacology. XXIX. Update on endothelin receptor nomenclature. Pharmacol Rev. 2002;54:219–26.
Davenport AP, Maguire JJ. Is endothelin-induced vasoconstriction mediated only by ETA receptors in humans? Trends Pharmacol Sci. 1994;15:9–11.
De Geus HRH, Betjes MGH, et al. Biomarkers for the prediction of acute kidney injury: a narrative review on current status and future challenges. Clin Kidney J. 2012;5:102–8.
de Nucci G, Thomas R, D'Orleans-Juste P, et al. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. Proc Natl Acad Sci U S A. 1988;85(24):9797–800.
Delanghe J, Speeckaert M. Preanalytical requirements of urinalysis. Biochem Med. 2014;24 (1):89–104.
Denault JB, Claing A, D'Orleans-Juste P, et al. Processing of proendothelin-1 by human furin convertase. FEBS Lett. 1995;362(3):276–80.
Dhaun N, Goddard J, Webb DJ. The endothelin system and its antagonism in chronic kidney disease. J Am Soc Nephrol. 2006;17:943–55.
Dhaun N, Lilithkarntakul P, Maclntyre IM, et al. Urinary endothelin-1 in chronic kidney disease and as a marker of disease activity in lupus nephritis. Am J Physiol Ren Physiol. 2009;296(6): F1477–83.
Dhaun N, MacIntyre IM, Kerr D, Melville V, Johnston NR, Haughie S, et al. Selective endothelin-A receptor antagonism reduces proteinuria, blood pressure, and arterial stiffness in chronic proteinuric kidney disease. Hypertension. 2011;57:772–779.
Dupuis J, Stewart DJ, Cernacek P, et al. Human pulmonary circulation is an important site for both clearance and production of endothelin-1. Circulation. 1996;94:1278–84.
Eid H, de Bold ML, Chen JH, et al. Epicardial mesothelial cells synthesize and release endothelin. J Cardiovasc Pharmacol. 1994;24:715–20.
Emoto N, Yanagisawa M. Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metallo-protease with acidic pH optimum. J Biol Chem. 1995;270:15262–8.
Ferguson M, Waikar SS. Established and emerging markers of kidney function. Clin Chem. 2012;58(4):680–9.
Findlay J, Levvy GA. Purification of β-*N*-acetylglucosaminidase from the pig epididymis. Biochem J. 1960;77(1):170–5.
Gasic S, Wagner OF, Vierhapper H, et al. Regional haemodynamic effects and clearance of endothelin-1 in humans: renal and peripheral tissues may contribute to overall disposal of the peptide. J Cardiovasc Pharmacol. 1992;19:176–80.
Gibey R, Dupond JL, Henry JC. Urinary *N*-acetyl-β-D-glucosaminidase (NAG) isozyme profiles: a tool for evaluating nephrotoxicity of aminoglycosides and cephalosporins. Clin Chim Acta. 1984;137:1–11.
Gonzalez F, Vincent F. Biomarkers for acute kidney injury in critically ill patients. Minerva Anestesiol. 2012;78(12):1394–403.
Gray GA, Webb DJ. The endothelin system and its potential as a therapeutic target in cardiovascular disease. Pharmacol Ther. 1996;72(2):109–48.
Gupta RK, Bang TJ. Prevention of Contrast-Induced Nephropathy (CIN) in Interventional Radiology Practice. Seminars in Interventional Radiology. 2010;27(4):348–359.
Han WK, Waikar SS, Johnson A, et al. Urinary biomarkers in the early diagnosis of acute kidney injury. Kidney Int. 2008;73(7):863–9.

Han WK, Wagener G, Zhu Y, et al. Urinary biomarkers in the early detection of acute kidney injury after cardiac surgery. Clin J Am Soc Nephrol. 2009;4(5):873–82.

Haynes WG, Webb DJ. Contribution of endogenous generation of endothelin-1 to basal vascular tone. Lancet. 1994;344:852–4.

Haynes WG, Webb DJ. Endothelin as a regulator of cardiovascular function in health and disease. J Hypertens. 1998;16(8):1081–98.

Haynes WG, Strachan FE, Webb DJ. Endothelin ETA and ETB receptors cause vasoconstriction of human resistance and capacitance vessels in vivo. Circulation. 1995;92:357–63.

Hibi Y, Uemura O, Nagai T, et al. The ratios of urinary β2-microglobulin and NAG to creatinine vary with age in children. Pediatr Int. 2014;20. doi:10.1111/ped.12470. [Epub ahead of print].

Hiruma T, Asada T, Yamamoto M, et al. Mortality prediction by acute kidney injury biomarkers in comparison with serum creatinine. Biomarkers. 2014;19(8):646–51.

Hong JD, Lim IS. Correlation between glomerular filtration rate and urinary *N*-acetyl-beta-D-glucosaminidase in children with persistent proteinuria in chronic glomerular disease. Korean J Pediatr. 2012;55(4):136–42.

Horak E, Hopfer SM, Sunderman FW. Spectrophotometric assay for urinary *N*-acetyl-beta-D-glucosaminidase activity. Clin Chem. 1981;27(7):1180–5.

Ikonne JU, Ellis RB. *N*-Acetyl-β-D-hexosaminidase component A. Different forms in human tissues and fluids. Biochem J. 1973;135(3):457–62.

Inoue A, Yanagisawa M, Kimura S, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci U S A. 1989a;86(8):2863–7.

Inoue A, Yanagisawa M, Takuwa Y, et al. The human preproendothelin-1 gene. Complete nucleotide sequence and regulation of expression. J Biol Chem. 1989b;264(25):14954–9.

Inoue T, Matsunaga R, Morooka S, et al. Serum *N*-acetyl-β-D-gulucosaminidase activity increases in association with insulin resistance in patients with coronary artery disease. Atherosclerosis. 2000;149(1):117–22.

Iqbal MP, Kazmi KA, Jafri HR, et al. *N*-acetyl-beta-D-glucosaminidase in acute myocardial infarction. Exp Mol Med. 2003;35(4):275–8.

Iqbal MP, Kazmi K, Mehboobali N, et al. *N*-acetyl-beta-D-glucosaminidase-a marker of reperfusion and a prognostic indicator in patients with acute myocardial infarction. Acta Cardiol. 2005;60 (5):509–13.

Itoh Y, Yanagisawa M, Ohkubo S, et al. Cloning and sequence analysis of cDNA encoding the precursor of a human endothelium-derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. FEBS Lett. 1988;231(2):440–4.

Itoh Y, Kimura C, Onda H, et al. Canine endothelin-2: cDNA sequence for the mature peptide. Nucleic Acids Res. 1989;17(13):5389.

Itoh Y, Numata Y, Morita A, et al. Varied value of urinary *N*-acetyl-beta-D-glucosaminidase isoenzyme B in males of reproductive age. Kidney Int Suppl. 1994;47:38–42.

Javed MH, Hussain AN. Estimation and separation of *N*-acetyl-B-D-glucosaminidase isoenzymes in urine. JPMA. 1992;42:64.

Jiang C, Qi K, Sun K, et al. Diagnostic value of *N*-acetyl-β-D-glucosaminidase for the early prediction of acute kidney injury after percutaneous nephrolithotripsy. Exp Ther Med. 2013;5 (1):197–200.

Karet FE, Davenport AP. Localization of endothelin peptides in human kidney. Kidney Int. 1996;49:382–7.

Khimji AK, Rockey DC. Endothelin-biology and disease. Cell Signal. 2010;22(11):1615–25.

Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group. KDIGO clinical practice guideline for acute kidney injury. Kidney Int Suppl. 2012;2:1–138.

Kohan DE. Endothelins in the normal and diseased kidney. Am J Kidney Dis. 1997;29:2–26.

Kohan DE, Rossi NF, Inscho EW, et al. Regulation of blood pressure and salt homeostasis by endothelin. Physiol Rev. 2011;91(1):1–77.

Komatsumoto S, Nara M. Changes in the level of endothelin-1 with aging. Nihon Ronen Igakkai Zasshi. 1995;32:664–9.
Komuro I, Kurihara H, Sugiyama T, et al. Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells [published erratum appears in FEBS Lett. 1989;244:509]. FEBS Lett. 1988;238:249–52.
Kowalczyk A, Kleniewska P, Kolodziejczyk M et al. The role of endothelin-1 and endothelin receptor antagonists in inflammatory response and sepsis. Arch Immunol Ther Exp (Warsz). 2015;63:41–52.
Koyama H, Tabata T, Nishzawa Y, et al. Plasma endothelin levels in patients with uremia. Lancet. 1989;333:991–2.
Kwiatkowska E, Domański L, Bober J, et al. *N*-acetyl-beta-glucosaminidase urine activity as a marker of early proximal tubule damage and a predictor of the long-term function of the transplanted kidneys. Acta Biochim Pol. 2014;61(2):275–80.
Leung KC, Tonelli M, James MT. Chronic kidney disease following acute kidney injury-risk and outcomes. Nat Rev Nephrol. 2013;9(2):77–85.
Levin ER. Endothelins. N Engl J Med. 1995;333(6):356–63.
Liangos O, Perianayagam MC, Vaidya VS, et al. Kidney injury molecule-1 level are associated with adverse outcomes in acute renal failure. J Am Soc Nephrol. 2007;18 (3):904–12.
Lin HY, Kaji EH, Winkel GK, et al. Cloning and functional expression of a vascular smooth muscle endothelin 1 receptor. Proc Natl Acad Sci U S A. 1991;88(8):3185–9.
Lockwood TD, Bosmann HB. the use of urinary *N*-acetyl-beta-D-glucosaminidase in human renal toxicology 1. Partial biochemical characterisation and excretion in humans and release from the isolated perfused rat kidney. Toxicol Appl Pharm. 1979;49:323–36.
Loutzenhiser R, Epstein M, Hayashi K, et al. Direct visualization of effects of endothelin on the renal microvasculature. Am J Physiol. 1990;258:F61–8.
Maeda S, Tanabe T, Miyauchi T, et al. Aerobic exercise training reduces plasma endothelin-1 concentration in older women. J Appl Physiol. 2003;95:336–41.
Mandić LM, Aćimović JM, Jovanović VB. The possibility of determining *N*-acetyl-beta-D-glucosaminidase isoenzymes under alkaline conditions. Clin Biochem. 2005;38(4):384–9.
Masaki T. Historical review: endothelin. Trends Pharmacol Sci. 2004;25(4):219–24.
Masaki T, Vane JR, Vanhoutte PM. International union of pharmacology nomenclature of endothelin receptors. Pharmacol Rev. 1994;46(2):137–42.
Matsumoto H, Suzuki N, Onda H, et al. Abundance of endothelin-3 in rat intestine, pituitary gland and brain. Biochem Biophys Res Commun. 1989;164(1):74–80.
McWilliam SJ, Antoine DJ, Sabbisetti V, et al. Mechanism-based urinary biomarkers to identify the potential for aminoglycoside-induced nephrotoxicity in premature neonates: a proof-of-concept study. PLoS ONE. 2012;7(8):e43809.
Milnerowicz H, Bukowski R, Jablonowska M, et al. The antioxidant profiles, lysosomal and membrane enzymes activity in patients with acute pancreatitis. Mediat Inflamm. 2014;2014:376518.
Mishra J, Dent C, Tarabishi R, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. Lancet. 2005;365:1231–8.
Miyauchi T, Suzuki N, Kurihara T, et al. Endothelin-1 and endothelin-3 play different roles in acute and chronic alterations of blood pressure in patients with chronic hemodialysis. Biochem Biophys Res Commun. 1991;178:276–81.
Miyauchi T, Yanagisawa M, Iida K, et al. Age and sex-related variation of plasma endothelin-1 concentration in normal and hypertensive subjects. Am Heart J. 1992;123:1092–3.
Mohkam M, Karimi A, Habibian S, et al. Urinary *N*-acetyl-beta-D-glucosaminidase as a diagnostic marker of acute pyelonephritis in children. Iran J Kidney Dis. 2008;2(1):24–8.
Moriguchi J, Inoue Y, Kamigama S, et al. *N*-Acetyl-β-D-glucosaminidase (NAG) as the most sensitive marker of tubular dysfunction for monitoring residents in non-polluted areas. Toxicol Lett. 2009;190:1–8.

Morita A, Numata Y, Kosugi Y, et al. Stabilities of *N*-acetyl-β-D-glucosaminidase (NAG) isoenzymes in urine: advantage of NAG isoenzyme B measurement in clinical applications. Clin Chim Acta. 1998;278:35–43.

Mueller PW, MacNeil ML, Steinberg KK. *N*-acetyl-beta-D-glucosaminidase assay in urine: urea inhibition. J Anal Toxicol. 1989;13(3):188–90.

Nakajima K, Kumagaye S, Nishio H, et al. Synthesis of endothelin-1 analogues, endothelin-3, and sarafotoxin S6b: structure-activity relationships. J Cardiovasc Pharmacol. 1989;13 Suppl 5: S8–12; discussion S18.

Nakamura S, Naruse M, Naruse K, et al. Immunocytochemical localization of endothelin in cultured bovine endothelial cells. Histochemistry. 1990;94:475–7.

Nakano A, Kishi F, Ninami K. Selective conversion of big endothelins to tracheal smooth muscle-constricting 31-amino acid-length endothelins by chymase from human mast cells. J Immunol. 1997;159(4):1987–92.

Nicot G, Lachâtre G, Gonnet C, et al. Rapid assay of *N*-acetyl-beta-D-glucosaminidase isoenzymes in urine by ion-exchange chromatography. Clin Chem. 1987;33(10):1796–800.

Noto A, Ogawa Y, Mori S, et al. Simple, rapid spectrophotometry of urinary *N*-acetylbeta-D-glucosaminidase, with use of a new chromogenic substrate. Clin Chem. 1983;29(10):1713–6.

Numata Y, Morita A, Kosugi Y, et al. New sandwich ELISA for human urinary *N*-acetyl- β-D-glucosaminidase isoenzyme B as a useful clinical test. Clin Chem. 1997;43(4):569–74.

Nussdorfer GG, Rossi GP, Malendowicz LK, et al. Autocrine-paracrine endothelin system in the physiology and pathology of steroid-secreting tissues. Pharmacol Rev. 1999;51:403–38.

Ohkita M, Wang Y, Nguyen ND, et al. Extrarenal ETB plays a significant role in controlling cardiovascular responses to high dietary sodium in rats. Hypertension. 2005;45 (5):940–6.

Oka M, Niwa Y, Mawatari K, et al. A novel peptide of endothelin family, 31 amino-acid length endothelin in patients with acute myocardial infarction. J Med Invest. 2014;61 (3–4):298–305.

Okada S, O'Brien JS. Tay-Sachs disease: generalized absence of a beta-D-*N*-acetylhexosaminidase component. Science. 1969;165(894):698–700.

Oláh AV, Price RG, Csáthy L, et al. Age dependence of serum beta-*N*-acetylhexosaminidase (NAG) activity. Clin Chem Lab Med. 2004;42(3):305–6.

Opgenorth TJ, Wu-Wong JR, Shiosaki K. Endothelin converting enzymes. FASEB J. 1992;6 (9):2653–9.

Orlando R, Mussap M, Plebani M, et al. Diagnostic value of plasma cystatin C as a glomerular filtration marker in decompensated liver cirrhosis. Clin Chem. 2002;48:850–8.

Plumpton C, Champeney R, Ashby MJ, et al. Characterization of endothelin isoforms in human heart: endothelin-2 demonstrated. J Cardiovasc Pharmacol. 1993;22 Suppl 8:S26–8.

Pollock DM, Pollock JS. Evidence for endothelin involvement in the response to high salt. Am J Physiol. 2001;281(1):F144–50.

Price RG. Measurement of *N*-acetyl-beta-glucosaminidase and its isoenzymes in urine methods and clinical applications. Eur J Clin Chem Clin Biochem. 1992;30(10):693–705.

Rabelink TJ, Kaasjager KAH, Boer P, et al. Effects of endothelin-1 on renal function in humans. Implications for physiology and pathophysiology. Kidney Int. 1994;46(2):376–81.

Richter CM. Role of endothelin in chronic renal failure-developments in renal involvement. Rheumatology. 2006;45:iii36–8.

Robinson BD, Stirling JL. *N*-acetyl-beta-glucosaminidases in human spleen. Biochem J. 1968;107 (3):321–7.

Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol Rev. 1994;46:325–415.

Saito S, Hirata Y, Imai T, et al. Autocrine regulation of the endothelin-1 gene in rat endothelial cells. J Cardiovasc Pharmacol. 1995;26:84–7.

Sakamoto A, Yanagisawa M, Sakurai T, et al. Cloning and functional expression of human cDNA for the ETB endothelin receptor. Biochem Biophys Res Commun. 1991;178(2):656–63.

Sakurai T, Yanagisawa M, Masaki T. Molecular characterization of endothelin receptor. Trends Pharmacol Sci. 1992;13:103–8.

Saleh MA, Boesen EI, Pollock JS, et al. Endothelin-1 increases glomerular permeability and inflammation independent of blood pressure in the rat. Hypertension. 2010;56(5):942–9.

Sandhoff K. Variation of beta-*N*-acetylhexosaminidase-pattern in Tay-Sachs disease. FEBS Lett. 1969;4(4):351–4.

Sawamura T, Kimura S, Shinmi O, et al. Analysis of endothelin related peptides in culture supernatant of porcine aortic endothelial cells: evidence for biosynthetic pathway of endothelin-1. Biochem Biophys Res Commun. 1989;162(3):1287–94.

Schildroth J, Rettig-Zimmermann J, Kalk P, et al. Endothelin type A and B receptors in the control of afferent and efferent arterioles in mice. Nephrol Dial Transplant. 2011;26(3):779–89.

Schmidt M, Kröger B, Jacob E, et al. Molecular characterization of human and bovine endothelin converting enzyme (ECE-1). FEBS Lett. 1994;356(2–3):238–43.

Semerci T, Cuhadar S, Akcay FA, et al. Comparing the renal safety of isoosmolar versus low-osmolar contrast medium by renal biomarkers *N*-acetyl-β-D-glucosaminidase and endothelin. Angiology. 2014;65(2):108–12.

Serneri GGN, Modesti PA, Cecioni I, et al. Plasma endothelin and renal endothelin are two distinct systems involved in volume homeostasis. Am J Physiol. 1995;268:H 1829–37.

Severinl G, Aiiberti LM, Glrolamo MDI. *N*-Acetyl-β-glucosaminidase isoenzymes in serum and urine of patients with diabetes mellitus. Clin Chem. 1988;34(12):2430–2.

Shah R. Endothelins in health and disease. Eur J Int Med. 2007;18(4):272–82.

Sharma RK. Nephrology biomarkers of acute kidney injury. Clin Queries. 2012;1(1):13–7.

Shinmi O, Kimura S, Sawamura T, et al. Endothelin-3 is a novel neuropeptide: isolation and sequence determination of endothelin-1 and endothelin-3 in porcine brain. Biochem Biophys Res Commun. 1989;164:587–93.

Skálová S.The diagnostic role of urinary N-acetyl-beta-D-glucosaminidase (NAG) activity in the detection of renal tubular impairment. Acta Medica. 2005;48(2):75–80.

Speed JS, Pollock DM. Endothelin, kidney disease, and hypertension. Hypertension. 2013;61:1142–55.

Suzaki Y, Yoshizumi M, Yamashita Y, et al. Determination of plasma concentrations of endothelin-1 (1-31) and endothelin-1 in healthy subjects and patients with atherosclerosis. J Cardiovasc Pharmacol. 2003;41:83–7.

Suzuki N, Matsumoto H, Kitada C, et al. A sensitive sandwich-enzyme immunoassay for human endothelin. J Immun Methods. 1989;118:245–50.

Thorin E, Clozel M. The cardiovascular physiology and pharmacology of endothelin-1. Adv Pharmacol. 2010;60:1–26.

Tonomura Y, Uehara T, Yamamoto E, et al. Decrease in urinary creatinine in acute kidney injury influences diagnostic value of urinary biomarker-to-creatinine ratio in rats. Toxicology. 2011;290(2–3):241–8.

Uchino S, Kellum JA, Bellomo R, et al. Acute renal failure in critically ill patients: a multinational, multicenter study. JAMA. 2005;294:813–8.

Verhaar MC, Strachan FE, Newby DE, et al. Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. Circulation. 1998;97:752–6.

Vidgoff J, Buist NRM, Brien JSO. Absence of β-*N*-Acetyl-D-Hexosaminidase A activity in a healthy woman. Am J Hum Genet. 1973;25:372–81.

Waikar SS, Curhan GC, Wald R, et al. Declining mortality in patients with acute renal failure, 1998 to 2002. J Am Soc Nephrol. 2006;17:1143–50.

Waikar SS, Betensky RA, Bonventre JV. Creatinine as the gold standard for kidney injury biomarker studies? Nephrol Dial Transplant. 2009;24(11):3263–5.

Wu I, Parikh CR. Screening for kidney diseases: older measures versus novel biomarkers. Clin J Am Soc Nephrol. 2008;3:1895–901.

Xu D, Emoto N, Giaid A, et al. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. Cell. 1994;78(3):473–85.
Xue JL, Daniels F, Star RA, et al. Incidence and mortality of acute renal failure in Medicare beneficiaries, 1992 to 2001. J Am Soc Nephrol. 2006;17(4):1135–42.
Yanagisawa M, Masaki T. Molecular biology and biochemistry of the endothelins. Trends Pharmacol Sci. 1989;10:374–8.
Yanagisawa M, Kurihara H, Kimura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988a;332:411–5.
Yanagisawa M, Inoue A, Ishikawa T, et al. Primary structure, synthesis and biological activity of rat endothelin, an endothelium-derived vasoconstrictor peptide. Proc Natl Acad Sci U S A. 1988b;85:6964–7.
Zoccali C, Leonardis D, Parlongo S, et al. Urinary and plasma endothelin-1 in essential hypertension and in hypertension secondary to renoparenchymal disease. Nephrol Dial Transplant. 1995;10(8):1320–3.

Methylated Arginines as Biomarkers in Renal Disease

18

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Abstract

The endogenous methylated analogues of the amino acid arginine, asymmetric dimethylarginine, symmetric dimethylarginine, and monomethylarginine are generated following the degradation of proteins containing methylated arginine residues. Research conducted over the last 25 years has convincingly demonstrated that methylated arginines, in particular asymmetric dimethylarginine and monomethylarginine, play a key role in vascular homeostasis by inhibiting the

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activity of the three isoforms of nitric oxide synthase, endothelial, neuronal, and inducible, with consequent reduced synthesis of nitric oxide. The increased synthesis and/or reduced catabolism or clearance of methylated arginines might contribute to the onset and progression of endothelial dysfunction, vascular damage, and atherosclerosis through relatively high local exposure in critical organs and tissues. The initial evidence of a detrimental effect of methylated arginines on vascular homeostasis originated from studies in patients with either chronic kidney disease or end-stage renal disease. As the kidney represents a major elimination route, it is not surprising that the plasma/serum concentrations of asymmetric dimethylarginine, symmetric dimethylarginine, and monomethylarginine are significantly increased in patients with renal disease. Methylated arginine concentrations have been shown to independently predict adverse clinical outcomes, e.g., cardiovascular morbidity and mortality and all-cause mortality, both in chronic kidney disease and in end-stage renal disease patients.

This review discusses the synthesis, transport, and elimination of methylated arginines in physiological conditions and their main pathophysiological effects in the cardiovascular system. It also discusses the role of methylated arginines as predictors of adverse outcomes in patients with renal disease and their potential clinical use as biomarkers.

Keywords
Methylated arginines • Chronic kidney disease • End-stage renal disease • Outcomes • Endothelium • Nitric oxide • Dialysis

Abbreviations

ADMA	Asymmetric dimethylarginine
AQC	6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
BNP	Brain natriuretic peptide
CE	Capillary electrophoresis
CKD	Chronic kidney disease
CRP	C-reactive protein
CVD	Cardiovascular disease
DAH	Dialysis-associated hypotension
DMA	Dimethylamine
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease
FITC	Fluorescein isothiocyanate
FLR	Fluorescence
GC	Gas chromatography
HD	Hemodialysis
HILIC	Hydrophilic interaction liquid chromatography
HMA	Homoarginine
HPLC	High-performance liquid chromatography
LCMS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoprotein

LIF	Laser-induced fluorescence
MI	Myocardial infarction
MMA	Monomethylarginine
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NDA	Naphthalene-2,3-dicarboxaldehyde
NO	Nitric oxide
NOS	Nitric oxide synthase
OPA	*O*-phthaldialdehyde
PFP	Pentafluoropropionyl
SDMA	Symmetric dimethylarginine
UPLC	Ultra-performance liquid chromatography

Key Facts of Methylated Arginines

- Methylated arginines are arginine analogues containing one or two methyl groups.
- The methylation of arginine residues in proteins is mediated by a family of enzymes known as protein arginine methyltransferases.
- Methylated arginines are released into the cytoplasm following protein degradation.
- Methylated arginines in the cytoplasm are either metabolized by the enzyme dimethylarginine dimethylaminohydrolase or transported into the extracellular space by means of a cationic transporter.
- Circulating plasma methylated arginines are either eliminated by the kidney or catabolized by dimethylarginine dimethylaminohydrolase.
- Different analytical methods have been developed to measure methylated arginine concentrations from plasma and other biological fluids.
- Reference ranges for methylated arginines concentrations have been published for healthy subjects. In this group, methylated arginines concentrations are primarily dependent on age and renal function.

Definitions

Cardiovascular events Cardiovascular events include a number of cardiovascular complications as clinical manifestations of the onset and progression of atherosclerosis, e.g., myocardial infarction, stroke, and peripheral arterial occlusive disease.

Dialysis Dialysis is treatment strategy consisting in the removal of excess water, toxins, and other substances from the blood or other body compartments. It is used primarily to artificially replace the function of the kidney in patients with renal disease.

Methylated arginines Methylated arginines are a group of endogenous arginine analogues generated by the methylation of arginine residues in protein and subsequent degradation of such proteins.

Nitric oxide Nitric oxide is an endogenous molecule that plays a key role in the physiological regulation of vascular tone, regional blood flow, and blood pressure in health and in disease states.

Renal disease Renal disease is a condition where the capacity of the kidney to eliminate water as well as toxic compounds is impaired. Different degrees of renal impairment predict the risk of complications and death.

Introduction

Cardiovascular disease (CVD) represents the main cause of morbidity and mortality in either chronic kidney disease (CKD) or end-stage renal disease (ESRD). At least two factors may explain the association between CVD and renal disease: (1) several CVD risk factors, particularly hypertension and diabetes, increase the risk of CKD and ESRD onset and progression, and (2) a good amount of clinical evidence has demonstrated that both CKD and ESRD independently increase the risk of CVD events, e.g., myocardial infarction (MI) and stroke (Gansevoort et al. 2013).

A number of functional and structural abnormalities of various components of the cardiovascular system, generally grouped under the term "cardiovascular remodeling," have been demonstrated in CKD/ESRD patients. They include endothelial dysfunction with impaired endothelium-dependent vasodilatation, increased intima-media thickness, arterial stiffening, and left ventricular hypertrophy (Gansevoort et al. 2013). Alterations in vascular homeostasis, mainly secondary to impaired synthesis of the endogenous vasodilator nitric oxide (NO) by the enzyme isoform endothelial NO synthase (eNOS), play a key role in this context (Napoli et al. 2006). Over the last 25 years or so, experimental and clinical studies have demonstrated the biological and pathophysiological relevance of endogenous methylated arginine analogues in modulating NO synthesis and vascular homeostasis. A significant number of human studies addressing this issue have been conducted in patients with CKD/ESRD.

Synthesis, Transport, and Metabolism of Methylated Arginines

Three methylated arginine analogues have been identified in mammalian cells: asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and monomethylarginine (MMA) (Vallance and Leiper 2004) (Fig. 1). ADMA, SDMA, and MMA are synthesized following the methylation of arginine residues in proteins by protein arginine methyltransferases (PRMTs). PRMTs use *S*-adenosylmethionine as methyl group donor (Vallance and Leiper 2004).

Following protein degradation, ADMA, SDMA, and MMA are released into the cytoplasm (Vallance and Leiper 2004). Intracellular ADMA and MMA, but not SDMA, are further metabolized to citrulline and dimethylamine by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (Fig. 2). DDAH is present in two isoforms, DDAH-1 and DDAH-2 (Vallance and Leiper 2004). DDAH-1 has

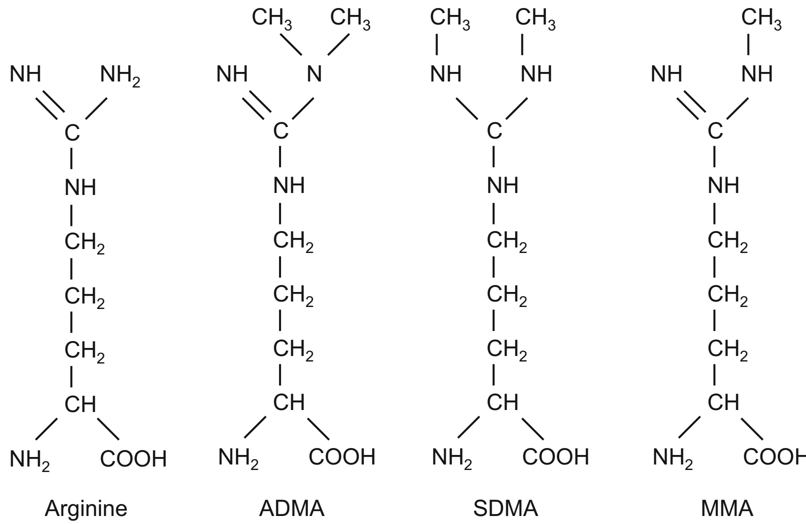

Fig. 1 Chemical structure of the amino acid arginine and the methylated arginines asymmetric dimethylarginine (*ADMA*), symmetric dimethylarginine (*SDMA*), and monomethylarginine (*MMA*)

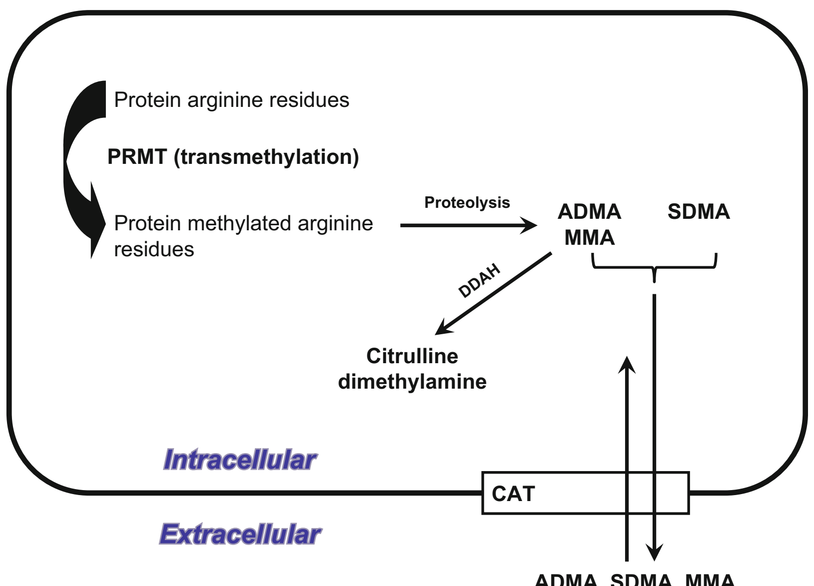

Fig. 2 Pathways of intracellular synthesis, metabolism, and transport of the methylated arginines asymmetric dimethylarginine (*ADMA*), symmetric dimethylarginine (*SDMA*), and monomethylarginine (*MMA*). *PRMT* protein arginine methyltransferases, *DDAH* dimethylarginine dimethylaminohydrolase, *CAT* cation transporter

been identified primarily in the liver, and the kidney, and brain (Vallance and Leiper 2004). By contrast, DDAH-2 is predominantly expressed in the endothelium, heart, placenta, and kidney (Vallance and Leiper 2004). ADMA and MMA metabolism by DDAH involves a nucleophilic attack on the guanidine carbon by a cysteine held in an activated state in the tertiary structure of the enzyme (Vallance and Leiper 2004). There is evidence that increased NO concentrations nitrosate DDAH, inhibiting its activity (Vallance and Leiper 2004). This suggests a negative feedback system whereby increased NO can inhibit further NO synthesis through increased concentrations of ADMA and MMA.

Although ADMA and MMA are extensively metabolized in the cytoplasm, a fraction is transported, together with SDMA, across the cell membrane into the extracellular space and the systemic circulation via the y^+ cationic amino acid transport system (Vallance and Leiper 2004) (Fig. 2). The fraction of ADMA and MMA that escapes intracellular metabolism is either metabolized in the liver or in the kidney through DDAH or eliminated unchanged by the kidney (Vallance and Leiper 2004). SDMA is not metabolized by DDAH. Instead, it undergoes renal clearance, although there is some evidence of additional uptake by the liver (Vallance and Leiper 2004). Therefore, it is not surprising that renal and/or liver disease states are characterized by a significant increase in plasma concentrations of methylated arginines (Vallance and Leiper 2004).

Determination of Methylated Arginines

ADMA is the methylated arginine most studied in human studies. Table 1 describes the methods used for its measurement in human plasma, including concentrations in apparently healthy subjects. The majority of methods utilize liquid chromatography (LC) with separation of analytes achieved on either reverse phase (C18) or hydrophilic interaction (HILIC) analytical columns and detection of analytes by either ultraviolet (UV), fluorescence (FLR), or mass spectrometry (MS) (MacAllister et al. 1996; Anderstam et al. 1997; Marescau et al. 1997; Pettersson et al. 1997; Pi et al. 2000; Teerlink et al. 2002; Marra et al. 2003; Heresztyn et al. 2004; Zhang and Kaye 2004; Sotgia et al. 2008; Blackwell et al. 2009; Jones et al. 2010; Ivanova et al. 2010; Vishwanathan et al. 2000; Martens-Lobenhoffer and Bode-Boger 2003, 2006, 2012; Martens-Lobenhoffer et al. 2004; Huang et al. 2004; Schwedhelm et al. 2007; Bishop et al. 2007; D'Apolito et al. 2008; Gervasoni et al. 2011; Yi et al. 2011; Davids et al. 2012; Hui et al. 2012; El-Khoury et al. 2012; Servillo et al. 2013; Di Gangi et al, 2010). To a lesser extent, various other technologies such as capillary electrophoresis (CE) (Zinellu et al. 2007a, 2011; Causse et al. 2000; Linz et al. 2012; Linz and Lunte 2013; Desiderio et al. 2010), gas chromatography (GC) (Tsikas et al. 2003; Albsmeier et al. 2004), and enzyme-linked immunosorbent assay (ELISA) (Schulze et al. 2004) have also been reported. Many of these methods can simultaneously measure also arginine, SDMA, and MMA concentrations. However, the simultaneous quantification of arginine and its methylated metabolites in biological fluids raises several analytical issues: (a) plasma concentrations of ADMA

Table 1 Determination of ADMA in human plasma. Description of the analytical methods developed to measure asymmetric dimethylarginine (ADMA) in human plasma including information on method of detection, reported ADMA concentrations, and detection limit

References	Apparatus	Detection	Derivative	Plasma ADMA concentration	Detection limit
(MacAllister et al. 1996)	HPLC	UV	None	0.39 ± 0.05 (n = 9)	ND
(Anderstam et al. 1997)	HPLC	FLR	AQC	0.36 ± 0.08 (n = 7)	ND
(Marescau et al. 1997)	HPLC	FLR	OPA	0.41 ± 0.09 (n = 66)[a]	ND
(Pettersson et al. 1997)	HPLC	FLR	OPA	0.58 ± 0.02 (n = 10)	0.015
(Pi et al. 2000)	HPLC	FLR	OPA	0.30 ± 0.05 (n = 7)	0.015 (LOQ)
(Teerlink et al. 2002)	HPLC	FLR	OPA	0.42 ± 0.06 (n = 53)	0.01 (LOQ)
(Marra et al. 2003)	HPLC	FLR	NDA	0.38–1.30 (range, n = 50)	0.01
(Heresztyn et al. 2004)	HPLC	FLR	AQC	0.44 ± 0.08 (n = 12)	0.1
(Zhang and Kaye 2004)	HPLC	FLR	OPA	0.76 ± 0.12 (n = 35)	0.0015 (LOQ)
(Sotgia et al. 2008)	HPLC	FLR	Ninhydrin	0.58 ± ND (n = 50)	0.004
(Blackwell et al. 2009)	HPLC	FLR	OPA	0.46 ± 0.08 (n = 70)	0.001
(Jones et al. 2010)	HPLC	FLR	AQC	0.45 ± 0.07 (n = 30)	0.20 (LOQ)
(Ivanova et al. 2010)	HPLC	FLR	OPA	0.36–0.63 (range, n = 225)	ND
(Vishwanathan et al. 2000)	HPLC	MS/MS	None	0.12 ± 0.05 (n = 20)	0.005
(Martens-Lobenhoffer and Bode-Boger 2003)	HPLC	MS	OPA	0.45 ± 0.13 (n = 15)[a]	0.20 (LOQ)
(Martens-Lobenhoffer et al. 2004)	HPLC	MS	OPA	0.36 ± 0.07 (n = 47)	0.15 (LOQ)
(Huang et al. 2004)	HPLC	MS	None	0.48 ± 0.07 (n = 40)	0.010
(Martens-Lobenhoffer and Bode-Boger 2006)	HPLC	MS/MS	None	0.37 ± 0.06 (n = 14)	0.02
(Schwedhelm et al. 2007)	HPLC	MS	1-butanol	0.46 ± 0.09 (n = 85)	0.0005
(Bishop et al. 2007)	HPLC	MS/MS	None	0.66 ± 0.12 (n = 15)	0.06

(*continued*)

Table 1 (continued)

References	Apparatus	Detection	Derivative	Plasma ADMA concentration	Detection limit
(D'Apolito et al. 2008)	HPLC	MS/MS	None	0.57 ± 0.11 (n = 30)	ND
(Gervasoni et al. 2011)	HPLC	MS/MS	None	0.46 ± 0.07 (n = 103)[a]	0.005
(Yi et al. 2011)	HPLC	MS	1-butanol	0.46 ± 0.17 (n = 21)	0.08 (LOQ)
(Davids et al. 2012)	HPLC	MS/MS	1-butanol	0.47 ± 0.06 (n = 100)	0.0004 (LOQ)
(Martens-Lobenhoffer and Bode-Boger 2012)	HPLC	MS/MS	None	0.45 ± 0.06 (n = 10)	0.003
(Hui et al. 2012)	HPLC	MS/MS	NDA	0.30 ± 011 (n = 123)	0.0026
(El-Khoury et al. 2012)	HPLC	MS/MS	None	0.36–0.67 (range, n = 51)	ND
(Servillo et al. 2013)	HPLC	MS/MS	None	0.67 ± 0.04 (n = 12)	0.06 (LOQ)
(Di Gangi et al. 2010)	UPLC	MS/MS	1-butanol	0.56 ± 0.10 (n = 20)	0.02
(Zinellu et al. 2007b)	CE	UV	None	ND (n = 77)	0.03
(Zinellu et al. 2011)	CE	UV	None	0.46 ± 0.11 (n = 50)	0.01
(Causse et al. 2000)	CE	LIF	FICT	0.34 ± 0.02 (n = 5)	0.05
(Linz et al. 2012)	CE	LIF	NDA	ND	0.02
(Linz and Lunte 2013)	CE	LIF	NDA	0.37 ± 0.05[b]	0.005
(Desiderio et al. 2010)	CE	MS/MS	None	0.42 ± 0.05 (n = 1)	0.02
(Tsikas et al. 2003)	GC	MS/MS	PFP	0.39 ± 0.06 (n = 12)	0.0001
(Albsmeier et al. 2004)	GC	MS	PFP	0.60 ± 0.08 (10)	0.0004
(Schulze et al. 2004)	ELISA	UV	None	0.65 ± 0.13 (n = 10)	0.05[c]

Concentrations, limits of detection (LOD), and limits of quantification (LOQ) are given in μmol/L
AQC 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, *CE* capillary electrophoresis, *ELISA* enzyme-linked immunosorbent assay, *FITC* fluorescein isothiocyanate, *FLR* fluorescence, *GC* gas chromatography, *LIF* laser-induced fluorescence, *MS* mass spectrometry, *ND* not declared, *NDA* naphthalene-2,3-dicarboxaldehyde, *OPA* o-phthaldialdehyde, *PFP* pentafluoropropionyl, *UPLC* ultra-performance liquid chromatography

[a]Values from serum
[b]Pooled serum
[c]Analytical sensitivity

and SDMA are, in fact, two orders of magnitude lower than those of arginine and an order of magnitude higher when compared to the concentration of MMA; (b) the discrimination between the chemically related compounds ADMA and its stereoisomer SDMA, which is produced in equivalent quantities, and MMA and homoarginine (HMA) represents a primary analytical challenge; and (c) arginine, ADMA, SDMA, and MMA are lacking of intrinsic chromophores, making direct detection by optical-based devices (UV or FLR) particularly challenging; however, the latter is overcome by the use of MS. For these reasons, assays of high specificity and sensitivity are desirable, and sample cleanup by solid-phase extraction is often required prior to analysis.

Underivatized ADMA has been successfully measured by HPLC-CE with UV detector (MacAllister et al. 1996; Zinellu et al. 2007b, 2011), high-performance liquid chromatography-mass spectrometry (HPLC/MS) (Huang et al. 2004), and HPLC-CE/MS/MS (Vishwanathan et al. 2000; Martens-Lobenhoffer and Bode-Boger 2006, 2012; Bishop et al. 2007; D'Apolito et al. 2008; Gervasoni et al. 2011; El-Khoury et al. 2012; Servillo et al. 2013; Zinellu et al. 2007a, 2011; Desiderio et al. 2010) detection. By CE, detection of underivatized analytes may also be accomplished by a pre-column sample concentration (Zinellu et al. 2007b) or by online sample concentration using the field-amplified sample injection (FASI) technique (Zinellu et al. 2011; Desiderio et al. 2010). Commonly, however, a pre-column derivatization is performed before the analysis (Anderstam et al. 1997; Marescau et al. 1997; Pettersson et al. 1997; Pi et al. 2000; Teerlink et al. 2002; Marra et al. 2003; Heresztyn et al. 2004; Zhang and Kaye 2004; Sotgia et al. 2008; Blackwell et al. 2009; Jones et al. 2010; Ivanova et al. 2010; Martens-Lobenhoffer and Bode-Boger 2003; Martens-Lobenhoffer et al. 2004; Schwedhelm et al. 2007; Yi et al. 2011; Davids et al. 2012; Hui et al. 2012; Di Gangi et al. 2010; Causse et al. 2000; Linz et al. 2012; Linz and Lunte 2013; Tsikas et al. 2003; Albsmeier et al. 2004). Ortho-phthaldialdehyde (OPA) has been widely used for fluorescence application in this context (Marescau et al. 1997; Pettersson et al. 1997; Pi et al. 2000; Teerlink et al. 2002; Blackwell et al. 2009; Ivanova et al. 2010). A major drawback of OPA is the instability of derivatives due to rapid decomposition, thus requiring online derivatization. More stable derivatives, even at room temperature, are obtained by using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (ACQ) as derivatization agent (Anderstam et al. 1997; Heresztyn et al. 2004; Jones et al. 2010). Other fluorescence probes used especially in CE/laser-induced fluorescence (LIF)-based methods are fluorescein isothiocyanate (FITC) (Causse et al. 2000) and naphthalene-2,3-dicarboxaldehyde (NDA) (Linz et al. 2012; Linz and Lunte 2013). Ninhydrin is also used for fluorescence detection. Unlike other derivatization reagents, it shows reaction specificity toward ADMA (Sotgia et al. 2008). An ELISA assay for the specific analysis of ADMA is also available (Schulze et al. 2004).

Mass spectrometric detection hyphenated with HPLC (Vishwanathan et al. 2000; Martens-Lobenhoffer and Bode-Boger 2003, 2006, 2012; Martens-Lobenhoffer

et al. 2004; Huang et al. 2004; Schwedhelm et al. 2007; Bishop et al. 2007; D'Apolito et al. 2008; Gervasoni et al. 2011; Yi et al. 2011; Davids et al. 2012; Hui et al. 2012; El-Khoury et al. 2012; Servillo et al. 2013), UPLC (Di Gangi et al. 2010), CE (Desiderio et al. 2010) or Gas Chromatography (GC) (Tsikas et al. 2003; Albsmeier et al. 2004) is increasingly used. Quantification is carried out by either MS (Martens-Lobenhoffer and Bode-Boger 2003; Martens-Lobenhoffer et al. 2004; Huang et al. 2004; Schwedhelm et al. 2007; Yi et al. 2011) or MS/MS (Vishwanathan et al. 2000; Martens-Lobenhoffer and Bode-Boger 2006, 2012; Bishop et al. 2007; D'Apolito et al. 2008; Gervasoni et al. 2011; Davids et al. 2012; Hui et al. 2012; El-Khoury et al. 2012; Servillo et al. 2013). Detection can be performed either for underivatized arginines (Vishwanathan et al. 2000; Huang et al. 2004; Martens-Lobenhoffer and Bode-Boger 2006, 2012; Bishop et al. 2007; D'Apolito et al. 2008; Gervasoni et al. 2011; El-Khoury et al. 2012; Servillo et al. 2013) or after derivatization with OPA (Martens-Lobenhoffer and Bode-Boger 2003; Martens-Lobenhoffer et al. 2004), NDA (Hui et al. 2012) or 1-butanol (Schwedhelm et al. 2007; Yi et al. 2011; Davids et al. 2012; Di Gangi et al. 2010).

As described previously, the major limitation of LC-based approaches used to quantify methylated arginines has been the requirement to achieve substantial chromatographic separation of these chemically similar compounds. However, the challenges associated with achieving chromatographic separation of ADMA and SDMA have been overcome, to some degree, in recent years through the use of HILIC chromatography or the in situ generation of unique analytes (mass fragments) for these compounds, using triple-quadrupole MS with multiple reaction monitoring (MRM) and collision-induced dissociation of underivatized analytes (Martens-Lobenhoffer and Bode-Boger 2012). The application of this approach to other chemically related arginine metabolites (MMA and HMA) remains to be considered. While HILIC chromatography offers significant advantages for the separation of chemically related highly polar compounds, such as arginines, the logistical and technical challenges associated with this type of chromatography continue to limit its application. It is likely that the different approaches utilized to overcome these technical challenges account for the large inter-assay variability in reported plasma ADMA concentrations.

Measurements of ADMA concentrations in human urine (Marescau et al. 1997; Pi et al. 2000; Teerlink et al. 2002; Martens-Lobenhoffer and Bode-Boger 2003, 2006; Servillo et al. 2013; Tsikas et al. 2003; Gopu et al. 2013; Markowski et al. 2007), cerebrospinal fluid (Martens-Lobenhoffer et al. 2007), exhaled breath condensate (Di Gangi et al. 2012), and human vascular endothelial cells (Davids et al. 2012; Shin et al. 2011) have been also reported. Moreover, evaluations of methylated arginines in animal plasma (Schwedhelm et al. 2007; Chae et al. 2011; Nonaka et al. 2005; Tsunoda et al. 2005) or tissues both as free (Ueno et al. 1992; Nonaka et al. 2006) or total form (free plus protein incorporated) (Teerlink 2005) have been described. A specific method for the evaluation of protein-incorporated arginine, ADMA, and SDMA by capillary electrophoresis UV detection has been recently reported (Zinellu et al. 2007a).

Pathophysiological Effects of Methylated Arginines in the Cardiovascular System

As previously described, NO is a pivotal endogenous modulator of vascular and cardiovascular homeostasis both in health and in disease states (Napoli et al. 2006). Its key role in mediating endothelium-dependent vasodilation, vascular smooth muscle relaxation, regional blood flow, and blood pressure control has been extensively characterized both in vitro and in vivo (Napoli et al. 2006). Research conducted over the last 10–15 years has demonstrated additional effects of NO on prevention of atherosclerosis onset and progression, e.g., reduction of arterial stiffness and intima-media thickness, anti-inflammatory effects, reduced platelet activity, and reduced cardiac remodeling (Napoli et al. 2006). Therefore, factors leading to an impaired NO synthesis are likely to exert detrimental effects to cardiovascular homeostasis, favoring pro-atherosclerotic processes (Fig. 3) (Napoli et al. 2006).

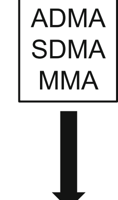

Fig. 3 Putative pathophysiological effects by which the methylated arginines asymmetric dimethylarginine (*ADMA*), symmetric dimethylarginine (*SDMA*), and monomethylarginine (*MMA*) modulate the onset and progression of altered vascular homeostasis, atherosclerosis, and thrombosis

A large number of experimental and human studies have investigated the effects of methylated arginines on established markers of cardiovascular homeostasis and remodeling, e.g., endothelial function, intima-media thickness, arterial stiffness, peripheral vascular resistance, blood pressure, and left ventricular hypertrophy (Fig. 3).

Endothelial Function

Studies have shown that the plasma concentrations of ADMA and MMA are independently associated with impaired endothelial function and endothelium-dependent vasodilation (Boger et al. 1998). Although SDMA does not directly inhibit eNOS activity, recent studies have reported independent associations between SDMA concentrations and impaired endothelial function. The latter might be mediated by indirect NO synthesis inhibition secondary to reduced arginine availability (Memon et al. 2013).

Intima-Media Thickness

Plasma ADMA, SDMA, and MMA concentrations have shown positive and independent associations with carotid intima-media thickness in patients with CKD or ESRD (Zoccali et al. 2002a; Nanayakkara et al. 2005).

Arterial Stiffness

An acute increase in arterial stiffness after MMA administration has been demonstrated both in animal and in human studies (Wilkinson et al. 2002). Population studies investigating associations between methylated arginine concentrations and measures of arterial stiffness have provided conflicting results. Some studies showed independent associations between ADMA concentrations and arterial stiffness (Schutte et al. 2010), whereas other studies did not (Chirinos et al. 2008).

Blood Pressure and Peripheral Vascular Resistance

Intravenous administration of ADMA and MMA has been shown to acutely increase blood pressure and renal, mesenteric, and peripheral vascular resistance in animal models (Gardiner et al. 1993). By contrast, SDMA administration was not associated with significant changes in blood pressure (Veldink et al. 2013). Increased plasma ADMA and SDMA, but not MMA, concentrations have been observed in hypertensive subjects (Wang et al. 2009).

Left Ventricular Hypertrophy

Significant associations between serum ADMA concentrations and echocardiographic measures of left ventricular hypertrophy have been demonstrated in CKD patients as well as ESRD patients (Shi et al. 2010; Zoccali et al. 2002b).

Methylated Arginines and Other Cardiovascular Risk Biomarkers

From a pathophysiological point of view, it seems unlikely that a single biomarker, or group of biomarkers, can fully account for the various molecular and biochemical

processes responsible for the onset and progression of atherosclerosis and its various clinical manifestations. In this context, there is good evidence of associations between methylated arginines and other established cardiovascular biomarkers, e.g., homocysteine, C-reactive protein (CRP), low-density lipoprotein (LDL) cholesterol concentrations, and insulin resistance. Notably, these biomarkers are also significantly raised in CKD/ESRD patients and independently predict adverse outcomes in this population.

Homocysteine

Positive associations between homocysteine, ADMA, and SDMA concentrations have been demonstrated in human studies (Meinitzer et al. 2011). These associations can be explained by a number of factors: (1) PRMTs utilize S-adenosylmethionine, an intermediate in the methionine-homocysteine pathway, as a methyl donor for the methylation of arginine residues in proteins (Vallance and Leiper 2004); and (2) homocysteine inhibits DDAH activity in vitro (Wadham and Mangoni 2009).

CRP

Human studies have shown positive associations between ADMA and CRP concentrations in CKD patients, although other studies have not reported a significant association (Zoccali et al. 2002a; Nanayakkara et al. 2005). Positive associations between SDMA and CRP have been reported in hyperuricemia (Tenderenda-Banasiuk et al. 2013). The inhibitory effect of pro-oxidative pathways on DDAH activity might explain the positive associations between ADMA, but not SDMA, and CRP (Wadham and Mangoni 2009).

Low-Density Lipoprotein (LDL) Cholesterol

Positive associations between ADMA and plasma LDL cholesterol concentrations have been reported in ESRD patients undergoing peritoneal dialysis (PD) (Gocmen et al. 2008). By contrast, no significant correlation between ADMA and LDL cholesterol was observed in patients with ischemic heart disease and in patients with polycystic ovary syndrome (Meinitzer et al. 2007). Positive associations between SDMA and plasma LDL cholesterol concentrations have been reported in patients with thyroid dysfunction (Arikan et al. 2007). Similarly to CRP, it is possible that oxidized-LDL-induced pro-oxidative pathways might reduce DDAH activity with consequent accumulation of ADMA, but not SDMA.

Insulin Resistance

There is good evidence of a strong, positive association between ADMA concentrations and markers of insulin resistance in healthy subjects and in patients with rheumatoid arthritis (Stuhlinger et al. 2002). By contrast, no relationship between ADMA and insulin resistance was demonstrated in young patients with metabolic syndrome (Garcia et al. 2007). Ethnic differences might explain, at least in part, the discrepancy in the results (Reimann et al. 2007). Plasma ADMA, but not SDMA, concentrations have been also shown to predict worsening of insulin resistance over time in patients with ischemic heart disease (Surdacki et al. 2013). Hyperglycemia-induced

prooxidant pathways might reduce DDAH activity (Wadham and Mangoni 2009). This phenomenon, similarly to CRP and LDL cholesterol, might explain the positive associations between ADMA and insulin resistance.

Methylated Arginines and Adverse Clinical Outcomes in Patients with Renal Disease

Several studies in CKD, ESRD, and post-kidney transplant patients have investigated the predictive capacity and/or association between plasma methylated arginine concentrations and adverse clinical outcomes (Table 2).

Dialysis-Associated Hypotension (DAH)

Two relatively small studies have investigated the association between methylated arginines and DAH (Bergamini et al. 2004; Mangoni et al. 2008). In one study, both pre-HD and post-HD ADMA, but not SDMA, concentrations were significantly higher in DAH versus non-DAH patients (Bergamini et al. 2004) (Table 2). In another study, pre-HD and HD-induced changes in SDMA, but not ADMA, concentrations independently predicted DAH (Mangoni et al. 2008). The limited available evidence suggests that higher concentrations of methylated arginines pre-HD, and/or their acute HD-induced reduction, might increase the risk of DAH through reduced inhibition of NO synthesis, with consequent vasodilatation and reduced blood pressure. Further research is needed to corroborate this hypothesis in larger study populations, including ESRD patients on PD.

Progression to ESRD

Three studies have investigated the impact of methylated arginines on the progression to ESRD, HD, or PD, in patients with CKD (Ravani et al. 2005; Lajer et al. 2008; Levin et al. 2014). Two studies demonstrated an independent effect of plasma ADMA concentrations in predicting a further deterioration of renal function (Ravani et al. 2005; Lajer et al. 2008). No associations between SDMA and progression to ESRD were observed in one of these studies (Lajer et al. 2008). In another larger study, ADMA did not independently predict progression to ESRD (Levin et al. 2014). Notably, the duration of follow-up in this study was relatively short, 12 months, compared with the first two studies, 27 months and 11.3 years, respectively.

Cardiovascular Events and Mortality

The impact of methylated arginines on the risk of fatal/nonfatal cardiovascular events, e.g., myocardial infarction (MI), stroke, and peripheral arterial occlusive events, in CKD/ESRD has been extensively studied. Eight studies demonstrated that higher plasma ADMA concentrations significantly predict cardiovascular events in this population (Lajer et al. 2008; Zoccali et al. 2001; Mallamaci et al. 2004, 2005; Testa et al. 2005; Young et al. 2009; Tripepi et al. 2011; Ignjatovic et al. 2013). Furthermore, two studies demonstrated an interaction effect between ADMA and other biomarkers, i.e., eNOS genetic polymorphism, C-reactive protein, and brain

natriuretic peptide (Mallamaci et al. 2005; Testa et al. 2005). As a result of this interaction, the predictive capacity was significantly improved when the biomarkers were considered together, rather than separately. By contrast, one study did not report significant associations (Zoccali et al. 2004), whereas another study did report an inverse association between plasma ADMA concentrations and risk of cardiovascular events (Busch et al. 2006). Although the results of the study from Busch et al. are intriguing, a mixed cohort of CKD, ESRD, and renal transplant patients was considered in the analysis. This is potentially relevant as there is evidence that ADMA and SDMA plasma concentrations significantly decrease in renal transplant patients, particularly in the early post-transplant phase (Claes et al. 2014). Finally, three studies have investigated whether SDMA plasma concentrations predict cardiovascular events. However, none of them reported significant associations (Lajer et al. 2008; Zoccali et al. 2001; Ignjatovic et al. 2013).

All-Cause Mortality

Eleven studies have investigated the predictive role of ADMA on all-cause mortality, either as separate or composite end-point, in CKD/ESRD patients. Positive independent associations between higher plasma ADMA concentrations and risk of all-cause mortality were reported in ten studies (Ravani et al. 2005; Levin et al. 2014; Zoccali et al. 2001; Mallamaci et al. 2004, 2005; Young et al. 2009; Tripepi et al. 2011; Ignjatovic et al. 2013; Aucella et al. 2009; Lu et al. 2011). An interaction between ADMA, C-reactive protein, and brain natriuretic peptide was also shown to significantly enhance the predictive capacity of each biomarker alone (Mallamaci et al. 2005). By contrast, one study reported no significant associations between ADMA and all-cause mortality (Lajer et al. 2008). Similarly to the studies on adverse cardiovascular outcomes, four studies failed to report significant associations between plasma SDMA concentrations and all-cause mortality (Lajer et al. 2008; Zoccali et al. 2001; Aucella et al. 2009).

Clinical Outcomes in Renal Transplant Patients

Two studies have specifically investigated the predictive role of methylated arginines in renal transplant patients. A small study reported a significant association between plasma ADMA concentrations and risk of acute rejection (Esposito et al. 2009). Another larger study with a relatively long follow-up showed that plasma ADMA concentrations independently predicted a number of adverse outcomes, including graft failure, fatal and nonfatal cardiovascular events, and all-cause mortality (Abedini et al. 2010).

Potential Applications to Prognosis, Other Diseases, or Conditions

The published evidence supports the role of methylated arginines, particularly ADMA, in predicting adverse clinical outcomes in CKD and ESRD patients. Higher plasma ADMA concentrations were associated with an increased risk of

Table 2 Prospective studies on methylated arginines, morbidity, and mortality in renal disease. Studies investigating the capacity of methylated arginine concentrations to predict morbidity and mortality in different cohorts of patients with renal disease. Information regarding the clinical and demographic characteristics of the patient population, the type of methylated arginines measured, the duration of follow-up, the type of end-points assessed, and a summary of the results is provided for each study

References	Population	Methylated arginines (determination)	Follow-up	End-points	Results
(Zoccali et al. 2001)	ESRD on HD	ADMA and SDMA (HPLC)	33.4 ± 14.6 months	CV events[#]	HR (95 % CI) per 1 μmol/L increase[a]
	$N = 225$			All-cause mortality[*]	ADMA 1.17 (1.04–1.33)[#]
	Age 59.9 ± 15.1 years				SDMA 1.00 (0.88–1.14)[#]
					ADMA 1.26 (1.11–1.41)[*]
					SDMA 1.06 (0.94–1.18)[*]
(Mallamaci et al. 2004)	ESRD on HD	ADMA	42.3 months (range 0.2–70.5)	CV events[#]	HR (95 % CI) per 1 μmol/L increase[b]
	$N = 224$	(HPLC)		All-cause mortality[*]	ADMA 1.19 (1.08–1.32)[#]
	Age not provided for total population				ADMA 1.22 (1.11–1.35)[*]
(Zoccali et al. 2004)	ESRD on HD or PD	ADMA	41 ± 22 months	CV events	HR (95 % CI) per 1 μmol/L increase [c]
	$N = 254$	(HPLC)			ADMA 1.06 (0.96–1.16)
	Age not provided for total population				
(Bergamini et al. 2004)	ESRD on HD	ADMA	–	DAH	ADMA higher in DAH versus non-DAH patients (6.23 ± 1.04 vs. 3.62 ± 0.62 μmol/L, $P < 0.05$)
	$N = 20$	SDMA			SDMA not significantly different between DAH and non-DAH patients
	Age not provided for total population	(HPLC)			(7.21 ± 1.02 vs. 6.30 ± 0.72 μmol/L) $P < 0.05$)

(Mallamaci et al. 2005)	ESRD on HD or PD	ADMA	34 ± 16 months	CV mortality[#]	Predictive value added[d]
	$N = 246$	(HPLC)		All-cause mortality[*]	ADMA: +3.3 %[#]
	Age 60.2 ± 15.3 years				CRP + ADMA: +8.4 %[#]
					CRP + ADMA + BNP: +10.5 %[#]
					Predictive value added[e]
					ADMA: +5.6 %[*]
					CRP + ADMA: +9.0 %[*]
					CRP + ADMA + BNP: +11.6 %[*]
(Ravani et al. 2005)	CKD	ADMA	27.0 months (range 3.4–36.0)	Progression to ESRD or all-cause mortality	HR (95 % CI) per 0.1 μmol/L increase[f]
	$N = 131$	(ELISA)			ADMA 1.20 (1.07–1.35)
	Age 71.1 ± 11.0 years				
(Testa et al. 2005)	ESRD on HD or PD	ADMA	42 ± 22 months	CV mortality	HR (95 % CI) per 1.0 μmol/L increase[g]
	$N = 261$	(HPLC)			ADMA 1.20 (1.07–1.34)
	Age not provided for total population				HR (95 % CI) of eNOS T allele ≥1 + ADMA >75th percentile versus eNOS T allele ≥1 + ADMA <75th percentile
					2.71 (1.38–5.35)
(Busch et al. 2006)	CKD + ESRD on HD + post-kidney transplant	ADMA	Median 24 months (range 1–52)	CV events[#]	ADMA highest versus lowest quartile 0.27 (0.10–0.71)[h#]
	$N = 200$	SDMA		Initiation or re-initiation of HD[*]	
	Age 57.6 ± 13.0 years	(HPLC)			HR (95 % CI) per 1.0 μmol/L increase[i]
					SDMA 1.63 (1.15–2.32)[*]

(*continued*)

Table 2 (continued)

References	Population	Methylated arginines (determination)	Follow-up	End-points	Results
(Lajer et al. 2008)	T1DN	ADMA	11.3 years (range 0.0–12.9)	Fatal and nonfatal CV events[#]	HR (95 % CI) above versus below median[l]
	$N = 397$	SDMA		Decline in GFR[*]	ADMA 2.05 (1.31–3.20)[#]
	Age 42.1 ± 10.5 years	(HPLC)		Progression to ESRD[^]	SDMA NS[#]
				All- cause mortality[~]	ADMA 0.9 (0.0–1.7)[*]
					SDMA NS[*]
					ADMA 1.85 (0.99–3.46)[^]
					SDMA NS[^]
					ADMA NS[~]
					SDMA NS[~]
(Mangoni et al. 2008)	ESRD on HD	ADMA	–	DAH	OR (95 % CI) per 0.10 μmol/L increase[m]
	$N = 52$	SDMA			SDMA 1.31 (1.04–1.65)
	Age 64.4 ± 13.4 years	(LCMS)			
(Young et al. 2009)	CKD	ADMA	9.5 years (range 0.2–11.6)	CV mortality[#]	HR (95 % CI) per 0.25 μmol/L increase[n]
	$N = 821$	(ELISA)		All-cause mortality[*]	ADMA 1.19 (1.00–1.42)[#]
	Age 52 ± 12 years				ADMA 1.09 (0.99–1.26)[*]
(Aucella et al. 2009)	ESRD on HD or PD	ADMA	56 ± 28 months	All-cause mortality	HR (95 % CI) per 1.0 μmol/L increase[o]
	$N = 288$	SDMA			ADMA 1.92 (1.16–3.16)
	Age 58 ± 16 years	(HPLC)			SDMA NS
(Esposito et al. 2009)	Post-kidney transplant	ADMA	6 months	Acute rejection	ADMA associated with acute rejection in multivariate analysis ($P < 0.05$, variables not provided)
	$N = 41$	(ELISA)			
	Age 49.9 ± 12.9 years				

(Abedini et al. 2010)	Post-kidney transplant	ADMA	5–6 years	GFDSC[#]	HR (95 % CI)[p]
	$N = 1{,}847$	(HPLC)		MACE[*]	ADMA 2.78 (1.22–5.82)[#]
	Age 30–75 years			CV mortality or nonfatal MI[^]	ADMA 2.61 (1.03–6.61)[*]
				Stroke[~]	ADMA 4.90 (1.70–14.10)[^]
				All-cause mortality[&]	ADMA 7.63 (2.52–23.13)[~]
					ADMA 4.87 (2.12–11.18)[&]
(Lu et al. 2011)	CKD	ADMA	2.9 ± 1.2 years	All-cause mortality, nonfatal MI or stroke[#]	HR (95 % CI) per 0.1 μmol/L increase[q]
	$N = 298$	(HPLC)		All-cause mortality[*]	ADMA 1.37 (1.09–1.73)[#]
	Age 73 ± 10 years				ADMA 1.25 (0.94–1.66)[*]
(Tripepi et al. 2011)	ESRD on HD	ADMA	156 months	Fatal and nonfatal CV events[#]	HR (95 % CI) per 1.0 μmol/L increase[r]
	$N = 225$	(HPLC)		All-cause mortality[*]	ADMA 1.18 (1.07–1.30)[#]
	Age 60 ± 15 years				ADMA 1.22 (1.12–1.34)[*]
(Ignjatovic et al. 2013)	ESRD on HD	ADMA	3 years	CV mortality[#]	HR (95 % CI) per 1.0 μmol/L increase[s]
	$N = 153$	SDMA		All-cause mortality[*]	ADMA 1.51 (0.99–2.28)[#]
	Age not provided for total population	(HPLC)			SDMA 0.21 (0.03–1.48)[#]
					ADMA 1.71 (1.32–2.21)[*]
					SDMA 1.41 (0.56–3.56)[*]

(*continued*)

Table 2 (continued)

References	Population	Methylated arginines (determination)	Follow-up	End-points	Results
(Levin et al. 2014)	CKD	ADMA	12 months	Progression to renal replacement therapy[#]	HR (95 % CI) per 0.11 μmol/L increase[t]
	$N = 2{,}544$	(ELISA)		All-cause mortality[*]	ADMA 1.03 (0.88–1.20)[#]
	Age 68.1 ± 12.7 years				ADMA 1.13 (1.01–1.29)[*]

CKD chronic kidney disease, *ESRD* end-stage renal disease, *ADMA* asymmetric dimethylarginine, *SDMA* symmetric dimethylarginine, *CRP* C-reactive protein, *BNP* brain natriuretic peptide, *HD* hemodialysis, *PD* peritoneal dialysis, *CV* cardiovascular, *HPLC* high-performance liquid chromatography, *HR* hazard ratio, *CI* confidence interval, *DAH* dialysis-associated hypotension, *ELISA* enzyme-linked immunosorbent assay, *eNOS* endothelial nitric oxide synthase, *T1DN* type 1 diabetic nephropathy, *GFR* glomerular filtration rate, *LCMS* liquid chromatography-mass spectrometry, *NS* nonsignificant, *GFDSC* graft failure or doubling of serum creatinine, *MACE* major adverse cardiovascular events, *MI* myocardial infarction

[a]Adjusted for arginine, SDMA, age, sex, previous cardiovascular events, systolic blood pressure, smoking, diabetes, total cholesterol, LDL cholesterol, fibrinogen, C-reactive protein, homocysteine, hemoglobin, calcium phosphate product, albumin, duration of dialysis, fractional urea clearance

[b]Adjusted for age, sex, smoking, diabetes, previous cardiovascular events, cholesterol, blood pressure, antihypertensive treatment, fractional urea clearance, duration of dialysis, hemoglobin, albumin, calcium phosphate product, C-reactive protein, homocysteine, plasma norepinephrine

[c]Adjusted for age, ejection fraction (or endocardial fractional shortening or mid-wall fractional shortening), previous cardiovascular events, smoking, dialysis modality, antihypertensive treatment, circumferential end-systolic stress, systolic blood pressure, diabetes, albumin, C-reactive protein

[d]Basic model including age, gender, smoking, diabetes, comorbidity score

[e]Basic model including age, gender, smoking, diabetes, albumin, dialysis modality, comorbidity score

[f]Adjusted for homocysteine, hemoglobin, proteinuria, glomerular filtration rate

[g]Adjusted for endothelial nitric oxide polymorphisms, age, gender, smoking, previous cardiovascular events, diabetes, pulse pressure, albumin, C-reactive protein

[h]Adjusted for hemodialysis, diabetes, age, body mass index, history of cardiovascular disease, albumin, C-reactive protein, hemoglobin, creatinine

[i]Adjusted for renal transplant, age, hypertension, albumin, C-reactive protein, hemoglobin, creatinine, LDL cholesterol

[l]Adjusted for sex, age, glycated hemoglobin, systolic blood pressure, glomerular filtration rate, cholesterol, smoking, previous cardiovascular events, antihypertensive treatment

[m]Adjusted for age, gender, baseline systolic blood pressure, hypertension, diabetes

[n]Adjusted for age, gender, race, randomization assignment, history of cardiovascular disease, diabetes, smoking, diastolic blood pressure, LDL cholesterol, HDL cholesterol, C-reactive protein, body mass index, glomerular filtration rate, proteinuria, cause of kidney disease

[o]Adjusted for diabetes, albumin, age, treatment modality, previous cardiovascular events, gender, duration of dialysis, hemoglobin, antihypertensive treatment, smoking, homocysteine, systolic blood pressure, cholesterol
[p]Adjusted for age, gender, systolic blood pressure, previous coronary artery disease, diabetes, LDL cholesterol, smoking, creatinine
[q]Adjusted for age, gender, hypercholesterolemia, diabetes, glomerular filtration rate, hypertension, smoking
[r]Adjusted for age, gender, smoking, diabetes, cholesterol, systolic blood pressure, cardiovascular comorbidities, antihypertensive treatment, duration of dialysis, albumin, hemoglobin, calcium phosphate product, homocysteine, C-reactive protein, interleukin-6
[s]Adjusted for age, gender, smoking, C-reactive protein, serum amyloid, albumin
[t]Adjusted for age, gender, history of cardiovascular disease, glomerular filtration rate, serum phosphate, albumin
[*]all-cause mortality, initiation of HD, decline in GFR, or MACE
[#]CV events, CV mortality, GFDSC, all-cause mortality, nonfatal MI or stroke, progression to renal replacement therapy
[^]progression to ESRD, CV mortality or nonfatal MI
[~]all-cause mortality
[&]all-cause mortality

cardiovascular morbidity and mortality and all-cause mortality in studies with different population characteristics, renal disease etiology, and follow-up. The predictive capacity of ADMA in these studies was accounted for a number of clinical, demographic, and biochemical variables associated per se with adverse outcomes in this patient group. Therefore, plasma ADMA concentrations can independently predict risk in renal disease patients. Some studies have also demonstrated that the inclusion of other biomarkers, e.g., C-reactive protein, in ADMA-based statistical models can further enhance risk prediction. By contrast, published studies failed to demonstrate a predictive role for SDMA, except for one study demonstrating independent associations with DAH and another study showing an increased risk of undergoing HD in a mixed cohort of CKD, ESRD, and renal transplant patients (Mangoni et al. 2008; Busch et al. 2006). Virtually no evidence is available on the predictive role of MMA in renal disease patients.

Generalizability of Findings

Several potential issues might limit the applicability of the findings of published studies on the prognostic role of methylated arginines in CKD/ESRD patients. First, as previously discussed, the concentrations of methylated arginines reported in these studies significantly depend on the technique used for their determination. Second, the majority of studies recruited either middle-aged or older patients <80 years (Table 2). As both CKD and ESRD affect pediatric patients and adolescents as well as patients ≥ 80 years, further clinical studies are required to ascertain whether plasma methylated arginines, particularly ADMA, have similar predictive capacity in these age groups. Of note, age is a significant determinant of plasma methylated arginine concentrations (Schwedhelm et al. 2009). Third, the available evidence has been largely derived from white Caucasian populations. Recent studies have highlighted significant differences in the concentrations of methylated arginines and other arginine metabolites between different ethnic groups (Sydow et al. 2010). This suggests an interaction between genetic polymorphisms and environmental factors in modulating arginine metabolic pathways. Fourth, although a limited number of studies failed to demonstrate a significant impact of SDMA in CKD/ESRD patients, there is increasing evidence of its predictive role in other related disease states (Meinitzer et al. 2011). Further larger studies in renal disease patients should better clarify the role of SDMA, and MMA, in risk stratification.

Methylated Arginines and Other Disease States

There is increasing evidence of the predictive capacity of methylated arginines in other disease states, particularly those related to the pathophysiology of atherosclerosis and thrombosis. ADMA and SDMA have been associated with adverse outcomes in patients with type 2 diabetes (Krzyzanowska et al. 2007) and ischemic heart disease (Meinitzer et al. 2011).

Applications in Clinical Practice

For its routine use in clinical practice, a biomarker should possess several key characteristics, e.g., (1) it should be easily measurable at population level by means of robust and relatively inexpensive techniques; (2) it should be independently associated with the risk of disease onset, progression, or outcomes in a predictable fashion; and (3) it should be modifiable by means of pharmacological and/or non-pharmacological interventions. The measurement of methylated arginines plasma/serum concentrations can be reliably accomplished by means of HPLC and LCMS methods. Although the determination of methylated arginines is currently limited to research, a widespread use in the clinical setting might be justified on the base of cost-effectiveness. The latter would require further confirmation of the role of methylated arginines in specific disease states as well as their capacity to significantly enhance risk stratification and reclassification. Both in vitro and in vivo studies have investigated the pharmacological modulation of methylated arginine concentrations, particularly ADMA. There is evidence that some lipid-lowering agents, angiotensin receptor blockers, and beta-blockers can enhance or restore DDAH activity in experimental studies (Wadham and Mangoni 2009). Both angiotensin-converting enzyme inhibitors and angiotensin receptor blockers have been shown to reduce plasma ADMA concentrations in human studies (Delles et al. 2002). However, other studies, also investigating the effects of beta-blockers, have not confirmed these findings (Kelly et al. 2012). As both angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are the cornerstone of management in patients with renal disease, further research is warranted to establish whether their renoprotective effects are mediated by their modulatory effects on methylated arginine concentrations.

Future Research Directions

Although there have been significant advances in knowledge regarding the pathophysiological and prognostic role of methylated arginines in renal disease, the available evidence has also identified important knowledge gaps. The latter should encourage further investigations in this area. For example, relatively little is known on the role of methylated arginines in predicting further deterioration in renal function, particularly in CKD patients, and the risk of acute and post-acute renal transplant rejection. The availability of data from published studies on the effect size in both CKD and ESRD patients should allow a robust power sample size calculation as well as the selection of appropriate clinical, demographic, and biochemical confounders for multivariate analyses, in such further studies.

Additional in vitro and in vivo studies should investigate the interaction between ADMA, MMA, and markers of NO synthesis. Although it is well established that ADMA and MMA are competitive inhibitors of NOS enzymes, positive associations between ADMA, MMA, and nitrite/nitrate concentrations have been reported in ESRD (Bouteldja et al. 2013). This supports the concept of NO-mediated inhibition

of DDAH activity to prevent excessive NO synthesis, primarily by the inducible NOS isoform iNOS, in the presence of a pro-inflammatory environment, such as CKD or ESRD.

Another opportunity for further research is the assessment of the potential enhancement of predictive capacity using combined versus single biomarkers. Two studies have already provided evidence that adding either specific genetic polymorphisms or biochemical surrogate markers can significant increase the predictive capacity of ADMA-based statistical models (Mallamaci et al. 2005; Testa et al. 2005). The increasing availability of analytical platforms able to perform combined measurements of one or more biochemical pathways from the same biological sample offer a number of opportunities in this context.

Summary Points

- This chapter focuses on the biology and the pathophysiology of the endogenous arginine analogues asymmetric dimethylarginine, symmetric dimethylarginine, and monomethylarginine and their role in renal disease.
- Asymmetric dimethylarginine, symmetric dimethylarginine, and monomethylarginine inhibit nitric oxide synthesis either directly or indirectly.
- Experimental and human studies have shown that the kidney is an important elimination site of methylated arginines.
- Plasma asymmetric dimethylarginine, symmetric dimethylarginine, and monomethylarginine concentrations are increased in patients with different degrees of renal disease.
- Plasma concentrations of methylated arginines, particularly asymmetric dimethylarginine, independently predict the risk of adverse cardiovascular events and all-cause mortality in patients with renal disease.

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References

Abedini S, Meinitzer A, Holme I, et al. Asymmetrical dimethylarginine is associated with renal and cardiovascular outcomes and all-cause mortality in renal transplant recipients. Kidney Int. 2010;77:44–50.

Albsmeier J, Schwedhelm E, Schulze F, et al. Determination of NG, NG-dimethyl-L-arginine, an endogenous NO synthase inhibitor, by gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2004;809:59–65.

Anderstam B, Katzarski K, Bergstrom J. Serum levels of NG, NG-dimethyl-L-arginine, a potential endogenous nitric oxide inhibitor in dialysis patients. J Am Soc Nephrol. 1997;8:1437–42.

Arikan E, Karadag CH, Guldiken S. Asymmetric dimethylarginine levels in thyroid diseases. J Endocrinol Invest. 2007;30:186–91.

Aucella F, Maas R, Vigilante M, et al. Methylarginines and mortality in patients with end stage renal disease: a prospective cohort study. Atherosclerosis. 2009;207:541–5.

Bergamini S, Vandelli L, Bellei E, et al. Relationship of asymmetric dimethylarginine to haemodialysis hypotension. Nitric Oxide. 2004;11:273–8.
Bishop MJ, Crow B, Norton D, et al. Direct analysis of un-derivatized asymmetric dimethylarginine (ADMA) and L-arginine from plasma using mixed-mode ion-exchange liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;859:164–9.
Blackwell S, O'Reilly DS, Talwar DK. HPLC analysis of asymmetric dimethylarginine (ADMA) and related arginine metabolites in human plasma using a novel non-endogenous internal standard. Clin Chim Acta. 2009;401:14–9.
Boger RH, Bode-Boger SM, Szuba A, et al. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. Circulation. 1998;98:1842–7.
Bouteldja N, Woodman RJ, Hewitson CL, et al. Methylated arginines and nitric oxide in end-stage renal disease: impact of inflammation, oxidative stress and haemodialysis. Biomarkers. 2013;18:357–64.
Busch M, Fleck C, Wolf G, et al. Asymmetrical (ADMA) and symmetrical dimethylarginine (SDMA) as potential risk factors for cardiovascular and renal outcome in chronic kidney disease – possible candidates for paradoxical epidemiology? Amino Acids. 2006;30:225–32.
Causse E, Siri N, Arnal JF, et al. Determination of asymmetrical dimethylarginine by capillary electrophoresis-laser-induced fluorescence. J Chromatogr B Biomed Sci Appl. 2000;741:77–83.
Chae JW, Baek IH, Kang W, et al. Simultaneous determination of L-arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in the plasma of rodents with LC-MS/MS. Arzneimittelforschung. 2011;61:340–6.
Chirinos JA, David R, Bralley JA, et al. Endogenous nitric oxide synthase inhibitors, arterial hemodynamics, and subclinical vascular disease: the PREVENCION Study. Hypertension. 2008;52:1051–9.
Claes KJ, Bammens B, Kuypers DR, et al. Time course of asymmetric dimethylarginine and symmetric dimethylarginine levels after successful renal transplantation. Nephrol Dial Transplant. 2014;29:1965–72.
D'Apolito O, Paglia G, Tricarico F, et al. Development and validation of a fast quantitative method for plasma dimethylarginines analysis using liquid chromatography-tandem mass spectrometry. Clin Biochem. 2008;41:1391–5.
Davids M, Swieringa E, Palm F, et al. Simultaneous determination of asymmetric and symmetric dimethylarginine, L-monomethylarginine, L-arginine, and L-homoarginine in biological samples using stable isotope dilution liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;900:38–47.
Delles C, Schneider MP, John S, et al. Angiotensin converting enzyme inhibition and angiotensin II AT1-receptor blockade reduce the levels of asymmetrical N(G), N(G)-dimethylarginine in human essential hypertension. Am J Hypertens. 2002;15:590–3.
Desiderio C, Rossetti DV, Messana I, et al. Analysis of arginine and methylated metabolites in human plasma by field amplified sample injection capillary electrophoresis tandem mass spectrometry. Electrophoresis. 2010;31:1894–902.
Di Gangi IM, Chiandetti L, Gucciardi A, et al. Simultaneous quantitative determination of N(G), N(G)-dimethyl-L-arginine or asymmetric dimethylarginine and related pathway's metabolites in biological fluids by ultrahigh-performance liquid chromatography/electrospray ionization-tandem mass spectrometry. Anal Chim Acta. 2010;677:140–8.
Di Gangi IM, Pirillo P, Carraro S, et al. Online trapping and enrichment ultra performance liquid chromatography-tandem mass spectrometry method for sensitive measurement of "arginine-asymmetric dimethylarginine cycle" biomarkers in human exhaled breath condensate. Anal Chim Acta. 2012;754:67–74.
El-Khoury JM, Bunch DR, Reineks E, et al. A simple and fast liquid chromatography-tandem mass spectrometry method for measurement of underivatized L-arginine, symmetric dimethylarginine, and asymmetric dimethylarginine and establishment of the reference ranges. Anal Bioanal Chem. 2012;402:771–9.

Esposito C, Grosjean F, Torreggiani M, et al. Increased asymmetric dimethylarginine serum levels are associated with acute rejection in kidney transplant recipients. Transplant Proc. 2009;41:1570–3.

Gansevoort RT, Correa-Rotter R, Hemmelgarn BR, et al. Chronic kidney disease and cardiovascular risk: epidemiology, mechanisms, and prevention. Lancet. 2013;382:339–52.

Garcia RG, Perez M, Maas R, et al. Plasma concentrations of asymmetric dimethylarginine (ADMA) in metabolic syndrome. Int J Cardiol. 2007;122:176–8.

Gardiner SM, Kemp PA, Bennett T, et al. Regional and cardiac haemodynamic effects of NG, NG, dimethyl-L-arginine and their reversibility by vasodilators in conscious rats. Br J Pharmacol. 1993;110:1457–64.

Gervasoni J, Bonelli F, Zuppi C, et al. Determination of asymmetric dimethyl arginine in human serum by liquid chromatography-tandem mass spectrometry: clinical application in hypertensive subjects. Clin Chem Lab Med. 2011;49:2109–15.

Gocmen AY, Sahin E, Kocak H, et al. Levels of asymmetric dimethylarginine, nitric oxide and lipid peroxidation markers in patients with end-stage renal disease having peritoneal dialysis treatment. Clin Biochem. 2008;41:836–40.

Gopu CL, Hari PR, George R, et al. Simultaneous determination of homocysteine and asymmetric dimethylarginine in human urine by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2013;939:32–7.

Heresztyn T, Worthley MI, Horowitz JD. Determination of l-arginine and NG, NG - and NG, NG' -dimethyl-L-arginine in plasma by liquid chromatography as AccQ-Fluor fluorescent derivatives. J Chromatogr B Analyt Technol Biomed Life Sci. 2004;805:325–9.

Huang LF, Guo FQ, Liang YZ, et al. Simultaneous determination of L-arginine and its mono- and dimethylated metabolites in human plasma by high-performance liquid chromatography-mass spectrometry. Anal Bioanal Chem. 2004;380:643–9.

Hui Y, Wong M, Kim JO, et al. A new derivatization method coupled with LC-MS/MS to enable baseline separation and quantification of dimethylarginines in human plasma from patients to receive on-pump CABG surgery. Electrophoresis. 2012;33:1911–20.

Ignjatovic AM, Cvetkovic TP, Pavlovic RM, et al. Endothelial dysfunction, inflammation and malnutrition markers as predictors of mortality in dialysis patients: multimarker approach. Int Urol Nephrol. 2013;45:1715–24.

Ivanova M, Artusi C, Boffa GM, et al. HPLC determination of plasma dimethylarginines: method validation and preliminary clinical application. Clin Chim Acta. 2010;411:1632–6.

Jones CE, Darcy CJ, Woodberry T, et al. HPLC analysis of asymmetric dimethylarginine, symmetric dimethylarginine, homoarginine and arginine in small plasma volumes using a Gemini-NX column at high pH. J Chromatogr B Analyt Technol Biomed Life Sci. 2010;878:8–12.

Kelly AS, Gonzalez-Campoy JM, Rudser KD, et al. Carvedilol-lisinopril combination therapy and endothelial function in obese individuals with hypertension. J Clin Hypertens (Greenwich). 2012;14:85–91.

Krzyzanowska K, Mittermayer F, Wolzt M, et al. Asymmetric dimethylarginine predicts cardiovascular events in patients with type 2 diabetes. Diabetes Care. 2007;30:1834–9.

Lajer M, Tarnow L, Jorsal A, et al. Plasma concentration of asymmetric dimethylarginine (ADMA) predicts cardiovascular morbidity and mortality in type 1 diabetic patients with diabetic nephropathy. Diabetes Care. 2008;31:747–52.

Levin A, Rigatto C, Barrett B, et al. Biomarkers of inflammation, fibrosis, cardiac stretch and injury predict death but not renal replacement therapy at 1 year in a Canadian chronic kidney disease cohort. Nephrol Dial Transplant. 2014;29:1037–47.

Linz TH, Lunte SM. Heat-assisted extraction for the determination of methylarginines in serum by CE. Electrophoresis. 2013;34:1693–700.

Linz TH, Snyder CM, Lunte SM. Optimization of the separation of NDA-derivatized methylarginines by capillary and microchip electrophoresis. J Lab Automot. 2012;17:24–31.

Lu TM, Chung MY, Lin CC, et al. Asymmetric dimethylarginine and clinical outcomes in chronic kidney disease. Clin J Am Soc Nephrol. 2011;6:1566–72.

MacAllister RJ, Rambausek MH, Vallance P, et al. Concentration of dimethyl-L-arginine in the plasma of patients with end-stage renal failure. Nephrol Dial Transplant. 1996;11:2449–52.
Mallamaci F, Tripepi G, Maas R, et al. Analysis of the relationship between norepinephrine and asymmetric dimethyl arginine levels among patients with end-stage renal disease. J Am Soc Nephrol. 2004;15:435–41.
Mallamaci F, Tripepi G, Cutrupi S, et al. Prognostic value of combined use of biomarkers of inflammation, endothelial dysfunction, and myocardiopathy in patients with ESRD. Kidney Int. 2005;67:2330–7.
Mangoni AA, Hewitson CL, Woodman RJ, et al. Symmetric dimethylarginine is an independent predictor of intradialytic hypotension. Am J Hypertens. 2008;21:955–9.
Marescau B, Nagels G, Possemiers I, et al. Guanidino compounds in serum and urine of nondialyzed patients with chronic renal insufficiency. Metabolism. 1997;46:1024–31.
Markowski P, Baranowska I, Baranowski J. Simultaneous determination of L-arginine and 12 molecules participating in its metabolic cycle by gradient RP-HPLC method: application to human urine samples. Anal Chim Acta. 2007;605:205–17.
Marra M, Bonfigli AR, Testa R, et al. High-performance liquid chromatographic assay of asymmetric dimethylarginine, symmetric dimethylarginine, and arginine in human plasma by derivatization with naphthalene-2,3-dicarboxaldehyde. Anal Biochem. 2003;318:13–7.
Martens-Lobenhoffer J, Bode-Boger SM. Simultaneous detection of arginine, asymmetric dimethylarginine, symmetric dimethylarginine and citrulline in human plasma and urine applying liquid chromatography-mass spectrometry with very straightforward sample preparation. J Chromatogr B Analyt Technol Biomed Life Sci. 2003;798:231–9.
Martens-Lobenhoffer J, Bode-Boger SM. Fast and efficient determination of arginine, symmetric dimethylarginine, and asymmetric dimethylarginine in biological fluids by hydrophilic-interaction liquid chromatography-electrospray tandem mass spectrometry. Clin Chem. 2006;52:488–93.
Martens-Lobenhoffer J, Bode-Boger SM. Quantification of L-arginine, asymmetric dimethylarginine and symmetric dimethylarginine in human plasma: a step improvement in precision by stable isotope dilution mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;904:140–3.
Martens-Lobenhoffer J, Krug O, Bode-Boger SM. Determination of arginine and asymmetric dimethylarginine (ADMA) in human plasma by liquid chromatography/mass spectrometry with the isotope dilution technique. J Mass Spectrom. 2004;39:1287–94.
Martens-Lobenhoffer J, Sulyok E, Czeiter E, et al. Determination of cerebrospinal fluid concentrations of arginine and dimethylarginines in patients with subarachnoid haemorrhage. J Neurosci Methods. 2007;164:155–60.
Meinitzer A, Seelhorst U, Wellnitz B, et al. Asymmetrical dimethylarginine independently predicts total and cardiovascular mortality in individuals with angiographic coronary artery disease (the Ludwigshafen Risk and Cardiovascular Health study). Clin Chem. 2007;53:273–83.
Meinitzer A, Kielstein JT, Pilz S, et al. Symmetrical and asymmetrical dimethylarginine as predictors for mortality in patients referred for coronary angiography: the Ludwigshafen Risk and Cardiovascular Health study. Clin Chem. 2011;57:112–21.
Memon L, Spasojevic-Kalimanovska V, Bogavac-Stanojevic N, et al. Assessment of endothelial dysfunction: the role of symmetrical dimethylarginine and proinflammatory markers in chronic kidney disease and renal transplant recipients. Dis Markers. 2013;35:173–80.
Nanayakkara PW, Teerlink T, Stehouwer CD, et al. Plasma asymmetric dimethylarginine (ADMA) concentration is independently associated with carotid intima-media thickness and plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) concentration in patients with mild-to-moderate renal failure. Kidney Int. 2005;68:2230–6.
Napoli C, de Nigris F, Williams-Ignarro S, et al. Nitric oxide and atherosclerosis: an update. Nitric Oxide. 2006;15:265–79.
Nonaka S, Tsunoda M, Imai K, et al. High-performance liquid chromatographic assay of N(G)-monomethyl-L-arginine, N(G), N(G)-dimethyl-L-arginine, and N(G), N(G)'-dimethyl-L-

arginine using 4-fluoro-7-nitro-2, 1,3-benzoxadiazole as a fluorescent reagent. J Chromatogr A. 2005;1066:41–5.

Nonaka S, Tsunoda M, Aoyama C, et al. Determination of NG, NG-dimethyl-L-arginine in rat plasma and dimethylarginine dimethylaminohydrolase activity in rat kidney using a monolithic silica column. J Chromatogr B Analyt Technol Biomed Life Sci. 2006;843:170–4.

Pettersson A, Uggla L, Backman V. Determination of dimethylated arginines in human plasma by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 1997;692:257–62.

Pi J, Kumagai Y, Sun G, et al. Improved method for simultaneous determination of L-arginine and its mono- and dimethylated metabolites in biological samples by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 2000;742:199–203.

Ravani P, Tripepi G, Malberti F, et al. Asymmetrical dimethylarginine predicts progression to dialysis and death in patients with chronic kidney disease: a competing risks modeling approach. J Am Soc Nephrol. 2005;16:2449–55.

Reimann M, Schutte AE, Malan NT, et al. Asymmetric dimethylarginine is associated with parameters of glucose metabolism in Caucasian but not in African women from South Africa. Exp Clin Endocrinol Diabetes. 2007;115:600–5.

Schulze F, Wesemann R, Schwedhelm E, et al. Determination of asymmetric dimethylarginine (ADMA) using a novel ELISA assay. Clin Chem Lab Med. 2004;42:1377–83.

Schutte AE, Schutte R, Huisman HW, et al. Dimethylarginines: their vascular and metabolic roles in Africans and Caucasians. Eur J Endocrinol. 2010;162:525–33.

Schwedhelm E, Maas R, Tan-Andresen J, et al. High-throughput liquid chromatographic-tandem mass spectrometric determination of arginine and dimethylated arginine derivatives in human and mouse plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;851:211–9.

Schwedhelm E, Xanthakis V, Maas R, et al. Asymmetric dimethylarginine reference intervals determined with liquid chromatography-tandem mass spectrometry: results from the Framingham offspring cohort. Clin Chem. 2009;55:1539–45.

Servillo L, Giovane A, D'Onofrio N, et al. Determination of homoarginine, arginine, NMMA, ADMA, and SDMA in biological samples by HPLC-ESI-mass spectrometry. Int J Mol Sci. 2013;14:20131–8.

Shi B, Ni Z, Zhou W, et al. Circulating levels of asymmetric dimethylarginine are an independent risk factor for left ventricular hypertrophy and predict cardiovascular events in pre-dialysis patients with chronic kidney disease. Eur J Intern Med. 2010;21:444–8.

Shin S, Fung SM, Mohan S, et al. Simultaneous bioanalysis of L-arginine, L-citrulline, and dimethylarginines by LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2011;879:467–74.

Sotgia S, Zinellu A, Pinna GA, et al. A new selective pre-column ninhydrin-based derivatization for a RP-HPLC determination of plasma asymmetric dimethyl-L-arginine (ADMA) by fluorescence detection. Amino Acids. 2008;34:677–82.

Stuhlinger MC, Abbasi F, Chu JW, et al. Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. JAMA. 2002;287:1420–6.

Surdacki A, Kruszelnicka O, Rakowski T, et al. Asymmetric dimethylarginine predicts decline of glucose tolerance in men with stable coronary artery disease: a 4.5-year follow-up study. Cardiovasc Diabetol. 2013;12:64.

Sydow K, Fortmann SP, Fair JM, et al. Distribution of asymmetric dimethylarginine among 980 healthy, older adults of different ethnicities. Clin Chem. 2010;56:111–20.

Teerlink T. Determination of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine in biological samples by HPLC. Methods Mol Med. 2005;108:263–74.

Teerlink T, Nijveldt RJ, de Jong S, et al. Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. Anal Biochem. 2002;303:131–7.

Tenderenda-Banasiuk E, Wasilewska A, Taranta-Janusz K, et al. Asymmetric and symmetric dimethylarginine in adolescents with hyperuricemia. Dis Markers. 2013;35:407–12.
Testa A, Spoto B, Tripepi G, et al. The GLU298ASP variant of nitric oxide synthase interacts with asymmetric dimethyl arginine in determining cardiovascular mortality in patients with end-stage renal disease. J Hypertens. 2005;23:1825–30.
Tripepi G, Mattace Raso F, Sijbrands E, et al. Inflammation and asymmetric dimethylarginine for predicting death and cardiovascular events in ESRD patients. Clin J Am Soc Nephrol. 2011;6:1714–21.
Tsikas D, Schubert B, Gutzki FM, et al. Quantitative determination of circulating and urinary asymmetric dimethylarginine (ADMA) in humans by gas chromatography-tandem mass spectrometry as methyl ester tri(*N*-pentafluoropropionyl) derivative. J Chromatogr B Analyt Technol Biomed Life Sci. 2003;798:87–99.
Tsunoda M, Nonaka S, Funatsu T. Determination of methylated arginines by column-switching high-performance liquid chromatography-fluorescence detection. Analyst. 2005;130:1410–3.
Ueno S, Sano A, Kotani K, et al. Distribution of free methylarginines in rat tissues and in the bovine brain. J Neurochem. 1992;59:2012–6.
Vallance P, Leiper J. Cardiovascular biology of the asymmetric dimethylarginine:dimethylarginine dimethylaminohydrolase pathway. Arterioscler Thromb Vasc Biol. 2004;24:1023–30.
Veldink H, Faulhaber-Walter R, Park JK, et al. Effects of chronic SDMA infusion on glomerular filtration rate, blood pressure, myocardial function and renal histology in C57BL6/J mice. Nephrol Dial Transplant. 2013;28:1434–9.
Vishwanathan K, Tackett RL, Stewart JT, et al. Determination of arginine and methylated arginines in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Biomed Sci Appl. 2000;748:157–66.
Wadham C, Mangoni AA. Dimethylarginine dimethylaminohydrolase regulation: a novel therapeutic target in cardiovascular disease. Expert Opin Drug Metab Toxicol. 2009;5:303–19.
Wang D, Strandgaard S, Iversen J, et al. Asymmetric dimethylarginine, oxidative stress, and vascular nitric oxide synthase in essential hypertension. Am J Physiol Regul Integr Comp Physiol. 2009;296:R195–200.
Wilkinson IB, Qasem A, McEniery CM, et al. Nitric oxide regulates local arterial distensibility in vivo. Circulation. 2002;105:213–7.
Yi L, Zhang P, Ji X, et al. Quantitation of L-arginine and asymmetric dimethylarginine in human plasma by LC-selective ion mode-MS for Type 2 diabetes mellitus study. Chem Pharm Bull (Tokyo). 2011;59:839–43.
Young JM, Terrin N, Wang X, et al. Asymmetric dimethylarginine and mortality in stages 3 to 4 chronic kidney disease. Clin J Am Soc Nephrol. 2009;4:1115–20.
Zhang WZ, Kaye DM. Simultaneous determination of arginine and seven metabolites in plasma by reversed-phase liquid chromatography with a time-controlled ortho-phthaldialdehyde precolumn derivatization. Anal Biochem. 2004;326:87–92.
Zinellu A, Sotgia S, Zinellu E, et al. High-throughput CZE-UV determination of arginine and dimethylated arginines in human plasma. Electrophoresis. 2007a;28:1942–8.
Zinellu A, Sotgia S, Scanu B, et al. Assessment of protein-incorporated arginine methylation in biological specimens by CZE UV-detection. Electrophoresis. 2007b;28:4452–8.
Zinellu A, Sotgia S, Usai MF, et al. Improved method for plasma ADMA, SDMA, and arginine quantification by field-amplified sample injection capillary electrophoresis UV detection. Anal Bioanal Chem. 2011;399:1815–21.
Zoccali C, Bode-Boger S, Mallamaci F, et al. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. Lancet. 2001;358:2113–7.
Zoccali C, Benedetto FA, Maas R, et al. Asymmetric dimethylarginine, C-reactive protein, and carotid intima-media thickness in end-stage renal disease. J Am Soc Nephrol. 2002a;13:490–6.

Zoccali C, Mallamaci F, Maas R, et al. Left ventricular hypertrophy, cardiac remodeling and asymmetric dimethylarginine (ADMA) in hemodialysis patients. Kidney Int. 2002b;62:339–45.
Zoccali C, Benedetto FA, Mallamaci F, et al. Prognostic value of echocardiographic indicators of left ventricular systolic function in asymptomatic dialysis patients. J Am Soc Nephrol. 2004;15:1029–37.

Albuminuria as a Biomarker of the Renal Disease

19

Visnja Lezaic

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Abstract

Albuminuria is an early and sensitive marker of kidney damage in diabetic patients, a good predictor of kidney outcome and cardiovascular disease. Screening for albuminuria is important to identify individuals at risk for renal outcome, i.e., developing end-stage renal disease, acute kidney injury, and progressive chronic kidney disease as well as cardiovascular disease and all-cause mortality in both general and high-risk population (diabetes, cardiovascular disease, hypertension, and older patients). Also, it is the most widely used clinical marker of diabetic nephropathy. The terminology of "early diabetic nephropathy" indicated diabetic subjects with albuminuria. In this early phase of diabetic

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nephropathy, the glomerular filtration rate is usually well preserved. In patients with several cardiovascular risks, albuminuria is predicting outcome, even below the level to be taken as normal. It seems that the pathophysiologic mechanisms linking albuminuria to cardiovascular and renal risk are generalized loss of vascular endothelial function in organs. Furthermore, albuminuria can be used as a target for treatment for primary and secondary prevention of renal and cardiovascular disease development. ACE inhibition in subjects with nondiabetic albuminuria may prevent future cardiovascular events. Measurement of albuminuria can help to determine whether or not the patient with hypertension should be treated, how aggressively they should be treated, and what medications we should treat them with.

Keywords
Albuminuria • Healthy subject • Diabetic nephropathy • Screening of chronic kidney disease • Prediction of renal progression • Cardiovascular mortality • Target for treatment • Hypertension

Abbreviations

ACR	Albumin-to-creatinine ratio
CKD	Chronic kidney disease
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
RAAS	Renin–angiotensin–aldosterone system
RRT	Renal replacement therapy
UAER = UAE	Urinary albumin excretion rate

Key Facts of Kidney Function

- Healthy kidneys regulate the body’s fluid levels and filter wastes and toxins from the blood into the urine. Also, they release a hormone that regulates blood pressure, activate vitamin D to maintain healthy bones, release the hormone that directs production of red blood cells, and keep blood minerals in balance (sodium, phosphorus, potassium).
- Patients at risk for kidney disease should have simple blood and urine tests to check if their kidneys are working properly. These include diabetes, high blood pressure, family history of kidney failure and being age 60 or older, kidney stones, smoking, obesity, and cardiovascular disease.
- The glomerular filtration rate is a measure of kidney work to remove wastes from the blood. To assess the glomerular filtration rate is necessary to determine the concentration of creatinine in the serum. Reduction of glomerular filtration rate and increased serum creatinine show renal failure.
- The presence of high albumin concentration in urine indicates renal damage most probably at an early stage. The presence of high protein in the urine may indicate the kidney disease.

- Part of routine health screening is urinalysis. Urine sample is collected from the patient in a specimen cup (about 50 ml). It is evaluated by its physical appearance (color, cloudiness, odor, clarity); macroscopic, chemical, and molecular properties; or microscopic assessment.
- Urine is used to diagnose a urinary tract or kidney infection, to evaluate causes of kidney failure, and to screen for progression of some chronic conditions such as diabetes mellitus and high blood pressure (hypertension).
- Patients with decreased glomerular filtration rate or proteinuria should be evaluated to determine its cause(s).

Definitions

Acute kidney injury (AKI) Acute kidney injury (AKI) is the sharp loss of kidney function, resulting in the retention of urea and other nitrogenous waste products and in the dysregulation of extracellular volume and electrolytes.

Albumin-to-creatinine ratio (ACR) Expression of albuminuria from the ratio of albumin to creatinine in urine, determined in individual sample of urine.

Angiotensin-converting enzyme inhibitors Are drugs that block the production of angiotensin II. The latter is a hormone that circulates in the blood and has many effects on the cardiovascular system; angiotensin II is a strong vasoconstrictor.

Angiotensin II receptor antagonists Are the drugs that modulate the renin–angiotensin–aldosterone system by blocking angiotensin II receptors.

Chronic kidney disease (CKD) Chronic kidney disease, also called **chronic kidney failure**, describes the gradual loss of kidney function. Its stages are based on the patient's level of glomerular filtration rate (GFR) which is a measure of filtering capacity of the glomeruli.

Diabetic nephropathy Is a slow progressive kidney disease that occurs in patients with diabetes.

End-stage renal disease (ESRD) Means glomerular filtration rate below 15 ml/min/1.73 m^2.

Estimated glomerular filtration rate (eGFR) Describes how much fluid filtered through the kidney. It is usually measured with creatinine clearance rate: it is the volume of plasma that is cleared of creatinine per unit time. Nowadays, glomerular filtration rate is estimated using different formulas based on creatinine or other markers such as cystatin C.

Framingham score for assessment of cardiovascular risk It gives an estimate of the probability that a person will develop cardiovascular disease within a specified amount of time, usually 10–30 years. It also indicates who is most likely to benefit from prevention.

Glomeruli and tubule Both represent part of the nephron, the basic structural and functional unit in the kidney.

Immunochemistry assays (Immunonephelometry, immunoturbidimetry, radioimmunoassay, enzyme-linked immunosorbent assay) Are based on the interaction of the urinary albumin with anti-albumin antibodies in the reagents.

Insulin resistance A decreased sensitivity to the action of insulin. Conditions in which the increased amount of insulin is inadequate to induce normal insulin responses in insulin-sensitive tissues (liver, skeletal muscles, adipose tissues).

Likelihood ratios Tell us how much we should shift our suspicion for a particular test result. The "positive likelihood ratio" (LR+) tells us how much to increase the probability of disease if the test is positive, while the "negative likelihood ratio" (LR−) tells us how much to decrease it if the test is negative.

Nonsteroidal anti-inflammatory drugs It is a group of medication with antipyretic, analgesic, and anti-inflammatory effects.

Progression of chronic kidney disease Worsening of kidney function, usually slow, which is determined by measuring glomerular filtration rate and albuminuria and/or proteinuria.

Renal replacement therapy (RRT) Refers to the three ways of replacing the lost kidney function: dialysis (hemo- or peritoneal) and kidney transplant.

Renin–angiotensin–aldosterone system Is a hormone system that regulates blood pressure and water balance in the body.

Sensitivity vs. specificity Sensitivity (the true positive rate) measures the proportion of actual positives which are correctly identified (the percentage of sick people who are correctly identified as having the condition). Specificity (the true negative rate) measures the proportion of negatives which are correctly identified (the percentage of healthy people who are correctly identified as not having the condition).

Type 1 diabetes mellitus It is a type of glucose control disturbance that results from the autoimmune destruction of insulin-producing cells in the pancreas. There is absolute lack of insulin. Type 2 diabetes mellitus It is characterized by hyperglycemia due to insulin resistance and relative lack of insulin.

Urinary albumin excretion (UAE) The presence of albumin in the urine as a consequence of high permeable glomerular membrane and inhibition of tubular cell reabsorption.

Introduction

Albuminuria refers to abnormal loss of albumin in the urine. Albumin is one fraction of plasma protein found in the urine in normal subjects but in larger quantity in patients with kidney disease. Although the measurement of urinary proteins has been a standard tool for nephrologists to diagnose kidney diseases for more than two centuries, the introduction of novel methods in 1980s enabled the measurement of small quantities of albumin in the urine. This triggered a series of investigations showing that increased urinary albumin excretion was an early and sensitive marker of diabetic nephropathy (Viberti 1982; Parving 1982), and an independent risk factor for cardiovascular disease in patients with hypertension and diabetes, cardiovascular and peripheral vascular disease (Parving 2001; Heart Outcomes Prevention Evaluation (HOPE) Study Investigators 2000) and in the general population (Hillege 2002; Romundstad 2003).

All these statements derived from the screening studies, which have shown that there are signs of kidney disease in apparently healthy individuals (Kiberd 2006; Levey 2007). Thus renal disease became epidemic worldwide. The overall prevalence of albuminuria in the general population has been reported to be 7.2 % in the PREVEND cohort from the Netherlands ($n = 40{,}548$) (Hillege 2002) and 10.7 % in the more racially diverse United States National Health and Nutrition Examination Survey (NHANES III) study ($n = 15{,}939$) (Mattix 2002). About 8 % of adults have microalbuminuria (30–300 mg of albumin per 24 h), and 1 % have macroalbuminuria (i.e., excretion of more than 300 mg of albumin per 24 h). Furthermore, albuminuria was detected in one of every three persons with diabetes, one of every seven persons with high blood pressure but no diabetes, and one of every six persons older than 60 years (Collins 2009; Levey 2009). Also, assessment of urinary albumin excretion rather than plasma creatinine or estimated glomerular filtration rate (eGFR) seems the utmost tool for recognizing the chronic kidney disease especially in the early stages (Brantsma 2008; de Jong 2008; van der Velde 2009). Finally, in the majority of guidelines, albuminuria as a marker of kidney damage joined the glomerular filtration rate (GFR) as a measure of kidney function for definition of chronic kidney disease stages in order to evaluate chronic kidney disease (CKD) (National Kidney Foundation 2002; NICE clinical guideline 73 http://guidance.nice.org.uk/cg73, American Diabetes Association 2014).

In this review, the etiology and physiologic mechanisms of albuminuria in renal disease are highlighted. Also, clinical impact of albuminuria as a factor in renal disease compromises and establishes the link toward cardiovascular diseases, diabetes, and hypertension is shown. Therapeutic possibilities influencing on albuminuria and consequences of this are shown, too. Finally, a few dilemmas will be set up as a proposal for further investigation.

Mechanisms of Albuminuria

Mechanisms underlying increased urinary albumin excretion are complex. Plasma albumin filtered in the glomeruli is considered to be the major source of urinary albumin. Most albumin passing (intact-unprocessed albumin) through the glomerular membrane is reabsorbed by proximal tubular cells (known as the *retrieval pathway*). The small amount of albumin not taken up by this pathway is destined for excretion through the *degradation pathway* in tubular cell lysosomes. Thus, normal protein excretion in humans is now recognized to involve the excretion of 1–3 g/day of albumin-derived fragments in combination with less than 25 mg/day of intact albumin (Greive 2001). In pathological states, the glomeruli may become increasingly permeable to circulating albumin due to disturbances in endothelial cell function, basement membrane abnormalities, or podocyte disorders. In addition, inhibition in proximal tubule reabsorption of filtered albumin can also contribute to albuminuria (Russo 2009).

Data suggest that the excreted albumin itself and/or bound ligands, such as fatty acids, initiate a series of events that eventually leads to fibrosis (Chen 2000; Thomas 2002; Arici 2002). In vitro studies have shown that albumin induces changes in tubular cells stimulating the expression of inflammatory and fibrogenic mediators (Wang 1997; Tang 2003; Zoja 1995; Yard 2001). Although inflammation appears to be an important pathogenic factor in a number of renal diseases characterized by albuminuria, the possible pathogenic role of albumin itself is not yet fully elucidated in humans.

Values and Categories of Albuminuria

Previously, the presence of albumin in the urine is called microalbuminuria. This name was changed in albuminuria, because this is not a small molecule of plasma albumin, but a low quantity of albumin present in the urine that could not be detected by standard dipstick test. The current definition of proteinuria, albuminuria, and microalbuminuria is shown in Table 1. Lately, albuminuria is categorized into the following stages: normal or $<$30 mg albumin/g creatinine ($<$3.4 mg/mmol), moderately increased (formerly called *microalbuminuria*) from 30 to 299 mg/g (3.4–34.0 mg/mmol), and severely increased (formerly called *macroalbuminuria* or nephrotic range) at $\geq$300 mg/g ($>$34.0 mg/mmol) (Levey UpToDate last visit 2015) (Table 2).

Urinary albumin excretion (UAE) can be measured in the urine collected during 24 h or overnight (8–12 h). As UAE changes throughout the day, the amount of albumin excreted over 24 h has been considered the "gold standard." Nevertheless,

Table 1 The terminology and the difference between albuminuria and microalbuminuria

Proteinuria	Abnormal excretion of protein by the kidney including any or all proteins excreted
Albuminuria	Abnormal excretion rate of albumin
Microalbuminuria	Abnormally increased excretion rate of albumin in the urine in the range of 30–299 mg/g creatinine

Table 2 Categories of albuminuria

Categories	UAER mg/24 h	Overnight UAER μg/min	ACR mg/mmol	ACR mg/g
Normal	<30	<20	<3	<30
			Male: <2.5	<17
			Female: <3.5	<25
Moderately increased (previous name *microalbuminuria*)	30–300	20–200	3–30	30–300
Severe increased (previous name *macroalbuminuria*)	>300	>200	>30	>300

Derived from KDIGO (2012) Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease 2013; Joint Specialty Committee of the Royal College of Physicians of London and the British Renal Association (2005, 20–3; Mattix 2002; and The CARI guidelines. Caring for Australasians with Renal Impairment. Sydney: Australian and New Zealand Society of Nephrology http://www.cari.org.au/guidelines.php
Levin 2008 http://www.cmaj.ca/cgi/data/179/11/1154/DC1
UAER urinary albumin excretion rate, *ACR* albumin-to-creatinine ratio

24-h urine collection is a cumbersome procedure. In daily practice albumin can be determined in any urine sample: first morning urine specimens or random spot urine collections. In such cases, its excretion can be estimated by measurement of the albumin alone or albumin-to-creatinine ratio (ACR). Comparisons among all albuminuria measurements and 24-h albuminuria showed that albuminuria from first morning voids is a more reliable alternative to 24-h urinary albumin excretion than spot urine samples for diagnosis of albuminuria and to monitor it over time. If it is decided to collect the first morning sample, then measurement of the ACR is to be preferred over urinary albumin concentration alone (Witte 2009; Jafar 2007).

The cutoff value that traditionally indicates the presence of albuminuria is considered to be albumin excretion equal or greater than 30 mg/24 h. For the diagnosis of kidney disease, guidelines suggest diverse values as the limit of normal albuminuria (Table 2). Some of them recommend different threshold values depending on the individual's sex, so albuminuria is defined as an ACR greater than 2.5 g/mmol creatinine for men and 3.5 g/mmol creatinine for women (Joint Speciality Committee of the Royal College of Physicians of London and the British Renal Association. Guidelines for identification, management and referral of adults with chronic kidney disease. London: Department of Health for England 2005;20–3. http://www.rcplondon.ac.uk/pubs/books/kidney/), or 17 mg/g creatinine for men and 25 mg/g creatinine for women (Mattix 2002), while a threshold value for albuminuria of 30 mg/g (3.4 mg/mmol) regardless of the sex of the patients is recommended by others (National Kidney Foundation 2002 http://www.kidney.org/professionals/KDOQI/guidelines_ckd/toc.htm

The CARI guidelines. Caring for Australasians with Renal Impairment. Sydney: Australian and New Zealand Society of Nephrology n.d.. http://www.cari.org.au/guidelines.php Levin 2008 http://www.cmaj.ca/cgi/data/179/11/1154/DC1). Individual laboratories express the ACR in milligrams of albumin per gram of creatinine, while others use albumin in milligrams per millimole of creatinine, so specified limit values are expressed in several units.

There are some limitations that must be considered to maximize the reliability of the ACR. Many conditions can cause a false-positive value for albuminuria. Thus, urine samples should not be collected during: marked acute hyperglycemia; urinary tract infection; marked hypertension; congestive cardiac failure; heavy exercise (due to increased protein catabolism and altered renal circulation); fever, immediately after surgery or after an acute fluid overload; and contamination with seminal or menstrual fluid (which contains more albumin). Also, the value of the ACR depends on the rate of urinary creatinine excretion that, in turn, reflects interindividual differences in muscle mass. Therefore, persons with low muscular mass often have the moderate ACR elevation used to define albuminuria in the absence of a true elevation in absolute albuminuria. That contributes to the difference in values for albuminuria between men and women (Joint Speciality Committee of the Royal College of Physicians of London and the British Renal Association. Guidelines for identification, management and referral of adults with chronic kidney disease. London: Department of Health for England 2005, 20–3. http://www.rcplondon.ac.uk/pubs/books/kidney/, Mattix 2002).

Furthermore, due to variability in urinary albumin excretion, two out of three specimens collected within a 3- to 6-month period should be abnormal before considering a patient to have developed increased UAE or to have exhibited progression in albuminuria.

Methods of Albuminuria Assessment

Collection of the urine sample is very important when albuminuria is measured, as many factors can alter the value and errors may occur due to inadequate aseptic precautions or improper storage and handling. After collection it is preferable to measure on the same day, but if urine albumin is not estimated immediately, then the urine can be stored at 4 °C. Specimens are stable for at least 2 weeks at 4 °C and 5 months at −70 °C. Freezing samples may decrease the albumin result but mixing immediately before assay eliminates this effect (David et al. 1999).

There are two types of assay used for assessment of albuminuria: colorimetric test strips (semiquantitative) and immunochemistry-based assays (quantitative) (Table 3). Traditionally, detection of albuminuria starts with the dipstick test. These test strips have been used in some countries for screening program of kidney damage (Iseki

Table 3 Methods of albuminuria assessment

Semiquantitative colorimetric assays
Dipstick test: Micral microalbumin urine test strip, CLINITEK Microalbumin
Quantitative immunochemistry-based assays
Immunoturbidimetry
Nephelometry
Radioimmunoassay (RIA)
Chemiluminescent immunoassay (CLIA)
Enzyme-linked immunosorbent assay (ELISA)

Derived from Martin (2011)

1996; Ležaić 2011). Some disadvantages are recognized. The test is semiquantitative and insensitive for reliable detection of albumin in concentration ranges around 300 mg/day. Furthermore, concentrated urines may give a color change in the positive range of a reagent strip device even though protein loss remains normal and vice versa. False-positive results may occur if the urine is alkalinized (e.g., due to urinary tract infection) or in the presence of quaternary ammonium compounds that alter the urine pH. The performance of reagent strips is operator dependent (Rumley 2000) and affected by the presence of colored compounds such as bilirubin and certain drugs (e.g., ciprofloxacin, quinine, and chloroquine) (Scotti da Silva-Colombeli 2007).

Immunochemical assays (immunonephelometry, immunoturbidimetry, radioimmunoassay, enzyme-linked immunosorbent assay) are based on the interaction of urinary albumin with anti-albumin antibodies in the reagents. With these methods it is possible to detect very small concentrations of albumin in the urine. Each method has advantages and disadvantages, and the choice depends on local experience and technical support. All methods have similar precision, sensitivity, and range. Currently urinary albumin is predominantly measured in diagnostic laboratories using immunoturbidimetric assays (Martin 2011). Radioimmunoassay (RIA) and chemiluminescent immunoassay (CLIA) are highly sensitive, specific, and reproducible methods. Disadvantages are unavailability, the cost factor, proper infrastructure needed, and radioactive hazards (Agarwal 2002).

Measuring albumin in the urine is complex, especially as multiple species of intact albumin (immunoreactive albumin and immuno-unreactive albumin) and albumin-derived fragments have been reported (Comper 2003). Albumin fragments are not detected by conventional immunoassays commonly used to measure urinary albumin. At present there is no diagnostic tool available to measure albumin fragments in urine routinely.

Potential Applications to Prognosis, Other Diseases, or Conditions

Albuminuria (ACR more than 3 mg/mmol) is a marker for kidney disease in apparently healthy subjects as well as in patients with different comorbidities such as diabetes, hypertension, or obesity and is widely used in clinical practice (Chronic kidney disease: early identification and management of chronic kidney disease in adults in primary and secondary care. NICE guidelines [CG182] Published date: July 2014). It may precede the appearance of type 2 diabetes mellitus, being present in the insulin resistance syndrome. Also albuminuria is considered to be a marker of cardiovascular risk in the general population (Mykkanen 1998; MacIsaac 2004; Thomas 2011). In addition, albuminuria has predictive significance, i.e., the higher the albuminuria the higher the risk for mortality (cardiovascular and all cause), progression of chronic kidney disease (CKD), and end-stage renal disease (ESRD) independent of eGFR as a measure of kidney function. No apparent threshold value was found in the general population and a population at high risk for kidney disease (Matsushita 2010; Gansevoort 2011; van der Velde 2011). All this derives from the results of large studies conducted over the last three decades.

Screening for Chronic Kidney Disease in the General Population

Screening for chronic kidney disease in the general population is usually performed using eGFR and albuminuria. Recent data have shown a positive correlation between UAE and rate of decline in eGFR, i.e., patients with higher levels of UAE had a more rapid decline in eGFR, especially beyond a UAE of above 150 mg/24 h. The average rate of renal function decline in the UAE category above 300 mg/24 h was four times more rapid than that for UAE less than 15 mg/24 h (van der Velde 2009). In Table 4 the predictive cutoff albuminuria values for renal replacement therapy (RRT) start during 6 years of follow-up was shown. During follow-up a UAE concentration above 20 mg/L identified individuals with the risk of RRT with 58 % sensitivity and 92 % specificity. The likelihood ratio of a positive test result showed that an individual at risk, i.e., with a history of hypertension or diabetes and a UAC >20 mg/L, has 24.3 times the previous odds to start RRT during follow-up; for a UAC >200 mg/L, this odds to start RRT is 118.7. These data suggest that screening for albuminuria might be effective to identify individuals at risk for developing ESRD (van der Velde 2009).

Albuminuria is a risk factor not only for ESRD but also acute kidney injury and progressive CKD in both general and high-risk populations (Gansevoort 2011). Hazard ratios for renal outcomes at different ACR presented in Table 5 show similarity between general population and high-risk cohorts. The patterns for ESRD were less steep in the high-risk cohorts compared with the general population, whereas the patterns for acute kidney injury and progressive CKD were similar in the general population cohorts and high-risk cohorts. These associations are independent of other cardiovascular risk factors (Gansevoort 2011) (Table 6).

Lower eGFR and higher albuminuria were each independently associated with mortality and ESRD as it was shown in the meta-analysis of 13 cohorts, including 21,688 individuals selected because of CKD (Astor et al. 2011). This risk increased progressively with every higher level of albuminuria: an eightfold higher ACR was

Table 4 Various cutoff values of albumin excretion rate in a single first morning void urine to identify individuals who started RRT during follow-up

AER category mg/L	Sensitivity %	Specificity %	PPV %	NPV %	LR+	LR−
General population						
>20	58	92	0.8	99.9	7.43	0.46
>200	36	99	5.7	99.9	54.44	0.65
With known HTA/DM						
>20	44	98	2.6	99.9	24.3	0.57
>200	31	99	11.6	99.9	118.7	0.69
Age >55 years						
>20	44	96	1.3	99.9	12.1	0.58
>200	27	99	7.1	99.9	68.9	0.74

Derived from van der Velde (2009)
AER albuminuria excretion rate, *LR+* likelihood ratio of a positive test, *LR−* likelihood ratio of a negative test, *NPV* negative predictive value, *PPV* positive predictive value
HTA arterial hypertension, *DM* diabetes mellitus

Table 5 Pooled hazard ratios for renal outcomes, i.e., end-stage renal disease, acute kidney injury, and progressive chronic kidney disease by albuminuria categories for the general and high-risk population

	Albumin-to-creatinine ratio 30–299 mg/g	Albumin-to-creatinine ratio ≥300 mg/g
General population		
ESRD	12 (7.9–18.1)	72.1 (64.3–121)
AKI	2.5 (1.7–3.7)	6.0 (4.5–8.0)
Progressive CKD	3.1 (2.5–3.8)	11.2 (5.8–21.5)
High-risk patients		
ESRD	4.3 (2.6–7.1)	38.1 (15.6–93.5)
AKI	2.7 (2.2–3.4)	7.4 (5.5–9.8)
Progressive CKD	2.2 (1.9–2.7)	9.9 (6.7–14.5)

Derived from Gansevoort (2011)
Pooled adjusted hazard ratios (95 % confidence interval) for ESRD and acute kidney injury and pooled adjusted odds ratios (95 % confidence interval) for progressive CKD
ESRD end-stage renal disease, *AKI* acute kidney injury, *CKD* chronic kidney disease

Table 6 Adjusted hazard ratio (95 % confidence interval) for mortality, by albuminuria category

	HR	95 % confidence interval
Albumin-to-creatinine ratio, mg/g		
30–299	1.5	1.28–1.75
300–999	1.85	1.08–3.16
>1,000	2.7	1.74–4.26
Estimated glomerular filtration rate, ml/min/1.73 m^2		
30–44	1.35	1.23–1.49
15–29	2.25	1.81–2.79
<15	3.74	2.69–5.20

Derived from Astor et al. (2011)
Adjusted for age, sex, race, previous cardiovascular disease, smoking status, diabetes mellitus, systolic blood pressure, and serum total cholesterol concentration

associated with an estimated 40 % higher risk of death and an estimated threefold higher risk of ESRD after adjustment for eGFR (Astor et al. 2011).

For evaluation of chronic kidney disease, albuminuria as a marker of kidney damage is combined with eGFR as a measure of kidney function. Both markers were used in the classification of chronic kidney disease (CKD) (National Kidney Foundation 2002; NICE clinical guideline 73 http://guidance.nice.org.uk/cg73, American Diabetes Association. Standards of Medical Care in Diabetes 2014). That is why regular screening for albuminuria is of utmost importance, especially at stages 1 and 2 of CKD, where therapeutic interventions may be of benefit to prevent or delay the development of renal injury.

Albuminuria is the most widely used clinical marker of diabetic nephropathy and has been recognized as a predictor of progression to ESRD in both type 1 and type 2 diabetes (Perkins 2010; Adler 2003). It is one of the earliest markers of diabetic

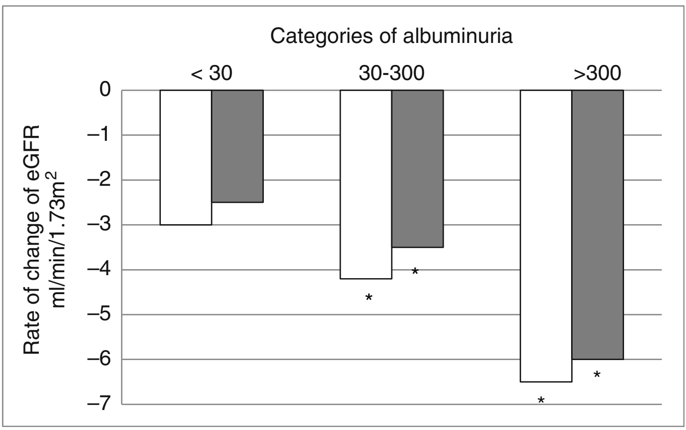

Fig. 1 Comparison of the rate of the change in estimated glomerular filtration rate (*eGFR*) monitored at least 5 years, adjusted for age and baseline eGFR in diabetic women and men, and classified according to the average urinary albumin excretion at baseline. *White* (women) and *gray* (men) bars; *$p < 0.001$ versus normal albuminuria value (Derived from Babazono (2009))

nephropathy that precede severely increased albuminuria (>300 mg/24 h). The impact of elevated UAE in predicting future renal function loss in subjects with diabetes was emphasized in the 1980s (Mogensen 1986; Parving 1982). During these years, the terminology of "early diabetic nephropathy" was introduced to indicate diabetic subjects with albuminuria. In this early phase of diabetic nephropathy, eGFR is usually well preserved. Early nephropathy contrasts with the later phase of overt nephropathy in which albuminuria increases into overt proteinuria and the eGFR falls below 60 ml/min finally progressing to the level of ESRD. Documentation of the early phase of kidney damage with albuminuria but still normal eGFR has helped us to understand better the impact of albuminuria during the loss of renal function in diabetes. Moreover, it led to the demonstration that early intervention by interfering in the renin–angiotensin–aldosterone system (RAAS) slows the progression of nephropathy in diabetic patients (Parving 2001; Ruggenenti 2004). The changes between these albuminuria states represent a hallmark of disease progression or regression (American Diabetes Association 2014). More recent studies have shown that an increase in albuminuria, even within the range that is currently considered normal, indicates higher renal risk (Babazono et al. 2009; Gansevoort 2011). In patients with type 2 diabetes monitored for at least 5 years, higher albuminuria at baseline was associated with a faster decline in renal function (Fig. 1), according to the data of Babazono T and coworkers' study (Babazono 2009). Importantly, although within the normal range, it was calculated that albuminuria of $\times 10$ mg/g in women or $\times 5$ mg/g in men was associated with a significantly greater rate of renal function decline (Babazono 2009). Not only the albuminuria level itself but also changes in albuminuria (ACR within 30–300 mg/g) over time predict renal or cardiovascular risk changes. In patients with type 2 diabetes and ACR 30–300 mg/g, those subjects in whom albuminuria decreased by more than

50 % over 2-year follow-up had a subsequent renal function decline of −1.8 ml/min per year. In contrast, in subjects without a 50 % reduction in albuminuria, long-term renal function decline was significantly greater, being −3.1 ml/min per year (Araki 2007).

During the 1990s, there was growing evidence that albuminuria is important for progressive reduction of renal function in nondiabetic renal disease. The findings from large clinical trials showed the beneficial effect of lowering albuminuria by using agents that interfere with the RAAS to prevent progression to ESRD (The Gruppo Italiano di Studi Epidemiologici in Nefrologia (GISEN) Group 1997; Jafar 2003). This focused attention on treatment of renal patients for albuminuria and not only for high blood pressure (De Jong 1999).

Albuminuria as a Cardiovascular Disease Marker

Some evidence suggested that high albuminuria is associated with cardiovascular risk. Three large studies conducted in the general population showed that high albuminuria predicts cardiovascular risk (Hillege 2001; Romundstad 2003; Yuyun 2004). The predictive power of albuminuria on cardiovascular risk is independent of other known cardiovascular risks. Hence the idea that albuminuria should be combined with Framingham scores for assessment of cardiovascular risk is suggested as a primary prevention strategy with higher efficiency (Asselbergs 2004b). In patients with several cardiovascular risk factors, albuminuria predicts outcome, even below the level taken as normal (Mann 2004).

Albuminuria and Hypertension

Albuminuria may not only be the consequence of but it may also precede the development of hypertension (Forman 2008; Scheven 2013). Albuminuria is found in 11–40 % of persons with hypertension, the prevalence increasing with age and the duration of hypertension (Rossa 2000; Özyilmaz 2013). According to the PREVEND study results, in subjects with elevated albuminuria and newly discovered hypertension or hypercholesterolemia, the cardiovascular risk exceeded by 20 % the risk in normoalbuminuric hypertensive patients. Thus, detecting albuminuria might also help the clinician decide when to initiate antihypertensive therapy. In addition, identification of target organ damage is an indication for treatment in patients with lower blood pressure (European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension 2003).

Link Between Albuminuria and Increased Cardiovascular and Renal Risk

The pathophysiological mechanisms linking albuminuria to cardiovascular and renal risk are still not fully understood, but much evidence appears to connect any level of

UAE to generalized loss of vascular endothelial function in many organs including kidneys (Solbu 2009; Foster 2008).

Albuminuria as a Target for Treatment

Albuminuria can be used as a target for treatment for primary and secondary prevention of renal and cardiovascular disease development. Previous studies showed that the degree of albuminuria reduction was associated with a more beneficial renal outcome in long-term follow-up (Apperloo 1994; Rossing 1994). Therefore, several measures were introduced in clinical practice in order to reduce albuminuria, such as dietary protein restriction (El Nahas 1984), nonsteroidal anti-inflammatory drugs (Vriesendorp 1986), angiotensin-converting enzyme inhibitors, and angiotensin II receptor antagonists (Gansevoort 1994). Thus far, no trials have shown that lowering albuminuria in the early phase (i.e., microalbuminuria with a GFR >60 ml/min, namely, stage 1 or 2 CKD) slows the progressive decline of renal function. On the other side, there is some evidence that ACE inhibition in subjects with nondiabetic albuminuria may prevent future cardiovascular events (Asselbergs 2004). Also, measurement of albuminuria can help to determine whether or not a patient with hypertension should be treated, how aggressive should the therapy be, and what medications should be used (European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension 2003).

Not Resolved Questions

Although the strong association between age and kidney function is well established, the clinical significance of CKD in older asymptomatic people remains disputable. In addition, it is unclear what portion of this decline is due to the higher prevalence of risk factors for kidney disease at older ages, such as hypertension, diabetes, and vascular disease. It seems to be worth investigating:

- The cutoff value of albuminuria that contribute to the accurate diagnosis of CKD in the elderly
- The introduction of standardized laboratory tests that will clearly separate the whole molecule, antibodies' recognizable molecules, and fragments of albumin in the urine

Summary Points

- This chapter focuses on albuminuria: definition, classification, importance in renal disease detection, prognostic significance, mechanism of occurrence, and methods of estimation.

- Currently, albuminuria categorizes into the following stages: normal, less than 30 mg/g (<3.4 mg/mmol); moderately increased, 30–299 mg/g (3.4–34.0 mg/mmol); and severely increased albuminuria, $\geq$300 mg/g (>34.0 mg/mmol).
- Albuminuria is a marker for kidney disease in apparently healthy subjects and in patients with different comorbidities.
- Screening for albuminuria is important to identify individuals at risk for developing end-stage renal disease, acute kidney injury, and progressive chronic kidney disease, as well as cardiovascular disease and all-cause mortality in both general and high-risk population (diabetes, cardiovascular disease, hypertension, and older patients).
- Albuminuria is the most widely used clinical marker of early stage of diabetic nephropathy when the glomerular filtration rate (GFR) is usually well preserved.
- The predictive power of albuminuria on cardiovascular risk is independent to other known cardiovascular risks.
- Albuminuria can be used as a target for treatment for primary and secondary prevention of renal and cardiovascular disease development.

References

Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA, Holman RR, UKPDS GROUP. Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). Kidney Int. 2003;63:225–32.

Agarwal S, Sandeep KB, Anuradha R, Vasudha K, Chadha KH, Doli P, et al. Microalbuminuria. Clin Lab Technol. 2002;3(1):14–22.

American Diabetes Association. Standards of medical care in diabetes—2014. Diabetes Care 2014;37:S14–S80.

Apperloo AJ, de Zeeuw D, de Jong PE. Short-term antiproteinuric response to antihypertensive treatment predicts long-term GFR decline in patients with non-diabetic renal disease. Kidney Int Suppl. 1994;45:S174–8.

Araki S, Haneda M, Koya D, et al. Reduction in albuminuria as an integrated indicator for renal and cardiovascular risk reduction in patients with type 2 diabetes. Diabetes. 2007;56:1727–30.

Arici M, Brown J, Williams M, et al. Fatty acids carried on albumin modulate proximal tubular cell fibronectin production: a role for protein kinase C. Nephrol Dial Transplant. 2002;17:1751–7.

Astor BC, Matsushita K, Gansevoort RT, et al. Lower estimated glomerular filtration rate and higher albuminuria are associated with mortality and end-stage renal disease. A collaborative meta-analysis of kidney disease population cohorts. Kidney Int. 2011;79(12):1331–40.

Asselbergs FW, Diercks GFH, Hillege HL, et al. Effects of fosinopril and pravastatin on cardiovascular events in subjects with microalbuminuria. Circulation. 2004a;110:2809–16.

Asselbergs FW, Hillege HL, van Gilst WH. Framingham score and microalbuminuria: combined future targets for primary prevention? Kidney Int Suppl. 2004b;92:S111–4.

Babazono T, Nyumura I, Toya K, et al. Higher levels of urinary albumin excretion within the normal range predict faster decline in glomerular filtration rate in diabetic patients. Diabetes Care. 2009;32:1518–20.

Brantsma AH, Bakker SJ, Hillege HL, et al. Cardiovascular and renal outcome in subjects with K/DOQI stage 1–3 chronic kidney disease: the importance of urinary albumin excretion. Nephrol Dial Transplant. 2008;23:3851–8.

Chen S, Cohen MP, Ziyadeh FN. Amadori-glycated albumin in diabetic nephropathy: pathophysiologic connections. Kidney Int Suppl. 2000;77:S40–4.

Chronic kidney disease: early identification and management of chronic kidney disease in adults in primary and secondary care. NICE guidelines [CG182] Published date: Jul 2014.
Collins AJ, Vassalotti JA, Wang C, Li S, Gilbertson DT, Liu J, Foley RN, Chen SC, Arneson TJ. Who should be targeted for CKD screening? Impact of diabetes, hypertension, and cardiovascular disease. Am J Kidney Dis. 2009;53(3 Suppl 3):S71–7.
Comper WD, Osicka TM, Jerums G. High prevalence of immuno-unreactive intact albumin in urine of diabetic patients. Am J Kidney Dis. 2003;41:336–42.
de Jong PE, Gansevoort RT. Fact or fiction of the epidemic of chronic kidney disease – let us not squabble about estimated GFR only, but also focus on albuminuria. Nephrol Dial Transplant. 2008;23:1092–5.
David BS, Burtis AC, Ashwood RE (eds). Tietz text book of clinical chemistry 3rd ed. Philadelphia: Saunders. 1999. p 798–801.
De Jong PE, Navis GJ, de Zeeuw D. Renoprotective therapy: titration against urinary protein excretion. Lancet. 1999;354:352–3 [commentary].
El Nahas AM, Masters-Thomas A, Brady SA, Farrington K, Wilkinson V, Hilson AJ, Varghese Z, Moorhead JF. Selective effect of low protein diets in chronic renal diseases. Br Med J (Clin Res Ed). 1984;289(6455):1337–41.
European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. J Hypertens. 2003;21:1011–53.
Forman JP, Fisher ND, Schopick EL, et al. Higher levels of albuminuria within the normal range predict incident hypertension. J Am Soc Nephrol. 2008;19:1983–8.
Foster MC, Keyes MJ, Larson MG, et al. Relations of measures of endothelial function and kidney disease: the Framingham Heart Study. Am J Kidney Dis. 2008;52:859–67.
Gansevoort RT, de Zeeuw D, de Jong PE. Is the antiproteinuric effect of ACE inhibition mediated by interference in the renin-angiotensin system? Kidney Int. 1994;45(3):861–7.
Gansevoort RT, Matsushita K, van der Velde M, et al. Lower estimated GFR and higher albuminuria are associated with adverse kidney outcomes. A collaborative meta-analysis of general and high-risk population cohorts. Kidney Int. 2011;80:93–104.
Greive KA, Balazs ND, Comper WD. Protein fragments in urine have been considerably underestimated by various protein assays. Clin Chem. 2001;47:1717–9.
Heart Outcomes Prevention Evaluation (HOPE) Study Investigators. Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-HOPE substudy. Lancet. 2000;355:253–9.
Hillege HL, Janssen WM, Bak AA, et al. Microalbuminuria is common, also in a nondiabetic, nonhypertensive population, and an independent indicator of cardiovascular risk factors and cardiovascular morbidity. J Intern Med. 2001;249(6):519–26.
Hillege HL, Fidler V, Diercks GF, et al. Prevention of renal and vascular end stage disease (PREVEND) study group. Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. Circulation. 2002;106:1777–82.
Iseki K, Iseki C, Ikemiya Y, et al. Risk of developing end-stage renal disease in a cohort of mass screening. Kidney Int. 1996;49:800–5.
Jafar TH, Stark PC, Schmid CH, et al. Progression of chronic kidney disease: the role of blood pressure control, proteinuria, and angiotensin-converting enzyme inhibition: a patient-level meta-analysis. Ann Intern Med. 2003;139:244–52.
Jafar TH, Chaturvedi N, Hatcher J, Levey AS. Use of albumin creatinine ratio and urine albumin concentration as a screening test for albuminuria in an Indo-Asian population. Nephrol Dial Transplant. 2007;22:2194–200.
Joint Specialty Committee of the Royal College of Physicians of London and the British Renal Association. Guidelines for identification, management and referral of adults with chronic kidney disease. London: Department of Health for England; 2005. p. 20–3. http://www.rcplondon.ac.uk/pubs/books/kidney/.
Kiberd B. The chronic kidney disease epidemic: stepping back and looking forward. J Am Soc Nephrol. 2006;17:2967–73.

Levey S, Inker L. Definition and staging of chronic kidney disease in adults. UpToDate last visit 2015.
Levey AS, Atkins R, Coresh J, Cohen EP, Collins AJ, Eckardt KU, Nahas ME, Jaber BL, Jadoul M, Levin A, Powe NR, Rossert J, Wheeler DC, Lameire N, Eknoyan G. Chronic kidney disease as a global public health problem: approaches and initiatives – a position statement from kidney disease improving global outcomes. Kidney Int. 2007;72(3):247–59.
Levey AS, Schoolwerth AC, Burrows NR, Williams DE, Stith KR, McClellan W. Comprehensive public health strategies for preventing the development, progression, and complications of CKD: report of an expert panel convened by the centers for disease control and prevention. Am J Kidney Dis. 2009;53:522–35.
Levin A, Hemmelgarn B, Culleton B, Canadian Society of Nephrology, et al. Guidelines for the management of chronic kidney disease. CMAJ. 2008;179(11):1154–62. http://www.cmaj.ca/cgi/data/179/11/1154/DC1.
Ležaić V, Dimković N, Peković GP, et al. Screening of a population at risk of chronic kidney disease: analysis of factors associated with low eGFR and microalbuminuria. Ren Fail. 2011;33(10):969–76.
MacIsaac RJ, Jerums G, Cooper ME. New insights into the significance of microalbuminuria. Curr Opin Nephrol Hypertens. 2004;13(1):83–91.
Mann JFE, Yi Q-L, Gerstein HC. Albuminuria as a predictor of cardiovascular and renal outcomes in people with known atherosclerotic cardiovascular disease. Kidney Int. 2004;66 Suppl 92: S59–62.
Martin H. Laboratory measurement of urine albumin and urine total protein in screening for proteinuria in chronic kidney disease. Clin Biochem Rev. 2011;32:97–102.
Matsushita K, van der Velde M, Astor BC, et al. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. Lancet. 2010;375:2073–81.
Mattix H, Hsu C-Y, Shaykevich S, Curhan G. Use of the albumin/creatinine ratio to detect microalbuminuria: implications of sex and race. J Am Soc Nephrol. 2002;13:1034–9.
Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity onset diabetes. N Engl J Med. 1986;310:356–60.
Mykkanen L, Zaccaro DJ, Wagenknecht LE, et al. Microalbuminuria is associated with insulin resistance in nondiabetic subjects: the insulin resistance atherosclerosis study. Diabetes. 1998;47:793–800.
National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis. 2002;39(2 suppl 1):S1–266.
NICE clinical guideline 73. Chronic kidney disease: early identification and management of chronic kidney disease in adults in primary and secondary care. http://guidance.nice.org.uk/cg73.
Ozyilmaz A, Bakker SJ, de Zeeuw D, de Jong PE, Gansevoort RT, PREVEND Study Group. Screening for albuminuria with subsequent screening for hypertension and hypercholesterolaemia identifies subjects in whom treatment is warranted to prevent cardiovascular events. Nephrol Dial Transplant. 2013;28(11):2805–15.
Parving HH, Oxenbøll B, Svendsen PA, et al. Early detection of patients at risk of developing diabetic nephropathy. A longitudinal study of urinary albumin excretion. Acta Endocrinol (Copenh). 1982;100:550–5.
Parving HH, Lehnert H, Bröchner-Mortensen J. The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. N Engl J Med. 2001;345:870–8.
Perkins BA, Ficociello LH, Roshan B, Warram JH, Krolewski AS. In patients with type 1 diabetes and new-onset albuminuria the development of advanced chronic kidney disease may not require progression to proteinuria. Kidney Int. 2010;77:57–64.
Romundstad S, Holmen J, Kvenild K, Hallan H, Ellekjaer H. Microalbuminuria and all-cause mortality in 2,089 apparently healthy individuals: a 4.4-year follow-up study. The Nord-Trøndelag Health Study (HUNT), Norway. Am J Kidney Dis. 2003;42(3):466–73.
Rossa TT, Palatini P. Clinical value of microalbuminuria in hypertension. J Hypertens. 2000;18:645–54.

Rossing P, Hommel E, Smidt UM, Parving HH. Reduction in albuminuria predicts a beneficial effect on diminishing the progression of human diabetic nephropathy during antihypertensive treatment. Diabetologia. 1994;37(5):511–6.
Ruggenenti P, Fassi A, Ilieva AP, et al. Preventing microalbuminuria in type 2 diabetes. N Engl J Med. 2004;351:1941–51.
Rumley A. Urine dipstick testing: comparison of results obtained by visual reading and with the Bayer CLINITEK 50. Ann Clin Biochem. 2000;37(Pt 2):220–1.
Russo LM, Sandoval RM, Campos SB, et al. Impaired tubular uptake explains albuminuria in early diabetic nephropathy. J Am Soc Nephrol. 2009;20:489–94.
Scheven L, Van der Velde M, Lambers Heerspink HJ, De Jong PE, Gansevoort RT. Isolated microalbuminuria indicates a poor medical prognosis. Nephrol Dial Transplant. 2013;28 (7):1794–801.
Scotti da Silva-Colombeli A, Falkenberg M. Analytical interferences of drugs in the chemical examination of urinary protein. Clin Biochem. 2007;40:1074–6.
Solbu MD, Jenssen TG, Eriksen BO, Toft I. Changes in insulin sensitivity, renal function and markers of endothelial dysfunction in hypertension – the impact of microalbuminuria: a 13 year follow-up study. Metabolism. 2009;58:408–15.
Tang S, Leung JC, Abe K, et al. Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells in vitro and in vivo. J Clin Invest. 2003;111:515–27.
The CARI guidelines. Caring for Australasians with renal impairment. Sydney: Australian and New Zealand Society of Nephrology. 2014. http://www.cari.org.au/guidelines.php
The Gruppo Italiano di Studi Epidemiologici in Nefrologia (GISEN) Group. Randomised placebo-controlled trial of effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric non-diabetic nephropathy. Lancet. 1997;349:1857–63.
Thomas ME, Harris KP, Walls J, et al. Fatty acids exacerbate tubulointerstitial injury in protein-overload proteinuria. Am J Physiol Renal Physiol. 2002;283:F640–7.
Thomas G, Sehgal AR, Kashyap SR, et al. Metabolic syndrome and kidney disease: a systematic review and meta-analysis. Clin J Am Soc Nephrol. 2011;6:2364–73.
van der Velde M, Halbesma N, de Charro FT, Bakker SJL, de Zeeuw D, de Jong PE, Gansevoort RT. Screening for albuminuria identifies individuals at increased renal risk. J Am Soc Nephrol. 2009;20:852–62.
van der Velde M, Matsushita K, Coresh J, et al. Lower estimated glomerular filtration rate and higher albuminuria are associated with all cause and cardiovascular mortality. A collaborative meta-analysis of high-risk population cohorts. Kidney Int. 2011;79:1341–52.
Viberti GC, Hill RD, Jarrett RJ, et al. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. Lancet. 1982;1:1430–2.
Vriesendorp R, Donker AJ, de Zeeuw D, de Jong PE, van der Hem GK, Brentjens JR. Effects of nonsteroidal anti-inflammatory drugs on proteinuria. Am J Med. 1986;81(2B):84–94.
Wang Y, Chen J, Chen L, et al. Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. J Am Soc Nephrol. 1997;8:1537–45.
Witte EC, Lambers Heerspink HJ, de Zeeuw D, Bakker SJL, de Jong PE, Gansevoort R. First morning voids are more reliable than spot urine samples to assess microalbuminuria. J Am Soc Nephrol. 2009;20:436–43.
Yard BA, Chorianopoulos E, Herr D, van der Woude FJ. Regulation of endothelin-1 and transforming growth factor-beta1 production in cultured proximal tubular cells by albumin and heparan sulphate glycosaminoglycans. Nephrol Dial Transplant. 2001;16:1769–75.
Yuyun MF, Khaw KT, Luben R, Welch A, et al. Microalbuminuria independently predicts all-cause and cardiovascular mortality in a British population: The European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) population study. Int J Epidemiol. 2004;33(1):189–98.
Zoja C, Morigi M, Figliuzzi M, et al. Proximal tubular cell synthesis and secretion of endothelin-1 on challenge with albumin and other proteins. Am J Kidney Dis. 1995;26:934–41.

Serum Cystatin C as a Biomarker

20

Serap Çuhadar

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Abstract

Cystatin C is a low molecular weight cationic protein produced by all nucleated cells which is a potent cysteine protease inhibitor. Its plasma concentration is proportional with glomerular filtration as it is synthesized at a constant rate, freely filtered through the glomerulus, and largely reabsorbed and catabolized in the proximal renal tubule with no tubular secretion which makes it ideal for GFR estimation. This protein has a

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capability to detect early renal failure as it gives reliable GFR estimation at the critical level of 60 ml/min/1.73 m^2. Though cystatin C is superior to serum creatinine, non-standardization and several clinical situations such as thyroid dysfunction and the use of high doses of glucocorticoid limit its acceptance as a GFR marker to replace creatinine, the current endogenous gold standard biomarker.

Keywords
Creatinine • Cystatin C • Cystatins • Cysteine proteases • Glomerular filtration rate • Kidney diseases • Renal functions

Abbreviations

Ahsg	Alpha 2HS glycoprotein
CRES	Cystatin-related epididymal spermatogenic
CRPs	Androgen-regulated cystatin-related proteins
CSF	Cerebrospinal fluid
Cys C	Cystatin C
HCHWA-1	Hereditary cerebral hemorrhage with amyloidosis of Icelandic type
HMWK	High molecular weight kininogen
HRG	Histidine-rich glycoprotein
LMWK	Low molecular weight kininogen
MDRD	Modification of diet in renal disease
SCr	Serum creatinine

Key Facts of Renal Biomarker Cystatin C

- Kidney filtration function is tested with serum creatinine-based equations named as estimated glomerular filtration rate (eGFR).
- Creatinine is affected by nonrenal factors: age, gender, muscle mass, volume status, and analytical interferences such as bilirubin, ketone, and protein.
- Cystatin C is an endogenous protein synthesized in all nucleated cells that its plasma level is proportional with renal filtration capacity.
- Cystatin C is catabolized in the tubules of the kidney, that its high concentration in urine reflects tubular injury.
- In contrary to serum creatinine, cystatin C is less effected from volume status and not affected from hepatic dysfunctions which make it more reliable in the state of cirrhosis.

Definitions

Cathepsin Lysosomal enzyme responsible for terminal protein degradation

Cystatin A low molecular weight protein that inhibits reversibly the lysosomal enzyme cysteine proteases

GFR A test to determine the filtration function of the kidney

Housekeeping gene A type of gene that indicates a stable production rate

Introduction

Cystatin C (Cys C), originally called as gamma-trace and post-gamma-globulin, is a non-glycosylated low molecular weight protein found in many body fluids and secretions. In 1961, it was isolated as a cerebrospinal fluid-specific protein in human and named as gamma-trace protein (γ-CSF) (Clausen 1961). In 1981, the complete amino acid sequence of human cystatin C was determined by Grubb and Löfberg (Grubb and Löfberg 1982). The amino acid sequence of Cys C was the first sequence to be determined among cystatins. Later on the sequence similarity of the isolated human protein and chicken egg white cystatin was shown, and Cys C was identified as an inhibitor of cysteine proteases (Turk et al. 1983).

The cystatins inhibit the cysteine peptidases, the papain-like proteases (cathepsins), classified as clan C1, and some also inhibit the asparaginyl endopeptidase/legumain as clan C13 enzymes, and parasite proteases like cruzipain, where they appear to provide protective functions. Also cystatins have a role in the defense mechanism against microbial infections (Magister and Kos 2013; Turk et al. 2000; Abrahamson et al. 2003). Known as the tightest binding inhibitor of lysosomal and extracellular cysteine proteases, Cys C inhibits papain, cathepsin L, and cathepsin S in pM range. Cys C neutralizes the protease activity strongly and rapidly (Turk and Turk 2008).

Lysosomal cysteine proteases, generally known as cathepsins (clan C1), are the papain family that are responsible for terminal protein degradation in the lysosomes (Magister and Kos 2013; Turk et al. 2000). Also they may be secreted to degrade extracellular matrix components (Turk and Turk 2008). The increased cysteine protease activity has been shown to be related to a variety of pathopysiological conditions such as bone resorption, chronic inflammation (rheumatoid arthritis, bronchiectasis), cancer progression and metastasis, viral and parasitic infections, and septic shock (Turk and Turk 2008; Ni et al. 1997). Likewise, failure in the function of the protease inhibition results in neurodegeneration, cardiovascular diseases, osteoporosis, arthritis, and cancer. In atherosclerotic lesions, overexpression of cathepsins was found (Dinic et al. 2014).

Cystatin Superfamily

Cystatins, a superfamily of cysteine protease inhibitors, are comprised of 12 different inhibitors. They provide protective functions for uncontrolled proteolysis and tissue damage which are found in a variety of human fluids and secretions (Turk et al. 2002).

Table 1 The human cystatin superfamily classification

Family 1 (stefins)	Family 2 (cystatins)	Family 3 (kininogens)
Intracellular	Extracellular/transcellular	Intravascular
Stefin A	Cystatin C	LMW kininogen
Stefin B	Cystatin D	HMW kininogen
	Cystatin E/M	
	Cystatin F	
	Cystatin G	
	Cystatin S	
	Cystatin SA	
	Cystatin SN	

The cystatin superfamily consists of three types classified on the basis of the presence of one, two, or three copies of cystatin-like segments and the presence or absence of disulfide bond (Table 1). The members of the cystatin superfamily are reversible competitive inhibitors of cysteine proteases (Rashid et al. 2006). In addition to the family 1, 2, and 3 cystatins, the proteins containing cystatin domains but lacking inhibitory activities are also the members of the cystatin superfamily.

Family 1 cystatins (stefins): Stefin A (also named cystatin A, acid cysteine protease inhibitor, epidermal SH-protease inhibitor) and stefin B (also named cystatin B, neutral cysteine protease inhibitor) are intracellular cystatins (Magister and Kos 2013). These single-chain proteins lack disulfide bonds and carbohydrate side chains and are composed of 98 amino acid residues with 11,175 Da and 11,006 Da, respectively (Machleidt et al. 1983; Ochieng and Chaudhuri 2010).

The structure of stefin molecule consists of a five-stranded antiparallel β-sheet wrapped around a five-turn α-helix with an additional C-terminal strand (Stubbs et al. 1990).

Stefin A and B are potent inhibitors of papain and cathepsins L, S, and H. Their genes do not encode signal peptides (Ni et al. 1997). These intracellular cytoplasmic proteins of many types of cells have been detected in extracellular fluids as well (Kos and Schweiger 2002).

Family 2 cystatins (cystatins): Cys C, D, E/M, F, G, S, SA, and SN are type 2 extracellular and/or transcellular proteins distributed in body fluids at high concentrations with molecular masses of 13–14 kDa. Cys C, D, E/M, F, G, S, SA, and SN are encoded by genes located on the chromosome 20 (Abrahamson et al. 1990). Some members of the family 2 are glycosylated (Ochieng and Chaudhuri 2010). They all contain characteristic intrachain disulfide bonds toward the C-terminal unlike stefins. S type (S, SA, and SN) were first isolated from human saliva (Magister and Kos 2013; Isemura et al. 1984). Cys D, S, SN, and SA, mainly found in saliva, are poorer inhibitors of cysteine proteases than Cys C which is the

most abundant human cystatin that strongly inhibits clan C1 and clan C13 (Magister and Kos 2013). Human Cys D, present in tears and saliva, strongly inhibits cathepsin H and S and weakly neutralizes cathepsin L, but not cathepsin B (Balbín et al. 1994). The target proteases of cystatin E/M are the papain-like cysteine proteases including cathepsin B, L, and V (Cheng et al. 2006). Legumain, the asparaginyl endopeptidase, is mostly inhibited by its potent inhibitor cystatin E/M (Abrahamson et al. 2003).

Cys C is a major local regulator of extracellular proteolytic activity that inhibits cysteine proteases belonging to the papain (C1) and legumain (C13) families and especially inhibits the cathepsin B, H, L, and S. Cys C controls the activity of cathepsins which have elastolytic and collagenolytic activities that contributes to atherosclerotic process (Dinic et al. 2014). The high activity of legumain and cathepsins has been shown to ease the invasion of tumor cells (Briggs et al. 2010). It has been suggested that in breast cancer, the loss of cystatin E/M activity leaded an increase in tumor cell growth and metastasis (Ni et al. 1997).

Family 3 cystatins (**kininogens**): Intravascular low molecular weight kininogen (LMWK; MW 50e80 kDa) and high molecular weight kininogen (HMWK; MW 120 kDa) are large extracellular proteins comprised of about 335 amino acid residues that contain three family 2-like domains. These glycosylated forms of human kininogens have additional disulfide bonds and differ in length of the C-terminal regions. Structurally both HMWK and LMWK are composed of a light chain, a heavy chain connected by disulfide bridges, and the kinin segment. The light chains of both kininogens are different; however, the heavy chain and the kinin segment have identical amino acid sequences (Kellermann et al. 1986). LMWK binds papain and cathepsin L and S; HMWK binds papain, cruzipain, and cathepsin S.

CRES (cystatin-related epididymal spermatogenic) proteins: cystatin-like proteins named as cystatin-related epididymal-specific proteins were firstly found in mouse epididymis. Though structural homology was shown with cystatins, CRES proteins have no inhibitory effect on cysteine proteases papain and cathepsin B (Cornwall and Hsia 2003).

Other cystatin-like proteins, which lack cysteine protease inhibitory properties, are fetuin A, alpha 2HS glycoprotein (ahsg), histidine-rich glycoprotein (HRG), and androgen-regulated cystatin-related proteins (CRPs), testatin, and cystatin T. Testatin and cystatin T, specifically expressed in the testis, have similar sequence with family 2 cystatins (Ochieng and Chaudhuri 2010; Eriksson et al. 2002).

Structure of Cys C

The structure of the Cys C is a composition of five antiparallel β-sheets wrapped around a central helix with the disulfide bonds of between residues 73 and 83 and between residues 97 and 117. The molecular mass of Cys C is 13,343 Da

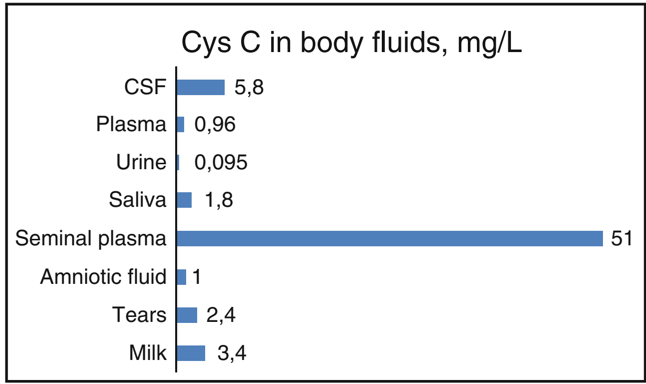

Fig. 1 Cystatin C concentrations (mg/L) in the body fluids

(nonhydroxylated) and 13,359 Da (hydroxylated proline residue at position 3). The isoelectric point of Cys C is 9.3 and thus positively charged in all body fluids (Filler et al. 2005).

Cys C is composed of 120 aa residues encoded by the "housekeeping type" CST3 gene located in the short arm of chromosome 20 at p.11.2 (Grubb and Löfberg 1982; Abrahamson et al. 1990). The protein is synthesized as a 146 aa pro-protein with a 26-residue hydrophobic signal peptide by all nucleated cells (Abrahamson et al. 1987). With the cleavage of the signal peptide, mature Cys C is released into the bloodstream in a short time. The N-terminal amino acid residue is the part of the Cys C molecule that has a high binding affinity to papain.

The amino acid sequence of the single polypeptide chain of human Cys C is SSPGK PPRLV GGPMD ASVEE EGVRR ALDFA VGEYN KASND MYHSR ALQVV RARKQ IVAGV NYFLD VELGR TTCTK TQPNL DNCPF HDQPH LKRKA FCSFQ IYAVP WQGTM TLSKS TCQDA (Grubb and Löfberg 1982).

The concentrations of Cys C in human biological fluids are figured (Fig. 1). Cystatin C is found at high concentrations in the body fluids, particularly in cerebrospinal fluid and seminal plasma (Filler et al. 2005; Magister and Kos 2013). The CSF concentration is five times more than in blood plasma which is the dominant cysteine proteinase inhibitor supplied mainly by the choroid plexus (Cole et al. 1989).

Detection method: PETIA (particle-enhanced turbidimetric immunoassay) and PENIA (particle-enhanced nephelometric immunoassay). Both methods make possible to measure routinely and rapidly, but the higher cost compared with SCr constraints its use routinely.

Specimens: Serum, plasma (EDTA, lithium heparin).

Assay Interferent Factors

Hemoglobin≤8 g/L, triglycerides ≤23 mmol/L, bilirubin ≤488 μL, and rheumatoid factor ≤2,000 kIU/L did not show any significant interference analyzing with nephelometric and turbidimetric methods (Finney et al. 1997; Delanaye et al. 2008).

Stability of cystatin C: Cys C is stable up to 48 h in the whole blood. Stored plasma samples are stable up to 4 years at −80 °C (Hoek et al. 2003).

Reference Range

Cys C production rate is constant throughout the ages of 1–50 years and increases significantly above the age of 50–60 in both gender which might be due to the physiological aging of the renal function (Finney et al. 2000; Galteau et al. 2001).

Plasma
<1 year: 0.59–1.97 mg/L (Finney et al. 2000)
1–50 years: 0.53–0.92 mg/L
>50 years: 0.58–1.02 mg/L

In premature infants, reference ranges are higher than adults (1.10–2.06 mg/L) (Schwartz and Work 2009).

Urine: 0.03–0.18 mg/L (Conti et al. 2005).

Cys C cannot be detected in urine under physiological conditions; however, in state of tubular injury, urine Cys C may become a measurable level, a potential biomarker for AKI (Slocum et al. 2012).

Cys C and Children

In pediatric population, Cys C is more advantageous than serum creatinine, especially under 4 years of age due to their lower muscle mass. Cys C levels are higher in the first weeks of life until 1 year of age, and the production rate is constant till 50–60 years unlike serum creatinine which increases until the early years of adolescence, due to muscle mass gain (Finney et al. 2000).

Cys C and Pregnancy

During the pregnancy, the serum Cys C concentrations were found as differed significantly with gestational age. In the first trimester, Cys C values were detected as higher than the second trimester and were increased in the third trimester which is

at the highest value after delivery. It was considered as a reliable GFR marker in pregnancy instead of serum creatinine that is unreliable (Babay et al. 2005).

Factors Affecting Cys C

Cystatins are involved in a number of immunomodulatory functions; it was shown to be associated in the pathophysiology of multiple sclerosis and Alzheimer' s disease (Bollengier 1987; Levy et al. 2001) During the inflammatory processes, Cys C release has been found as downregulated, contributing to increased cysteine protease activities in the macrophage microenvironment (Chapman et al. 1990) However, contrary to earlier suggestions, no significant effect of systemic inflammation on plasma Cys C concentrations was detected in a recent study by Grubb et al. (2011).

Cys C levels were found as affected by visceral obesity and insulin resistance (Ognibene et al. 2006). In fact, visceral obesity and insulin resistance are frequently associated with GFR increase and lower Cys C concentrations. Thyroid hormones increase Cys C levels; thus, hypothyroidism has been associated with lower Cys C concentrations and hyperthyroidism with higher Cys C concentrations (Schmid et al. 2012; Fricker et al. 2003).

It has been shown that moderate or high doses of glucocorticoids increase the Cys C production (Risch et al. 2001).

Advantages of Cys C

Cys C is a promising marker due to its proven satisfactory criteria, thus gaining popularity. Cys C has a short half-life (1.5 h) compared with serum creatinine (4 h with normal GFR) (Sjostrom et al. 2004). Serum creatinine is distributed in whole body water, whereas Cys C is distributed in extracellular part. Therefore Cys C rises more rapidly than serum creatinine and advantageous for early contrast-induced renal injury detection (Briguori et al. 2010). Less than 10 % increase in Cys C at 24 h can be a reliable marker for ruling out contrast-induced acute renal injury, and more than $\geq$10 % increase in serum Cys C at 24 h is an independent predictor of 1-year major adverse events such as death and dialysis (Briguori et al. 2010).

Although there are some conflicting data regarding greater intraindividual variability of Cys C than serum creatinine, in a recent study intraindividual variability of Cys C has been confirmed as similar to serum creatinine that Cys C seems as accurate as SCr for longitudinal patient follow-up (Delanaye et al. 2008). Interindividual variations of Cys C and creatinine were found as similar about 15.1 % and 14.4 %, respectively (Reinhard et al. 2009).

Cys C plasma concentration is inversely correlated with GFR as it is only catabolized in the kidney. Its renal clearance cannot be measured. Urine Cys C can

Table 2 Concentrations of human cystatin c in body fluids of healthy adults (mg/L; mean and range) (Filler et al. 2005)

Body fluid
Blood plasma 0.96, 0.57–1.79
Cerebrospinal fluid 5.8, 3.2–12.5
Urine 0.095, 0.033–0.29
Saliva 1.8, 0.36–4.8
Seminal plasma 51.0, 41.2–61.8
Amniotic fluid 1.0, 0.8–1.4
Tears 2.4, 1.3–7.4
Milk 3.4, 2.2–3.9

only be detected in case of renal proximal tubular impairment, which is more specific than serum Cys C (Koyner et al. 2008). Rapid testing is available with commercial automated assay procedures.

Disadvantages of Cys C

The test is relatively expensive; in daily routine, a large volume of testing requires more evidences clinically and careful consideration.

Cys C levels are lower in the hypothyroid and higher in hyperthyroid state. Very high doses of glucocorticoids increase the Cys C production, whereas low or medium doses of glucocorticoids decrease the Cys C production. Some evidence suggests that inflammation, osteoporosis, and diabetes affect the cystatin levels. In a study authors found 8.5 % higher levels of Cys C in patients with diabetes mellitus. However, that effect might be due to the interaction with proteinuria and Cys C. As in a study with type 1 diabetic patients with normal renal function without proteinuria, no influence of diabetes was found on the relationship between Cys C and GFR (Hofstra et al. 2009). The advantages and disadvantages are summarized in Table 2.

Cys C and GFR

GFR estimation is a widely used measurement protocol instead of the invasive methods based on exogenously injection of substances such as inulin, $^{125-}$Iothalamate, iohexol, ^{51}Cr-EDTA which are traumatic for patients, especially for pediatric population. Moreover some techniques imply exposure to radiation.

Creatinine is produced by the muscle from creatine; thus, the muscle mass affects its concentration. Besides serum creatinine differs by age, gender, rhabdomyolysis, and dietary meat (Ochieng and Chaudhuri 2010). Moreover creatinine is secreted by renal tubules in a varied amount, and drugs may influence the tubular secretion of creatinine (Grubb et al. 2012). Therefore a new endogenous biomarker is being searched for GFR estimation that Cys C seems ideal in this aspect (Table 3).

Table 3 Advantages and disadvantages of cystatin C measurement

Advantages of Cys C
Less effected from nonrenal factors: gender, ethnicity (Caucasian, Afro-American and Asian), and muscle mass
Cys C production rate is constant from 1 year of age to 50 years
Cys C secretion does not have a circadian rhythm (Larsson et al. 2008)
The absence of a circadian rhythm for Cys C allows its quantification in a single urine sample (Conti et al. 2005)
Interindividual variation of Cyc C is about 15.1 % (Reinhard et al. 2009), intraindividual variation 4.5 % (Delanaye et al. 2008; Bandaranayake et al. 2007)
Short half-life (1.5 h) and extracellular distribution cause a rapid rise in serum concentration that is an advantage over serum creatinine
Less effected from volume status
Rapid and precise testing is available with automated methods
Sensitive to small changes in GFR
Reliable in liver disease
More convenient in the pediatric population
Specific to proximal renal tubule injury; only small amounts of Cys C can be found in urine (tenfold lower than in plasma), and increased concentrations in single-void urine samples directly reflect tubular damage (Conti et al. 2005)
Disadvantages of Cys C
Non-standardized (lack of international standard)
Expensive
Levels altered by thyroid dysfunctions
High doses of corticosteroids increase Cys C production
Malignancy increases serum concentrations

To overcome the creatinine-based limitations, several formulas have been developed and some parameters have been added to the GFR prediction equations (Cockcroft and Gault 1976; Levey et al. 1999). However, neither Cockcroft and Gault equation that produces GFR values in ml/min nor the modification of diet in renal disease (MDRD) equation that gives results as ml/min (1.73 $m^2)^{-1}$ is suitable for children or for adults especially at eGFR >90 ml/min (1.73 $m^2)^{-1}$ level. Instead, Schwartz equation or Counahan-Barratt equation is preferred for children (Schwartz et al. 1976; Counahan et al. 1976; Grubb et al. 2005).

Cys C is present in many biological fluids at higher concentrations, and its low molecular weight and positive net charge facilitate its glomerular filtration. Its plasma concentration is proportional with glomerular filtration as it is produced constantly by all nucleated cells. Cys C is later largely reabsorbed and catabolized in the proximal renal tubule with no tubular secretion which makes it ideal for GFR estimation (Grubb et al. 1985, 1992). Cys C has been proposed as an alternative marker of renal function because of its possible advantages over serum creatinine (Dharnidharka et al. 2002; Grubb et al. 2005). This protein has a capability to detect early renal failure as it gives reliable GFR estimation at the critical level of 60 ml/min/1.73 m^2 (Bargnoux et al. 2012). Grubb et al. concluded that instead of

Table 4 Ideal GFR marker characteristics (Ochieng and Chaudhuri 2010; Swan 1997)

Ideal GFR marker characteristics
Endogenously produced at a constant rate
Filtered and excreted only by the kidney
Unaffected by nonrenal factors such as age, gender, weight, nutrition, disease state, etc.
Freely filtered through the glomerulus
Neither reabsorbed nor secreted by the renal tubules
Undergo no extrarenal elimination
Stable in urine for further analysis

creatinine, Cys C-based prediction equation for GFR estimation that uses only serum concentration and a prepubertal factor (GFR [ml/min^{-1}.(1.73 m^2)$^{-1}$] = 84.69 × cys c (mg/L)$^{-1.680}$ × 1.384 (if <14 years)) might replace both the MDRD prediction equation for adults and the Schwartz and Counahan-Barratt prediction equations for children. In that formula, a prepubertal factor added to compensate the prepubertal concentrations that is higher than the older individuals where 13–14 years of age limit represents the start of the puberty (Grubb et al. 2005). Ideal GFR marker characteristics are listed in Table 4.

The gold standard exogenous marker ^{51}Cr-EDTA has been compared with Cys C to estimate GFR; this endogenous marker gave excellent correlation with GFR (Simonsen et al. 1985). Cys C is more sensitive to small changes in the so-called creatinine-blind GFR (40–70 ml/min) (Schwartz and Work 2009). It was reported that in children, the serum Cys C is better correlated with GFR than serum creatinine (Grubb et al. 2005).

There are equations recommended to estimate GFR from Cys C concentration:

(A) Grubb's equation : $\mathrm{GFR_{Grubb}} = 84.69 \times \mathrm{Cystatin\ C}^{-1.680} (\times 1.384 \text{ if child} < 14 \text{ years})$ (Grubb et al. 2005).
(B) Larsson's equation : $\mathrm{GFR_{Larsson}} = 99.43 \times \mathrm{Cystatin\ C}^{-1.5837}$; cystatin C is measured in mg/L (Larsson et al. 2004).

The problem that restricts the widespread use of Cys C is the lack of standardization. Though recently a certified reference material (ERM-DA471/IFCC) has been released, the different assay systems, differences in the established reference intervals for different populations are the factors that affect the reliability of Cys C-based equations inevitably (Grubb et al. 2005). Besides the use of large doses of corticosteroids, thyroid dysfunction reduces the performance of Cys C-based equations. In those cases the diagnostic performance of creatinine-based equation is more reliable.

The use of Cys C in combination with SCr to calculate eGFR strengthens the risk classification of chronic kidney disease. The risk of death was found as increased below the threshold of ~85 ml/min/1.73 m^2 when using both Cys C and creatinine-based eGFR (Shlipak et al. 2013). It has been concluded that Cys C is more sensitive to small changes in GFR than serum creatinine in contrast-induced acute kidney injury (Briguori et al. 2010).

Although Cys C-based equations offer significant advantages over creatinine-based equations, Cys C cannot replace creatinine as it also has some limitations mentioned above and not perfectly tested in some clinical situations. Consequently Cys C-based equations cannot replace gold standard methods (Grubb et al. 2005; Andersen et al. 2009).

Cys C and Cirrhosis

In patients with liver cirrhosis, renal dysfunction is associated with poor prognosis. In these patients, SCr may be influenced directly by nonrenal factors such as protein-calorie malnutrition, muscle wasting, and increased tubular secretion, and impaired liver function caused reduced creatinine production. And indirectly, hemodynamic changes will affect serum creatinine concentrations. In cirrhosis, GFR estimation with creatinine has been shown to overestimate the true GFR by up to 200 % (Sherman et al. 2003); consequently the renal failure is greatly underestimated.

Inulin clearance was considered as the gold standard in cirrhosis (Caregaro et al. 1994). However, this method is very cumbersome to perform in clinical practice. Moreover, urine collection is difficult to execute in clinical practice because of urinary losses and incomplete urinary bladder emptying. ^{51}CR-EDTA and ^{99}TC-DPTA are other measurement techniques that imply exposure to radiation. Conversely, Cys C is independent of gender, age, and muscle mass, and not influenced by serum bilirubin, inflammation, or malignancy. Accordingly, Cys C has been proposed as a specific marker of GFR and an early indicator of impaired renal function in patients with cirrhosis (Ćulafić et al. 2014; Ustundag et al. 2007).

Cys C and Malignancy

Cys C is shown to be increased in malignancies in some studies irrespective of kidney function. High concentrations of cathepsin B and H are detected in the sera of patients with colorectal cancer and malign melanoma which are associated with increased serum Cys C (Kos et al. 1998). Imbalance between cysteine proteinases and cystatins is associated with tumoral cell metastasis that is considered to facilitate tumor cell invasion and metastasis. Though high levels of Cys C may inhibit cathepsin activities, increased levels of Cys C was found to be associated with poor prognosis (Kos et al. 2000). Increase in the inhibitory functions of cystatin could lead to a harmful impairment of the antitumor response of cysteine cathepsins (Magister and Kos 2013).

Cysteine proteases take part in tumoral metastasis that involves local invasion and angiogenesis. Cathepsin B, detected to be present on the surface of the tumor cell (Mai et al. 2000), plays a key role in tumoral cell invasion (Ochieng and Chaudhuri 2010) which is particularly inhibited by Cys C. Though increases in Cys C concentration were determined in malignant diseases such as colorectal cancer or melanoma, in those studies, GFR measurement was not performed with a reference

technique (Kos et al. 1997, 1998). In myeloma, serum Cys C was found as more sensitive than SCr for GFR estimation that detected moderate GFR reductions. Additionally, Cys C also correlated with advanced disease and provided important information for prognosis (Terpos et al. 2009; Nückel et al. 2012).

Amyloid Angiopathy

Cys C is a protein with amyloidogenic properties that aggregates in the brain arteries of elderly people with amyloid angiopathy. A more severe disease massive amyloidosis is associated with the L68Q mutant of human Cys C that leads to death in young adults with massive cerebral hemorrhage. This autosomal dominant disorder, associated with mutation in the Cys C gene CST3, is known as Icelandic type. A point mutation that replaces leucine amino acid with glutamine at position codon 68 of the Cys C gene is a disorder known as hereditary cerebral hemorrhage with amyloidosis of Icelandic type (HCHWA-1) (Ghiso et al. 1986; Levy et al. 1989; Revesz et al. 2009). This less stable deposit of mutant Cys C (ACys) aggregates mainly in the brain arteries to form plaques called amyloid deposits that impairs the elasticity of the arterial wall by replacing the muscle fibers and elastic fibers (Pezzini et al. 2009).

Summary Points

- This chapter reviews cystatin C and its significance for renal function.
- Cystatins are cysteine protease inhibitors where they appear to provide protective functions.
- Cystatin C is a protein synthesized by all nucleated cells and secreted shortly after its synthesis and not affected by nonrenal factors.
- Cystatin C is freely filtered through the glomerulus and almost completely catabolized by tubules that are an advantage for GFR estimation.
- Low molecular weight and positive net charge of Cys C facilitate its glomerular filtration. The plasma concentration is proportional with glomerular filtration as it is produced constantly.
- Cystatin C is an endogenous GFR marker that can be easily detected in serum with automated methods.
- Urine concentration is very low (tenfold lower than plasma concentration), and increased concentrations in urine samples directly reflect tubular damage.
- Cys C is a protein with amyloidogenic properties that aggregates in the brain arteries of elderly people with amyloid angiopathy.
- Lysosomal cysteine proteases, generally known as cathepsins (clan C1), are the papain family that are responsible for terminal protein degradation in the lysosomes.

- The cystatin superfamily consists of three types classified on the basis of the presence of one, two, or three copies of cystatin-like segments and the presence or absence of disulfide bond.
- Cystatin C is found at high concentrations in the body fluids, particularly in the cerebrospinal fluid and seminal plasma.

References

Abrahamson M, Grubb A, Olafsson I, Lundwall A. Molecular cloning and sequence analysis of cDNA coding for the precursor of the human cysteine proteinase inhibitor cystatin C. FEBBS Lett. 1987;216:229–33.

Abrahamson M, Olafsson I, Palsdottir A, et al. Structure and expression of the human cystatin C gene. Biochem J. 1990;268(2):287–94.

Abrahamson M, Alvarez-Fernandez M, Nathanson CM. Cystatins. Biochem Soc Symp. 2003;70:179–99.

Andersen TB, Eskild-Jensen A, Frøkiaer J, et al. Measuring glomerular filtration rate in children; can cystatin C replace established methods? A review. Pediatr Nephrol. 2009;24(5):929–41.

Babay Z, Al-Wakeel J, Addar M, et al. Serum cystatin C in pregnant women: reference values, reliable and superior diagnostic accuracy. Clin Exp Obstet Gynecol. 2005;32(3):175–9.

Balbín M, Hall A, Grubb A, et al. Structural and functional characterization of two allelic variants of human cystatin D sharing a characteristic inhibition spectrum against mammalian cysteine proteinases. J Biol Chem. 1994;269(37):23156–62.

Bandaranayake N, Ankrah-Tetteh T, Wijeratne S, Swaminathan R. Intra-individual variation in creatinine and cystatin C. Clin Chem Lab Med. 2007;45(9):1237–9.

Bargnoux AS, Servel AC, Piéroni L, et al. SFBC Biology of renal function and renal failure working group. Accuracy of GFR predictive equations in renal transplantation: validation of a new turbidimetric cystatin C assay on Architect c8000. Clin Biochem. 2012;45(1–2):151–3.

Bollengier F. Cystatin C, alias post-gamma-globulin: a marker for multiple sclerosis? J Clin Chem Clin Biochem. 1987;25:589–93.

Briggs JJ, Haugen MH, Johansen HT, et al. Cystatin E/M suppresses legumain activity and invasion of human melanoma. BMC Cancer. 2010;10 (17):1–13.

Briguori C, Visconti G, Rivera NV, et al. Cystatin C and contrast-induced acute kidney injury. Circulation. 2010;121:2117–22.

Caregaro L, Menon F, Angeli P, et al. Limitations of serum creatinine level and creatinine clearance as filtration markers in cirrhosis. Arch Intern Med. 1994;154:201–5.

Chapman Jr HA, Reilly Jr JJ, Yee R, et al. Identification of cystatin C, a cysteine proteinase inhibitor, as a major secretory product of human alveolar macrophages in vitro. Am Rev Respir Dis. 1990;141(3):698–705.

Cheng T, Hitomi K, van Vlijmen-Willems I, et al. Cystatin M/E is a high affinity inhibitor of cathepsin V and cathepsin L by a reactive site that is distinct from the legumain-binding site – a novel clue for the role of cystatin M/E in epidermal cornification. J Biol Chem. 2006;281 (23):15893–9.

Clausen J. Proteins in normal cerebrospinal fluid not found in serum. Proc Soc Exp Biol Med. 1961;107:170–2.

Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron. 1976;16:31–41.

Cole T, Dickson PW, Esnard F, et al. The cDNA structure and expression analysis of the genes for the cysteine proteinase inhibitor cystatin C and for beta 2-microglobulin in rat brain. Eur J Biochem. 1989;186(1–2):35–42.

Conti M, Zater M, Lallali K, et al. Absence of circadian variations in urine cystatin C allows its use on urinary samples. Clin Chem. 2005;51(1):272–3.

Cornwall GA, Hsia N. A new subgroup of the family 2 cystatins. Mol Cell Endocrinol. 2003;200 (1–2):1–8.
Counahan R, Chantler C, Ghazali S, et al. Estimation of glomerular filtration rate from plasma creatinine concentration in children. Arch Dis Child. 1976;51:875–8.
Ćulafić D, Štulić M, Obrenović R, et al. Role of cystatin C and renal resistive index in assessment of renal function in patients with liver cirrhosis. World J Gastroenterol. 2014;20(21):6573–9.
Delanaye P, Pieroni L, Abshoff C, et al. Analytical study of three cystatin C assays and their impact on cystatin C-based GFR-prediction equations. Clin Chim Acta. 2008;398(1–2):118–24.
Dharnidharka VR, Kwon C, Stevens G. Serum cystatin C is a superior to serum creatinine as a marker of kidney function: a meta-anlysis. Am J Kidney Dis. 2002;40:221–6.
Dinic M, Maillard N, Thibaudin D, et al. Serum cystatin C is a determinant of central pressure augmentation index measured by oscillometric method in renal transplant recipients. BMC Nephrol. 2014;15:196.
Eriksson A, Tohonen V, Wedell A, Nordqvist K. Isolation of the human testatin gene and analysis in patients with abnormal gonadal development. Mol Hum Reprod. 2002;8:8–15.
Filler G, Bökenkamp A, Hofmann W, et al. Cystatin C as a marker of GFR-history, indications, and future research. Clin Biochem. 2005;38:1–8.
Finney H, Newman DJ, Gruber W, et al. Initial evaluation of cystatin C measurement by particle-enhanced immunonephelometry on the Behring nephelometer systems (BNA, BN II). Clin Chem. 1997;43:1016–622.
Finney H, Newman DJ, Price CP. Adult reference ranges for serum cystatin C, creatinine and predicted creatinine clearance. Ann Clin Biochem. 2000;37:49–59.
Fricker M, Wiesli P, Barndle M, et al. Impact of thyroid dysfunction on serum cystatin C. Kidney Int. 2003;63:1944–7.
Galteau MM, Guyon M, Gueguen R, Siest G. Determination of serum cystatin C: biological variation and reference values. Clin Chem Lab Med. 2001;39(9):850–7.
Ghiso J, Jensson O, Frangione B. Amyloid fibrils in hereditary cerebral hemorrhage with amyloid-osis of Icelandic type is a variant of gamma-trace basic protein (cystatin C). Proc Natl Acad Sci U S A. 1986;83(9):2974–8.
Grubb A. Diagnostic value of analysis of cystatin C and protein HC in biological fluids. Clin Nephrol. 1992;38:S20–7.
Grubb A, Löfberg H. Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. Proc Natl Acad Sci U S A. 1982;79:3024–7.
Grubb A, Simonsen O, Sturfelt G, et al. Serum concentration of cystatin C, factor D and beta 2-microglobulin as a measure of glomerular filtration rate. Acta Med Scand. 1985;218(5):499–503.
Grubb A, Nyman U, Björk J, et al. Simple cystatin C-based prediction equations for glomerular filtration rate compared with the modification of diet in renal disease prediction equation for adults and the Schwartz and the Counahan-Barratt prediction equations for children. Clin Chem. 2005;51(8):1420–31.
Grubb A, Björk J, Nyman U, et al. Cystatin C, a marker for successful aging and glomerular filtration rate, is not influenced by inflammation. Scand J Clin Lab Invest. 2011;71(2):145–9.
Grubb A, Nyman U, Björk J. Improved estimation of glomerular filtration rate (GFR) by compar-ison of eGFR cystatin C and eGFR creatinine. Scand J Clin Lab Invest. 2012;72(1):73–7.
Hoek FJ, Kemperman FA, Krediet RT. A comparison between cystatin C, plasma creatinine and the Cockcroft and Gault formula for the estimation of glomerular filtration rate. Nephrol Dial Transplant. 2003;18(10):2024–31.
Hofstra JM, Vervoort G, Willems JL, Wetzels JF. Cystatin C levels are unaltered in patients with diabetes mellitus and normal renal function. Kidney Int. 2009;76(4):462.
Isemura S, Saitoh E, Ito S, et al. Cystatin S: a cysteine proteinase inhibitor of human saliva. J Biochem. 1984;96(4):1311–4.

Kellermann J, Lottspeich F, Henschen A, Müller-Esterl W. Completion of the primary structure of human high-molecular-mass kininogen. The amino acid sequence of the entire heavy chain and evidence for its evolution by gene triplication. Eur J Biochem. 1986;154(2):471–8.

Kos J, Schweiger A. Cathepsins and cystatins in extracellular fluids-useful biological markers in cancer. Radiol Oncol. 2002;36(2):176–9.

Kos J, Stabuc B, Schweiger A, et al. Cathepsins B, H, and L and their inhibitors stefin A and cystatin C in sera of melanoma patients. Clin Cancer Res. 1997;3(10):1815–22.

Kos J, Stabuc B, Cimerman N, Brünner N. Serum cystatin C, a new marker of glomerular filtration rate, is increased during malignant progression. Clin Chem. 1998;44(12):2556–7.

Kos J, Krasovec M, Cimerman N, et al. Cysteine proteinase inhibitors stefin A, stefin B, and cystatin C in sera from patients with colorectal cancer: relation to prognosis. Clin Cancer Res. 2000;6 (2):505–11.

Koyner JL, Bennett MR, Worcester EM, et al. Urinary cystatin C as an early biomarker of acute kidney injury following adult cardiothoracic surgery. Kidney Int. 2008;74(8):1059–69.

Larsson A, Malm J, Grubb A, Hansson LO. Calculation of glomerular filtration rate expressed in mL/min from plasma cystatin C valuesin mg/L. Scand J Clin Lab Invest. 2004;64(1): 25–30.

Larsson A, Akerstedt T, Hansson LO, Axelsson J. Circadian variability of cystatin C, creatinine, and glomerular filtration rate (GFR) in healthy men during normal sleep and after an acute shift of sleep. Chronobiol Int. 2008;25(6):1047–61.

Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of diet in renal disease study group. Ann Intern Med. 1999;130:461–70.

Levy E, Lopez-Otin C, Ghiso J, et al. Stroke in Icelandic patients with hereditary amyloid angiopathy is related to a mutation in the cystatin C gene, an inhibitor of cysteine proteases. J Exp Med. 1989;169(5):1771–8.

Levy E, Sastre M, Kumar A, Gallo G, Piccardo P, Ghetti B, Tagliavini F. Codeposition of cystatin C with amyloid-beta protein in the brain of Alzheimer disease patients. J Neuropathol Exp Neurol. 2001;60:94–104.

Machleidt W, Borchart U, Fritz H, et al. Protein inhibitors of cysteine proteinases. II. Primary structure of stefin, a cytosolic protein inhibitor of cysteine proteinases from human polymorphonuclear granulocytes. Hoppe Seylers Z Physiol Chem. 1983;364(11):1481–6.

Magister Š, Kos J. Cystatins in immune system. J Cancer. 2013;4(1):45–56.

Mai J, Waisman DM, Sloane BF. Cell surface complex of cathepsin B/annexin II tetramer in malignant progression. Biochim Biophys Acta. 2000;1477(1–2):215–30.

Ni J, Abrahamson M, Zhang M, et al. Cystatin E is a novel human cysteine proteinase inhibitor with structural resemblance to family 2 cystatins. J Biol Chem. 1997;272(16):10853–8.

Nückel H, Langer C, Herget-Rosenthal S, et al. Prognostic significance of serum cystatin C in multiple myeloma. Int J Hematol. 2012;95(5):545–50.

Ochieng J, Chaudhuri G. Cystatin superfamily. J Health Care Poor Underserved. 2010;21:51–70.

Ognibene A, Mannucci E, Caldini A, et al. Cystatin C reference values and aging. Clin Biochem. 2006;39(6):658–61.

Pezzini A, Del Zotto E, Volonghi I, et al. Cerebral amyloid angiopathy: a common cause of cerebral hemorrhage. Curr Med Chem. 2009;16(20):2498–513.

Rashid F, Sharma S, Bano B. Detailed biochemical characterization of human placental cystatin (HPC). Placenta. 2006;27(8):822–31.

Reinhard M, Erlandsen EJ, Randers E. Biological variation of cystatin C and creatinine. Scand J Clin Lab Invest. 2009;69(8):831–6.

Revesz T, Holton JL, Lashley T, et al. Genetics and molecular pathogenesis of sporadic and hereditary cerebral amyloid angiopathies. Acta Neuropathol. 2009;118(1):115–30.

Risch L, Herklotz R, Blumberg A, Huber AR. Effects of glucocorticoid immunosuppression on serum cystatin C concentrations in renal transplant patients. Clin Chem. 2001;47:2055–9.

Schmid C, Ghirlanda-Keller C, Zwimpfer C, Zoidis E. Triiodothyronine stimulates cystatin C production in bone cells. Biochem Biophys Res Commun. 2012;419:425–30.
Schwartz GJ, Work DF. Measurement and estimation of GFR in children and adolescents. Clin J Am Soc Nephrol. 2009;4(11):1832–43.
Schwartz GJ, Haycock GB, Edelmann Jr CM, Spitzer A. A simple estimate of glomerular filtration rate in children derived from body length and plasma creatinine. Pediatrics. 1976;58:259–63.
Sherman DS, Fish DN, Teitelbaum I. Assessing renal function in cirrhotic patients: problems and pitfalls. Am J Kidney Dis. 2003;41:269–78.
Shlipak MG, Coresh J, Gansevoort RT. Cystatin C versus creatinine for kidney function-based risk. N Engl J Med. 2013;369(25):2459.
Simonsen O, Grubb A, Thysell H. The blood serum concentration of cystatin C (gamma-trace) as a measure of the glomerular filtration rate. Scand J Clin Lab Invest. 1985;45(2):97–101.
Sjostrom P, Tidman M, Jones I. The shorter T1/2 of cystatin C explains the earlier change of its serum level compared to serum creatinine. Clin Nephrol. 2004;62:241–2.
Slocum JL, Heung M, Pennathur S. Marking renal injury: can we move beyond serum creatinine? Transl Res. 2012;159(4):277–89.
Stubbs MT, Laber B, Bode W, et al. The refined 2.4 A X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction. EMBO J. 1990;9(6):1939–47.
Swan SK. The search continues-an ideal marker of GFR. Clin Chem. 1997;43:913–4.
Terpos E, Katodritou E, Tsiftsakis E, Greek Myeloma Study Group, et al. Cystatin-C is an independent prognostic factor for survival in multiple myeloma and is reduced by bortezomib administration. Haematologica. 2009;94(3):372–9.
Turk V, Turk B. Lysosomal cysteine proteases and their protein inhibitors: recent developments. Acta Chim Slov. 2008;55:727–38.
Turk V, Brzin J, Longer M, et al. Protein inhibitors of cysteine proteinases. III. Amino-acid sequence of cystatin from chicken egg white. Hoppe Seyler Z Physiol Chem. 1983;364:1487–96.
Turk B, Turk D, Turk V. Lysosomal cysteine proteases: more than scavengers. Biochim Biophys Acta. 2000;1477(1–2):98–111.
Turk B, Turk D, Salvesen GS. Regulating cysteine protease activity: essential role of protease inhibitors as guardians and regulators. Curr Pharm Des. 2002;8(18):1623–37.
Ustundag Y, Samsar U, Acikgoz S, et al. Analysis of glomerular filtration rate, serum cystatin C levels, and renal resistive index values in cirrhosispatients. Clin Chem Lab Med. 2007;45(7): 890–4.

Homocysteinemia as a Biomarker in Kidney Disease 21

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Abstract

Homocysteine is an amino acid containing sulfur in the form of the thiol group. Homocysteine is a metabolite of the essential amino acid methionine. First, methionine is converted to S-adenosylmethionine, which is then converted to S-adenosylhomocysteine, wherein transmethylation processes take place.

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Homocysteine finally emerges from S-adenosylhomocysteine. It then enters either the transsulfuration pathway when converted to cystathionine or the remethylation pathway when converted back into methionine in the presence of the 5-methyltetrahydrofolate reductase, cystathionine β-synthase, and methionine synthase and with the participation of vitamin B_{12} and folic acid. Therefore, the decreased activities of these enzymes, as well as the deficit of the vitamin B_{12} and folic acid, are frequent causes of hyperhomocysteinemia. In addition, chronic kidney disease and the use of certain drugs are also very important factors that cause hyperhomocysteinemia.

In the bloodstream, homocysteine is found in one of two forms, the bound and the free form, which together makes up the total homocysteine. The most suitable sample for determining the circulating levels of total homocysteine is plasma. Enzymatic and immunochemical methods are most commonly used in routine clinical practice. Reference values of the total homocysteine vary among different countries and even among different laboratories as a result of the use of different methods for determining the levels of total homocysteine but also the different lifestyles of the residence.

The plasma total homocysteine level is positively correlated with the serum creatinine values, and it is in a high inverse correlation with the glomerular filtration rate, so it could be used as a marker of renal hypofunction. In addition, the kidneys possess enzymes involved in metabolic pathways. That is why chronic kidney disease is one of the most common causes of hyperhomocysteinemia. The level of homocysteine in patients with chronic kidney disease can be elevated even in the initial stages. Hyperhomocysteinemia is present in most patients with glomerular filtration rate <60 ml/min/1.73 m^2, with the highest values in patients on chronic dialysis treatment. Hyperhomocysteinemia occurs in 50–60 % of patients with transplanted kidneys. On the other hand, hyperhomocysteinemia is an important factor that contributes to the structural damage of the kidney, both glomerular and tubular, along with damage to the interstitial tissue. Therefore, when there are hyperhomocysteinemia and absence of the deficiency of the vitamin B_{12} and folic acid, it is necessary to examine the renal function.

Homocysteine is a vascular toxin, and it is one of the risk factors for the development of coronary heart disease, cerebrovascular disease, and peripheral artery disease. Also, it has a role in the development of venous thrombosis. Therefore, in patients with chronic kidney disease, the plasma concentration of homocysteine is a significant determinant of the risk of morbidity and mortality from cardiovascular disease. The measurement of homocysteine in patients with renal dysfunction is necessary for making decisions about therapeutic treatment, especially if the patient has not yet had signs of development of cardiovascular disease.

In addition, it is assumed that homocysteine has a role in the pathogenesis of other pathological conditions, such as neurodegenerative diseases, dementia, cognitive disorders, osteoporosis, epigenetic changes, hypertension, as well as the stimulation of an immune response.

Keywords
Homocysteine (Hcy) • Vascular disease in patients with chronic kidney disease • Determination • Diabetes mellitus and diabetic nephropathy • Diagnostics and monitoring of renal dysfunction • Metabolism • Nephrotic syndrome • Potential applications • Regulation • Renal function • Structure and origin • Terminal renal failure and in transplanted patients • Hyperhomocysteinemia • Biological effects and mechanisms • Causes • Nephrotic syndrome • Homocysteine

Abbreviations

5-MTHF	Methyltetrahydrofolate
5-MTHFR	Methyltetrahydrofolate reductase
ADMA	Asymmetric dimethylarginine
apo A-I	Apolipoprotein A-I
ATP	Adenosine triphosphate
bHcy	Bound homocysteine
BHL	Bleomycin hydrolase
BHMT	Betaine-homocysteine methyltransferase
CBS	Cystathionine β-synthase
CKD	Chronic kidney disease
CMIS	Chronic malnutrition-inflammation state
CSE	Cystathionine γ-lyase
CVD	Cardiovascular disease
DM	Diabetes mellitus
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
eNOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease
fHcy	Free homocysteine
GFR	Glomerular filtration rate
H2S	Hydrogen sulfide
Hcy	Homocysteine
HDL	High-density lipoprotein
HHcy	Hyperhomocysteinemia
HTL	Homocysteine thiolactone
IL	Interleukin
Km	Michaelis constant
LDL	Low-density lipoprotein
MAT	Methionine adenosyltransferase
MCP-1	Monocyte chemoattractant protein-1
Met	Methionine
MIP-2	Macrophage inflammatory protein-2
MMP	Matrix metalloproteinase
MS	Methionine synthase
NAC	*N*-acetylcysteine

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NS	Syndroma nephroticum
oxLDL	Oxidized low-density lipoprotein
PON1	Paraoxonase-1
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
TAFI	Thrombin activatable fibrinolysis inhibitor
TGF-β1	Transforming growth factor β1
tHcy	Total homocysteine
t-PA	Tissue plasminogen activator
tRNA	Transfer ribonucleic acid
VSMC	Vascular smooth muscle cells

Key Facts of Homocysteine as a Biomarker in Kidney Disease

- The kidneys are a major regulator of the circulating levels of homocysteine (Hcy), as they take part in its excretion and metabolism.
- In patients with chronic kidney disease (CKD), the glomerular filtration rate (GFR) is the main determinant of the total Hcy levels. Hyperhomocysteinemia (HHcy) is present in renal transplant patients as well.
- Hemodialysis treatment leads to a transient reduction in the levels of tHcy.
- HHcy in patients with diabetes mellitus mainly occurs with the development of diabetic nephropathy.
- HHcy contributes to the progression of renal dysfunction. Therefore, in all patients with HHcy and without deficit of folic acid and vitamin B_{12}, it is necessary to evaluate the functional status of the kidneys.
- HHcy in CKD may contribute to the development of vascular comorbidity and comortality.
- In patients with CKD, the determination of the tHcy levels has significance in the assessment of the risk for the morbidity and mortality due to CVD, then in the assessment of the progression of renal dysfunction, the estimation of the total mortality in CKD patients with malnutrition-inflammation syndrome, as well as for making a decision regarding the therapeutic treatment.

Definitions

Homocysteinylation The reaction in which the Hcy in the form of homocysteine thiolactone binds to amino groups of amino acids, leading to a dysfunction of the proteins.

Hyperhomocysteinemia Increasing levels of total homocysteine (tHcy), which implies an increase in the level of the free (fHcy) and bound Hcy (bHcy).

Hypomethylation Reduction in the volume of the transmethylation reactions in hyperhomocysteinemia (HHcy), due to the inhibition of the methyltransferase by the increased levels of S-adenosylhomocysteine (SAH).

Remethylation The conversion of Hcy into methionine by the enzyme methionine synthase, the folic acid as a donor of the methyl groups, and of vitamin B_{12} as a cofactor.

Transmethylation The reaction of the methyl group transfer from the donor to the acceptor. This reaction takes place during the conversion of methionine into Hcy, and it is dependent on the intermediates S-adenosylmethionine (SAM) and SAH.

Transsulfuration Reaction to the conversion of Hcy into cystathionine with the participation of vitamin B_6. Cystathionine, after being converted into various compounds, is excreted by the kidneys, which are the pathways for eliminating Hcy from the body.

Introduction

Homocysteine (Hcy) was discovered in the fourth decade of the twentieth century. However, only when its role in the pathogenesis of vascular diseases was realized, in the last 30 years, has it received greater attention. In addition, an increasing number of scientific papers indicate a possible causal relationship between hyperhomocysteinemia (HHcy) and other pathological conditions.

The kidneys play an important role in the metabolic homeostasis of Hcy, because they participate in Hcy metabolism, and on the other hand, they are involved in Hcy excretion. Hyperhomocysteinemia is almost an inevitable phenomenon in patients with kidney disease, particularly chronic.

The Structure and Origin of Homocysteine

Homocysteine is a nonessential amino acid which contains sulfur in the form of thiol (-SH) group (Fig. 1). Hcy is produced in all human cells, as a product of the intracellular metabolism of the essential amino acid methionine (Met). When Hcy production is beyond the scope of its intracellular metabolic processing, Hcy released from the cells is increased which might lead to the development of HHcy.

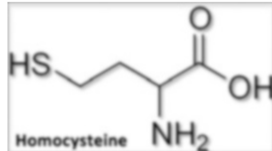


Fig. 1 Homocysteine – the structure. Homocysteine is an amino acid which contains sulfur in the form of -SH group

Hcy is eliminated from the bloodstream in a number of ways, the most important being the reuptake by cells and excretion in the urine.

Hcy is present in two forms in the bloodstream. Approximately 70–80 % of Hcy is in the bound form (bHcy), by disulfide bond to plasma proteins, mainly albumin. About 10–30 % is in the free form (fHcy), which can be in one of the following forms: the reduced (1–2 % of the total Hcy (tHcy)) or the oxidized form, as a disulfide that is bounded by disulfide bond to cysteine or another Hcy molecule (homocysteine) (Nekrassova et al. 2003; Medina et al. 2001).

The Metabolism of Homocysteine

Homocysteine is an intermediate metabolite of the Met. The process begins with the submission of an adenosyl group from the adenosine triphosphate (ATP) to a sulfur atom of Met, with the participation of the methionine adenosyltransferase (MAT), which results in the conversion of Met into S-adenosylmethionine (SAM). SAM is the donor in most transfer reactions of the methyl group (transmethylation) (Fig. 2). Most of the methyl groups originating from SAM bind with guanidinoacetic acid and in that form is used for the synthesis of creatine (about 70 %). The rest of the methyl group acceptors and their methylation products are deoxyribonucleic acids (DNA) adenine or cytosine (DNA-*N*-methyladenine-DNA or 5-methylcytosine),

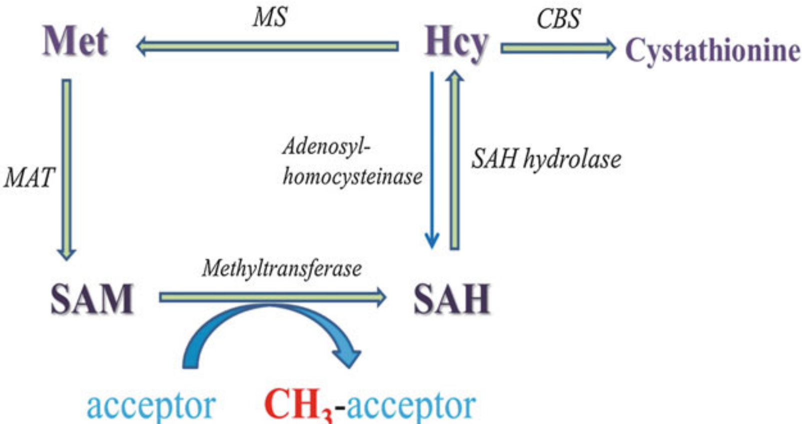


Fig. 2 The main metabolic pathways of homocysteine (*Hcy*). Hcy is the product of methionine (*Met*) metabolism. First, the amino acid is converted into S-adenosylmethionine (*SAM*) in the presence of the methionine adenosyltransferase (*MAT*). Then SAM is converted into S-adenosylhomocysteine (*SAH*) by the methyltransferase, and the released methyl group is submitted to various acceptors (transmethylation). From SAH by the SAH hydrolase, Hcy is generated. Hcy can then be reconverted to Met by the methionine synthase (*MS*), or it can be converted into cystathionine in the presence of the cystathionine β-synthase (*CBS*), which is excreted in the urine after subsequent conversion. In conditions of hyperhomocysteinemia, Hcy can be converted into SAH by the adenosylhomocysteinase

transfer ribonucleic acid (tRNA) bases (tRNA-methylated bases), L-arginine (asymmetric dimethylarginine (ADMA)), etc. After submitting a methyl group to an acceptor, with the participation of methyltransferase, SAM is converted into S-adenosylhomocysteine (SAH). Hcy is generated from SAH, under the influence of SAH hydrolase; and thereafter Hcy is the substrate for the synthesis of cystathionine and then cysteine (transsulfuration of Hcy) and resynthesis of Met (remethylation). However, in conditions of HHcy, in the presence of adenosine, Hcy can be converted to SAH, which disrupts the SAM/SAH ratio. The concentration of SAH is an important determinant of the methyltransferase activity in tissues; it acts as an inhibitor of these enzymes as they interact with higher affinity with SAH than with SAM. Therefore, in conditions of HHcy, due to an increase in the concentration of SAH, there is a decrease in the intensity of the transmethylation processes, or so to say, it leads to hypomethylation.

In healthy individuals, the Hcy clearance from plasma is constant, and the transsulfuration pathway is the only way to eliminate Hcy from the body. Within this pathway, in reactions that are dependent on pyridoxal phosphate, and with the participation of the cystathionine β-synthase (CBS), Hcy is converted into cystathionine, which is then converted into cysteine, under the influence of cistationase (Fig. 3). The kidneys, liver, intestine, and pancreas possess both enzymes of the transsulfuration pathway (Hirsch et al. 2002). Activation of these two enzymes is induced, among other factors, by Met originating from food, which potentiates the clearance of Hcy. Interruption of dietary protein rich with Met leads to favoring remethylation pathway. Remethylation can occur with the participation of the betaine-homocysteine methyltransferase, which happens almost exclusively in the liver and has a small role in the metabolism of Hcy, also with the participation of MS or 5-methyltetrahydrofolate-homocysteine methyltransferase, when Hcy receives a methyl group from the monocarbonyl pool, and the 5-methyltetrahydrofolate (5-MTHF) is used as a donor of the methyl group. Vitamin B_{12} is required as a cofactor in this reaction of transmethylation.

The biological functions of Hcy are as follows: it is the main substance for the synthesis of cystathionine, cysteine, and other metabolites of the transsulfuration pathway; it participates in the conservation of Met; it participates in the catabolism of choline, as a receptor of the methyl group from betaine, which is a product of the choline degradation process; and it serves as a substrate for the recycling of tissue folate (Finkelstein and Martin 2000; Wu et al. 2012).

The Regulation of Homocysteine Metabolism

The level of Hcy in the plasma depends on the balance between the release of Hcy from the cells into the bloodstream and its elimination from the plasma. The mechanisms involved in regulating the metabolism of Hcy include difference in the affinity of enzymes to the substrate that determines in which pathway Hcy will be included, as well as the influence of SAM, SAH, and 5-MTHF. In addition, the Hcy

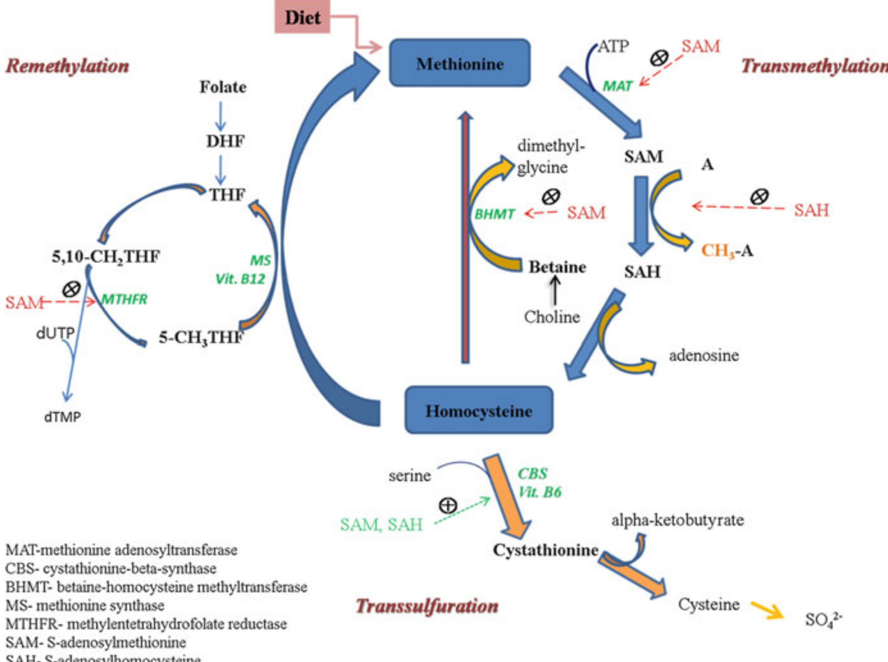


Fig. 3 The regulation of homocysteine (*Hcy*) metabolism. After the intake of food rich in proteins, the methionine (*Met*) is converted to S-adenosylmethionine (*SAM*), which is converted into S-adenosylhomocysteine (*SAH*), by the reaction of transmethylation. SAH is also the inhibitor of this reaction. Hcy is generated through the conversion of SAH, and then Hcy can be converted again into Met through the reaction of remethylation that depends on the presence of folate and vitamin B_{12}. Hcy can also be converted to Met in a reaction with betaine. Additionally, Hcy is converted into cystathionine and cysteine by transsulfuration reactions which depend on vitamin B_6, which potentiated by SAM and SAH. Transsulfuration pathway is the only way of eliminating Hcy. SAM is an inhibitor of the folic acid cycle, the conversion of Met into SAM, as well as the conversion of Hcy to Met via betaine. SAH is an inhibitor of the transmethylation processes, and that is why hypomethylation occurs in the case of decrease in the SAM/SAH ratio; *dUTP* deoxyuridine triphosphate, *dTMP* deoxythymidine monophosphate, *A* acceptor, *DHF* dihydrofolate, *THF* tetrahydrofolate

metabolism is affected by the oxidative state in the cell and by the distribution of enzymes in the body.

The enzymes can be divided into two groups: those which participate in the conservation of Met (they have a low Michaelis constant (Km) value), and enzymes that are involved in the catabolism of Met (which have a higher Km value). Whether Hcy will go through transsulfuration or remethylation pathway depends on the Km enzymes value versus Hcy. Km value for CBS is about 100 times higher than the Km value of the methylase, which means that the affinity of CBS is significantly lower than the affinity of the methylase. That is why under physiological conditions, the remethylation pathway is favored. Activation of the transsulfuration pathway of Hcy occurs only when there is a maximum capacity utilization of the methylation

pathways of Hcy. Apart from CBS, cistationase and MAT – the III form – have a high Km value (Finkelstein and Martin 2000; Ramakrishnan et al. 2006; Finkelstein 2007). MAT forms I and II, MS, and betaine-homocysteine methyltransferase (BHMT) have a low Km value. While CBS is active in the oxidative form, oxidation inactivates MS, and that is how oxidative stress favors the transsulfuration pathway of Hcy, generating cysteine or glutathione. Therefore, in conditions of oxidative stress, Hcy release from the cells is reduced (Durand et al. 2001).

Intermediate metabolites of Met, primarily SAM and SAH, are significant regulatory factors of Hcy metabolism. An increased concentration of SAM inhibits the activity of MTHFR and the synthesis of 5-MTHF, as well as the activity of BHMT, all of which have a purpose to reduce converting Hcy into Met. Simultaneously, it also leads to the activation of CBS, favoring the transsulfuration pathway. Furthermore, SAM inhibits its own synthesis by acting as an inhibitor to the forms I and II of MAT (extrahepatic forms) and activating the form III of MAT (the hepatic form). Also, SAM activates some methyltransferases and potentiates the transmethylation pathway. The ultimate effect of SAM is to increase the catabolism of Hcy and to prevent its accumulation. Adequate folate intake provides sufficient amounts of 5-MTHF, which inhibit the alternative transmethylation pathways. The final effect is an increase in the level of SAM which consequently favors the clearance of Hcy (Wu et al. 2012). On the other hand, a high level of SAM leads to the inhibition of 5,10-MTHF conversion into 5-MTHF. In case of the deficiency of folates, which are essential for the proper flow of the Hcy remethylation process, there is a deficit of SAM, whose synthesis is dependent on the Hcy remethylation, especially in conditions of insufficient intake of Met. Consequently, there is a decreased activity of CBS and HHcy is induced. In addition, a reduction of 5-MTHF contributes to the increase of SAH or Hcy, because it leads to a reduced inhibition of some methyltransferase enzymes which induces alternative transmethylation pathways.

Methods for the Determination of Homocysteine and Reference Values and Factors Affecting the Level of Homocysteine in the Blood

The main indications for determining homocysteinemia are diagnostics on homocystinuria, examination of the deficiency of vitamin B_{12} and folic acid, and the assessment of cardiovascular risk (Refsum et al. 2004). In determining the levels of tHcy, it is necessary to determine the level of folates and vitamin B_{12}.

The most suitable sample for determining the circulating levels of Hcy is potassium EDTA or lithium heparin plasma. Citrate as an anticoagulant is not recommended because it has a dilution effect and lowers the tHcy levels by 5–15 % in the individual samples (Refsum et al. 2004; Rasmussen and Møller 2000). After blood sampling, it is necessary to immediately put the test tube on ice, in order to prevent an increase in the concentration of Hcy due to its synthesis and release from erythrocytes. The separation of plasma or serum is preferably carried out after 30–60 min but no longer than 6 h. The concentration of tHcy in

serum samples is about 5–10 % higher than in plasma because the coagulation of the serum is slower on ice (Rasmussen and Møller 2000). Because samples should not contain fibrin, red blood cells, or particles, special caution is needed in patients receiving anticoagulant or thrombolytic therapy. The stability of Hcy in plasma or serum is 4 days at 20–25 °C, 14 days at 2–8 °C, and at least 12 months at −20 °C.

Available routine methods usually allow us to determine the levels of tHcy, and the first step usually involves treating the sample of plasma or serum with a reducing agent and converting all forms of Hcy into the reduced form whose level is then measured. The methods used to determine levels of tHcy levels are chromatographic methods, gas chromatography-mass spectrometry, capillary electrophoresis with fluorescence detection, as well as enzymatic and immunochemical methods (Rafii et al. 2009). The development of the immunochemical and enzymatic methods has facilitated the determination of tHcy levels in the general clinical practice. The most commonly used methods in this group are the fluorescence polarization immunoassay and the chemiluminescent immunoassay (Ramakrishnan et al. 2006).

Increasingly there is a need to identify other forms of Hcy. For example, it has been found that the reduced form of Hcy has the strongest toxic effects on blood vessels (Chambers et al. 2001). However, determining the levels of the fHcy with the most current methods is not suitable for routine clinical practice (Refsum et al. 2004; Nekrassova et al. 2003).

The reference values for tHcy vary among different countries and even among different laboratories. The use of different methods for determining the level of tHcy and people's different life habits (especially folate intake habits, or the use of fruits and vegetables in general diet, then smoking, and consumption of coffee and tea) are most likely the cause for that. The use of coffee, as well as smoking, was positively correlated, while the consumption of tea was negatively correlated with the level of tHcy (Golbahar et al. 2004; Grubben et al. 2000). In smokers, vitamin B_6 interacts with carbon monoxide from cigarette smoke, which leads to a disruption in the transsulfuration pathway; also, nicotine disrupts the methylation processes and affects the activity of MS (Bergmark et al. 1997). In premenopausal women, the circulating tHcy level was slightly lower than that in men, which is mainly attributed to the effect of estrogen which leads to a decrease in tHcy levels probably by influencing the catabolism of Met, as well as the possible influence of estrogen on the level of folates (Refsum et al. 2004; Stanger et al. 2003; Zappacosta et al. 2013; Stanislawska-Sachadyn et al. 2008). During pregnancy, there is a reduction in homocysteinemia, due to hemodilution, reduced albumin concentration in the plasma, and because of the renal hyperfiltration (Walker et al. 1999). The levels of tHcy increase along with the body aging, and the values practically double from childhood to old age (Refsum et al. 2004); also, in older people the values of tHcy are 2–5 μmol/L higher than in people of middle age, who have the same level of creatinine in the serum (Elshorbagy et al. 2007). Possible causes of this trend are decrease in renal clearance with aging, reduction in the CBS activity, reduced intake of folate, and the intracellular functional deficiency of vitamin B_{12}, as well as the use of different drugs (Refsum et al. 2004; Elshorbagy et al. 2007). In the supine position, homocysteinemia is about 10 % lower than in the sitting position, probably

due to the reduction of plasma albumin concentration in this position. In addition, 6–8 h after a meal rich in proteins, an increase may occur in the plasma levels of tHcy by 10–15 % (Nurk et al. 2001). This may be the cause of the daily individual differences of the tHcy levels which are lower in the morning than in the second half of the day.

In most European countries, the upper limit for the reference value of tHcy does not exceed 15 μmol/L (Refsum et al. 2004; Nekrassova et al. 2003). In addition, in a significant number of laboratories, the upper limit for the reference value is 12 μmol/L (Stanger et al. 2003; Finkelstein and Martin 2000). It is considered that it would be best to establish the limit values of tHcy in relation to the intake of food enriched with folates, the intake of beverages, and smoking, so that in a population with favorable living habits, this limit should be 12 μmol/L, while in a population with unhealthy lifestyle, the upper limit should be 15 μmol/L (Refsum et al. 2004). The given values are related to individuals aged 15–65 years old. For the elderly with adequate intake of folates, tHcy upper limit should be set at 15 μmol/L or 20 μmol/L in patients with poor intake of folates. Hcy level above 15 μmol/L is present in approximately 5 % of the general population and in about 50 % of patients who suffered a stroke or have a cardiovascular disease (CVD) (Guthikonda and Haynes 2006). In any case, tHcy levels up to 30 μmol/L are considered as a moderate HHcy; values between 30 and 100 μmol/L are considered as an intermediate HHcy, while values above 100 μmol/L are considered as an extreme or severe HHcy (Stanger et al. 2003).

However, since HHcy is associated with an increased cardiovascular risk, the question is whether there is a need for establishing upper limits for "normal" homocysteinemia. Namely, tHcy concentration below 10 μmol/L is considered to be "ideal" when it comes to low risk of CVD (Dhonukshe-Rutten et al. 2009). Moreover, when reducing the tHcy level by 3 μmol/L, it leads to a reduction in the risk for ischemic heart disease by 16 %, deep vein thrombosis by 25 %, and stroke by 24 % (Wald et al. 2002). It has been established that in countries in which the average values of tHcy are closer to "ideal," there is lower incidence of vascular disease (Golbahar 2004).

The Causes of Hyperhomocysteinemia

HHcy implies an increase in the level of fHcy and bHcy. HHcy is caused by different factors. The most common factors are genetic factors, nutritional factors, the use of drugs, and renal disease.

5-MTHFR and CBS deficits are the most common enzyme deficiencies that lead to HHcy and homocystinuria (increased Hcy excretion in urine). So far, 60 different mutations have been described in patients with 5-MTHFR deficiency (Iacobazzi et al. 2014), which is characterized by a decrease in SAM/SAH ratio and impairments in the methylation processes. The most common mutations that are associated with reduced activity of 5-MTHFR are C677T and A1298C. C677T mutation leads to the manifestation of a thermo-sensitive form of 5-MTHFR, which is frequently

associated with coronary artery disease. In heterozygotes, the activity of 5-MTHFR is reduced by 40 %, while in monozygotes, it is reduced by more than 70 % (Iacobazzi et al. 2014). However, there is an interaction between the genotype and the nutritional status. In homozygotes for the thermo-sensitive form of 5-MTHFR, an adequate folate intake may lead to the maintenance of homocysteinemia within the reference range (McLean et al. 2004). The deficiency of CBS, another very important enzyme involved in the metabolism of Hcy, particularly in the homozygous form, can be the cause of severe HHcy.

A vitamin B_{12} and folic acid deficit is one of the most common causes of HHcy, and on the other side, HHcy can be a very sensitive marker of the intracellular deficiency of these vitamins (Ramakrishnan et al. 2006). Vitamin B_{12} is a cofactor for MS, and folic acid participates in the donation of a methyl group to Hcy in the process of remethylation. Because of the impairment in the remethylation process caused by the deficiency of these vitamins, Hcy conversion into Met is reduced, as well as the synthesis of SAM, and due to HHcy there is an increase in the reversible synthesis of the SAH, leading to a disorder in the methylation processes. Apart from malnutrition, deficiency of these vitamins may also occur due to their malabsorption or increased consumption, or it may be caused by the use of some medications. Those who have an increased risk for deficiency of these vitamins are vegetarians or people with reduced intake of fresh fruits and vegetables, then the elderly, persons with malignancies or bowel diseases, pregnant women, and alcoholics (Stanger et al. 2003). In order to reduce the cardiovascular risk and achieve the "ideal" values of homocysteinemia ($<$10 μmol/L), it is necessary to establish an adequate folate ($>$15 nmol/L) and vitamin B_{12} status ($>$350 pmol/L) (Dhonukshe-Rutten et al. 2009).

Various medications have an influence on cofactors in the metabolic pathways of Hcy. HHcy is caused by folate metabolic antagonists (methotrexate, trimethoprim, antiepileptics), hypolipemics which are antagonists of vitamin B_6 (niacin), inhibitors of vitamin B_{12} and folic acid absorption (colestipol/cholestyramine), as well as those which lead to renal damage (fibrates), then drugs that reduce the absorption of vitamin B_{12} (metformin and omeprazole); cyclosporine A, probably due to its effects on the renal function; isoniazid as an antagonist of B_6 vitamin; and others.

The effect of renal function on the Hcy metabolic homeostasis will be discussed in detail hereinafter.

The Biological Effects and Mechanisms of the Toxicity of Hyperhomocysteinemia

Given the high reactivity of Hcy in biological systems, in HHcy, this amino acid can be converted to various metabolites such as Hcy-thiolactone (HTL), S-nitroso-Hcy, Hcy-containing disulfides, adenosylhomocysteine, etc., which take a part in various pathophysiological processes.

In the plasma, Hcy is constantly subjected to oxidation to form homocysteine, Hcy-mixed disulfide bonds, and thiolated proteins. During the oxidation of -SH groups, free radicals are generated, such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2-), which exert adverse effects on lipids and proteins in the cell membrane, thus resulting in endothelial dysfunction (Medina et al. 2001). They also react with nitric oxide (NO) and reduce the bioavailability of this potent vasodilator and inhibitor of platelet adhesion (Fischer et al. 2003). In addition, HHcy leads to a decreased activity of enzymes involved in the catabolism of ADMA, primarily the dimethylarginine dimethylaminohydrolase. This causes an increase in the level of ADMA, which is an inhibitor of NO synthase, thus resulting in a reduced NO production (Sen et al. 2014).

In the milieu of HHcy, especially in situations where the transsulfuration and remethylation pathways are impaired, for example, in conditions of CBS or 5-MTHFR deficiency, the production of highly reactive HTL is induced (Ramakrishnan et al. 2006). The level of HTL in plasma is up to 0.3 % of tHcy (Chwatko and Jakubowski 2005), and one of the indicators of an increased production of HTL is an elevated Hcy/Met ratio. In the form of HTL, Hcy reacts with the -NH2 group of the amino acids, especially lysine. This so-called process of *N*-homocysteinylation of proteins, which is specific for Hcy, leads to structural changes in the proteins which may ultimately result in their denaturation. In addition, as a result of the homocysteinylation, modification occurs on lipoproteins, hemoglobin, and other plasma proteins. The *N*-homocysteinylation of fibrinogen leads to an increased thrombogenesis (Karolczak and Olas 2009); homocysteinylation of the high-density lipoprotein (HDL) reduces their defensive role (Ferretti et al. 2003), while homocysteinylation of the endothelial NO synthase (eNOS) causes a decrease in the NO production (Sen et al. 2014). Also, as a result of protein homocysteinylation, vascular damage may occur due to the fact that these modified proteins accumulate on the surface of the blood vessel wall. Then they are identified directly by macrophages, or they can first be recognized by the anti-Hcy antibodies with which they form antigen-antibody complexes that are then recognized by macrophages. In both cases the result is phagocytosis with the consequent destruction of endothelial cells (Jakubowski 1999). The enzymes involved in the organism's defense from proteins' homocysteinylation are the paraoxonase, which is a component of the HDL, and the bleomycin hydrolase (BHL) (Zimny et al. 2006). Both enzymes lead to HTL hydrolysis.

Due to the increased level of SAH, which is an inhibitor of the methyltransferases, in HHcy, there is a hypomethylation of the DNA, RNA, and other substances. Hypomethylation is associated with vascular damage, malignant diseases, the aging process, and other pathological conditions (Perez et al. 2007). Other effects are being considered as well, such as genotoxicity, endoplasmic reticulum stress, unfolded protein response, stimulation of the immune response, activation of the metalloproteinases, mitochondrial and telomerase damage, stimulation of the vascular smooth muscle cell proliferation (VSMC), etc. (Perła-Kajàn et al. 2007; Jakubowski 2007; Jacobsen et al. 2005; Perez et al. 2007).

However, there is still a dilemma whether Hcy is only a biomarker of a certain pathological condition or an active participant in the processes that lead to these pathological conditions.

Homocysteine and the Renal Function

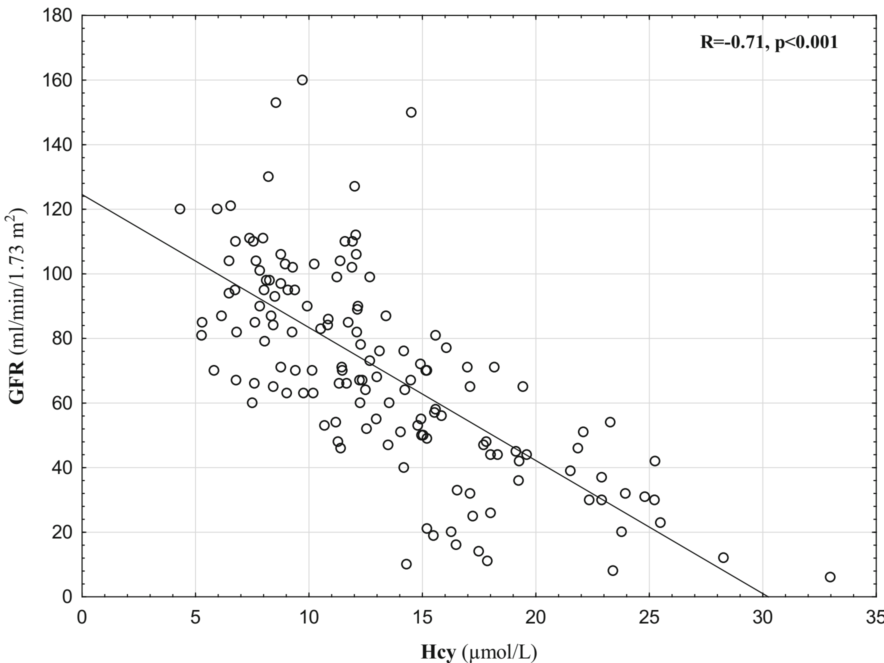
The processes of filtration, reabsorption, degradation, and urinary excretion of amino acids take place in the kidneys, and that is why the kidneys have an important role in the amino acids' homeostasis (Garibotto et al. 2010). The kidneys are, in addition to the liver, the main places for eliminating Hcy from the plasma. The kidneys eliminate about 70 % of plasma Hcy, so it is not surprising that kidney failure is one of the most common causes of HHcy.

In patients with chronic kidney disease (CKD), HHcy is most prominent in end-stage renal disease (ESRD) patients, i.e., patients on dialysis (85–100 %), while in patients with CKD who are not on dialysis, HHcy is present in 36–89 % of them (in most cases, between 12 and 30 μmol/L) (Čabarkapa 2007; Jardine et al. 2012). Even when a slight reduction occurs in the glomerular filtration rate (GFR), it results in an increase in the Hcy level, if compared to the level before the reduction in GFR, which may actually be within the reference range (Refsum et al. 2004). Hcy level >12 μmol/L is present in about 10 % of CKD patients with GFR >60 ml/min/1.73 m^2, in about 90 % of CKD patients with GFR <60 ml/min/1.73 m^2, and in patients on dialysis (Čabarkapa et al. 2007). The plasma tHcy level is in positive correlation with the plasma creatinine level and in an inverse correlation with the GFR ($R = -0.71$, $p < 0.001$) (Čabarkapa et al. 2012) (Graph 1). Therefore, tHcy level could be used as a marker of renal hypofunction, particularly in patients with GFR <60 ml/min/1.73 m^2.

Only a small fraction of tHcy is subjected to glomerular filtration, and it is that which is not bound to proteins (fHcy). Most of the filtered Hcy is reabsorbed in the tubules, and only a small portion of it is eliminated in the urine, about 6–10 μmol per day (Van Guldener and Stehouwer 2003). Arginine and lysine inhibit the Hcy reabsorption, because they share the same path of reabsorption with Hcy.

Since the amount of Hcy that is normally excreted in the urine is relatively small, it is assumed that the main factor that leads to HHcy in renal insufficiency is a disorder in the intrarenal cellular metabolism. The kidneys are a place of intense Hcy metabolism (Sen et al. 2014). They play an active role in the remethylation and transsulfuration metabolic pathways of Hcy, because they have the enzymes (CSB, MS, and BHMT) involved in the mentioned metabolic pathways. After entering the cell, Hcy is reduced and becomes accessible for action of these enzymes. Therefore, to maintain the levels of tHcy within the limits of the reference range, in addition to the preserved functional ability of the kidneys, a preserved function and level of the enzymes involved in Hcy metabolism is essential.

In CKD, it is most likely that the remethylation pathway is one that is primarily disturbed (i.e., the metabolic clearance of Hcy), while the transsulfuration metabolic



Graph 1 The correlation between the glomerular filtration rate (*GFR*) and the plasma levels of total homocysteine (*tHcy*). A statistically significant inverse correlation was determined (correlation coefficient $R = -0.71, p < 0.001$) in a sample of 142 chronic kidney disease patients (100 diabetics and 42 patients without diabetes) (Čabarkapa et al. 2007; Čabarkapa 2011)

pathway of Hcy is preserved in the initial stages, but as the renal damage progresses, there is a consequent reduction in the functioning of that metabolic pathway as well (de Koning and Hu 2010). The kidneys are also the main routes for the removal of SAH from the blood, which indicate their very important role in the regulation of the transmethylation reactions in the body (Garibotto et al. 2009). In CKD, especially in ESRD, due to increasing levels of SAH and the consequent reduction in the SAM/SAH ratio in the plasma and erythrocytes, as well as delays in the methyltransferase activity, there is a reduction in the extent of transmethylation. The altered expression of certain genes, altered cell differentiation, changes in the properties and structure of cell membranes, damaged chemotaxis and phagocytosis, reduced repair of damaged proteins, and reduced synthesis of neurotransmitters are all just some of the consequences of hypomethylation in these patients. Moreover, hypomethylation in CKD/ESRD patients is a very important factor that contributes to the development of vascular diseases, which are the leading causes of mortality in these patients. Besides that, particularly in the pronounced reduction in the functional abilities of the kidneys, in addition to the renal, the extrarenal metabolism of Hcy is damaged as well. This is due to presence of uremic toxins that inhibit the

intracellular metabolism of Hcy, which leads to an increased Hcy release from the cells (de Koning and Hu 2010; Elshorbagy et al. 2007), as well as due to the disrupted hepatic metabolism in chronic renal failure and the presence of inhibitors of various enzymes in the circulation. Although the liver has a significant capacity for remethylation and transsulfuration of Hcy, it still fails to remove the excess of Hcy in patients with a significant reduction in the GFR, especially in ESRD.

Apart from the fact that the decrease in renal function leads to HHcy, HHcy itself can contribute to the damage in the kidney structures, the glomerulus, the tubules, and the corresponding interstitium (Mao et al. 2014). The mechanisms that may be involved in the pathogenesis of the glomerular damage are changes in renal hemodynamics, endoplasmic reticulum stress, hypomethylation, homocysteinilation, apoptosis of the mesangial cells, and increase in the levels of ADMA, which reduce NO production and lead to a consequent endothelial dysfunction (Finocchiaro and Zoccali 2005). In addition, HHcy causes oxidative stress, which may play a role in the damage of kidney structures.

Also, one of the mechanisms by which HHcy leads to the progression of the renal dysfunction is an induction of the inflammatory molecules in the mesangial cells, such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) (Sen et al. 2009, 2010). Cytokines and chemokines contribute to the recruitment of monocytes and their deposition in the wall of the glomerular blood vessels, which leads to deposition of extracellular matrix and ultimately to the occurrence of glomerulosclerosis (Sen et al. 2010), a decline in renal function and development of chronic renal failure. The result is the expression of the matrix metalloproteinases in the kidneys (MMPs) (Tan and Liu 2012), and HHcy contributes to their activation. Increased activity of MMP-2 and MMP-9 leads to the dysregulation of the synthesis and degradation of the extracellular matrix (Sen et al. 2014) which results in the development of glomerular-tubulointerstitial fibrosis.

HHcy also stimulates a hydrogen sulfide (H_2S), which is one of the molecules that play an essential role in the regulation of the inflammatory response. Hcy is a precursor in the synthesis of H_2S. However, the inhibition of the cystathionine γ-lyase (CSE) occurs in HHcy. CSE participates in the transsulfuration pathway and catalysis of H_2S formation, thus its inhibition leading to a decreased production of H_2S in the mesangial cells. This could be one of the most important mechanisms by which HHcy contributes to kidney damage and the eventual progression of the renal dysfunction in patients with CKD. H_2S somewhat inhibits the production of MCP-1 and MIP-2, which is stimulated by HHcy, thus preventing renal damage associated with HHcy (Sen et al. 2009) and contributing to the reparation of lesions caused by HHcy. Also, the renoprotective effect of H_2S is reflected in the regulation of the extracellular matrix remodeling and inhibition of the renal glomerular fibrosis (Sen et al. 2010). In addition to the direct renoprotective effects, H_2S also exhibits an antioxidant, vasodilator, and antihypertensive effects. A reduction in the level of this substance contributes to the pathogenesis of neurodegenerative diseases, hypertension, inflammation, and atherosclerosis (Sen et al. 2014). Due to its antioxidant and

vasodilator characteristics which oppose the harmful effects of HHcy, H_2S could be a very useful therapeutic agent in patients with HHcy.

Homocysteine in Patients with Terminal Renal Failure and in Transplanted Patients

HHcy is almost an inevitable companion of patients on dialysis. The type of dialysis affects the plasma tHcy levels. HHcy is more common and more pronounced in patients on hemodialysis (approximately 90 %) than those on peritoneal dialysis (approximately 70 %). Plasma levels of Hcy in ESRD patients are typically two to three times higher than in the healthy population and range, in most cases, between 16 and 100 μmol/L, with a median of 30 μmol/L (Perna et al. 2007). The hemodialysis treatment leads to a reduction in tHcy levels by 30–40 %, but the Hcy levels do not usually return back to reference range. In addition, the tHcy level increases back rapidly after the hemodialysis treatment and within 24 h reaches the predialysis level. Hcy bound to proteins cannot be removed by hemodialysis, only the fHcy, which makes about 20 % of the tHcy. That is why the amount of Hcy that could be eliminated by dialysis treatment is very small when compared to the influx of Hcy in the plasma. However, uremic toxins are efficiently removed by hemodialysis treatment which contributes to the reduction of tHcy levels in ESRD patients, especially when using the superflux dialysis, which significantly reduces the level of Hcy (Van Guldener and Stehouwer 2003). In patients on peritoneal dialysis, the intensity of removing excess Hcy via dialysate is low, due to the predominance of Hcy fraction which is bound to proteins and the possible reabsorption of Hcy from the dialysate, as well as the application of dialysis solutions containing amino acids including Met. The extent of Hcy removal significantly depends on its predialysis level in the bloodstream; the higher the level of Hcy in the plasma is associated with, the higher the percentage of reduction of the homocysteinemia. Therefore, in these patients, it would be preferable to put the emphasis on the reparation of the Hcy metabolic pathways, which can be achieved by using folates.

If there is a lowering of levels of Hcy with ESRD patients with malnutrition-inflammation syndrome (CMIS), it indicates disturbed nutritional status, and it is associated with increased mortality in these patients (Perna et al. 2007; Ingrosso and Perna 2009; Kalantar-Zadeh et al. 2004; Marcus et al. 2007).

HHcy appears in about 50–60 % of patients with a transplanted kidney with a median of 16–19 μmol/L (Einollahi et al. 2011; Sengoegle et al. 2003), even though it would be expected that the transplantation leads to a significant reduction in the levels of tHcy. Immediately after transplantation, the tHcy level is reduced averagely by about one-seventh of levels before transplantation, although almost a third of patients experience an increase in the levels of tHcy. In addition to the reduced renal function and the impaired vitamin status, HHcy in transplant patients is also induced

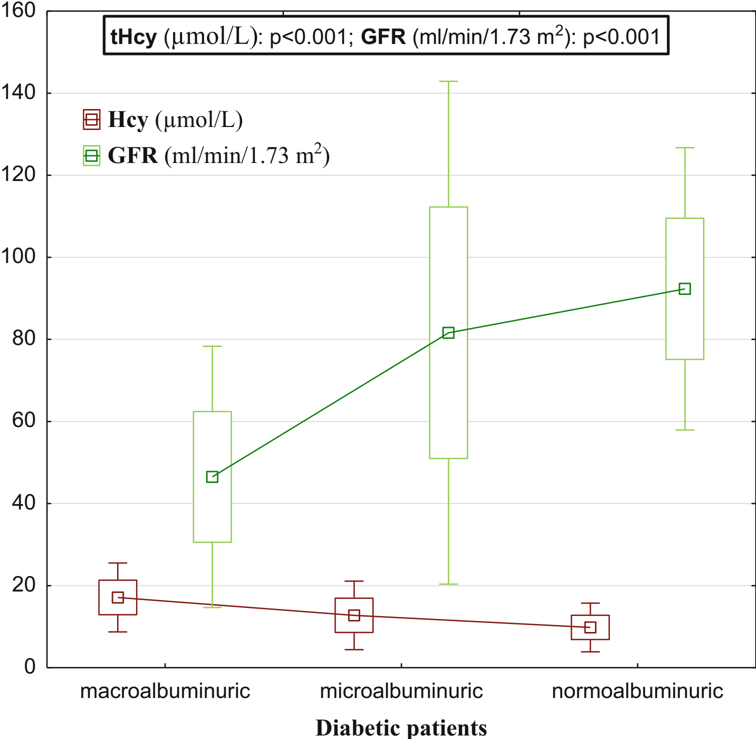
by the immunosuppressive therapy, especially cyclosporine, as well as increased expression of the transforming growth factor beta 1 (TGF-β1). This cytokine stimulates the release of Hcy from the cells thus leading to HHcy in these patients.

Diabetes Mellitus, Diabetic Nephropathy, and Homocysteine

HHcy is not characteristic for patients with diabetes mellitus (DM) until it comes to the development of diabetic nephropathy (DN). In patients with DM, tHcy level can be increased, decreased, or within the reference range. For patients with DM and without DN, it is characteristic to have lower plasma concentrations of tHcy compared to people who are not suffering from diabetes or renal disease (Čabarkapa 2007). The cause for this is the activation of the transsulfuration pathway enzymes in conditions of chronic hyperglycemia, and a contributory factor is the glomerular hyperfiltration. However, when DN is developed, even in the initial stages, it is characterized by the appearance of microalbuminuria (urinary albumin excretion of 30–300 mg/day), and especially after the manifestation of macroalbuminuria (urinary albumin excretion $>$300 mg/day) and with the decrease in the GFR, impaired renal function is the main factor that affects the level of tHcy and that leads to the appearance of HHcy (Čabarkapa et al. 2012; Mao et al. 2014) (Graph 2). In addition to the high inverse correlation between tHcy and the GFR, there is also a significant correlation between albuminuria and homocysteinemia. The level of tHcy is significantly higher in diabetic patients with macroalbuminuria than in diabetic patients with microalbuminuria and normoalbuminuria (Graph 2), which confirms the significant connection between the functional status of the kidneys and the homocysteinemia. Given the fact that albuminuria is also a risk factor for the development of cardiovascular disease (CVD), the simultaneous presence of HHcy and pathologic albuminuria significantly increases this risk. In addition, insulin therapy may increase the level of tHcy, because of the inhibition of the CBS activity.

Although the underlying disease and DN lead to an increased inflammatory response, Hcy has a contributory role as well, because HHcy is associated with stimulation of the inflammatory response. It is most likely that the increase in the inflammatory response is one of the major factors that lead to the development and progression of the DN (Wada and Makino 2013). The level of oxidized low-density lipoprotein (oxLDL) is higher in diabetic patients with HHcy than in patients with tHcy levels within the reference range. Oxidized LDL may have a role in the progression of the renal dysfunction in diabetic patients, because it stimulates the production of collagen in the mesangial cells. In fact, patients with DM and tHcy levels $>$10 μmol/L have a significantly higher risk of progression of the renal disease (OR 3.4, $p < 0.001$) (Čabarkapa 2007). Therefore, the homocysteinemia may serve as a predictor of the development of DN (Mao et al. 2014), as well as the progression of the renal failure in patients with DN.

HHcy may contribute to the development and progression of microvascular complications in DM due to the fact that Hcy exerts toxic effects on the blood vessels, mainly in its free, reduced form, whose level is increased in the bloodstream



Graph 2 The level of total homocysteine (*tHcy*) in macroalbuminuric ($n = 34$), microalbuminuric ($n = 46$), and normoalbuminuric ($n = 52$) diabetic patients. Glomerular filtration rate (*GFR*) and tHcy levels are significantly different between all groups of patients ($p < 0.001$), wherein the lowest GFR is characteristic for the macroalbuminuric patients and consequently the highest tHcy (Čabarkapa et al. 2012; Čabarkapa 2011)

of patients with DM. The cause of this increase is the condition of chronic hyperglycemia, which is associated with the nonenzymatic glycation of albumin, which results in a decrease in the Hcy fraction that is bound to albumin, and which increases its free fraction. HHcy, combined with the mutation of MTHFRC667T, is a predictive factor for the development of microvascular complications in type 2 diabetes, especially in DN (Mtiraoui et al. 2007).

Homocysteine in the Nephrotic Syndrome

The tHcy level in the nephrotic syndrome (NS) can be normal, increased, or even decreased, and the level of tHcy does not correlate with the proteinuria, so the GFR is the main determinant of the tHcy level. Increased tHcy level in the NS is a contributing risk factor for thrombosis of the blood vessels.

Homocysteine and Vascular Disease in Patients with Chronic Kidney Disease

The progressive reduction in the functional ability of the kidneys in patients with CKD is accompanied by numerous complications, including CVD which is the leading cause of mortality (Garibotto et al. 2010). CKD patients with GFR <60 ml/min/1.73 m^2 have more than a tenfold increased risk, while CKD patients with GFR 60–90 ml/min/1.73 m^2 have twice the risk of developing a CVD when compared with the healthy population (Francis et al. 2004). In fact, more than 50 % of CKD patients die of CVD even before starting dialysis. In about one-third of the patients with CKD, the incidence of cardiovascular complications cannot be linked to any of the traditional risk factors, so Hcy could be that connection.

Hcy is an independent risk factor for the development of coronary heart disease, cerebrovascular disease, and peripheral arterial disease. Increasing the levels of Hcy by 5 μmol/L increases the risk of developing a coronary heart disease to the same extent as the increase in total cholesterol level by 0.5 mmol/L (Di Minno et al. 2010). Pro-atherogenic effect of the Hcy is manifested already in the mild to moderate increase in the plasma concentration of Hcy (Sarwar et al. 2007). An increase in the plasma level of tHcy by 3 μmol/L increases the risk for coronary artery disease by 10 %, and for cerebral infarction by 20 % (Nakao et al. 2014), and a reduction in the level of Hcy up to 25 % leads to a reduction in the risk for myocardial infarction by 20 % (Cheng 2013). In ESRD patients, an increase of the tHcy level by 5 μmol/L leads to an increase in the mortality rate by 7 % (Heinz et al. 2009), and a reduction in the level of Hcy level in patients on dialysis by 10 μmol/L leads to a reduction in the risk for cardiovascular events by 20 % (Zoccali et al. 2000).

The mechanisms linking HHcy and vascular damage are the endothelial dysfunction due to a decreased NO bioavailability, the proliferation of VSMC and their impaired function, the accelerated degradation of elastin and the imbalance in the elastin/collagen relations with the consequent reduction in the blood vessel elasticity, the metalloproteinase activation, the H_2S inhibition, the interleukin-8 (IL-8) stimulation, the stimulation of the oxidative transformation of LDL, the increased adhesion of platelets, and the inhibited synthesis of apolipoprotein A-I (apo A-I) (Doronzo et al. 2010; Sen et al. 2010; Cheng 2013). HHcy leads to the thickening of the intima media of blood vessels (Sarwar et al. 2007). Due to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) via reactive oxygen species (ROS), HHcy leads to the proliferation of the VSMC, which is accompanied by an increased production of collagen and VSMC calcification. Hcy is also an important marker of the oxidative stress in CKD and ESRD patients (Wu et al. 2012). During the oxidation of Hcy into HTL and homocysteine, these newly created substances mediate in the oxidative damage of the endothelial cells of blood vessels, as well as in the oxidative modification of LDL, which leads to the formation of foam cells that release inflammatory cytokines and ROS (Wu et al. 2012).

Despite that, in CKD patients, there is a reduced activity of the paraoxonase 1 enzyme (PON1). PON1 in the bloodstream is bound to the HDL, and it plays a

role in the protection of LDL from oxidation (in the presence of apo A-I) and from the detrimental effect of HTL. Also, PON1 is important for protecting proteins from *N*-homocysteinylation which would lead to their damage and may play a role in the occurrence of CVD. In addition, the process of protein homocysteinylation contributes to the development of glomerulosclerosis which in turn leads to impairment in the renal homeostasis (Suszynska-Zajczyk et al. 2014). Therefore, this enzyme represents a link between the HDL and the Hcy metabolism. In CKD, there is an increased clearance of apo A-I from the circulation, which lowers the HDL levels and consequently reduces the PON1 activity. Given the cardioprotective role of PON1, the existence of a reduction of its activity is associated with an increased risk for CVD and with an increase in the mortality rate (Suszynska-Zajczyk et al. 2014). The use of the recombinant human erythropoietin, which is primarily used in patients on dialysis in the treatment of anemia, leads to an increase in the PON1 activity, which certainly has a favorable effect on the risk for CVD.

In addition to HHcy, increased levels of SAH and the consequent DNA hypomethylation are typical for patients with CKD, especially in those with vascular complications (Stenvinkel et al. 2007). DNA hypomethylation is associated with the development of atherosclerosis, and it is in correlation with the level of the circulating Hcy, not only with HHcy (Ingrosso and Perna 2009). In hypomethylation, due to the weakened repair process of damaged proteins, there is a consequent cell damage which favors the formation of atherosclerotic lesions.

Furthermore, Hcy may be considered as an inflammatory marker in patients with CKD, as it contributes to the induction of inflammation due to the increased oxidative stress, NF-κB activation, and the inhibition of the H_2S production in cases of HHcy (Wu et al. 2012). In patients on hemodialysis, in the absence of the chronic malnutrition-inflammation state (CMIS), characteristic is the existence of HHcy, and Hcy is an independent risk factor for CVD. However, in the presence of CMIS, the lower Hcy level reflects the disturbed nutritional status, especially the reduction of the amino acid pool and hypoalbuminemia. Lower levels of Hcy are associated with an increased mortality rate in these patients, independent from other CVD risk factors (Ingrosso and Perna 2009; Kalantar-Zadeh et al. 2004; Marcus et al. 2007).

The intake of folates in patients with CKD may lead to improvements in the transmethylation and remethylation pathways, or so to say, it may lead to the reduction of hypomethylation. However, due to the permanent impairment of the transsulfuration pathway, the normalization of Hcy levels, especially in ESRD patients, is very difficult. In patients with ESRD, the MTHFR genotype may affect the response of these patients to folate substitution. The hemodialysis patients with MTHFR677TT require higher doses of folic acid than the patients with CC or CT genotype, to achieve reduction in the tHcy levels (Wu et al. 2012). Also, diabetic patients with ESRD are less responsive to the treatment with folates and require higher doses (15 mg/day) than the ESRD patients without diabetes (5 mg/day) (Wu et al. 2012). In addition to the folate substitution in the attempt to restore the remethylation pathway, for an improved regulation of the intracellular metabolism of Hcy, it is required to use 500 mg of methylcobalamin.

Despite clear evidence of the association between HHcy and CVD, the questions whether Hcy is a marker or a risk factor for cardiovascular complications, and whether therapeutic lowering of the Hcy level using folates, $B_{12,}$ and other vitamins leads to a significant reduction in the risk for CVD, still remain open. The HOPE (Jamison et al. 2007) and the RCT study (Mann et al. 2008) showed that the use of folic acid, vitamin B_6, and B_{12} in patients with HHcy in different stages of CKD, including the ESRD patients, did not show satisfactory effects in reducing the cardiovascular complications and in improving the rate of survival in these patients, even though there has been a reduction in the level of Hcy. A meta-analysis, from 2012 (Pan et al. 2012), showed that the therapeutic lowering of Hcy level does not reduce the rate of the repeated cardiovascular events and stroke and the rate of total mortality in CKD patients. When the clinical manifestations of atherosclerosis are already present or there is a confirmation of a positive history of vascular diseases, treatment with vitamins of the B group could even have adverse effects and thus nullify the beneficial effects of lowering the Hcy levels (Cheng 2013). The use of folic acid could induce pro-atherogenic changes in the endothelial cells of the blood vessels, and the use of a combination of folates and vitamin B_{12} and the consequent stimulation of the DNA synthesis could stimulate the cells' proliferation and neointimal proliferation. In patients with DN, the application of high doses of vitamin B may even lead to a reduction in the GFR and increase the rate of vascular complications (Wu et al. 2012). Although the therapeutic lowering of the Hcy level has not led to the expected favorable results in the secondary prevention of vascular events, it also does not have to be a factor that excludes the causal relationship between HHcy and CVD. Unlike in secondary prevention, in the primary prevention, when there are still no significant signs of atherosclerotic lesions, lowering the tHcy could have beneficial effects.

The poor efficiency of the folic acid and B vitamins in lowering the levels of Hcy in ESRD patients can be partly explained by the Hcy trait to bind with high affinity to proteins in the circulation. Therefore, new therapeutic measures are aimed at liberating the Hcy from its protein complexes, because the fHcy can be removed from the circulation by hemodialysis thus achieving a significant reduction in the tHcy level. Agents that are used for that are the *N*-acetylcysteine (NAC) and thiol-containing drug sodium 2-mercaptoethanesulfonate (Mesna) (Scholze et al. 2004; Urquhart et al. 2007).

The Potential Use of Homocysteine in the Diagnostics and Monitoring of Renal Dysfunction

Because of the significant inverse correlation between Hcy levels and GFR, Hcy could be used as a marker of renal hypofunction. Therefore, in all patients with HHcy, unless they have a folic acid or vitamin B_{12} deficiency, it is necessary to carry out an assessment of the functional status of the kidneys.

In patients with CKD, the determination of tHcy levels would be significant in the assessment of the overall risk for morbidity and mortality from CVD, followed by

the assessment of progression of renal dysfunction, and Hcy level is a prognostic factor for overall mortality assessment in CKD patients with CMIS.

The measurement of Hcy in patients with renal dysfunction is also necessary for making a decision about the necessary therapeutic treatment, especially if the patient has not yet suffered from the development of CVD.

Potential Applications of Homocysteine in Other Diseases or Conditions

First of all, Hcy is a significant marker of vitamin B_{12} and folic acid deficiency. Hcy, i.e., HHcy, may be also an important risk factor for the development of some neurodegenerative diseases, osteoporosis, epigenetic changes, and arterial hypertension and may have a role in the stimulation of the immune response. In addition, the screening for HHcy is a significant test for assessing the risk for developing venous thrombosis.

Neurotoxic effects of HHcy are the results of hypomethylation of proteins and/or genes and the increased production of ROS. Hyperhomocysteinemia stimulates the inflammation, and it is well known that inflammation, which is almost mandatory present in many neurodegenerative diseases, contributes significantly to the development and progression of disease.

Due to the induction of hypomethylation, especially of the DNA, HHcy is associated with genetic instability and malignant diseases. Hypomethylation induced by HHcy plays also a part in the aging processes (Perez et al. 2007). In addition, HHcy leads to increased oxidative stress, which leads to the damage of proteins.

Due to the activation of osteoclasts, the stimulation of bone resorption, and the disturbance of collagen cross-links, as well as the induction of pre-osteoblastic cell apoptosis, HHcy is a risk factor for developing osteoporosis and for the occurrence of bone fractures (Herrmann et al. 2007). Since that the thyroid hormones influence the activity of the enzymes involved in the processes of remethylation, particularly in the liver, hypothyroidism is characterized by the presence of HHcy; on the other hand, in hyperthyroidism, the level of Hcy is reduced.

The fact that HHcy inhibits the production of H_2S, which in turn leads to vasoconstriction, an increased arterial stiffness and stimulation of the inflammatory response, HHcy may be associated with the pathogenesis of hypertension. Increased plasma concentrations of Hcy by 5 μmol/L was associated with an increase in systolic blood pressure by 0.7–1.2 mmHg and the diastolic blood pressure by 0.5–0.7 mmHg, regardless of the vitamin status and the functional status of the kidneys (Stehouwer and van Guldener 2003).

HHcy has a role in the development of arterial and venous thrombosis. It leads to changes in the platelet function, reduces the bioavailability of NO, and increases expression and activity of coagulation factors II, V, and VIII; it increases the activity of thrombin activatable fibrinolysis inhibitor (TAFI). It also leads to a decrease in the levels of protein C and antithrombin III deficiency, reduces the expression of thrombomodulin, and reduces the binding sites for the activator of the tissue

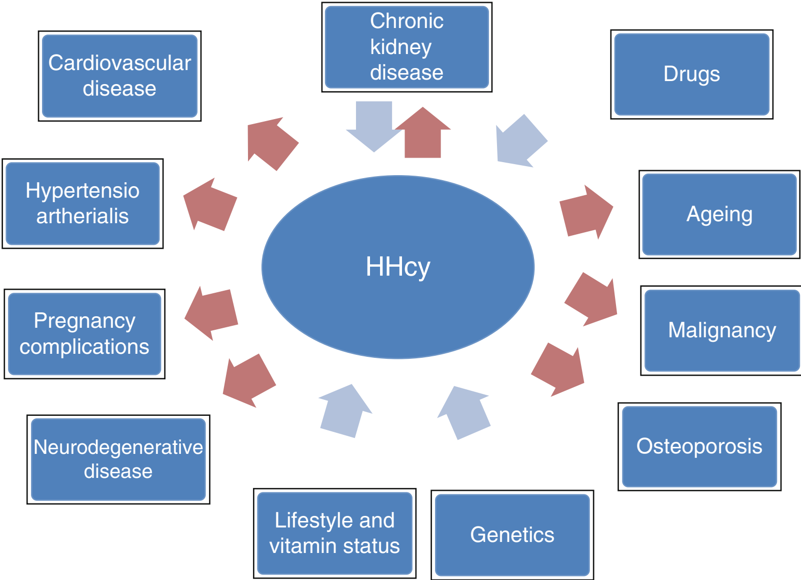


Fig. 4 The most common causes of hyperhomocysteinemia (*HHcy*) (*blue arrows*) and the condition in whose pathogenesis of HHcy (*red arrows*) has an important role. The most common factors that can cause HHcy are genetic factors, nutritional factors, the use of certain medications, and chronic kidney disease, and, the conditions in whose pathogenesis of HHcy has an important role are some neurodegenerative diseases, osteoporosis, arterial hypertension, cardiovascular disease, malignancy, pregnancy complications, and aging

plasminogen (t-PA). The fibrin clot that is formed in the milieu of HHcy is more resistant to fibrinolysis (Di Minno et al. 2010).

Because of hypervolemia and glomerular hyperfiltration, Hcy level is lower during pregnancy than in the general population. However, a disorder of metabolism of Hcy and an increase in its plasma values during pregnancy are associated with an increased incidence of preeclampsia, placental abruption, and recurrent pregnancy loss in the mother, as well as neural tube defects in the fetus. These complications can be overcome by the use of folates and other dietetic supplements, especially omega-3 fatty acids (Kulkarni et al. 2011) (Fig. 4).

Summary Points

- This chapter is focused on the importance of homocysteine as a marker in renal diseases.
- Homocysteine is synthesized from methionine and metabolized with the participation of vitamin B_{12} and folic acid.
- The concentrations of total homocysteine (free and bound) are routinely measured in the plasma, and the reference values are still not precisely determined.

- The increase in the plasma concentrations of homocysteine is caused by genetic factors, nutritional factors, the use of certain medications, and chronic kidney disease.
- Homocysteine is a significant risk factor for the development of numerous pathological conditions, especially cardiovascular disease.

References

Bergmark C, Mansoor MA, Svardal A, et al. Redox status of plasma homocysteine and related aminothiols in smoking and non smoking young adults. Clin Chem. 1997;43:1997–9.

Čabarkapa V. Examination of relationship between homocysteinemia and functional status of kidneys in patients with chronic renal failure. Master thesis. Medical Faculty, University of Novi Sad, 2007. (In Serbian).

Čabarkapa V. Relationship of specific biomarkers and progression of chronic renal insufficiency in patients with diabetic nephropathy. Ph.D. thesis. Medical Faculty, University of Novi Sad, 2007. (In Serbian).

Čabarkapa V, Stošić Z, Žeravica R, et al. The importance of homocysteinemia measurement in chronic renal failure. Med Pregl. 2007;60 Suppl 2:81–3.

Čabarkapa V, Djeric M, Stošić Z, et al. Determining relationship between homocysteinemia and biomarkers of inflammation, oxidative stress and functional kidney status in patients with diabetic nephropathy. J Med Biochem. 2012;32:131–9.

Chambers JC, Ueland PM, Wright M, et al. Investigation of relationship between reduced, oxidized, and protein-bound homocysteine and vascular endothelial function in healthy human subjects. Circ Res. 2001;89:187–92.

Cheng X. Updating relationship between hyperhomocysteinemia lowering therapy and cardiovascular disease. Cardiovasc Ther. 2013;31:19–26.

Chwatko G, Jakubowski H. The determination of homocysteine/thiolactone in human plasma. Anal Biochem. 2005;337:271–7.

De Koning L, Hu FB. Homocysteine lowering in end-stage renal disease: is there any cardiovascular benefit? Circulation. 2010;121:1379–81.

Dhonukshe-Rutten R, de Vries J, de Bree A, et al. Dietary intake and status of folate and vitamin B12 and their association with homocysteine and cardiovascular disease in European population. Eur J Clin Nutr. 2009;63:18–30.

Di Minno M, Tremoli E, Coppola A, et al. Homocysteine and arterial thrombosis: challenge and opportunity. Thromb Haemost. 2010;103:942–61.

Doronzo G, Russo I, Del Mese P, et al. Role of NMDA receptor in homocysteine-induced activation of mitogen-activated kinase and phosphatidyl inositol 3-kinase pathways in cultured human vascular muscle cells. Thromb Res. 2010;125:23–32.

Durand P, Prost M, Loreau N, et al. Impaired homocysteine metabolism and atherothrombotic disease. Lab Invest. 2001;81:645–72.

Einollahi B, Lessan-Pezeshki M, Kalantar E, et al. Hyperhomocysteinemia after kidney transplantation. Transplant Proc. 2011;43:685–587.

Elshorbagy A, Oulhaj A, Konstatinova S, et al. Plasma creatinine as a determinant of plasma total homocysteine concentrations in the hordaland homocysteine study: use of statistical modeling to determine reference limits. Clin Biochem. 2007;40:1209–18.

Ferretti G, Bacchetti T, Marotti E, et al. Effect of homocysteinylation on human high-density lipoproteins: a correlation with paraoxonase activity. Metabolism. 2003;52:146–51.

Finkelstein J. Metabolic regulatory properties of S-adenosylmethionine and S-adenosylhomocysteine. Clin Chem Lab Med. 2007;45:1694–9.

Finkelstein J, Martin J. Homocysteine. Int J Biochem Cell Biol. 2000;32:385–9.

Finocchiaro P, Zoccali C. Hyperhomocysteinemia and progression of renal disease. G Ital Nefrol. 2005;22:590–6.

Fischer PA, Dominguez GN, Cunibreti LA, et al. Hyperhomocysteinemia induces renal hemodynamic dysfunction: is nitric oxide involved? J Am Soc Nephrol. 2003;14:653–60.

Francis M, Eggers P, Hostetter T, et al. Association between serum homocysteine and markers of impaired kidney function in adults in US. Kidney Int. 2004;66:303–12.

Garibotto G, Valli A, Anderstam B, et al. The kidney is a major site of S-adenosylhomocysteine disposal in humans. Kidney Int. 2009;76:293–6.

Garibotto G, Sofia A, Saffioti S, et al. Amino acid and protein metabolism in the human kidney in patients with chronic kidney disease. Clin Nutr. 2010;29:424–33.

Golbahar J, Rezaian G, Bararpour H. Distribution of plasma homocysteine concentrations in the healthy Iranians. Clin Biochem. 2004;37:149–51.

Grubben MJ, Boers GH, Blom HJ, et al. Unfiltrated coffee increases plasma homocysteine concentrations in healthy volunteers: a randomized trial. Am J Clin Nutr. 2000;71:480–4.

Guthikonda S, Haynes WG. Homocysteine: role and implication in atheroscleorsis. Curr Atheroscler Rep. 2006;8:100–6.

Heinz J, Kropf S, Luley C, et al. Homocysteine as a risk factor for cardiovascular disease in patients treated by dialysis: a meta-analysis. Am J Kidney Dis. 2009;54:478–89.

Herrmann M, Schmidt JP, Umanskaya N, et al. The role of hyperhomocysteinemia as well as folate, vitamin B6 and B12 deficiencies in osteoporosis- a systematic review. Clin Chem Lab Med. 2007;45:1621–32.

Hirsch S, Pia De La Maza M, et al. Hyperhomocysteinemia and endothelial function in young subjects- effects of vitamin supplementation. Clin Cardiol. 2002;25:495–501.

Iacobazzi V, Infantino V, Castenga A, et al. Hyperhomocysteinemia: related genetic diseases and congenital defects, abnormal DNA methylation and newborn screening issues. Mol Genet Metab. 2014. doi:10.1016/j.ymgme.2014.07.016. Accessed 10 Nov 2014.

Ingrosso D, Perna A. Epigenetics in hyperhomocysteinemic states. A special focus on uremia. Biochim Biophys Acta. 2009;1790:892–9.

Jacobsen D, Catanescu O, DiBello P, et al. Molecular targeting by homocysteine: a mechanism for vascular pathogenesis. Clin Chem Lab Med. 2005;43:1076–83.

Jakubowski H. Protein homocysteinylation: possible mechanism underlying pathological consequences of elevated homocysteine levels. FASEB J. 1999;13:2277–83.

Jakubowski H. The molecular basis of homocysteine thiolactone-mediated vascular disease. Clin Chem Lab Med. 2007;45:1704–16.

Jamison RI, Hartigan P, Kaufman JS, et al. Effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease and end-stage renal disease: a randomized controlled trial. J Am Med Assoc. 2007;298:1163–70.

Jardine M, Kang A, Zoungas S, et al. The effect of folic acid based homocysteine lowering on cardiovascular events in people with kidney disease: systematic review and meta-analysis. BMJ. 2012;344:e3533. doi:10.1136/bmj.e3533. Accessed 21 Oct 2014.

Kalantar-Zadeh K, Block G, Humpheys MH, et al. A low, rather than a high, total plasma homocysteine is an indicator of poor outcome in hemodialysis patients. J Am Soc Nephrol. 2004;15:442–53.

Karolczak K, Olas B. Mechanism of action of homocysteine and its thiolactone in hemostasis system. Physiol Res. 2009;58:623–33.

Kulkarni A, Mehendale S, Pisal H, et al. Association of omega-3 fatty acids and homocysteine concentrations in pre-eclampsia. Clin Nutr. 2011;30:60–4.

Mann JF, Sheridan P, McQueen MJ, et al. Homocysteine lowering with folic acid and B vitamins in people with chronic kidney disease-results of the renal hope-2 study. Nephrol Dial Transplant. 2008;23:645–53.

Mao S, Xiang W, Huang S, et al. Association between homocysteine status and the risk of nephropathy in type 2 diabetes mellitus. Clin Chim Acta. 2014;431:206–10.

Marcus J, Sarnak MJ, Menon V. Homocysteine lowering and cardiovascular disease risk: lost in translation. Can J Cardiol. 2007;23:707–10.

McLean R, Karasik D, Selhub J, et al. Association of a common polymorphism in methylenetetrahydrofolate reductase (MTHFR) gene with bone phenotypes depends on plasma folate status. J Bone Miner Res. 2004;19:410–8.
Medina MA, Urdiales JL, Amores-Sanchez MI. Roles of homocysteine in cell metabolism. Eur J Biochem. 2001;268:3871–82.
Mtiraoui N, Ezzidi I, Chaieb M, et al. MTHFR C677T and A1298C gene polymorphisms and hyperhomocysteinemia as risk factor of diabetic nephropathy in type 2 diabetes patients. Diabetes Res Clin Pract. 2007;75:99–106.
Nakao A, Suzuki H, Ueno H, et al. Discovery of S-adenosyl-L-homocysteine hydrolase inhibitors based on non-adenosine analogs. Bioorg Med Chem Lett. 2014;24:4336–40.
Nekrassova O, Lawrence N, Compton R. Analytical determination of homocysteine: a review. Talanta. 2003;60:1085–95.
Nurk E, Tell GS, Nygard O, et al. Plasma total homocysteine is influenced by prandial status in humans: the Hordaland homocysteine study. J Nutr. 2001;131:1214–6.
Pan Y, Guo LL, Cai LL, et al. Homocysteine-lowering therapy does not lead to reduction in cardiovascular outcomes in chronic kidney disease patients: a meta-analysis of randomised, controlled trials. Br J Nutr. 2012;16:1–8.
Perez F, Ilie J, Zhou X, et al. Pathomolecular effects of homocysteine on the aging process: a new theory of aging. Med Hypotheses. 2007;69:149–60.
Perła-Kajàn J, Twardowski T, Jakubowski H. Mechanisms of homocysteine toxicity in humans. Amino Acids. 2007;32:561–72.
Perna A, Acanfora F, Luciano MG, et al. Plasma protein homocysteinylation in uremia. Clin Chem Lab Med. 2007;45:1678–82.
Rafii M, Elango R, House J, et al. Measurement of homocysteine and related metabolites in human plasma and urine by liquid chromatography electrospray tandem mass spectrometry. J Chromatogr B. 2009;877:3282–91.
Ramakrishnan S, Sulochana KN, Lakshmi S, et al. Biochemistry of homocysteine in health and diseases. Ind J Biochem Biophys. 2006;43:275–83.
Rasmussen K, Møller J. Total homocysteine determination in clinical practice. Ann Clin Biochem. 2000;37:627–48.
Refsum H, Smith D, Ueland P, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. Clin Chem. 2004;50:3–32.
Sarwar AB, Sarwar A, Rosen B, et al. Measuring subclinical atherosclerosis: is homocysteine relevant? Clin Chem Lab Med. 2007;45:1667–77.
Scholze A, Rinder C, Beige J, et al. Acetylcysteine reduces plasma homocysteine concentration and improves pulse pressure and endothelial function in patients with end-stage renal failure. Circulation. 2004;109:369–74.
Sen U, Basu P, Abe OA, et al. Hydrogen sulfide ameliorates hyperhomocysteinemia-associated chronic renal failure. Am J Physiol Ren Physiol. 2009;297:410–9.
Sen U, Munjal C, Qipshidze N, et al. Hydrogen sulfide regulates homocysteine-mediated glomerulosclerosis. Am J Nephrol. 2010;31:442–55.
Sen U, Pushpakumar S, Amin M, et al. Homocysteine in renovascular complications: hydrogen sulfide is a molecular and plausible anaerobic ATP generator. Nitric Oxide. 2014. doi:10.1016/i.niox.2014.06.006. Accessed 5 Nov 2014.
Sengoegle G, Kletzmayr J, Papagiannopoulos M, et al. TGF-B1 impairs homocysteine metabolism in human renal cells: possible implications for transplantation. Transplant Int. 2003;16:843–8.
Stanger O, Herrmann W, Pietrzik K, et al. DACH-LIGA homocysteine (German, Austrian and Swiss Homocysteine Society): consensus paper on the rational clinical use of homocysteine, folic acid and B-vitamins in cardiovascular and thrombotic disease: guidelines and recommendations. Clin Chem Lab Med. 2003;41:1392–403.
Stanislawska-Sachadyn A, Woodside J, Brown K, et al. Evidence for sex differences in the determinants of homocysteine concentrations. Mol Genet Metabol. 2008;93:355–62.

Stehouwer C, van Guldener C. Does homocysteine cause hypertension? Clin Chem Lab Med. 2003;41:1408–11.
Stenvinkel P, Karimi M, Johansson S, et al. Impact of inflammation on epigenetic DNA methylation-a novel risk factor for cardiovascular disease? J Intern Med. 2007;261:488–99.
Suszynska-Zajczyk J, Sikora M, Jakubowski H. Paraoxonase 1 deficiency and hyperhomocysteinemia alter the expression of mouse kidney proteins involved in renal disease. Mol Genet Metab. 2014. doi:10.1016/j.ymgme.2014.07.011. Accessed 7 Nov 2014.
Tan R, Liu Y. Matrix metalloproteinases in kidney homeostasis and diseases. Am J Physiol Ren Physiol. 2012;302:1351–61.
Urquhart BL, Freeman DJ, Spence JD, et al. The effect of mesna on plasma total homocysteine concentration in hemodialysis patients. Am J Kidney Dis. 2007;49:109–17.
Van Guldener C, Stehouwer C. Homocysteine metabolism in renal disease. Clin Chem Lab Med. 2003;41:1412–7.
Wada J, Makino H. Inflammation and the pathogenesis of diabetic nephropathy. Clin Sci. 2013;124:139–52.
Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from meta-analysis. Br Med J. 2002;325:1202–6.
Walker MC, Smith GN, Perkins SL, et al. Changes in homocysteine levels during normal pregnancy. Am J Obstet Gynecol. 1999;180:660–4.
Wu CC, Zheng CM, Lin YF, et al. Role of homocysteine in end-stage renal disease. Clin Biochem. 2012;45:1286–94.
Zappacosta B, Persichilli S, Iacoviello L, et al. Folate, vitamin B12 and homocysteine status in an Italian blood donor population. Nutr Metab Cardiovasc Dis. 2013;23:473–80.
Zimny J, Sikora M, Guranowski A, et al. Protective mechanisms against homocysteine toxicity: the role of bleomycin hydrolase. J Biol Chem. 2006;281:22485–92.
Zoccali C, Benedetto FA, Mallamaci F, et al. Inflammation is associated with carotid atherosclerosis in dialysis patients. Creed investigators. Cardiovascular risk extended evaluation in dialysis patients. J Hypertens. 2000;18:1207–13.

Fetal Beta2-Microglobulin as a Biomarker of Kidney Disease

22

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Abstract

Ultrasound diagnosis of fetal kidney and urinary tract anomalies is generally performed at 18–20 WG, showing renal agenesis, cystic kidneys, and urinary tract enlargement. Prognosis of the renal function and its postnatal outcome relies on the US follow-up of the amniotic fluid volume and renal parenchyma. In case of lower urinary tract obstruction, bilateral hydronephrosis, and nephropathies, fetal puncture may be useful to analyze β_2-microglobulin. It is a small protein which circulates in a free and stable soluble form in biological fluids and can be

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measured using different immunoassay. As β_2-microglobulin is entirely filtered and reabsorbed and degraded by the nephron, serum levels rise when glomerular filtration is impaired, while urinary levels increase when tubular reabsorption is affected. Viral fetal infections may also increase serum levels, activating the lymphocyte turnover and thus β_2-microglobulin release. An increase in β_2-microglobulin in serum (above 5–6 mg/L) and to some extent in urine (above 4–5) constitutes an accurate marker to predict a postnatal renal failure in case of urinary tract obstruction and bilateral hydronephrosis. In urines, measurement of Na and Ca if possible has to be associated. In contrast to uropathies, measurement of β_2-microglobulin is poorly informative in nephropathies. However, discrepancies remain due to the difficulty in defining a good postnatal outcome and choosing of a reliable cutoff value and to the fact that nephrogenesis is a dynamic complex process in which injury is not entirely reflected by β_2-microglobulin.

Keywords

Beta-2 microglobulin • Human fetus • Kidney • Microproteins • Nephropathies • Prognosis • Renal function • Serum • Urine • Uropathies

Abbreviations

ACE	Angiotensin-converting enzyme
β_2-m	Beta-2 microglobulin
Ca	Calcium
Da	Dalton
HLA	Human leukocyte antigens
IgG	Immunoglobulin G
LuTO	Lower urinary tract obstruction
MCDK	Multicystic dysplastic kidney disease
MHC-I	Class I major histocompatibility complex
Na	Sodium
NK	Natural killer cells
NMR	Nuclear magnetic resonance spectroscopy
NSAIDs	Nonsteroidal antiinflammatory drugs
PKD	Polycystic kidney disease
PUV	Posterior urethral valve
RAS	Renin-angiotensin system
US	Ultrasound
WG	Weeks of gestation

Key Facts

- Human nephrogenesis occurs in the renal parenchyma from 5 to 35 WG and leads to the formation of functional nephrons in terms of glomerular filtration and tubular secretion and reabsorption.

- It can be altered in case of lower urinary tract obstruction, bilateral hydronephrosis, and some nephropathies.
- Finding specific and sensitive markers of the renal parenchyma injury is still a challenge.
- Serum β_2-m is an index of glomerular filtration, while urinary β_2-m reflects tubular reabsorption.
- β_2-m levels is the most reliable biochemical marker to predict postnatal outcome when fetal ultrasound findings are doubtful.
- There may be a poor prognosis of the postnatal renal function if β_2-m levels increase in the urine or serum.
- However, there is still a gray zone in which prognosis is difficult to establish.

Definitions

The nephron is the functional unit of the kidney. It consists in the glomerulus responsible for the plasma filtration and the tubules where secretion and reabsorption take place, leading to the definitive urine which is thus an ultrafitra of the plasma.

Renal failure can be defined as impaired plasma clearance by the nephron. It can be functional or organic and rapidly lethal if there is no treatment requiring dialysis and kidney transplantation.

Nephropathies are purely kidney diseases affecting the development of the kidney (agenesis, hypoplasia), its location (ectopia, abnormal rotation), or the nephrogenesis and the differentiation of the renal parenchyma (defective corticomedullary differentiation, parenchymal dysplasia, cysts).

Uropathies are urinary tract disease which can lead to an alteration of the renal parenchyma and kidney functions due to urinary impaired excretion and reflux.

Introduction

Thanks to advances in the field of ultrasound scanning, the diagnosis of fetal renal and urinary tract anomalies has widely improved. However, the assessment of fetal renal functions and the prognosis of their postnatal outcome (i.e., renal failure) are still a matter of challenge. Thus, fetal sampling can help to analyze biochemical markers reflecting the renal parenchyma injury. Several studies pointed out the interest of β_2-microglobulin, a small protein that is entirely filtered and reabsorbed and degraded by the nephron. We aim in this chapter to make an overview of the past and the current knowledge on β_2-microglobulin as a biomarker of fetal kidney disease.

Physiology and Analysis of Beta-2 Microglobulin

Structure, Synthesis, and Function

Human beta-2 microglobulin (β_2-m) was first identified in urines from patients with chronic kidney dysfunction at the end of the 1960s (Berggård and Bearn 1968). It is a highly conserved protein among species (Grooves and Greenberg 1982). Human β_2-m is a small monomeric protein of 11,800 Da which is devoid of carbohydrate and has a pH of 5.7. It is made up of 100 residues with a seven-stranded β-sandwich fold typical of the Ig superfamily and a single inter-sheet disulfide bond between cysteine residues 25 and 80 in β-strands B and F. β_2-m displays partial homology with immunoglobulin constant region domains and particularly the $C_{H\ III}$ domain (Trinh et al. 2002). It is encoded by a single gene on chromosome 15 which is expressed in numerous tissues in fetus and adult (Cejka et al. 1975; Güssow et al. 1987). It is synthesized and expressed on the surface of nucleated cells. Lymphocytes release β_2-m during cell division and after stimulation. Indeed, different cytokines (e.g., interferons) can stimulate β_2-m synthesis in hepatocytes and T lymphocytes (Vraetz et al. 1999). On the cell surface, β_2-m forms the small invariable light chain of the class I major histocompatibility complex (MHC-I) bound to the heavy chain (also called the α-chain) extracellular region (Grey et al. 1973). Its expression is regulated in a coordinated manner with that of MHC-I antigens (also referred to as human leukocyte antigens [HLA]) It binds non-covalently to these antigens which form the α-chain of the MHC-I. β_2-m interacts with and stabilizes the tertiary structure of the MHC-I α-chain to present antigens (e.g., antigens present on cancer cells) to cytotoxic T lymphocytes (CD8+) and NK cells (Yokoyama 1998; Hill et al. 2003). It is probable that β_2-m not only reflects but also modulates cell turnover, i.e., proliferation, apoptosis, and the immune response, particularly in the setting of cancer. No receptor for β_2-m has yet been identified, and it acts via different pathways: cytokines, growth factors or hormones and their receptors, and different kinases (cAMP-dependent protein kinase A, phosphatidylinositol 3-kinase, etc.) (Rowley et al. 1995; Zhao et al. 2013; Nomura et al. 2014).

Release, Excretion, and Catabolism

β_2-m is principally a cell membrane protein. However, because it is non-covalently associated with MHC-I antigens and not directly attached to the cell membrane, β_2-m can be released from a cell in a free soluble form. Such forms of β_2-m are detectable in many body fluids, such as blood, urine, and amniotic fluid but also in cerebrospinal fluid, seminal fluid, pleural fluid, and ascites (Berggård et al. 1968; Cejka et al. 1975; Puolakka et al. 1982; Lutz et al. 1991) (Table 1). Its rate of production in normal adult subjects is quite constant at around 0.13 mg/h.kg with relatively stable serum levels of 1–3 mg/L, while it is excreted in urine at low levels of less than 0.5 mg/L. Levels are not age or gender dependent (Filler et al. 2002). Catabolism occurs in the kidney. β_2-m is a low-molecular-weight protein (≤40,000 Da) like α1-acid glycoprotein, retinol-binding protein, and α1-microglobulin (Regeniter et al. 2009). It is thus totally filtered through

Table 1 Levels of β_2-m in different human body fluids. β_2-m was measured in body fluids from healthy children, adults, and mothers using different assays and antibodies. Fetal fluids were also analyzed in fetus devoided of any renal impairment at the US or with no neonatal renal failure

	β_2-m (mg/L)	Range	Reference
Non pregnant			
Serum	1.4	1–2.3	Ikezumi et al. 2013
	3.09	2.88–3.3	Filler et al. 2002
Urine	<0.5		Davey and Gosling 1982
Cerebrospinal fluid	1	0.6–2	Lutz et al. 1991
Pregnancy			
Maternal blood	1.72 ± 0.42		Ferreira et al. 1991
	1.83 ± 0.48		Nolte et al. 1991
	1.2 ± 0.3		Jauniaux et al. 1998
	1.6 ± 0.51		J Guibourdenche, ANR Perinat Collection, personnal data
Coelomic fluid	4.5 ± 2.1		Jauniaux et al. 1998
Amniotic fluid	6.7	1–24.7	Burghard et al. 1988
	3.5 ± 2.0		Jauniaux et al. 1998
	5.1 ± 2.1	12–1.2	Oliveira et al. 2002
Fetal blood	3.6	0–5.8	Berry et al. 1995
	3.4	2–4.9	Tassis et al. 1996
	2.93	0.64–7.94	Cobet et al. 1996
	3.6 ± 0.4		Jauniaux et al. 1998
	4.28	2.95–6.61	Bökenhamp et al. 2001
	3.2	1.5–4.7	Dommergues et al. 2000
	3.7	3.1–4.8	Fabbri et al. 2011
	3.4 ± 1.5		J Guibourdenche, ANR Perinat Collection, personnal data
Fetal urines	1.22	0.41–2.04	Muller et al. 1993
	0.96	0–3.3	Muller et al. 1996
	1.4	0.1–6.8	Spaggiari et al. 2012
	1.6	<4	J Guibourdenche, ANR Perinat Collection, personnal data
Fetal ovarian cysts	3.1	2–4.1	Lecarpentier et al. 2012
Over intra abdominal cysts	7.3	0.1–41	Lecarpentier et al. 2012
Cord blood	2.78 ± 0.84		Ferreira et al. 1991
	3.3	2.1–4.5	Nolte et al. 1991

the glomeruli, reabsorbed at 99.9 %, and then degraded by lysosomes in the proximal tubules (Fig. 1). Around 5 µg/h of the protein is excreted in the final urine. The plasma disappearance curve of 125I-β_2-m displays a rapid turnover (t1/2 = 2.1 h; range: 1.1–2.8 h in normal healthy patients) (Karlsson et al. 1980). Its levels in blood reflect the rates of both nucleated cell turnover and glomerular filtration, whereas in urine they

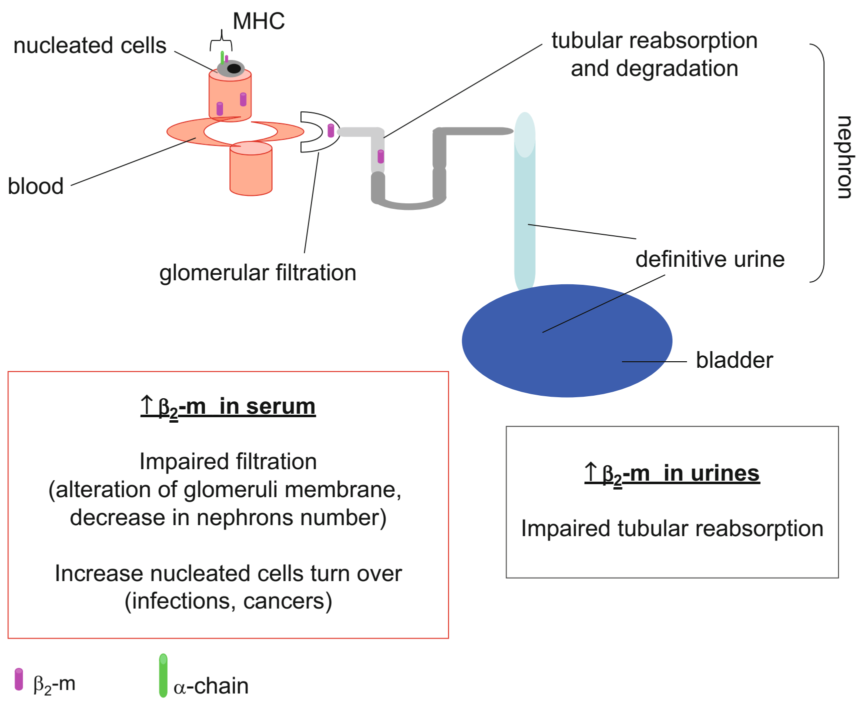

Fig. 1 Renal physiology and β_2-microglobulin in the human nephron. β_2-m is mainly a nucleated cell membrane protein associated with MHC α-chain, which is also released in free soluble form. This form is entirely filtered through the glomeruli and reabsorbed and degraded at 99.9 % in the proximal tubules. Serum β_2-m levels rise mainly when glomerular filtration is impaired, while urinary β_2-m levels increase when tubular reabsorption is affected

are indicative of tubular reabsorption and degradation. Soluble β_2-m can aggregate and polymerize into insoluble fibrils in pathological situations such as chronic renal failure and acidic conditions (Vincent et al. 1994). β_2-m is the major protein component in amyloid plaques and forms filamentous structures in the joints and connective tissue. Samples collected from dialysis-related amyloidosis patients exhibit such fibrils which are mostly formed by full-length β_2-m together with a few additional proteolytic split products (Rosano et al. 2005).

Sampling and Assay of β_2-microglobulin in Human Fluids

In serum, β_2-m circulates physiologically as a free monomer protein. In pathological situations, dimeric or insoluble forms may occur. Truncated forms have also been characterized in pathological conditions, resulting in enzymatic cleavage in the N-terminal part. Under acidic conditions ($pH<5$–6), which may affect the urine in pathological situations, β_2-m is spontaneously unstable and in vitro may form

Table 2 Urinary proteins in renal disease. Troubles within the glomeruli filtration and the tubular reabsorption can result in different types of proteinuria. Diagnosis can be made by measuring the excreted urinary proteins or performing an electrophoresis of urinary proteins. Tubular proteinuria is characterized by an increased excretion of low-molecular-weight proteins ($\leq$40,000 Da) such as β_2-m, whereas glomerular proteinuria is associated with the excretion of high-molecular-weight proteins ($\geq$66,000 Da)

Protein	Molecular mass (Da)	Normal urine concentration mg/L	Proteinuria
IgG	150,000	<10	Glomerular
Transferrin	79,550	<2.5	Glomerular
Albumin	66,460	<30	Mixed
α1-microglobulin	33,000	<12	Tubular
Retinol-binding protein	20,960	<0.5	Tubular
β_2-microglobulin	11,800	<0.3	Tubular

amyloid-like fibrils (Davey and Gosling 1982; Vincent et al. 1994). The stability of β_2-m in the urine decreases when the temperature rises, so urine specimens should be alkalinized if necessary and stored at +4 °C before being rapidly frozen if not arranged quickly. Froze and thaw cycles should be avoided even if the molecule seems quite stable (Juraschek et al. 2012).

β_2-m levels can be measured by different immunoassays using a variety of antibodies and detection systems (e.g., immunochemiluminescence, immunoturbidimetry) (Lutz et al. 1991; Terrier et al. 2004). These assays mainly recognize the monomer protein in serum, plasma, or urine. Most assays are correlated but with differences fluctuating from 2 % to 35 %, which may influence cutoff values. In addition, some assays present matrix effects so they cannot be applied to all biological fluids. There is no significant difference between plasma and serum levels. Assay values can range from 0 to 16 mg/L, depending on the type of sample.

Clinical Usefulness in Children and Adults

Diseases affecting glomerular filtration and tubular reabsorption may result in different types of proteinuria (Peterson et al. 1969; Gorriz and Martinez-Castelao 2012) (Table 2, Fig. 2). Important diagnostic information can be obtained by determining excreted urinary proteins in order to locate both the site and extent of renal injury. The pathological excretion of high-molecular-weight proteins, i.e., IgG, transferrin ($\geq$66,000 Da), reflects an alteration of glomerular filtration. The increased excretion of low-molecular-weight proteins ($\leq$40,000 Da) such as β_2-m is an index of tubular proteinuria indicative of impaired tubular reabsorption.

In serum, any elevation of β_2-m levels may result from either increased production and release due to a greater nucleated cell turnover or impaired renal filtration and clearance (Fig. 1). However, in the latter case, creatinine is still the most available indicator used for the diagnosis and follow-up of renal

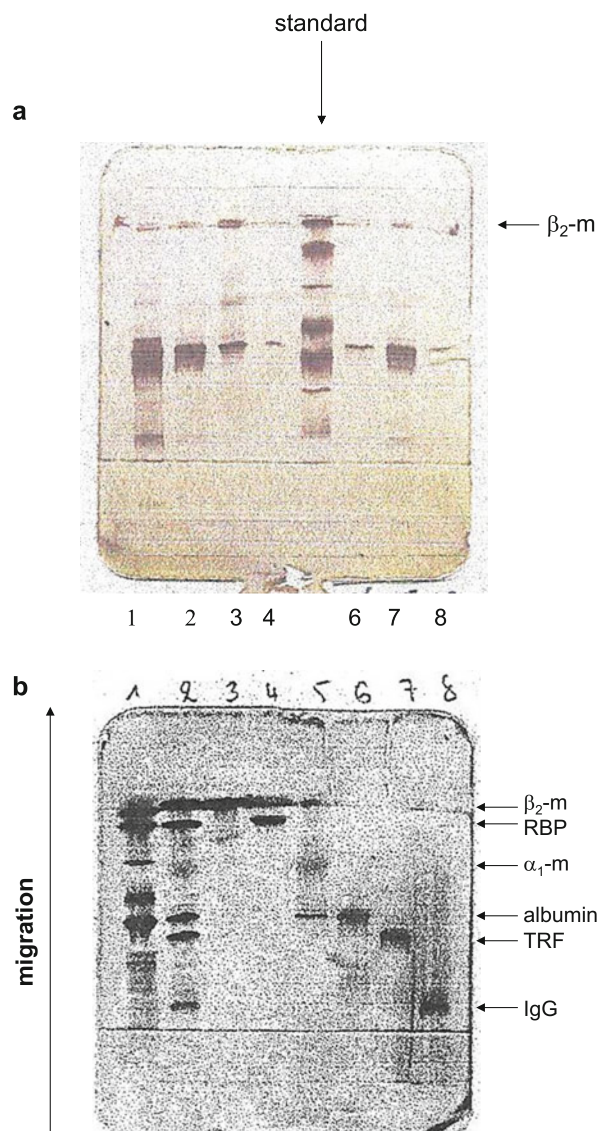

Fig. 2 Electrophoresis of fetal urinary proteins (1: mixte of standards; 2: amniotic fluid; 3: b2-m standard; 4: RBP standard; 5: a1-m standard; 6: albumin standard; 7: TRF standard; 8: IgG standard). Fetal urines (**a**) and protein standards (**b**) were applied for sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by an argentic detection (**b**). This allowed the separation of those proteins according to their apparent molecular size: low-molecular-weight proteins (β_2-m; α_1-m; RBP), albumin, high-molecular-weight proteins (IgG; TRF), *β_2-m* β_2-microglobulin, *α_1-m* α_1-microglobulin, *RBP* retinol-binding globulin, *IgG* immunoglobulin G, *TRF* transferrin

insufficiency in a daily practice in children and adults (Ikezumi et al. 2013; Juraschek et al. 2013; Reichel 2014). An increase in β_2-m may provide information on the progression of infection and the lymphoid response, in a setting such as HIV infection (Fabbri et al. 2011; Chiltra et al. 2011). It may also be useful to the prognosis and treatment of tumors such as lymphoma, reflecting an advanced disease stage, tumor mass, and its malignant environment (Changhoon et al. 2014; Nomura et al. 2014).

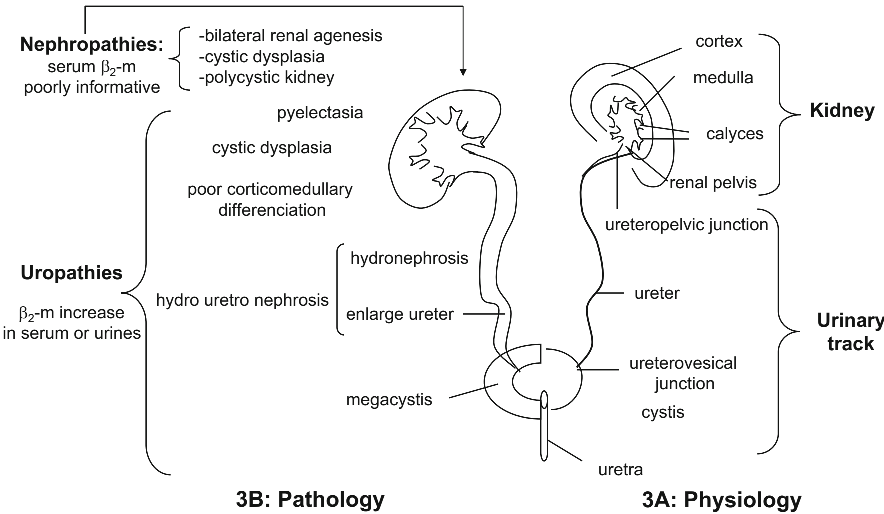

Fig. 3 Normal and pathological structures of the fetal kidney and the urinary tract. This is a schematic representation of the normal kidney and normal urinary tract (**a**) and the most frequent pathological findings discovered at the US (**b**)

Fetal Kidney and Urinary Tract: Normal Development and Pathological Situations

The placenta ensures clearance of the fetal compartment, while the fetal kidneys and excretory system develop during pregnancy (Fig. 3a).

Anatomical Development

Fetal kidneys develop from three successive embryonic structures of mesodermal origin. The intermediate mesoderm gives rise to the nephrogenic cord which in turn forms the pronephros (3–5 WG) that is never functional and will regress; the mesonephros (5–12 WG), a transient structure which can produce urine; and the metanephros (as from 6 WG) which will lead to the definitive kidney (Wolff and Winyard 2002; Dressler 2009). This results from an interaction between the ureteric bud which will lead to the urinary excretory tract and the nephrogenic blastema that will become the nephrons. The metanephros migrates from the pelvis to the lumbar region and then rotates. It produces urine as from 10 WG through the establishment of glomerular filtration. Nephrogenesis is a complex process that combines cell growth and differentiation. It occurs from 5 to 35 WG, with an intense phase between 18 and 32 WG. It occurs centrifugally, with newly formed nephrons being located in the peripheral cortex. This results in the typical US appearance with renal corticomedullary differentiation (Fig. 4a). At birth, each fetal kidney contains around one million nephrons. Nephrogenesis is induced by penetration of the ureteric bud into the nephrogenic blastema. Renal vesicles form and develop into S-shaped tubules. The end of these S tubules opposite the ureteric bud will

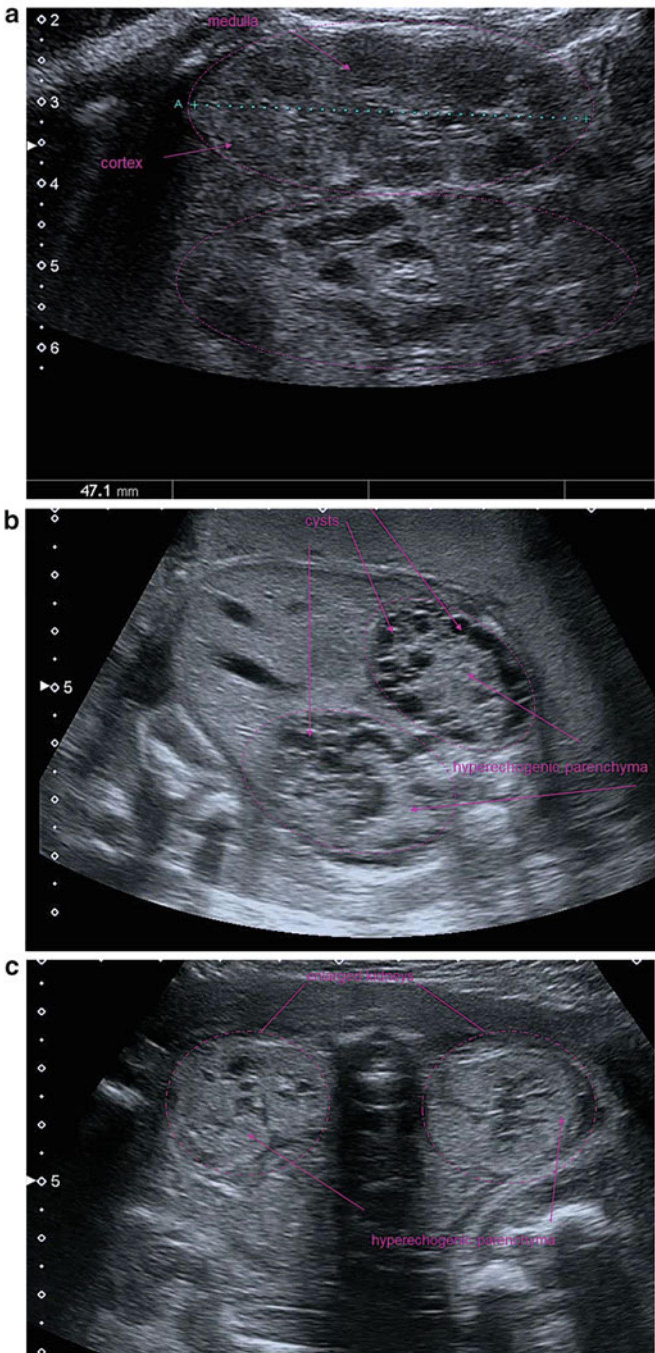

Fig. 4 (continued)

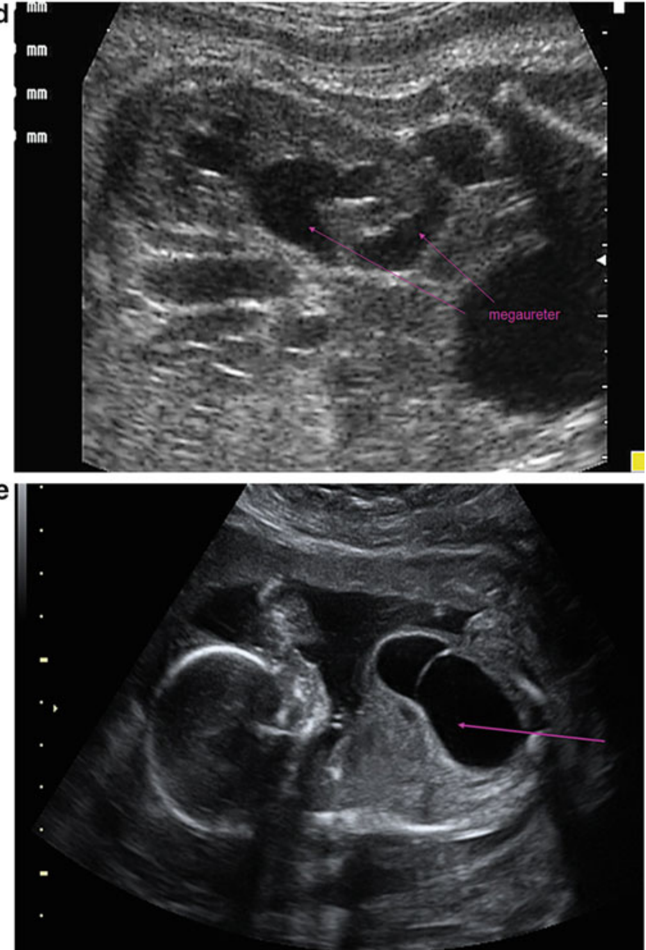

Fig. 4 Ultrasound scanning of the fetal kidney and the urinary tract in normal and pathological situations (Gilles Grangé, personal data). (**a**) Normal appearance of fetal kidney at the end of pregnancy. Its outline is bumpy and corticomedullary differentiation is clearly visible. (**b**) Multicystic fetal kidneys with oligohydramnios. The kidney carries numerous irregular cysts on the periphery with a loss of corticomedullary differentiation and hyperechogenic appearance. (**c**) Renal dysplasia and oligohydramnios within a Meckel syndrome. The kidneys are large with a hyperechogenic appearance of the parenchyma due to renal dysplasia. It should look for associated encephalocele. (**d**) Obstructive megaureter. The ureter appears as a dilated tortuous fluid-filled tubular structure located between the bladder and the renal pelvis. (**e**) Severe megacystis at the first trimester. The urinary bladder is enlarged, full of urines, and occupies most of the fetal abdomen

differentiate into the glomerulus. The remainder of the tubule extends and differentiates into proximal and distal convoluted tubules and the loops of Henle, connecting to the collecting ducts. The ureter arises from the lower ureteric bud which grows toward the nephrogenic blastema and undergoes repeated dichotomous branching to

form the collecting system (i.e., pelvis, calyces, collecting ducts). Branching of the collecting system is completed by 20 WG. The bladder derives mainly from the cloacal endoderm. At 6 WG, the cloaca divides into two separate chambers: the anorectal chamber and the urogenital sinus. The latter will form the major part of the bladder and the urethra.

Renal Function Development

Morphological development of the kidney and urinary tract can be followed by fetal ultrasound scanning (US) from the end of the first trimester and is associated with functional maturation (Quigley 2012). Glomerular filtration is likely to be initiated at around 9–10 WG and then rapidly increases with gestational age, reflecting the rise in the number of nephrons up to 35 WG. This filtration is the first step in the formation of urine which becomes the principal source of amniotic fluid from around 20 WG. From the second half of gestation, amniotic fluid volume reflects fetal diuresis so that evaluation of its volume by US constitutes a reliable index of renal function (Shackelford et al. 1992; Yamamoto et al. 2007). Its biochemical analysis, but preferentially that of fetal urine or blood whenever possible, can provide valuable information on functional development and renal maturation. Amniotic fluid is vital for lung and skeletal development. Decreased amniotic fluid volume, i.e., oligohydramnios and anhydramnios, causes lung hypoplasia and soft tissue deformities when it occurs at an early stage of pregnancy affecting the canalicular phase (16–25 WG) of lung development. At birth, the glomerular filtration rate is low but increases rapidly in line with postnatal hemodynamic changes. Fetal creatinine is cleared by the placenta. At birth, serum creatinine levels are equal to those of the mother, at around 70 μmol/L, and then fall during the first week of life (Nolte et al. 1991). Biochemical analysis of amniotic fluid (Burghard et al. 1988; Oliveira et al. 2002) and mainly fetal urine (Nicolaides et al. 1992; Muller et al. 1996) has suggested that glomerular filtration is functional as from 20 WG due to the decreasing low levels of total proteins in amniotic fluid and urine (Fig. 5). Tubular functions, i.e., secretion and reabsorption, are initiated later at around 12–14 WG leading to hypotonic fetal urine relative to plasma, maturing in late pregnancy. In utero, sodium reabsorption is low and glucose reabsorption is also observed at around 22 WG with an initially low threshold, increasing during pregnancy. The absence of glucose and phosphorus after 20 WG and the increase in calcium in urines are in favor of the maturation of tubular reabsorption and secretion. The fetal kidney seems able to acidify the urine and to reabsorb bicarbonate.

Different regulatory systems have been identified in the fetus and develop gradually during pregnancy. Arginine vasopressin is present but the fetal kidney only becomes sensitive to it toward the end of pregnancy. The renin-angiotensin system (RAS) is also expressed early and is independent of the maternal RAS (Schutz et al. 1996). Renin does not cross the placental barrier. The fetus produces renin and angiotensin II at higher levels than in the mother; they are involved in regulating the fetal circulation and in kidney development. Several prostaglandins are also produced by the kidney and are involved in modulating the fetal RAS.

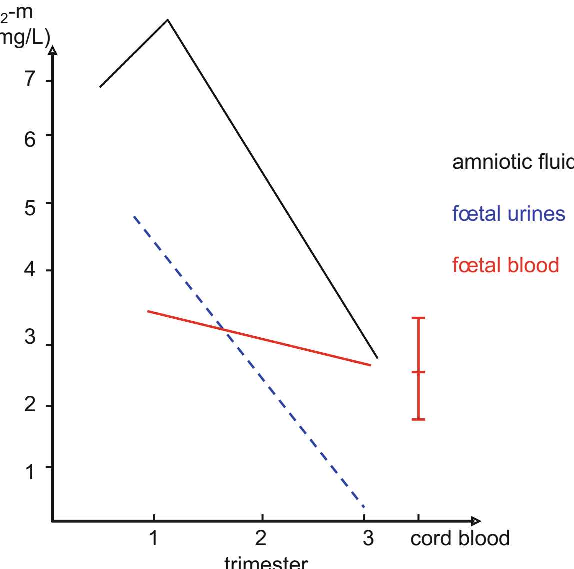

Fig. 5 Fetal β_2-m levels during pregnancy. β_2-m can be measured in amniotic fluid since the end of the first trimester and in fetal urine and blood in the second half of pregnancy. Levels are higher in amniotic fluid compared to fetal urine and blood. They decreased significantly during the third trimester of pregnancy (Muller et al. 1996; Burghard et al. 1988). In cord blood, levels were increased in preterms compared to full-term neonates (Nolte et al. 1991)

Renal Impairment in the Fetus

Fetal renal impairment may have several causes; they may be renal or extrarenal or endogenous or exogenous (Vanderheyden et al. 2003; Fig. 3b). Some can be detected by US scanning but it may be difficult to pronounce upon renal functionality and a future prognosis. As exogenous causes, drugs or medicines taken by the mother may cross the placenta and impair fetal renal function, leading to a reduction in amniotic fluid volume. Angiotensin-converting enzyme inhibitors (ACE inhibitors) can directly disrupt the fetal RAS which can impair both prenatal and postnatal renal functions. Renal tubular dysgenesis is often noted which may have long-term consequences such as renal failure and hypertension (Plazanet et al. 2014). Nonsteroidal antiinflammatory drugs (NSAIDs) can decrease prostaglandin synthesis by cyclooxygenase inducing transient or irreversible renal impairment (Phadke et al. 2012).

Fetal Nonrenal Causes

Intrauterine growth restriction (IUGR) of placental origin may be associated with chronic hypoxemia inducing the redistribution of blood flow to vital organs, i.e., the brain and the heart, at the expense of others such as the kidneys. This may disturb the physiological increase in the number of glomeruli and their functional maturation and regulation. Fetuses may therefore develop oligohydramnios and be at risk of decreased glomerular filtration rates during childhood (Bacchetta et al. 2009). In twin-twin transfusion syndrome, a major complication of monochorionic twin pregnancies, the donor twin may develop reduced renal perfusion. This upregulates the production of renin and the secretion of angiotensin II which can be transferred to the recipient twin, thus activating its own RAS (Mahieu-Caputo et al. 2005).

Renal Causes: Nephropathies

Nephropathy can result from abnormal kidney development. Except in a familial context where previous index cases are confirmed, they may be discovered fortuitously by ultrasound scanning (Avni and Hall 2006).

Bilateral renal agenesis can be detected in the case of oligohydramnios or anhydramnios as the absence of both kidneys, non-visualization of the bladder, or a failure to visualize the renal arteries using color Doppler US. It is less common than unilateral agenesis but has a fatal outcome. It is sporadic and usually isolated but may sometimes form part of a genetic syndrome or abnormal development sequence (e.g., Fraser syndrome). When unilateral, the bladder can be seen and the amniotic fluid volume is normal.

Ultrasound scanning is able to detect the abnormal presence of cysts in the kidney which may indicate a diagnosis of multicystic kidneys or cystic dysplasia (Fig. 4b). Multicystic dysplastic kidney disease (MCDK) results from non-coordinated development of the metanephros and the branching ureteric bud. It usually affects only one of the two kidneys which becomes enlarged and sometimes deformed, with an abnormal renal parenchyma and the presence of irregular cysts. When isolated the prognosis is good and the amniotic fluid volume is normal. The prognosis depends on the state of the contralateral kidney, which is generally normal or hypertrophic.

The presence of two enlarged and echogenic kidneys with numerous small cortical cysts is suggestive of infantile polycystic kidney disease (PKD) also referred to as autosomal recessive cystic kidney disease. It can also be part of few syndromes as Meckel (Fig. 4c). The prognosis is variable but usually fatal if this condition appears early in pregnancy, leading to oligohydramnios. Among the rare fetuses which survive, it often results in chronic renal failure.

Fetal Excretory Tract Abnormalities: Uropathies

Uropathies essentially arise from the obstruction and enlargement of the urinary tract (Figs. 3a, 4d). This leads to cystic renal dysplasia if a complete obstruction occurs at an early stage. If the obstruction is intermittent, or develops later in the second half of pregnancy, long-standing hydronephrosis can cause renal cystic dysplasia, the severity of which depends on both the degree and duration of the obstruction (Morris 2008; Longpre et al. 2012).

On an US, dilatation of the renal pelvis (i.e., pyelectasis) or of the calyces may be indicative of impaired urinary flow. Hydronephrosis refers to dilatation of the renal pelvis which may be transient and resolve at birth. Unilateral hydronephrosis is more common than its bilateral counterpart. It can result from an abnormal ureteropelvic or ureterovesical junction or from urethral obstruction. In the latter case, the obstruction is bilateral and involves some or all of the urinary tract. The prognosis for upper urinary tract obstruction is relatively good if it is found in isolation. By contrast, obstruction in the lower urinary tract (LuTO) may be associated with cystic renal dysplasia, oligohydramnios, or pulmonary hypoplasia, with a variable prognosis.

Hydroureteronephrosis combines hydronephrosis and an enlarged ureter, which is common in the case of megacystis. This enlargement may be moderate or severe or unilateral or bilateral.

Urethral obstruction can result in an enlarged bladder (megacystis) which can be detected from the end of the first trimester (Fig. 4e). Posterior urethral valve (PUV) is the principal cause in male fetuses, associating oligohydramnios, a large thick-walled bladder, and different degrees of bilateral hydronephrosis and hydroureter. The prognosis is poor for fetuses diagnosed antenatally when an oligohydramnios is present. In rare cases, it may be associated with normal or increased amniotic fluid volume, suggestive of megacystis microcolon intestinal hypoperistalsis syndrome. Megacystis may also be observed in the context of prune belly syndrome.

Assessment of Fetal Renal Function: Usefulness of β2-Microglobulin

The assessment of fetal renal function is based on ultrasound findings which help to diagnose and monitor nephropathies and uropathies and can be indicative of renal impairment. Depending on the gestational age, the US should provide detail on amniotic fluid volume, kidney size and appearance, renal parenchyma, corticomedullary differentiation, renal dysplasia (presence of cortical cysts and echogenicity of the renal parenchyma), collecting system, and bladder aspect and size (Fig. 4). Poor prognostic factors include dilatation of the upper tract, increased bladder wall thickness, oligohydramnios, echogenic renal cortex, and cysts, particularly before 24 weeks (Winyard and Chitty 2008; Morris 2008; Longpre et al. 2012). In certain cases, the biochemical analysis of fetal fluids can be a useful adjunct to ultrasound findings.

Amniotic Fluid

The analysis of amniotic fluid has been suggested because fetal urine is the principal source of amniotic fluid as from 20 WG. β_2-m is detectable in amniotic fluid, and its concentration rises after 10 WG when glomerular filtration becomes operational, picks at around 20–24 WG and then falls (Burghard et al. 1988; Gulbis et al. 1996; Jauniaux et al. 1998; Oliveira et al. 2002) (Table 1, Fig. 5). The reduction in its concentration during the third trimester is likely to be related to the maturation of tubular function. However, because oligohydramnios is often present in the event of impaired renal function, an amniotic puncture is rarely performed. Furthermore, because the origin of β_2-m in amniotic fluid is not unequivocal, its measurement is of little value (Kim et al. 2012).

Fetal Blood

The biochemical assay of fetal blood enables the evaluation of glomerular filtration. Serum creatinine cannot be used because the molecule is very small so it can easily cross the placenta and be cleared by the mother. Analyses of low-molecular-weight proteins such as α1-microglobulin and β_2-m have been proposed because they cannot cross the placenta and are entirely filtered and reabsorbed by the nephron (Nolte et al. 1991). Levels in fetal blood are quite stable around 3–4 mg/l with a trend to decrease at the end of pregnancy (Table 1, Fig. 5). There is no correlation between cord blood and maternal blood so that the assessment of maternal blood is of no

Table 3 Cutoff values for fetal serum β_2-m in obstructive uropathies. β_2-m was measured using different assays in the serum of fetuses affected by LuTO. Cutoff values were established to distinguish between LuTO leading to termination of pregnancy or neonatal renal failure, from LuTO associated with normal neonatal renal function or no renal failure in the first year of life

Fetal serum β_2m (mg/L)	
5.6	Berry et al. 1995
4.1	Cobet et al. 1996
4.9	Tassis et al. 1997
5	Dommergues et al. 2000
5.6	Bökenhamp et al. 2001
5	Spaggiari et al. 2012
5	Nguyen et al. 2013

interest in case of fetal nephropathies or uropathies. Elevation of these low-molecular-weight proteins in fetal blood is therefore likely to reflect a reduction in the number of nephrons and/or an alteration of the glomerular filtration. The first observation was based on the relationship between serum β_2-m levels and neonatal renal function in 15 affected fetuses with urinary tract or renal malformation, compared with 64 "unaffected" fetuses (Berry et al. 1995). By applying a cutoff value of 5.6 mg/L, elevated β_2-m levels were found to be both sensitive (80.0 %) and specific (98.6 %) in predicting a poor outcome (termination of pregnancy or renal failure at birth). These findings were confirmed by subsequent studies which nevertheless noted an overlap in β_2-m levels between the controls and fetuses with urinary tract anomalies of bad prognosis (Tassis et al. 1997; Nicolini and Spelzini 2001). Other micro-proteins such as α1-microglobulin had little (Cobet et al. 1996; Bökenhamp et al. 2001; Nguyen et al. 2013). α1-microglobulin is likely to be more independent of the gestational age than β_2-m which tends to decrease at the end of pregnancy. Cystatin C could be more specific than β_2-m which remains more sensitive in predicting impaired renal function. A significant correlation has been found between α1-microglobulin and β_2-m and cystatin C. Other studies have confirmed the usefulness of a cutoff value at around 4–6 mg/L for β_2-m (Table 3). It is now clear that fetal serum β_2-m levels rise markedly above 5 mg/L in the context of bilateral renal agenesis (median: 9.5 mg/L) and uropathies ending in terminal renal failure after 1 year of life (i.e., serum creatinine >50 μmol/L) (median 5.3–7.4 mg/L) (Muller et al. 2004; Nguyen et al. 2013). In newborns, fetal β_2-m is higher compared to young children with a decrease which is age but not gender dependent, while serum creatinine increases (Ikezumi et al. 2013). All survivors with normal postnatal creatinine values (≤ 50 μmol/L) had fetal β_2-m levels <5 mg/L (Dommergues et al. 2000). The same team confirmed that with this cutoff value, elevated serum β_2-m levels were still sensitive in the event of obstructive uropathy complicated by urinary ascites (Spaggiari et al. 2013a). The potential usefulness of sequential measurement of fetal serum β_2-m to enable improvements to the course of the disease or its treatment has not been clearly established, especially when compared to the potential additional risk to the fetus resulting from the invasiveness

blood sample collection (Craparo et al. 2007). Indeed, data regarding sampling intervals are still arbitrary and based on US follow-up.

Very few studies have focused specifically on hypoplastic kidney in which fetal serum β_2-m is likely to be increased (8.3 mg/L, $n = 7$; Muller et al. 2004). Using the cutoff value of 5 mg/L, this team showed that β_2-m has displayed a positive predictive value of 87.1 % ($n = 31$) in the case of hypoplasia in the second half of pregnancy, identical to those of amniotic fluid volume evaluation (Spaggiari 2013). β_2-m increases with the severity of the oligohydramnios. In the context of cystic dysplasia, amniotic fluid volume has the same prognostic value as β_2-m, which was elevated (8.8 mg/L, $n = 9$, Muller et al. 2004) but lack sensitivity. In the case of nephropathies, the sensitivity and specificity of β_2-m have been shown to be poor to predict renal function, with an important overlap with normal ranges in contrast to uropathies (Muller et al. 2004; Nguyen et al. 2013). Thus, a normal serum β_2-m value cannot exclude postnatal renal failure. In fetuses exposed to RAS blockers who present signs of renal impairment, β_2-m values ≥ 5 mg/L are as reliable as persistent severe oligohydramnios in predicting poor postnatal renal function during the first 18 months of life (Spaggiari et al. 2012).

In addition, as fetal serum β_2-m may increase in case of viral infection, the interest of the sampling should be discussed facing the US findings in such a situation (Fabbri et al. 2011). Results should then be cautiously interpreted.

Fetal Urine

Although amniotic fluid volume depends on fetal diuresis, its composition does not reflect strictly fetal renal function because its composition fluctuates during pregnancy, depending on renal and fecal excretion, respiratory secretion, and placental exchange. Any contamination of the urinary sample by amniotic fluid enables any analyses and interpretation (Spaggiari 2013). Fetal urine production has been documented as early as 10–12 WG. An increase in β_2-m urine excretion is a good indicator of tubular proteinuria resulting from changes in tubular reabsorption (Fig. 2). The determination of fetal urine parameters has been proposed since the 1980s in the case of uropathies because its collection may lower the pressure in the urinary tract and enables biochemical analysis (Cromblebome et al. 1990). Different technologies such as high-field proton nuclear magnetic resonance spectroscopy (NMR) have shown that LuTO may be associated with glycosuria, aminoaciduria, and organic aciduria (Foxall et al. 1995). The levels of biochemical fetal urine compounds fluctuate during pregnancy. Different publications concluded that current evidence is insufficient to pronounce upon the clinical usefulness of analyzing fetal urine alone (Clark et al. 2003; Morris 2008). However, it confirmed that two tests provided more accurate pointers: Ca >95th percentile for gestational age and Na >95th percentile for gestational age. Threshold values of urinary Na >100 meq/L and Ca >2 mmol/L are widely accepted as being predictive of a poor outcome, with variable sensitivity and specificity (Nicolini et al. 1992). The usefulness of measuring β_2-m in urine was highlighted in 1993 (Lipitz et al. 1993). “Normal” ranges for several urinary markers in a significant cohort of patients were published in 1996 (Muller et al. 1996) (Table 1, Fig. 5). Forty-one fetuses presenting with

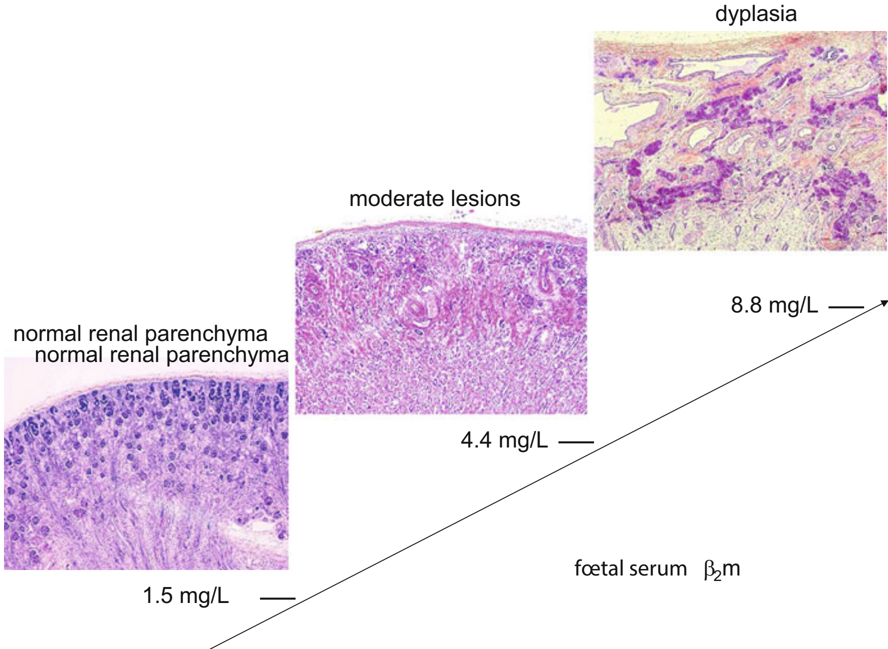

Fig. 6 Histological section of the fetal kidney in relation with β_2-m levels in LuTO (Luton et al. 2013 with the permission of J Guibourdenche). The fetal kidneys were removed just after termination of pregnancy. After formalin fixation, 5 μ medio-sagittal paraffin sections were stained with hematein-eosin-safran for a histological evaluation of the renal parenchyma, i.e., the blastema, the number of mature glomeruli, the mesangial glomerular fibrosis, the cortical tubules density, and primitive ducts medullar fibrosis

bilateral obstructive uropathy but no oligohydramnios who subsequently displayed normal postnatal renal function at age 1–2 years (i.e., serum creatinine <50 μmol/L) were used as controls. A drop in urinary β_2-m levels as from 20WG was correlated with a reduction in Na values. The normal β_2-m median value was 0.96 ± 1.2 mg/L and levels higher than 4–6 mg/L were predictive of tubular injury. Urinary β_2-m is both specific (0.83) and sensitive (0.80) in predicting elevated serum creatinine at the age of 2 years. Urinary glucose, calcium, phosphorus, ammonium, and total proteins were specific but lacked sensitivity in the context of obstructive uropathies (Muller et al. 1993). Urinary Na and β_2-m predicting poor prognosis are correlated with the severity of the renal injury but to a smaller extent compared to serum β_2-m (Daïkha-Dahmane et al. 1997; Luton et al. 2013) (Fig. 6). Fetal urinary β_2-m, but not Na, is correlated with postnatal clearance at ages 10–17 years, thus helping to predict the long-term outcome of bilateral uropathies. However, several limitations regarding the use of urine parameters are often discussed. First of all, cutoff values should be analyzed more accurately with respect to gestational age because urinary β_2-m levels decrease physiologically and are largely independent of renal function before

20–22 WG. Secondly, it is not always possible to puncture the calyces, and most samples are collected from the bladder. Unfortunately, urine from the bladder reflects the renal function of both kidneys. It does not allow to state on the unaffected kidney on which the prognosis is dependent in the event of urinary tract obstruction. In addition, the first urine void does not accurately reflect renal function, as the urine may have been present in the bladder for some time which could influence its composition. Sequential sampling has been proposed to improve diagnosis and prenatal management, such as vesicoamniotic shunting, but such procedures are invasive and do not significantly improve outcome (Biard et al. 2005)

Conclusion

Antenatal investigations to determine fetal renal function and predict postnatal renal outcome present different degrees of accuracy. Ultrasound findings and their evolution during pregnancy can be set against biochemical results. Biochemical analysis of amniotic fluid is of little value, and the benefits of performing an invasive procedure to collect fetal urine or blood are still a matter of debate. Its indication is limited to isolated cases of LuTO, bilateral hydronephrosis, and nephropathies. β_2-m is a low-molecular-weight protein that is entirely filtered by the glomeruli and reabsorbed and degraded by the renal tubules. High β_2-m levels in serum are not unequivocal but mainly reflect a reduction in the number of glomeruli or their impaired capacity for filtration. They are well correlated to the severity of renal parenchymal injury. Their increase in urine is due to impaired reabsorption but the accuracy of this assay is questionable because of the heterogeneity of samples and its variable correlation with parenchymal injury. The results obtained in fetuses suspected of being affected may overlap those of normal fetuses because of the difficulties encountered in establishing normal fetal ranges and defining a good postnatal renal outcome. This may be due to the fact that there is no linear relation between β_2-m levels and the number of nephrons that remain functional and maintain normal β_2-m levels. To date, β_2-m especially in fetal serum has been shown to be the most reliable biochemical marker for the prediction of postnatal impairment when fetal US follow-up is doubtful. However, because β_2-m does not constitute a gold standard, it is likely that new and more reliable biomarkers may emerge in the future (Taranta-Janusz et al. 2014).

Summary Points

- We aim to update the current knowledge on fetal β2-microglobulin in the human kidney and urinary tract diseases.
- Renal and urinary tract anomalies can be identified during a routine fetal ultrasound scan at 18–20 WG.

- Once diagnosed, the prognosis is dependent on monitoring the amniotic fluid volume and renal parenchyma.
- Biochemical markers partially reflect the renal parenchyma injury and can improved the prognosis of the renal function.
- Serum β_2-m levels rise when glomerular filtration is impaired, while urinary β_2-m levels increase when tubular reabsorption is affected.
- β_2-m determinations reflect renal function at the time of sampling.
- Its measurements in serum and urine can be useful in the context of lower urinary tract obstruction, bilateral hydronephrosis, and sometimes in nephropathies.
- Discrepancies remain because of the difficulty in defining a good postnatal outcome, the heterogeneity of the cases studied, the lack of prenatal reference values, the invasive collection procedures, and the assay to be used.
- However, by applying a cutoff value around 5 mg/L, β_2-m constitutes an accurate marker to predict postnatal renal function that is stable and easy to measure especially in fetal serum.

References

Avni FE, Hall M. Fetal nephropathies. Arch Pediatr. 2006;13:721–3.

Bacchetta J, Harambat J, Dubourg L, et al. Both extrauterine and intrauterine growth restriction impair renal function in children born very preterm. Kidney Int. 2009;76:445–52.

Berggård I, Bearn AG. Isolation and properties of a low molecular weight beta-2-globulin occurring in human biological fluids. J Biol Chem. 1968;243:4095–103.

Berry SM, Lecolier B, Smith RS, et al. Predictive value of serum β2-microglobulin for neonatal renal function. Lancet. 1995;345:1277–8.

Biard JM, Johnson MP, Carr MC, et al. Long-term outcomes in children treated by prenatal vesicoamniotic shunting for lower urinary tract obstruction. Obstet Gynecol. 2005;106:503–8.

Bökenhamp A, Dieterich C, Dressler F, et al. Fetal serum concentrations of cystatin C and β2-microglobulin as predictors of postnatal kidney. Am J Obstet Gynecol. 2001;1185:468–75.

Burghard R, Leititis JU, Brandis M. Studies on fetal renal function. Contr Nephrol. 1988;67:193–199.

Cejka J, Peterson Jr WE, Belamaric J, et al. In vitro synthesis of beta2-microglobulin by human fetal tissues. Dev Biol. 1975;43:200–3.

Changhoon Y, Dok Hyun Y, Cheolwon S. Serum beta-2 microglobulin in malignant lymphomas: an old but powerful prognostic factor. Blood Res. 2014;49:148–53.

Chiltra P, Bakthavatsalam B, Palvannan T. Beta-2 microglobulin as an immunological marker to assess the progression of human immunodeficiency virus infected patients on highly active antiretroviral therapy. Clin Chim Acta. 2011;412:1151–4.

Clark TJ, Martin WL, Divakaran TG, et al. Prenatal bladder drainage in the management of fetal lower urinary tract obstruction: a systematic review and meta-analysis. Obstet Gynecol. 2003;102:367–82.

Cobet G, Gummelt T, Bollmann R, et al. Assessment of serum levels of alpha-1-microglobulin, beta-2-microglobulin, and retinol binding protein in the fetal blood. A method for prenatal evaluation of renal function. Prenat Diagn. 1996;16:299–305.

Craparo FJ, Rustico M, Tassis B, et al. Fetal serum β2-microglobulin before and after bladder shunting: a 2-step approach to evaluate fetuses with lower urinary tract obstruction. J Urol. 2007;178:2576–9.

Cromblebome TM, Harrison MR, Golbus MS, et al. Fetal intervention in obstructive uropathy: prognostic indicators and efficacy of intervention. Am J Obstet Gynecol. 1990;162:1239–44.

Daïkha-Dahmane F, Dommergues M, Muller F, et al. Development of human fetal kidney in obstructive uropathy: correlations with ultrasonography and urine biochemistry. Kidney Int. 1997;52:21–32.
Davey PG, Gosling P. β2-microglobulin instability in pathological urine. Clin Chem. 1982;28:1330–3.
Dommergues M, Muller F, Ngo S, et al. Fetal serum β2-microglobulin predicts postnatal renal function in bilateral uropathies. Kidney Int. 2000;58:312–6.
Dressler GR. Advances in early kidney specification, development and patterning. Development. 2009;136:3863–74.
Fabbri E, Revello MG, Furione M, et al. Prognostic markers of symptomatic congenital human cytomegalovirus infection in fetal blood. Br J Obstet Gynecol. 2011;118:448–56.
Ferreira A, Garda Rodriguez MC, Omenaca F, Jimenez A, Villar LM, Gonzalez-Porque P, Fontan G. Soluble class I histocompatibility antigens (s-HLA) and beta 2-microglobulin at delivery. Clin Exp lmmunol. 1991;84:167–9.
Filler G, Priem F, Lepage N, Sinha P, Vollmer I, Clark H, Keely E, Matzinger M, Akbari A, Althaus H, Jung K. Beta-trace protein, cystatin C, beta(2)-microglobulin, and creatinine compared for detecting impaired glomerular filtration rates in children. Clin Chern. 2002;48:729–36.
Foxall PJ, Bewley S, Neild GH, et al. Analysis of fetal and neonatal urine using proton nuclear magnetic resonance spectroscopy. Arch Dis Child Fetal Neonatal. 1995;73:153–7.
Gorriz JL, Martinez-Castelao A. Proteinuria: detection and role in native renal disease progression. Transplant Rev. 2012;26:3–13.
Grey HM, Kubo RT, Colon SM, et al. The small subunit of HLA-antigens is b2-microglobulin. Exp Med J. 1973;138:1608–12.
Groves ML, Greenberg R. Complete amino acid sequence of bovine beta 2-microglobulin. J Biol Chem. 1982;257:2619–26.
Gulbis B, Jauniaux E, Jurkovic D, et al. Biochemical investigation of fetal renal maturation in early pregnancy. Ped Res. 1996;39:731–5.
Güssow D, Rein R, Ginjaar I, et al. The human b2-micoglobulin gene. J Immunol. 1987;139:3132–8.
Hill DM, Kasliwal T, Schwarz E, et al. A dominant negative mutant beta 2-microglobulin blocks the extracellular folding of a major histocompatibility complex class I heavy chain. J Biol Chem. 2003;278:5630–8.
Ikezumi Y, Honda M, Matsuyama T, et al. Establishment of a normal reference value for serum β2 microglobulin in Japanese children: reevaluation of its clinical usefulness. Clin Exp Nephrol. 2013;17:99–105.
Jauniaux E, Gulbis B, Hyett J, Nicolaides KH. Biochemical analyses of mesenchymal fluid in early pregnancy. Am J Obstet Gynecol. 1998;178:765–9.
Juraschek SP, Coresh J, Inker LA, et al. The effects of freeze-thaw on β-trace protein and β2-microglobulin assays after long-term sample storage. Clin Biochem. 2012;45:694–6.
Juraschek SP, Coresh J, Inker LA, et al. Comparison of serum concentrations of β-trace protein, β2-microglobulin, cystatin C, and creatinine in the US population. Clin J Am Soc Nephrol. 2013;8:584–92.
Karlsson FA, Goth T, Sege K, et al. Turnover in humans of beta 2-microglobulin: the constant chain HLA-antigens. Eur J Clin Invest. 1980;10:293–300.
Kim JY, Park SC, Lee JK, et al. Novel antibacterial activity of β−2microglobulin in human amniotic fluid. Plos One. 2012;7:e47642.
Lecarpentier E, Dreux S, Blanc T, Schaub B, Ville Y, Mandel brat L, El Ghoneimi A, Oury JF, Muller F. Biochemical analysis of cystic fluid in the diagnosis of fetal intra-abdominal masses. Prenatal diagnosis, 2012; 32:627–31
Lipitz S, Ryan G, Samuell C, Haeusler MCH, et al. Fetal urine analysis for the assessment of renal function in obstructive uropathy. Am J Obstet Gynecol. 1993;168:174–9.
Longpre M, Nguan A, MacNeily AE, et al. Prediction of the outcome of antenatally diagnosed hydronephrosis: a multivariable analysis. J Ped Urol. 2012;8:135–9.

Luton D, Delezoide AL, Leguy MC, et al. Foetal serum but not urinary β2-microglobulin correlates with histological injury to the kidney. Clin Biochem. 2013;46:1607–10.

Lutz CT, Cornell SH, Goeken JA. Establishment of a reference interval for b2-microglobulin in cerebrospinal fluid with the use of two commercial assays. Clin Chem. 1991;37:104–7.

Mahieu-Caputo D, Meulemans A, Martinovic J, et al. Paradoxic activation of the renin-angiotensin system in twin-twin transfusion syndrome: an explanation for cardiovascular disturbances in the recipient. Pediatr Res. 2005;58:685–8.

Morris RK. Congenital urinary tract obstruction. Best Pract Res Clin Obstet Gynecol. 2008;22:97–122.

Muller F, Dommergues M, Mandelbrot L, et al. Fetal urinary biochemistry predicts postnatal renal function in children with bilateral obstructive uropathies. Obstet Gynecol. 1993;82:813–20.

Muller F, Dommergues M, Bussieres L, et al. Development of human renal function: reference intervals for 10 biochemical markers in fetal urine. Clin Chem. 1996;42:1855–60.

Muller F, Dreux S, Audibert F, et al. Fetal serum b2-microglobulin and cystatin C in the prediction of post-natal renal function in bilateral hypoplasia and hyperechogenic enlarged kidneys. Prenat Diagn. 2004;24:327–32.

Nguyen C, Dreux S, Heider L, et al. Fetal serum α-1 microglobulin for renal function assessment: comparison with β2-microglobulin and cystatin C. Prenat Diagn. 2013;33:775–81.

Nicolaides KH, Cheng HH, Snidjers RJM, et al. Fetal urine biochemistry in the assessment of obstructive uropathy. Am J Obstet Gynecol. 1992;166:932–7.

Nicolini U, Spelzini F. Invasive assessment of fetal renal abnormalities: urinalysis, fetal blood sampling and biopsy. Prenat Diagn. 2001;21:964–9.

Nicolini U, Fisk NM, Beacham J, et al. Fetal urine biochemistry: an index of renal maturation and dysfunction. Bj J Obstet Gynecol. 1992;99:46–50.

Nolte S, Mueller B, Pringsheim WW. Serum alpha I – microglobulin and beta2-microglobulin for the estimation of fetal glomerular renal function. Pediatr Nephrol. 1991;5:573–7.

Nomura T, Huang WC, Zhau HE, et al. β2-Microglobulin-mediated signaling as a target for cancer therapy. Anticancer Agents Med Chem. 2014;14:343–52.

Oliveira FR, Barros EG, Magalhaes JA. Biochemical profile of amniotic fluid for the assessment of fetal and renal development. Braz J Med Biol Res. 2002;35:215–22.

Peterson PA, Evrin PE, Berggård I. Differentiation of glomerular, tubular, and normal proteinuria: determinations of urinary excretion of beta-2-macroglobulin, albumin, and total protein. J Clin Invest. 1969;48:1189–98.

Phadke V, Bhardwaj S, Sahoo B, et al. Maternal ingestion of diclofenac leading to renal failure in newborns. Pediatr Nephrol. 2012;27:1033–6.

Plazanet C, Arrondel C, Chavant F, et al. Fetal renin-angiotensin-system blockade syndrome: renal lesions. Pediatr Nephrol. 2014;29:1221–30.

Puolakka J, Ylöstalo P, Tuimala R, et al. Amniotic fluid beta-2-microglobulin in normal and complicated pregnancies. Correlation with gestational age, creatinine concentration and L/S ratio. Gynecol Obstet Invest. 1982;13:129–34.

Quigley R. Developmental changes in renal function. Curr Opin Pediatr. 2012;24:184–90.

Regeniter A, FreidanK H, Dickenman M, et al. Evaluation of proteinuria and GFR to diagnose and classify kidney disease: systematic review and proof of concept. Eur J Intern Med. 2009;20:556–61.

Reichel RR. Acute kidney injury: quoi de neuf? Ochsner J. 2014;14:359–68.

Rosano C, Zuccotti S, Bolognesi M. The three-dimensional structure of β2 microglobulin: results from X-ray crystallography. Biochem Biophys Acta. 2005;1753:85–91.

Rowley DR, Dang TD, McBride L, et al. Beta-2 microglobulin is mitogenic to PC-3 prostatic carcinoma cells and antagonistic to transforming growth factor beta 1 action. Cancer Res. 1995;55:781–6.

Schutz S, Le Moullec JM, Corvol P, et al. Early expression of all components of the renin-angiotensin-system in human development. Am J Pathol. 1996;149:2067–79.

Shackelford GD, Kees Folts D, Cole BR. Imaging the urinary tract. Clin Perinatol. 1992;19:85–119.

Spaggiari E, Heider L, Grange G, et al. Prognosis and outcome of pregnancies exposed to renin-angiotensin system blockers. Prenat Diagn. 2012;32:1071–6.
Spaggiari E, Dreux S, Czerkiewicz I, et al. Fetal obstructive uropathy complicated by urinary ascites: outcome and prognostic value of fetal serum -2microglobulin. Ultrasound Obstet Gynecol. 2013a;41:185–9.
Spaggiari E, Stirnemann JJ, Heidet L, et al. Outcome following prenatal diagnosis of severe bilateral renal hypoplasia. Prenat Diagn. 2013b;33:1167–72.
Taranta-Janusz K, Zalewska-Szajda B, Goscik E, et al. New tubular injury biomarkers in children with a solitary functioning kidney. Pediatr Nephrol. 2014;29:1599–605.
Tassis BM, Trespidi L, Tire IIi AS, Bocconi L, Zoppini C, Nicolini U. In fetuses with isolated hydronephrosis, urinary beta 2-microglobulin and N-acetyl-beta-D-glucosaminidase (NAG) have a limited role in the prediction of postnatal renal function. Prenat Diagn. 1996;16:1087–93.
Tassis BM, Trespidi L, Tirelli AS, et al. Serum b2-microglobulin in fetuses with urinary tract anomalies. Am J Obstet Gynecol. 1997;176:54–7.
Terrier N, Bonardet A, Descomps B, et al. Determination of beta-2-microglobulin in biological samples using immunochemiluminescence or immunoturbidimetry: comparison with radio-immunoassay. Immunol Anal Biol Spe. 2004;19:219–24.
Trinh CH, Smith DP, Kalverda AP, et al. Crystal structure of monomeric human beta-2-microglobulin reveals clues to its amyloidogenic properties. Proc Natl Acad Sci U S A. 2002;99:9771–6.
Vanderheyden T, Kumar S, Fisk NM. Fetal renal impairment. Semin Neonatol. 2003;8:279–89.
Vincent C, Dennoroy L, Revillard JP. Molecular variants of β2-microglobulin in renal insufficiency. Biochem J. 1994;298:181–7.
Vraetz T, Ittel TH, Van Mackelenbergh MG, et al. Regulation of β2-microglobulin expression in different human cell lines by proinflammatory cytokines. Nephrol Dial Transplant. 1999;14:2137–43.
Winyard P, Chitty LS. Dysplastic kidneys. Semin Fetal Neonatal Med. 2008;13:142–51.
Woolf AS, Winyard PJ. Molecular mechanisms of human embryogenesis: developmental pathogenesis of renal tract malformations. Pediatr Dev Pathol. 2002;5:108–29.
Yamamoto M, Essaoui M, Nasr B, et al. Three-dimensional sonographic assessment of fetal urine production before and after laser surgery in twin-to-twin transfusion syndrome. Ultrasound Obstet Gynecol. 2007;30:972–6.
Yokoyama WM. Natural killer cell receptors. Curr Opin Immunol. 1998;10:298–305.
Zhao L, Purandare B, Zhang J, et al. β2-microglobulin-free HLA-G activates natural killer cells by increasing cytotoxicity and proinflammatory cytokine production. Hum Immunol. 2013;74:417–24.

Proteinuric Biomarkers in Chronic Kidney Disease

23

Claudio Bazzi and Omran Bakoush

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Abstract

Risk stratification of patients with chronic kidney disease is crucial for early identification of patients at risk of disease progression and for timely initiation of treatments to prevent progression and the associated cardiovascular morbidity and mortality. Proteinuria and its components have been widely used for diagnosis and staging system of chronic kidney diseases. Albuminuria is used for diagnosis of kidney disease in the general population and kidney involvement in systemic diseases such as diabetes. However, albuminuria is not the most powerful predictor of disease outcome, and the urinary excretion of proteins larger than albumin such as IgM and IgG shows a higher predictive value: IgM-uria could be helpful for kidney and cardiovascular risk assessment of diabetic patients, and IgG-uria could be helpful for risk assessment of glomerulonephritis patients. This chapter presents a concise review of the clinical value of proteinuria profile for prediction of functional outcome and responsiveness to treatments in patients with chronic kidney disease.

Keywords

Proteinuric patterns • CKD • Diabetic nephropathy • Glomerulonephritis • Progression • Remission • Treatment responsiveness

Abbreviations

ACEi	Angiotensin-converting enzyme inhibitors
α2m/C	α2-macroglobulin/urinary creatinine ratio
Albumin/C	Urinary albumin/urinary creatinine ratio
ANCA	Antineutrophil cytoplasmic antibody
Anti-PLA2R	Anti-phospholipase A2 receptor antibody
α1m	α1-microglobulin
β2m	β2-microglobulin
β-NAG	N-acetyl-β-D-glucosaminidase
CKD	Chronic kidney disease
CSA	Cyclosporine A
CYC	Cyclophosphamide
CV	Cardiovascular
DM	Diabetes mellitus
DKD	Diabetic kidney disease
eGFR	Estimated glomerular filtration rate
ESKD	End-stage kidney disease
FE	Fractional excretion
FE α1m	Fractional excretion of α1-microglobulin
FE IgG	Fractional excretion of immunoglobulin G
FSGS	Focal segmental glomerulosclerosis
GBM	Glomerular basement membrane
GCW	Glomerular capillary wall
GFB	Glomerular filtration barrier
GGS	Global glomerular sclerosis

GN	Glomerulonephritis
HR	Hazard ratio
hsCRP	High-sensitive C-reactive protein
IgA	Immunoglobulin A
IgAN	IgA nephropathy
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMN	Idiopathic membranous nephropathy
MCD	Minimal change disease
MPGN	Membranoproliferative glomerulonephritis
MW	Molecular weight
NS	Nephrotic syndrome
PTECs	Proximal tubular epithelial cells
RAS	Renin-angiotensin system
ROC analysis	Receiver-operating characteristic analysis
RTX	Rituximab
sCr	Serum creatinine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SG	Surviving glomeruli: glomeruli without global sclerosis
SI	IgG/transferrin selectivity index
TID	Tubulointerstitial damage
TGF-β	Transforming growth factor β

Key Facts of Proteinuria in CKD

- Proteinuria is the presence in urine of amount of proteins exceeding the normal value (150 mg/24 h).
- Methods of measurement of proteinuria:
  - Total proteinuria measured in 24-h urine collection
  - Protein/creatinine ratio: ratio between total or single proteins and urinary creatinine expressed as g or mmol in morning urine sample
  - Fractional excretion of single proteins: clearance of protein related to creatinine clearance
- Determinants of proteinuria in CKD: increased loss of proteins across damaged glomerular wall (glomerular filtration barrier, GFB) and reduced reabsorption by proximal tubular epithelial cells (PTECs).
- Proteinuric pattern: the presence in urine of proteins with different molecular weight (MW) may allow a classification of proteinuria: glomerular (only middle and high MW proteins); tubular, low MW proteins and trace amount of albumin; and mixed, the presence of high, middle, and low MW proteins.
- Clinical significance of proteinuria: proteinuria is a marker of disease severity (defined nephrotic when it exceeds 3.5 g/24 h); persistent proteinuria is itself responsible of further renal damage at tubulointerstitial and glomerular level triggering a vicious circle of nephron loss and worsening renal function.

- Risk stratification by amount and type of proteinuria: in each CKD the variable amount and type of proteinuria between patients allow a risk stratification identifying patients with high probability of progression to renal failure and patients with low probability of progression.
- Responsiveness to treatments: the ability of risk stratification to predict renal outcome and in some CKD to identify responsiveness to treatments may be useful to guide decisions on the start and type of treatment.

Introduction

Patients with chronic kidney disease (CKD) frequently progress to end-stage kidney disease (ESKD) or succumb to cardiovascular disease. This is a costly worldwide public health problem (Jha et al. 2012). In the USA and Europe, about 10 % of adults suffer from CKD, mostly due to diabetes mellitus (DM), hypertension, and glomerulonephritis (Levey et al. 2009; Eckardt et al. 2013). In clinical practice, proteinuria is not only a biomarker for the diagnosis and severity of CKD but is also associated with increased risk of progression to ESKD, ischemic heart disease, heart failure, cerebrovascular insults, and cardiovascular death (Liu et al. 2003; Jafar et al. 2009). This association exists even after adjustment for other traditional atherosclerotic risk factors (diabetes, hypertension, dyslipidemia, and smoking) and nontraditional risk factors, such as elevated plasma levels of C-reactive protein (Arnlov et al. 2005; Stehouwer and Smulders 2006).

Determinants of Proteinuria

The glomerular filtration barrier (GFB) normally does not allow the passage of high molecular weight (MW) proteins such as IgG (150 kDa), α2-macroglobulin (720 kDa), and IgM (900 kDa), while the low MW proteins [α1-microglobulin (α1m, 31.8 kDa), β2-microglobulin (β2m, 11.8 kDa), and retinol-binding protein (20 kDa)] are freely filtered and almost completely reabsorbed by proximal tubular epithelial cells (PTECs). The middle MW protein albumin (67 kDa) passes through GFB in small amounts (2−6 g/day) and is also almost completely reabsorbed by PTECs. The main known mediators of protein reabsorption by PTECs are megalin (Srorm et al. 2013), cubilin (Amsellem et al. 2010), amnionless, neonatal Fc receptor (FcRn) (Rath et al. 2013), and CD36 (Baines et al. 2012).

The pathologically damaged GFB allows the passage of increased amounts of middle and high MW proteins according to the type and severity of damage (D'Amico and Bazzi 2003a). Maintenance of GFB integrity depends on structural and functional interactions among its three components: the fenestrated endothelium and its cell surface layer, the glomerular basement membrane (GBM), and the visceral epithelial cell layer (podocytes) (Haraldsson et al. 2008; Lowik et al. 2009; Toblli et al. 2012; Menon et al. 2012). The endothelial cell layer is characterized by fenestrae of 50–100 nm in size covered by negatively charged

proteoglycans (glycocalyx) that contribute to size and charge perm-selectivity and restrict the passage of the negatively charged albumin. The GBM consists of type IV collagen and laminin along with sulfated proteoglycans (Miner 2011). Podocytes and their slit diaphragms contribute to the size selectivity of the barrier. The molecular structure of podocytes has been elucidated mainly in familiar and experimental forms of focal segmental glomerulosclerosis (Gbadegesin et al. 2011). Several molecules are responsible for maintenance of podocyte integrity: nephrin, podocin, alpha-actinin 4, CD2-associated protein, transient receptor potential cation channel type 6, Wilms' tumor 1, phospholipase C epsilon-1, and lamininβ2. Alteration of the GFB is characterized by the opening of nonselective channels (pores) that allow high and middle MW proteins to escape into the urinary space.

The molecular machinery for reabsorption cannot handle the elevated tubular load of proteins passing through the damaged GFB. This leads to increased urinary excretion of albumin and high MW proteins. Also, the urinary excretion of LMW proteins is increased (tubular proteinuria) due to the competition for tubular reabsorption between high, middle, and low MW proteins and due to the functional and structural alteration of PTECs (Baines and Brunskill 2011). The proteinuria and the functional/structural alterations in PTECs are also responsible for the increased urinary excretion of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (β-NAG), a reliable marker of tubular damage (Bazzi et al. 2002; D'Amico and Bazzi 2003b). Thus, the quantity and composition of proteinuria in final urine depend on a complex interplay between filtration and reabsorption.

Kidney Damage Consequent to Proteinuria

Proteinuria is strongly associated with the risk of progression to ESKD not only as marker of the degree of structural alterations of GFB and PTECs but also because proteinuria itself is responsible of further renal damage at different levels.

Tubulointerstitial Compartment Damage

Regardless of the underlying cause, various types of proteinuric CKD may share a common pathogenetic mechanism of kidney disease progression. Brenner and coworkers, using the "remnant kidney" model, proposed a hypothesis for the mechanism of kidney disease progression. Brenner hypothesized that nephron loss increases glomerular hydraulic pressure and stretches the glomerular capillary wall (GCW), impairing its selectivity and increasing the protein content of the glomerular filtrate (Olson et al. 1982). PTECs persistently exposed to urinary proteins increase their expression of chemokines, cytokines, growth factors, and vasoactive molecules and activate complement, which together induce the infiltration of interstitial inflammatory cells (Ruggenenti 2012a; Cravedi and Remuzzi 2013) (Fig. 1). This leads to renal scarring and initiation of a vicious circle of further nephron loss and worsening renal function. Several studies showed a strong correlation between some proteinuria

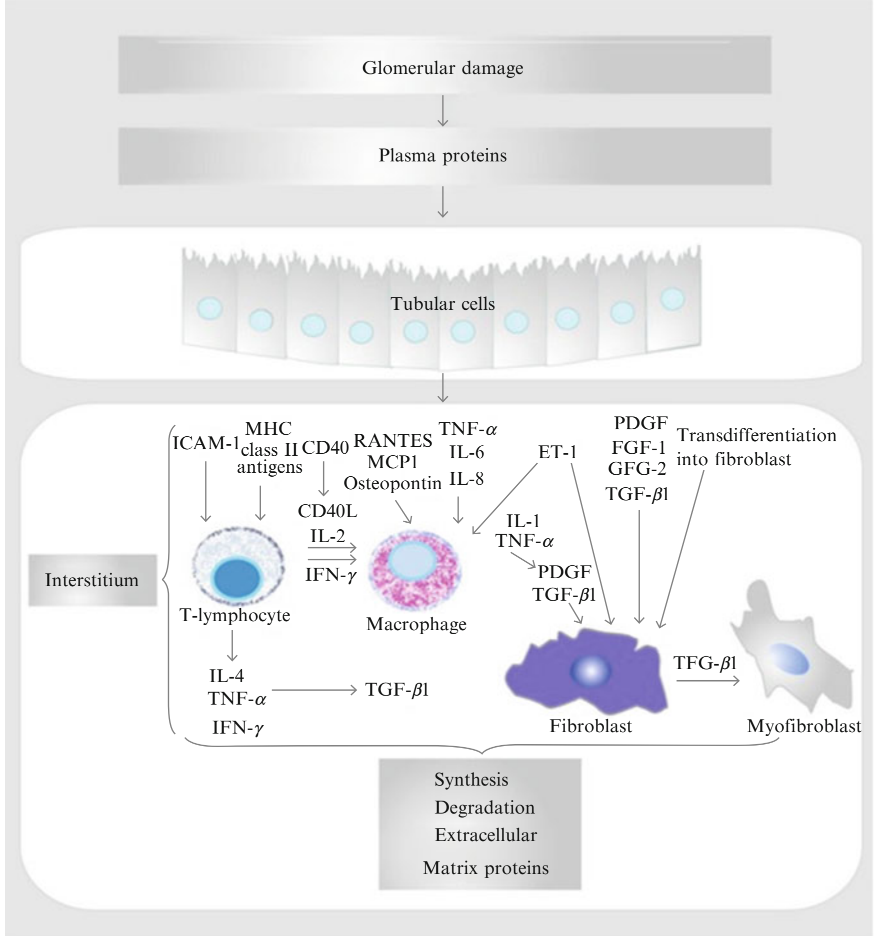

Fig. 1 Molecular and cellular features associated with tubulointerstitial compartment damage (From Jorge Toblli et al., Int J Nephrol, 2012 with permission. Courtesy of Dr. Jorge Toblli)

components and the extent of tubulointerstitial damage (TID) evaluated histologically or by proteinuric markers of TID. It has been demonstrated that the extent of TID depends not only on the amount but also on the type of proteinuria, as it is significantly associated with increased excretion of high MW proteins such as IgG (Bazzi et al. 2000). Lowering capillary pressure by RAS inhibition reduces proteinuria and the decline in kidney function, as has been demonstrated in several experimental and clinical studies in diabetic and nondiabetic patients (van der Meer et al. 2010). Another important determinant of TID is interstitial hypoxia caused by reduction of blood flow in the interstitial compartment due to vasoconstriction of efferent arterioles due to local activation of the renin-angiotensin system, increased synthesis of endothelin, and reduced synthesis of nitric oxide. Global

glomerular sclerosis and interstitial fibrosis reduce the number of peritubular capillaries and increase the distance between capillaries and tubular cells further compromising the reabsorption of proteins by PTECs (Nangaku 2004, 2006; Nangaku et al. 2007).

Glomerular Damage

Proteinuria is associated not only with TID but also with glomerular damage. Excessive protein load on podocytes induces overexpression of TGF-β (Loeffler and Wolf 2014; Ghayur and Margetts 2013), which may lead to GBM thickening, podocyte apoptosis and detachment, differentiation of mesangial cells into myofibroblasts, increased extracellular matrix production, and dysregulation of the balance between extracellular matrix deposition and breakdown with consequent development of glomerulosclerosis.

Thus the amount and persistence of proteinuria is one of the main factors responsible for progressive kidney damage and worsening renal function. In the last two decades, several studies evaluated the value of urinary excretion of high and low molecular weight (MW) proteins for predicting disease outcome, responsiveness to treatment, and performing risk stratification aimed at improving clinical practice.

Measurement of Proteinuria and Risk Stratification

Critical to reducing the kidney disease burden of ESKD is early diagnosis and timely initiation of treatments to prevent disease progression (Levey et al. 2009). Dipstick is a semiquantitative method used mainly for preliminary diagnostic assessment of suspected kidney disease (White et al. 2011; Samal and Linder 2013). Twenty-four-hour proteinuria had been widely measured in the past, but is now rarely used because urine collection is cumbersome and frequently incorrect. The protein/creatinine ratio in the first or second morning urine sample, which is highly correlated with 24-h proteinuria (Ruggenenti et al. 1998), is at present the most frequently used method for measurement of proteinuria (Mc Taggart et al. 2012). Fractional excretion (FE) of single proteins in relation to creatinine clearance is a better proteinuric marker combining two predictors of kidney function decline: increased protein excretion and GFR, a marker of functioning nephron mass (Park and Kim 2011).

Depending on the type of kidney disease, different agents are used to prevent kidney disease progression. They are mainly immunosuppressive (IS) agents for glomerulonephritis (GN) patients and RAS inhibitors for diabetic and GN patients. Unfortunately, the response to treatment is heterogeneous in CKD patients for both unknown and known reasons, including incomplete knowledge of the etiology and pathogenetic mechanisms of lesions and the different clinical features at presentation. Thus, it is of paramount importance in the management of CKD to identify the risk factors that can accurately predict the functional outcome (Halbesma et al. 2011; Tangri et al. 2013) and can be used for risk

stratification in order to assess drug responsiveness and develop more targeted treatment. Risk stratification could also be useful for the planning of controlled trials to avoid the confounding effect of including different percentages of low- and high-risk patients in the trial arms. Knowledge of the pathogenetic mechanisms of kidney damage has greatly increased in the last decades. Genetic and molecular studies have elucidated several damage mechanisms at the glomerular, tubular, interstitial, and vascular levels, but assessment of these markers is often limited to research laboratories and is frequently rather expensive and not available in the clinical practice. Conversely, laboratory devices for measuring the components of proteinuria, such as nephelometry and turbidimetry, are available in most hospital laboratories.

Here, we review the potential use of different urine proteins, such as albuminuria, IgG-uria, and IgM-uria, for diagnosis, risk stratification, and prediction of disease outcome in CKD patients.

Albuminuria

Measurement of urinary albumin excretion in the general population can identify patients at risk of development of kidney failure (Astor et al. 2011). A low eGFR and albuminuria are associated with adverse kidney outcomes (Gansevoort et al. 2011). Values of eGFR <60 mL/min/1.73 m^2 and albumin/creatinine ratio ≥ 10 mg/g could predict a high risk for cardiovascular (CV) mortality in the general population (Matsushita et al. 2010). Mild albuminuria may progress to heavier albuminuria, so its follow-up is indicated. Although albuminuria is important for early diagnosis of kidney diseases, not all glomerulonephritis patients with albuminuria progress to kidney failure, and not all diabetic patients with kidney disease develop albuminuria (Cohen-Bucay and Viswanathan 2012). Up to 30 % of diabetic patients with diabetic kidney disease (DKD) have normal urine albumin excretion despite the decline in kidney function and progressive vascular endothelial damage (Marshall 2014). Many recent longitudinal epidemiological studies of patients with diabetes and glomerulonephritis have pointed out the inferiority of albuminuria compared to other high MW proteins such as IgG and IgM for prediction of kidney disease outcome (Bakoush et al. 2001a, b, 2003, 2006).

Diabetic Kidney Disease (DKD)

The pathophysiological mechanisms of DKD could differ between type 1 and type 2 diabetes (Fioretto et al. 2008; Bakoush et al. 2002; Steinke and Maurer 2008). In type 1 diabetes, hyperglycemia could be the main mechanism for DKD, whereas in type 2 diabetes, the metabolic syndrome (obesity, hypertension, and dyslipidemia) and hyperglycemia are major contributors to DKD (Altemtam et al. 2012) (Fig. 2). In DKD, urinary excretion of albumin is used to differentiate normo-albuminuria (<30 mg/g uC) from microalbuminuria (≥ 30 and <300 mg/g uC) and

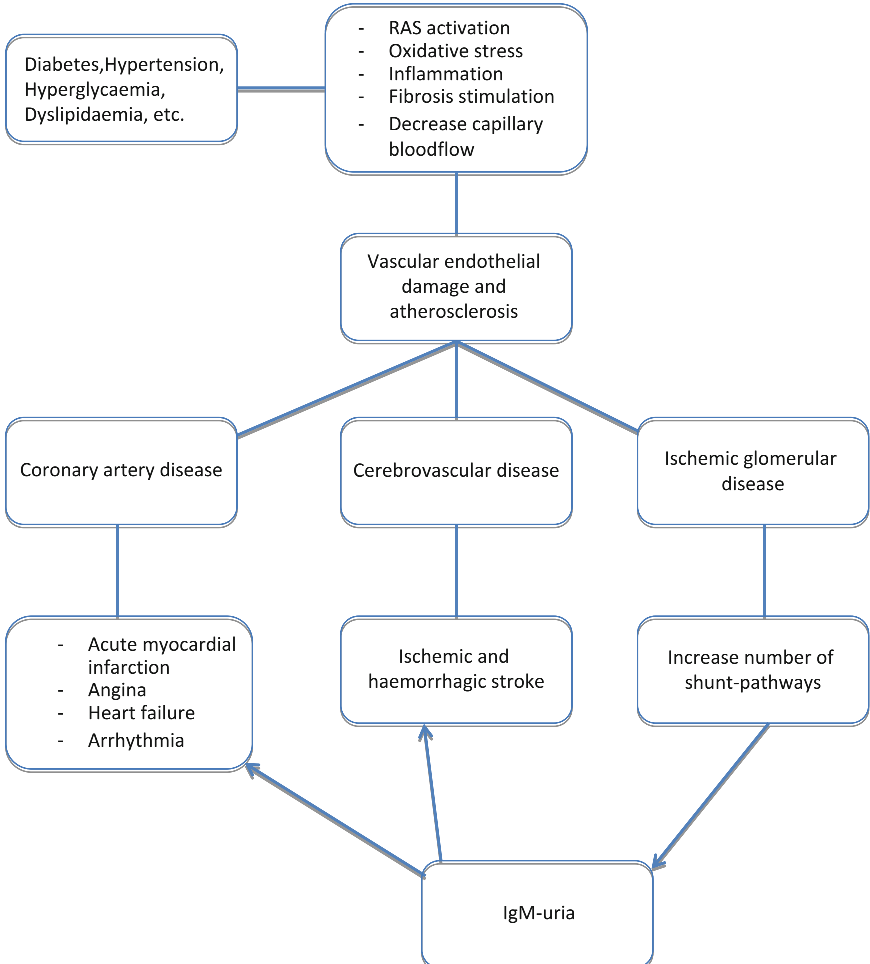

Fig. 2 A proposed mechanism for association of IgM-uria with cardiovascular disease

macroalbuminuria (≥300 mg/g uC) (Cohen-Bucay and Viswanathan 2012). In recent years it has been found that high values of normo-albuminuria are also associated with DKD progression and cardiovascular morbidity and mortality. Consequently, it has been suggested that the category of microalbuminuria should be eliminated (Ruggenenti and Remuzzi 2006). However, irrespective of the level of albuminuria, increased urine IgM excretion in patients with type 1 or type 2 diabetes was associated with significantly lower kidney and patient survival. The presence of IgM-uria increased the risk of kidney and cardiovascular death threefolds (Tofik et al. 2009) in patients with micro- or macroalbuminuria (Fig. 2). IgM-uria was found to be associated with a risk of the occurrence of subsequent cardiovascular events in patients with coronary artery disease with acute chest pain (Tofik et al. 2013). Markers of

general inflammation, such as pro-inflammatory cytokines and high-sensitive C-reactive protein (hsCRP), are also associated with cardiovascular disease (Kaptoge et al. 2010; Tedgui and Mallat 2006). However, recent studies on large cohorts raised doubts about the clinical value of these markers in the estimation of cardiovascular risk in nondiabetic and diabetic patients (Schottker et al. 2013; Kaptoge et al. 2012).

Although the strength of the association between IgM-uria and the degree of atherosclerosis should be studied, the presence of IgM-uria might reflect severe size-selectivity dysfunction of the GFB, secondary to generalized atherosclerosis and systemic inflammation. Atherosclerosis is associated with elevated glomerular vascular wall resistance, resulting in glomerular ischemia and a markedly increased population of highly unselective glomerular shunt pathways (Bakoush et al. 2004, 2006; Rippe et al. 2006).

Glomerulonephritis

More than 50 years after the introduction of kidney biopsy and identification of several types of primary and secondary GN, the etiology and pathogenesis of GN are still largely unknown. There is no standard first-line treatment that is effective in all patients of each type of GN. Several clinical, functional, laboratory, and histologic features have been evaluated to assess disease severity and predict functional outcome and responsiveness to treatment.

Studies of Patient Cohorts Including Different Types of GN

In 89 patients with nephrotic syndrome [9 minimal change disease (MCD), 29 primary focal segmental glomerulosclerosis (FSGS), 51 idiopathic membranous nephropathy (IMN)] (Bazzi et al. 2000), the outcome was predicted by a combination of IgG/transferrin selectivity index and the TID marker fractional excretion of α1-microglobulin (FE α1m). In 45 patients with selectivity index >0.10 and FE α1m above a cutoff value, the remission rate was 6 %, and progression rate was 69 %. In the 30 patients who were treated with steroids alone or in combination with cyclophosphamide (CYC), the remission rate was 75 % in low-risk patients, and 10 % in high-risk patients. In a cohort of 97 patients with various types of GN but without nephrotic syndrome (NS) (Mc Quarrie et al. 2011), fractional excretion of IgG and albumin was a good predictor of kidney disease progression and was more accurate than several other proteinuric markers. In a cohort of 189 patients with various types of GN (Tofik et al. 2011), urinary IgG/creatinine ratio of more than 5 mg/mmol was associated with the highest rate of kidney disease progression to ESKD.

Idiopathic Membranous Nephropathy (IMN)

In a study of 57 patients with IMN and NS (Branten et al. 2005), renal survival varied significantly with urinary β2m (< vs ≥0.5 μg/min) and urinary IgG (< vs ≥250 mg/24 h). The authors concluded that excretion of β2m and IgG accurately predicts renal

Table 1 Predictive value of progression and remission by fractional excretion of IgG alone (IMN, FSGS, MPGN) or divided by percentage of glomeruli without global sclerosis (*SG* surviving glomeruli) (IgAN and crescentic IgAN)

Diagnosis	Reference	No. of patients	Proteinuric biomarkers	Progression	Remission
IMN	Bazzi et al. 2014	84	FE IgG <0.020 vs. ≥0.020	3 % vs. 43 % P = 0.003	90 % vs. 24 % P < 0.001
		Untreated n. 35 vs. treated with ST + CYC n. 37	FE IgG <0.020	12 % vs. 0 % p: ns	94 % vs. 85 % p: ns
			FE IgG ≥0.020	72 % vs. 43 % P = 0.014	0 % vs. 36 % P = 0.025
FSGS	Bazzi et al. 2013	38	FE IgG <0.112 vs. ≥0.112	0 % vs. 75 % P < 0.0001	77 % vs. 25 % P = 0.016
			FE IgG <0.112 and α2m/C <4.79 vs. FE IgG ≥0.112 and α2m/C ≥ 4.79	0 % vs. 89 % P < 0.0001	83 % vs. 11 % P = 0.008
IgAN	Bazzi et al. 2012	68 ACEi −	FE IgG/SG < vs. ≥0.00010	2 % vs. 87 % P < 0.0001	
		64 ACEi +	FE IgG/SG < vs. ≥ 0.00010	2 % vs. 36 % P < 0.0001	
Crescentic IgAN	Bazzi et al. 2009	34	FE IgG/SG < vs. ≥0.00034	0 % vs. 89 % P < 0.0001	
		23 treated steroids + cycloph treated	FE IgG/SG <0.00034 + sCr vs. <1.74 mg/dL vs. FE IgG/SG ≥ 0.00034 + sCr ≥ 1.74	0 % vs. 100 % P < 0.0001	
MPGN	Bazzi et al. 2002	18	FE IgG < vs. ≥0.180	9 % vs. 86 % P = 0.002	

outcome and can be used to guide decisions on the start of immunosuppressive treatment. In a subsequent study on 129 patients with IMN and NS, the same group (van der Brand et al. 2011) confirmed the predictive value of the progression of urinary excretion of the tubular markers α1-microglobulin and β2-microglobulin. In IMN with NS, the efficacy of various IS agents remains controversial, as evidenced in the last meta-analysis published in 2013 (Chen et al. 2013). In a study of 86 patients with IMN and NS (Bazzi et al. 2014), the proteinuric marker with the highest sensitivity and specificity for progression was FE IgG (cutoff, 0.020). Kidney survival and remission rate were significantly higher in low- (FE IgG <0.020) versus high-risk patients (FE IgG ≥0.020) (Table 1, Fig. 3). Comparison of 35 untreated patients with 37 patients treated with steroids and cyclophosphamide (CYC)

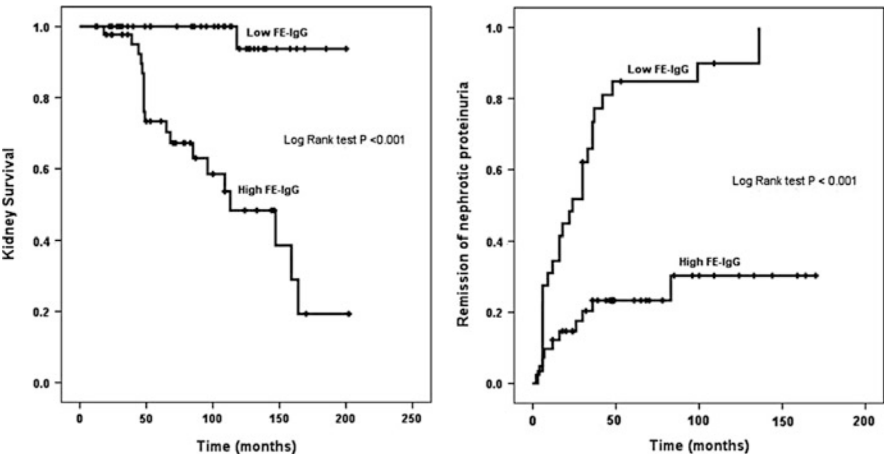

Fig. 3 Kidney survival and remission rate in 84 patients with IMN and NS according to FE IgG < vs ≥0.020

according to a published protocol (Ponticelli et al. 1998) showed that in untreated low-risk patients, progression is very low (12 %), and spontaneous remission is very high (94 %). In high-risk patients, treatment reduced progression from 72 % to 43 % and increased the remission rate from 0 % to 36 % (Table 1). Rituximab (RTX), a new and promising immunosuppressive agent for treatment of patients with IMN with NS, induced complete or partial remission in 65 of 100 treated patients: in 32 patients RTX was used as rescue treatment because of previous unresponsivity to various immunosuppressive or anti-proteinuric agents (Ruggenenti et al. 2012b). In a study of 20 patients with IMN and NS (Irazabal et al. 2013), fractional excretion of IgG and α1m was associated with the response to rituximab at 12 months, but not at 24 months (overall remission rate was 89 % at 24 months). In these patients, a decrease in anti-PLA2R antibodies preceded the decline in proteinuria, but baseline anti-PLA2R levels did not correlate with the response to therapy. Thus, in IMN patients, FE IgG may be used to guide the treatment: conservative treatment is recommended for low-risk patients, unless severe NS is present with risk of complications. Immunosuppressive agents or rituximab therapy of high-risk patients should be started soon after diagnosis to avoid the inefficacy of late treatment, when kidney disease damage may become irreversible.

Primary Focal Segmental Glomerulosclerosis (FSGS)

Very few studies evaluated the predictive value of proteinuric patterns in primary focal segmental glomerulosclerosis (Bazzi et al. 2003; Deegens and Wetzels 2007). In the largest and more recent study on 38 patients with primary FSGS and NS (Bazzi et al. 2013), FE IgG and α2-macroglobulin/creatinine ratio showed the

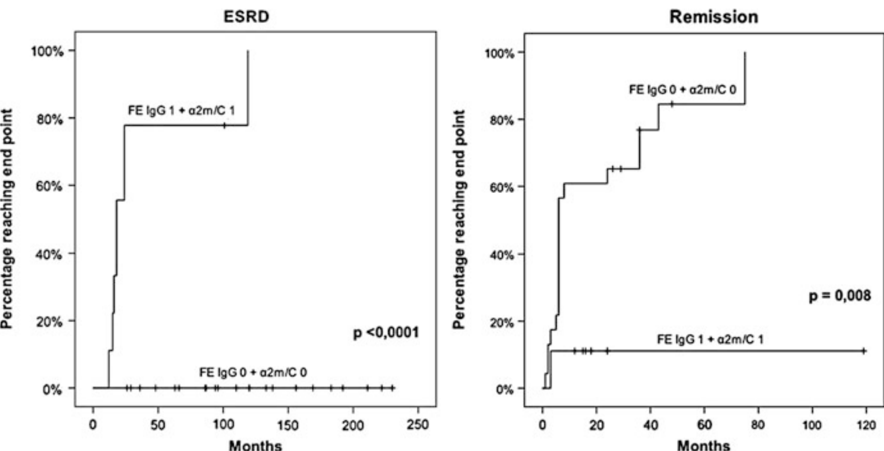

Fig 4 Probability of ESRD and remission in 38 patients with FSGS and NS according to FE IgG and α2-macroglobulin/Cr < vs ≥ respective cutoffs

highest sensitivity and specificity for progression to ESKD or remission (Table 1, Fig. 4). In the low-risk group with a combination of FE IgG <0.112 and α2m/C <4.79, 89 % of the patients had remission as first event and 78% of them had sustained remission after 104 ± 54 months. In 22 % of these cases, remission was obtained with steroids alone and in 61 % with steroids in combination with CYC; 17 % were unresponsive to steroids and CYC and remitted after treatment with other agents (mycophenolate mofetil, pentoxifylline, cyclosporine A). In the high-risk group (FE IgG ≥0.112 and α2m/C ≥4.79), 89 % of the patients progressed to ESKD after 32 ± 35 months; one patient was treated with steroids alone and eight were treated with steroids and CYC. Other markers (urinary protein/creatinine ratio, eGFR, FE α1m, segmental sclerosis, global sclerosis, and TID score) did not predict remission and had a lower predictive value of ESKD. The best predictor for ESKD was α2m/creatinine ratio and for disease remission it was FE IgG. The disease outcome predicted by these proteinuric markers is more accurate than that of the old long-term predictor of favorable outcome that was steroid-induced remission (about 30 %). These proteinuric biomarkers could be useful for individualizing treatment of patients with FSGS with NS.

IgA Nephropathy (IgAN)

In the last two decades, several laboratory methods for evaluating different features of proteinuria have been tested for their ability to predict outcome in IgAN (SDS-PAGE pattern, selectivity index, and proteomic analysis) (Woo et al. 1991, 1997; Haubitz et al. 2005). In a recent study of 132 patients with non-crescentic IgA nephropathy (Bazzi et al. 2012), global glomerular sclerosis (GGS) varied from 0 % to more than 50 %. To assess glomerular loss and tubular load of proteins more precisely, the

proteinuric markers were divided by the percentage of glomeruli without GGS, conventionally referred to as "surviving glomeruli" (SG). The fractional excretion of IgG and α1m and 24-h proteinuria were, respectively, 40-, 13-, and ninefold higher in patients with <50 % of SG than in those with 100 % of SG. These results validate the experimentally observed association between nephron loss and increased proteinuria. FE IgG/SG proved to be the best predictor of kidney disease progression to kidney failure (Table 1). In patients with FE IgG/SG above the cutoff, treatment with ACE inhibitors reduced kidney disease progression from 87.5 % to 36 %. In patients with FE IgG/SG below the cutoff, the increase in eGFR over time was significantly higher in ACEi-treated patients than in untreated patients.

Crescentic IgA Nephropathy

In a study of 37 patients with crescentic IgA nephropathy (Bazzi et al. 2009) (cellular crescents in 19 ± 15 % of glomeruli), fractional excretion of IgG was strongly correlated with baseline sCr ($r = 0.749$), percentage of global glomerular sclerosis (GGS) ($r = 0.536$), cellular crescents ($r = 0.414$), and TID score ($r = 0.725$). The values of proteinuric markers were divided by the percentage of glomeruli without GGS, conventionally defined as "surviving glomeruli" (SG). In 34 patients FE IgG/SG below or above a cutoff level assessed by ROC analysis was the most powerful predictor of kidney disease progression to kidney failure: 5 % vs. 83 %. In 23 patients treated with steroids and CYC soon after kidney biopsy, FE IgG/SG in combination with serum creatinine, both below and above their respective cutoffs, predicted kidney disease progression in 0 % versus 100 % of patients (Table 1). These data suggest that high-risk patients should be treated with alternative agents.

Membranoproliferative Glomerulonephritis (MPGN)

Only one study evaluated the predictive value of proteinuric markers in MPGN. Bazzi et al. (Abstract, J Am Soc Nephrol 2002; 13: SA-P905; Int. Congress Am. Soc. Nephrol. 2002) studied a cohort of 30 patients with membranoproliferative glomerulonephritis (type I, $n = 23$, type II $n = 1$, type III $n = 4$, fibrillary type $n = 2$). They reported that FE IgG <0.180 predicted progression in 9 % of 18 patients with NS, whereas FE IgG ≥0.180 predicted progression in 86 % of them.

ANCA-Associated Vasculitis

In 83 patients with ANCA-associated renal vasculitis (Bakoush et al. 2006), IgM excretion was a strong predictor of ESKD, and in addition to age, it predicted the risk of cardiovascular death. IgM-uria reflects a markedly increased population of shunts and large defects in the GFB and thereby is a good indicator of the severity of glomerular damage. Serum creatinine is a late kidney biomarker and is associated

with cardiovascular mortality only in cases with severe kidney impairment. In that study, IgM-uria was able to predict the risk in patients with relatively good kidney function and thus was a better predictor of disease outcome.

Summary

In CKD patients, the first physiopathological alteration is the dysfunction of the GFB, which allows passage of proteins normally absent in urine. Later, proteinuria itself causes further kidney damage in the glomerular and tubulointerstitial compartments. The characteristics of the overall proteinuric pattern thus depend on the interplay of glomerular and tubulointerstitial damage. The urinary proteins more frequently evaluated for prediction of kidney disease progression are albumin, IgG, IgM, α2-macroglobulin, and low MW proteins. Albuminuria in combination with low eGFR predicts the risk of adverse kidney outcomes, cardiovascular diseases, and death. In diabetes, IgM-uria is associated with progression of DKD and risk of cardiovascular events. In nine studies collectively covering 674 patients with glomerulonephritis (GN), IgG-uria (expressed as IgG/creatinine ratio or more frequently as fractional excretion) seems to be the best predictor of disease outcome. In non-crescentic IgA nephropathy, FE IgG predicts responsiveness to ACE inhibitors. In IMN, treatment of FE IgG high-risk patients with steroids and CYC reduced the progression rate from 72 % to 43 % and increased the remission rate from 0 % to 36 %. In primary FSGS, 89 % of FE IgG and α2m/creatinine ratio high-risk patients were unresponsive to treatment with steroids and CYC, and 78 % of low-risk patients had sustained remission. In crescentic IgA nephropathy, none of the patients assessed as high risk according to FE IgG and sCr responded to steroids and CYC. So, in glomerulonephritis the urinary excretion of IgG is not only a strong predictor of functional outcome but also a valuable predictor of responsiveness to some treatments. We recommend evaluation of proteinuric patterns at diagnosis of kidney disease because of its low cost and its potential influence on outcome through early identification and management of patients at high risk of kidney disease progression.

Summary Points

- The frequent progression of CKD to end-stage kidney failure and cardiovascular morbidity and mortality is a costly worldwide public health problem.
- Risk stratification of patients with CKD disease is crucial for early identification of patients at risk of kidney disease progression and cardiovascular morbidity and mortality and for timely initiation of preventive treatments.
- This chapter focuses on the usefulness of proteinuric patterns for prediction of the functional outcome (progression, remission) and responsiveness of CKD to some treatments.
- The glomerular filtration barrier (GFB) normally does not allow the passage of high molecular weight (MW) proteins (IgG, IgM, α2-macroglobulin); low

amounts of the middle MW albumin pass through and are almost completely reabsorbed by proximal tubular epithelial cells (PTECs); low MW proteins (α1-microglobulin, β2-microglobulin, retinol-binding protein) pass through freely and are almost completely reabsorbed by PTECs.

- Proteinuria is dependent on alteration of glomerular filtration barrier and functional or structural alteration of PTECs; the urinary protein amount and composition measured in final urine are consequent to complex interplay between filtration and reabsorption.
- Measurement of albuminuria is useful for preliminary diagnosis of suspected kidney disease.
- However, albuminuria is not as strong predictor of kidney disease progression as the urinary excretion of proteins larger than albumin such as IgM and IgG.
- IgM-uria could be helpful for the assessment of kidney and cardiovascular disease risk in diabetic patients.
- IgG-uria could be helpful for assessment of risk in glomerulonephritis. Fractional excretion of IgG, a marker that combines two predictors of kidney function decline, is a much better predictor of the functional outcome and responsiveness of primary glomerulonephritis to treatment.

References

Altemtam N, Russell J, El Nahas M. A study of the natural history of diabetic kidney disease (DKD). Nephrol Dial Transplant. 2012;27:1847–54.

Amsellem S, Gburek J, Hamard G, et al. Cubilin is essential for albumin reabsorption in the renal proximal tubule. J Am Soc Nephrol. 2010;21:1859–67.

Arnlov J, Evans JC, Meigs JB, Wang TJ, Fox CS, Levy D, Benjamin EJ, D'Agostino RB, Vasan RS. Low-grade albuminuria and incidence of cardiovascular disease events in nonhypertensive and nondiabetic individuals: the Framingham Heart Study. Circulation. 2005;112:969–75.

Astor BC, Matsushita K, Gansevoort RT, van der Velde M, Woodward M, Levey AS, Jong PE, Coresh J, et al. Lower estimated glomerular filtration rate and higher albuminuria are associated with mortality and end-stage renal disease. A collaborative meta-analysis of kidney disease population cohorts. Kidney Int. 2011;79:1331–440.

Baines RJ, Brunskill NJ. Tubular toxicity of proteinuria. Nat Rev Nephrol. 2011;7:177–80.

Baines RJ, Chana RS, Hall M, et al. CD36 mediates proximal tubular binding and uptake of albumin and is up-regulated in proteinuric nephropathies. Am J Physiol Renal Physiol. 2012;303:1006–14.

Bakoush O, Torffvit O, Rippe B, Tencer J. High proteinuria selectivity index based upon IgM is a strong predictor of poor renal survival in glomerular diseases. Nephrol Dial Transplant. 2001a;16:1357–63.

Bakoush O, Grubb A, Rippe B, Tencer J. Urine excretion of protein HC in proteinuric glomerular diseases correlates to urine IgG but not to albuminuria. Kidney Int. 2001b;60:1904–9.

Bakoush O, Tencer J, Tapia J, Rippe B, Torffvit O. Higher urinary IgM excretion in type 2 diabetic nephropathy compared to type 1 diabetic nephropathy. Kidney Int. 2002;61:203–8.

Bakoush O, Torffvit O, Rippe B, Tencer J. Renal function in proteinuric glomerular diseases correlates to the changes in urine IgM excretion but not to the changes in the degree of albuminuria. Clin Nephrol. 2003;59:345–52.

Bakoush O, Tencer J, Torffvit O, Tenstad O, Skogvall I, Rippe B. Increased glomerular albumin permeability in old spontaneously hypertensive rats. Nephrol Dial Transplant. 2004;19:1724–31.
Bakoush O, Segelmark M, Torffvit O, Ohlsson S, Tencer J. Urine IgM excretion predicts outcome in ANCA-associated renal vasculitis. Nephrol Dial Transplant. 2006;21:1263–9.
Bazzi C, Petrini C, Rizza V, et al. A modern approach to selectivity of proteinuria and tubulointerstitial damage in nephrotic syndrome. Kidney Int. 2000;58:1732–41.
Bazzi C, Petrini C, Rizza V, et al. Urinary N-acetyl-β-glucosaminidase excretion is a marker of tubular dysfunction and a predictor of outcome in primary glomerulonephritis. Nephrol Dial Transplant. 2002;17:1890–6.
Bazzi C, Petrini C, Rizza V, Napodano P, Paparella M, Arrigo G, Pisano L, D'Amico G. Fractional excretion of IgG predicts renal outcome and response to therapy in primary focal segmental glomerulosclerosis: a pilot study. Am J Kidney Dis. 2003;41:328–35.
Bazzi C, Rizza V, Raimondi S, et al. In crescentic IgA nephropathy fractional excretion of IgG in combination with nephron loss is the best predictor of progression and responsiveness to immunosuppression. Clin J Am Soc Nephrol. 2009;4:929–35.
Bazzi C, Rizza V, Casellato D, et al. Validation of some pathophysiological mechanisms of the CKD progression theory and outcome prediction in IgA nephropathy. J Nephrol. 2012;25:810–8.
Bazzi C, Rizza V, Casellato D, et al. Urinary IgG and α2-macroglobulin are powerful predictors of outcome and responsiveness to steroids and cyclophosphamide in idiopathic focal segmental glomerulosclerosis with nephrotic syndrome. Biomed Res Int. 2013. doi:10.1155/2013/941831.
Bazzi C, Rizza V, Casellato D, Tofik R, Berg AL, Gallieni M, D'Amico G, Bakoush O. Fractional excretion of IgG in idiopathic membranous nephropathy with nephrotic syndrome: a predictive marker of risk and drug responsiveness. BMC Nephrol. 2014. doi:10.1186/1471-2369-15-74.
Branten AJ, du BUF-Vereijken, Klasen IS, Bosch FH, Feith GW, Hollander DA, Wetzels JF. Urinary excretion of beta2-microglobulin and IgG predict prognosis in idiopathic membranous nephropathy: a validation study. J Am Soc Nephrol. 2005;16:169–74.
Chen Y, Schieppati A, Cai G, et al. Immunosuppression for membranous nephropathy: a systematic review and meta-analysis of 36 clinical trials. Clin J Am Soc Nephrol. 2013;8:787–96.
Cohen-Bucay A, Viswanathan G. Urinary markers of glomerular injury in diabetic nephropathy. Int J Nephrol. 2012. doi:10.1155/2012/146987.
Cravedi P, Remuzzi G. Pathophysiology of proteinuria and its value as outcome measure in CKD. Br J Clin Pharmacol. 2013;13:1159–67.
D'Amico G, Bazzi C. Pathophysiology of proteinuria. Kidney Int. 2003a;63:809–25.
D'Amico G, Bazzi C. Urinary protein and enzyme excretion as markers of tubular damage. Curr Opin Nephrol Hypertens. 2003b;12:639–43.
Deegens JK, Wetzels JF. Fractional excretion of high- and low-molecular weight proteins and outcome in primary focal segmental glomerulosclerosis. Clin Nephrol. 2007;68:201–8.
Eckardt KU, Coresh J, Devuyst O, Johnson RJ, Kottgen A, Levey AS, Levin A. Evolving importance of kidney disease: from subspecialty to global health burden. Lancet. 2013;382(9887):158–69.
Fioretto P, Caramori ML, Mauer M. The kidney in diabetes: dynamic pathways of injury and repair. The Camillo Golgi Lecture 2007. Diabetologia. 2008;51:1347–55.
Gansevoort RT, Matsushita K, van der Velde M, et al. Lower estimated GFR and higher albuminuria are associated with adverse kidney outcomes. A collaborative meta-analysis of general and high-risk population cohorts. Kidney Int. 2011;80:93–104.
Gbadegesin R, Lavin P, Foreman J, Winn M. Pathogenesis and therapy of focal segmental glomerulosclerosis. Pediatr Nephrol. 2011;26:1001–15.
Ghayur A, Margetts PJ. Transforming growth factor-beta and the glomerular filtration barrier. Kidney Res Clin Pract. 2013;32:3–10.
Halbesma N, Jansen DF, Heymans MW, et al. Development and validation of a general population renal risk score. Clin J Am Soc Nephrol. 2011;6:1731–8.

Haraldsson B, Nystrom J, Deen WM. Properties of the glomerular barrier and mechanisms of proteinuria. Physiol Rev. 2008;88:451–87.

Haubitz M, Wittke S, Weissinger EM, et al. Urine protein patterns can serve as diagnostic tools in patients with IgA nephropathy. Kidney Int. 2005;67:2313–20.

Irazabal MW, Eirin A, Lieske J, et al. Low- and high-molecular weight proteins as predictors of response to rituximab in patients with membranous nephropathy: a prospective study. Nephrol Dial Transplant. 2013;28:137–46.

Jafar TH, Qadri Z, Hashmi S. Prevalence of microalbuminuria and associated electrocardiographic abnormalities in an Indo-Asian population. Nephrol Dial Transplant. 2009;24:2111–6.

Jha V, Wang AY, Wang H. The impact of CKD identification in large countries: the burden of illness. Nephrol Dial Transplant. 2012;27 Suppl 3:iii32–8.

Kaptoge S, Di Angelantonio E, Lowe G, Pepys MB, Thompson SG, Collins R, Danesh J. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. Lancet. 2010;375(9709):132–40.

Kaptoge S, Di Angelantonio E, Pennells L, Wood AM, White IR, Gao P, Walker M, Thompson A, Sarwar N, et al. C-reactive protein, fibrinogen, and cardiovascular disease prediction. N Engl J Med. 2012;367:1310–20.

Liu JE, Robbins DC, Palmieri V et al. Association of albuminuria with systolic and diastolic left ventricular dysfunction in type 2 diabetes: the Strong Heart Study. J Am Coll Cardiol. 2003;41:2022–2028.

Levey AS, Schoolwerth AC, Burrows NR, et al. Comprehensive public health strategies for preventing the development, progression, and complications of CKD: report of an expert panel convened by the Centers for Disease Control and Prevention. Am J Kidney Dis. 2009;53:522–35.

Loeffler I, Wolf G. Transforming growth factor-β and the progression of renal disease. Nephrol Dial Transplant. 2014;29 Suppl 1:i37–45.

Lowik MM, Groenen PJ, Levtchenko EN, et al. Molecular genetic analysis of podocyte genes in focal segmental glomerulosclerosis. Eur J Pediatr. 2009;168:1291–304.

Marshall SM. Natural history and clinical characteristics of CKD in type 1 and type 2 diabetes mellitus. Adv Chron Kidney Dis. 2014;21:267–72.

Matsushita K, van der Velde M, Astor BC, et al. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. Lancet. 2010;375(9731):2073–81.

Mc Quarrie EP, Shakerdi L, Jardine AG, et al. Fractional excretions of albumin and IgG are the best predictors of progression in primary glomerulonephritis. Nephrol Dial Transplant. 2011;26:1563–9.

Mc Taggart MP, Price CP, Pinnock RG, et al. The diagnostic accuracy of a urine albumin-creatinine ratio point-of-care test for detection of albuminuria in primary care. Am J Kidney Dis. 2012;60:787–94.

Menon MC, Chuang PY, He CJ. The glomerular filtration barrier: components and crosstalk. Int J Nephrol. 2012. doi:10.1155/2012/749010.

Miner JH. Glomerular basement membrane composition and filtration barrier. Pediatr Nephrol. 2011;26:1413–7.

Nangaku M. Mechanisms of tubulointerstitial injury in the kidney: final common pathways to end-stage renal failure. Intern Med. 2004;43:9–17.

Nangaku M. Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. J Am Soc Nephrol. 2006;17:17–25.

Nangaku M, Inagi R, Miyata T, Fujita T. Angiotensin-induced hypoxia in the kidney: functional and structural changes of the renal circulation. Adv Exp Med Biol. 2007;618:85–99.

Olson JL, Hostetter TH, Rennke HG, Brenner BM, Venkatachalam A. Altered glomerular permselectivity and progressive sclerosis following extreme ablation of renal mass. Kidney Int. 1982;22:112–26.

Park EY, Kim TY. Fractional excretion of protein may be a useful predictor of decline of renal function in pan-chronic kidney diseases. Nephrol Dial Transplant. 2011;26:1746–7.

Ponticelli C, Altieri P, Scolari F, et al. A randomized study comparing methylprednisolone plus chlorambucil versus methylprednisolone plus cyclophosphamide in idiopathic membranous nephropathy. J Am Soc Nephrol. 1998;9:444–50.
Rath T, Kuo TT, Baker K, et al. The immunologic functions of the neonatal Fc receptor for IgG. J Clin Immunol. 2013;33 Suppl 1:S9–17.
Rippe C, Rippe A, Larsson A, Asgeirsson D, Rippe B. Nature of glomerular capillary permeability changes following acute renal ischemia-reperfusion injury in rats. Am J Physiol Renal Physiol. 2006;291:F1362–8.
Ruggenenti P, Remuzzi G. Time to abandon microalbuminuria? Kidney Int. 2006;70:1214–22.
Ruggenenti P, Gaspari F, Perna A, Remuzzi G. Cross sectional longitudinal study of spot urine protein:creatinine ratio, 24 hour urine protein excretion rate, glomerular filtration rate, and end stage renal failure in chronic renal disease in patients without diabetes. BMJ. 1998;316:504–9.
Ruggenenti P, Cravedi P, Chianca A, et al. Rituximab in idiopathic membranous nephropathy. J Am Soc Nephrol. 2012a;23:1416–25.
Ruggenenti P, Cravedi P, Remuzzi G. Mechanism and treatment of CKD. J Am Soc Nephrol. 2012b;23:1917–28.
Samal L, Linder JA. The primary care perspective on routine urine dipstick screening to identify patients with albuminuria. Clin J Am Soc Nephrol. 2013;8:131–5.
Schottker B, Herder C, Rothenbacher D, Roden M, Kolb H, Muller H, Brenner H. Proinflammatory cytokines, adiponectin, and increased risk of primary cardiovascular events in diabetic patients with or without renal dysfunction: results from the ESTHER study. Diabetes Care. 2013;36:1703–11.
Srorm T, Tranebjaerg L, Frykholm C, et al. Renal phenotypic investigations of megalin-deficient patients: novel insights into tubular proteinuria and albumin on filtration. Nephrol Dial Transplant. 2013;28:585–91.
Stehouwer CD, Smulders YM. Microalbuminuria and risk for cardiovascular disease: analysis of potential mechanisms. J Am Soc Nephrol. 2006;17:2106–11.
Steinke JM, Mauer M, International Diabetic Nephropathy Study G. Lessons learned from studies of the natural history of diabetic nephropathy in young type 1 diabetic patients. Pediatr Endocrinol Rev. 2008;5 Suppl 4:958–63.
Tangri N, Kitsios GD, Inker LA, et al. Risk prediction models for patients with chronic kidney disease. Ann Intern Med. 2013;158:596–603.
Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev. 2006;86:515–81.
Toblli JE, Bevione P, Di Gennaro F, et al. Understanding the mechanisms of proteinuria: therapeutic implications. Int J Nephrol. 2012. doi:10.1155/2012/546039.
Tofik R, Torffvit O, Tippe B, Bakoush O. Increased urine IgM excretion predicts cardiovascular events in patients with type 1 diabetic nephropathy. BMC Med. 2009. doi:10.1186/1741-7015-7-39.
Tofik R, Aziz R, Reda A, et al. The value of IgG-uria in predicting renal failure in idiopathic glomerular diseases. A long-term follow up study. Scand J Clin Lab Invest. 2011;71:123–8.
Tofik R, Ekelund U, Torffvit O, Sward P, Rippe B, Bakoush O. Increased urinary IgM excretion in patients with chest pain due to coronary artery disease. BMC Cardiovasc Disord. 2013. doi:10.1186/1471-2261-13-72.
van der Brand JAJG, Hofstra JM, Wetzels JFM. Low-molecular-weight proteins as prognostic markers in idiopathic membranous nephropathy. Clin J Am Soc Nephrol. 2011;6:2846–53.
van der Meer I, Cravedi P, Remuzzi G. The role of renin angiotensin system inhibition in kidney repair. Fibrogenesis Tissue Repair. 2010;3:7.
White SL, Yu R, Craig JC, et al. Diagnostic accuracy of urine dipstick for detection of albuminuria in the general community. Am J Kidney Dis. 2011;58:19–28.
Woo KT, Lau YK, Lee GSL, et al. Pattern of proteinuria in IgA nephritis by SDS-PAGE: clinical significance. Clin Nephrol. 1991;36:6–11.
Woo KT, Lau YK, Lee GSL, et al. Pattern of proteinuria in IgA nephropathy. Nephrology. 1997;3:31–4.

Apelin and Copeptin as Biomarkers of Kidney Disease

24

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Abstract

The need for faster diagnosis, more accurate prognostic assessment, and treatment decisions in renal diseases has led to the investigations of new biomarkers. Arginine vasopressin (AVP) is one of the main hormones of the hypothalamic–pituitary–adrenal axis, and it is mainly stimulated by hyperosmolarity.

AVP plays deleterious renal effects, inducing hypertension, glomerular hyperfiltration, albuminuria, and glomerulosclerosis, whereas its inhibition, by drinking water or by V2 antagonism, led to a renoprotection. The direct measurement of AVP in humans is problematic, due to its bond with platelets and its instability in isolated plasma.

However, copeptin, the C-terminal portion of the AVP precursor and released into the circulation from the posterior pituitary gland in equimolar amounts with AVP, represents a measurable substitute. In many studies copeptin mimed AVP levels and its behavior, modified by plasma osmolality, stress, and various disease states, revealing some of the various physiologic and pathophysiologic conditions associated with increased or decreased AVP.

Apelin, belonging to a signaling pathway implicated in the regulation of cardiovascular function and fluid homeostasis, is highly expressed in hypothalamic nuclei, is co-localized with AVP, and exerts an active diuretic action, counteracting the effects of vasopressin.

In this chapter, we examine the diagnostic and prognostic role of copeptin and apelin, reporting the last data about their physiological functions, their receptor interactions, and their functions in nephrologic field.

Conclusion: Copeptin and apelin represent prognostic marker for nephropathic patients and predictive factors for progression to end-stage renal disease. Their measurement may be included in a "renal" work-up for patients at high risk, and their evaluation may be helpful also to predict a therapy response. However, further studies are needed in order to make these results more reliable and applicable in larger populations.

Keywords

Apelin • Copeptin • Vasopressin • APJ signaling • Aquaretic • Tolvaptan • CKD progression • Kidney biomarkers • ADPKD

Abbreviations

ACR	Albumin-to-creatinine ratio
ADH	Antidiuretic hormone
ADPKD	Autosomal dominant polycystic kidney disease
Ang II	Angiotensin II
APJ	Angiotensin I receptor-related protein J receptor
AQP	Aquaporin
AVP	Arginine vasopressin
BNP	Brain natriuretic peptide
CKD	Chronic kidney disease
DM	Type 2 diabetes mellitus

ESRD	End-stage renal disease
GFR	Glomerular filtration rate
HD	Hemodialysis
HIF	Hypoxia-inducible factor
MI	Myocardial infarction
PD	Peritoneal dialysis
RAS	Renin–angiotensin system
SIADH	Syndrome of inappropriate antidiuretic hormone
TKV	Total kidney volume
VR	Vasopressin receptor
UAE	Urinary albumin excretion

Key Facts of Glomerular Filtration Rate

- Glomerular filtration rate (GFR) describes the flow rate of filtered fluid through the kidney.
- GFR is the best test to measure the level of kidney function and determine the stage of kidney disease.
- According to the National Kidney Foundation, normal results range from 90 to 120 mL/min/1.73 m^2.
- There are recommended equations for estimating GFR from serum creatinine, such as the Modification of Diet in Renal Disease (MDRD) Study equation and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.
- If GFR estimates are likely inaccurate, a measured GFR is an important confirmatory test.

Key Facts of Vasopressin

- Arginine vasopressin (AVP) is a peptide hormone formed in the hypothalamus and then transported via axons to, and released from, the posterior pituitary into the blood.
- AVP has two principle sites of action: the kidney and blood vessels.
- At renal level, AVP regulates extracellular fluid volume acting on the collecting ducts via V2 receptors and leading to decreased urine formation.
- For this reason it is also called antidiuretic hormone.
- A secondary function of AVP is exerted in vessels, binding V1 receptors on the vascular smooth muscle and causing vasoconstriction.

Key Facts of Autosomal Dominant Polycystic Kidney Disease

- Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common forms of polycystic kidney disease.

- It is a genetic disorder characterized by the growth of numerous cysts in both kidneys.
- The cysts are filled with fluid, and their progressive expansion slowly replaces much of the normal mass of the kidneys, reducing kidney function and leading to kidney failure.
- When ADPKD causes kidneys to fail, the patient requires dialysis or kidney transplantation.
- ADPKD can be diagnosed using ultrasound, computed tomography scan, or magnetic resonance imaging studies of the kidneys.
- A genetic test can detect mutations in the PKD1 and PKD2 genes.

Key Facts of End-Stage Renal Disease

- End-stage renal disease (ESRD) occurs when kidney function is permanently lost.
- According to the National Kidney Foundation classification, it is defined by a glomerular filtration rate <15 mil/min.
- Most cases of ESRD are caused by diabetes or high blood pressure.
- Dialysis or a kidney transplant is necessary to live.
- Possible complications of ESRD include heart and blood vessel problems, malnutrition, or anemia.

Key Facts of Aquaretics

- They are drugs that selectively bind vasopressin receptors in the renal collecting duct promoting excretion of solute-free water.
- These drugs are also called vaptans.
- Tolvaptan is an orally active selective V2 receptor antagonist approved for the treatment of the syndrome of inappropriate antidiuretic hormone secretion.
- Tolvaptan may rapidly increase the level of sodium in the blood.
- Tolvaptan would become the first pharmaceutical therapy in Europe for patients with autosomal polycystic kidney disease.

Definitions

Biomarker Biomarker is an objectively measured and evaluated indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.

Brain natriuretic peptide BNP is a substance secreted from the ventricles or lower chambers of the heart in response to changes in pressure that occur when heart failure develops and worsens.

Cellular receptor A receptor is a structure on the surface of a cell (or inside a cell) that selectively recognizes and binds with specific molecules, producing a specific effect in the cell.

Hemodialysis Hemodialysis is the most common method used to treat advanced and permanent kidney failure. This medical procedure is based on the removal of fluids and waste products from the blood, correcting electrolyte imbalances. This is accomplished using a machine and a dialyzer, also referred to as an "artificial kidney."

Peritoneal dialysis Peritoneal dialysis represents another renal replacement therapy, using, as a filter, the lining of the abdominal cavity (peritoneal membrane) and a solution (dialysate), infused through a specific catheter.

Renin–angiotensin–aldosterone system The renin–angiotensin–aldosterone system (RAAS) is a signaling pathway responsible for regulating the body's blood pressure. As the name implies, there are three important components to this system: (1) renin, (2) angiotensin, and (3) aldosterone.

Test sensitivity It evaluates the probability that a test result will be positive when the event is present.

Test specificity It measures the probability that a test result will be negative when the event is not present.

Total kidney volume Total kidney volume (TKV) growth is considered the best surrogate marker predicting the decline of renal function in autosomal dominant polycystic kidney disease. This datum is obtained by magnetic resonance imaging scans with an optimized T1-weighted acquisition protocol without gadolinium-based contrast agents.

Introduction

Kidney disease is characterized by progressive destruction of the renal parenchyma and loss of functional nephrons, leading to chronic renal failure. In clinical practice, the diagnosis is dependent on levels of unreliable biomarkers, such as blood urea, nitrogen, and serum creatinine, characterized by low sensitivity and specificity. In fact, the time relationship between changes in serum creatinine and concomitant changes in glomerular filtration rate (GFR) does not allow accurately estimating timing of injury and severity of renal dysfunction (Lacquaniti et al. 2013a).

Moreover, creatinine levels are affected by several confounding factors, such as diet and muscle mass, and, despite the use of weighting for age, gender, and race in the GFR estimating equations, this can result in misclassification of patients (Tangri et al. 2011).

Arginine vasopressin (AVP), also known as antidiuretic hormone (ADH), plays an important role in the regulation of volume status. It is secreted into the blood if

dehydration (increase in plasma osmolality) or volume loss occur, acting through a water reabsorption in the nephron tubules by binding to the AVP V_2 receptor (VR) (Birnbaumer 2000).

In various experimental models, including rodent models of diabetes, it has been shown that AVP infusion induces hypertension, glomerular hyperfiltration, albuminuria, and glomerulosclerosis, playing deleterious renal effects (Bankir et al. 2001), whereas its inhibition, by drinking water or by V_2 antagonism, led to a decrease in proteinuria in rats with renal failure (Okada et al. 1994). The direct measurement of vasopressin in humans is problematic. More than 90 % in the circulation is bound to platelets, it is unstable in isolated plasma, and most assays have relatively limited sensitivity (Preibisz et al. 1983).

Recently, copeptin, the C-terminal portion of the precursor of vasopressin and stoichiometrically secreted with it, becomes a reliable marker and a useful substitute to detect circulating concentration in clinical routine. Moreover, several studies in healthy subjects demonstrated a close relation between plasma copeptin, AVP concentrations, and a wide range of osmolalities (Szinnai et al. 2007; Balanescu et al. 2011).

However, the interpretation of copeptin levels must take into account potential confounding factors, such as gender, renal impairment, and the fluid status of the subject. Hence, a single reference range for normal copeptin will not be valid considering the need to adjust for the independent effects of gender and renal function (Bhandari et al. 2009).

Whereas water retention in the kidney is known to be an active phenomenon, controlled by AVP, water excretion is not a passive process, as a result of AVP release blockade, because this event is also controlled by a diuretic peptide, called apelin.

It belongs to a signaling pathway which is implicated in the regulation of cardiovascular function and fluid homeostasis. It is highly expressed in the supraoptic and paraventricular hypothalamic nuclei, co-localized with AVP in a subset of magnocellular neurons (Fig. 1).

Moreover, water deprivation, increasing systemic AVP release and causing depletion of hypothalamic AVP stores, decreases plasma apelin concentrations, indicating that AVP and apelin are conversely regulated to prevent additional water loss at kidney level (Llorens-Cortes and Moos 2012).

Until now, efforts to retard (or even prevent) progression of chronic kidney disease (CKD) have mainly focused on reducing protein consumption and lowering blood pressure and renin–angiotensin system (RAS) blockade. The outcomes from these approaches, however, remain far from satisfactory, and additional strategies are therefore welcome.

The issues of optimal water intake and vasopressin receptor antagonism may therefore represent novel frontiers in the effort to slow progression of renal pathologies.

In this chapter, we examine the diagnostic and prognostic role of copeptin and apelin, reporting the last data about their physiological functions, their receptor interactions, and their functions in nephrologic field.

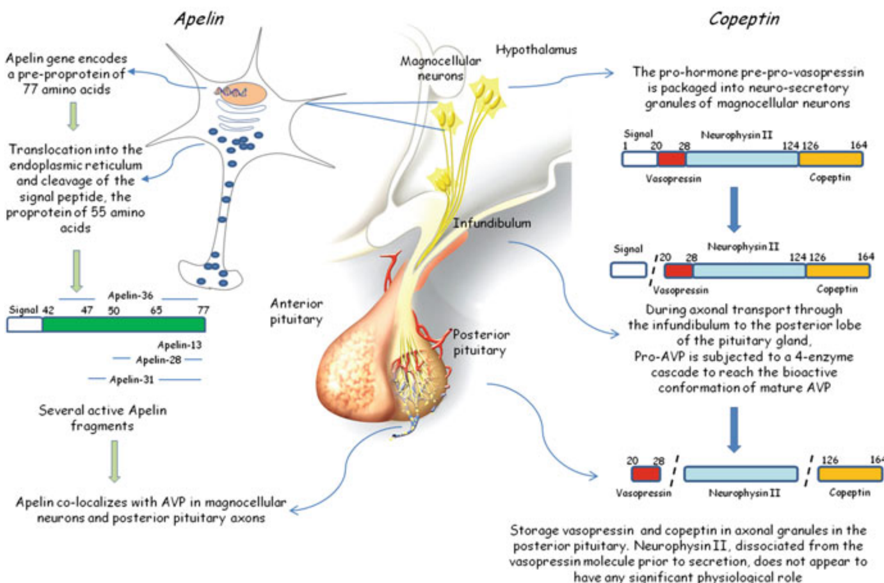

Fig. 1 Vasopressin/copeptin and apelin release and their systemic effects

Physiologic Point of View

Copeptin physiologically contributes to the correct structural formation of AVP prior to release into the circulation. The measurement of copeptin appears to be a clinically relevant method for reliably assessing AVP plasma concentrations, which cannot be determined in routine practice. This would be particularly helpful in diseases where disturbances of the vasopressinergic system contribute to or are the immediate result of the pathogenesis. The kinetics of copeptin, however, has not been well explored, and it is not yet known whether copeptin-specific receptors exist nor whether copeptin exerts biological functions.

Target organs and cells perceive hormonal stimuli mediated by AVP through three distinct AVP receptors, V1a, V1b, and V2, all of which belong to a family of heptahelical guanine nucleotide-binding protein-coupled receptors (Jard 1998).

In the kidney, AVP exerts its antidiuretic effect by regulating sodium and water transport via the V2 receptor, which is expressed in the basolateral membrane of the thick ascending limb of Henle’s loop, distal tubules, and collecting ducts. Through the V2 receptors, AVP stimulates the Gs protein and adenylate cyclase to increase intracellular cAMP, stimulating the translocation of aquaporin (AQP)-2 and the amiloride-sensitive ENaC in the principal cells of the collecting duct and thereby increasing water reabsorption (Imbert et al. 1975).

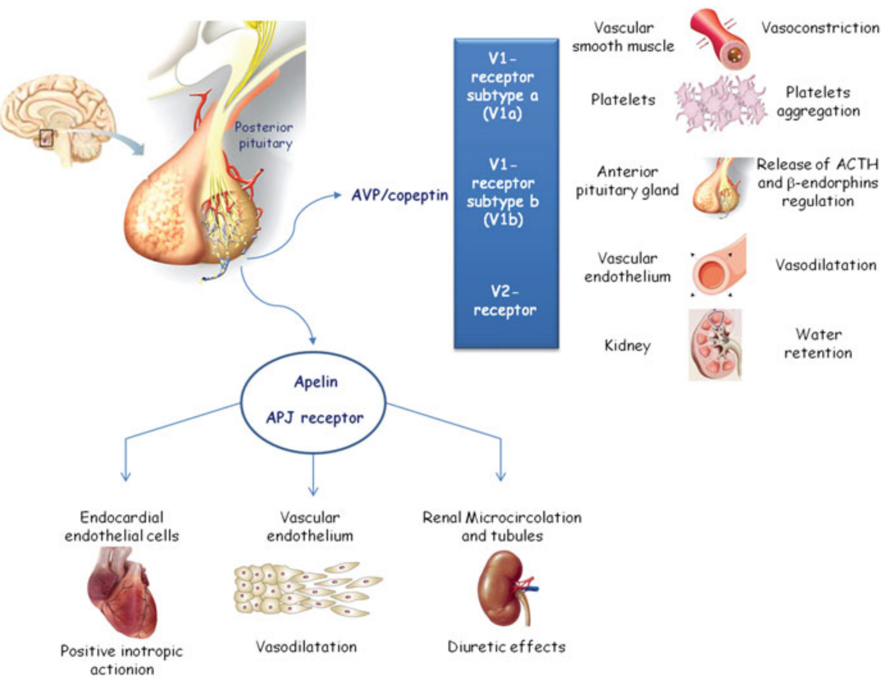

Fig. 2 Copeptin and apelin generation and maturation

The discovery of apelin constitutes an important advance in both fundamental research and clinical medicine. Experimental data have shown that apelin has diuretic effects via its central and renal actions: by inhibiting the phasic activity of vasopressinergic neurons and systemic AVP secretion and by its direct effect on the renal microcirculation and probably tubular function. Besides its diuretic action, when injected into the blood stream, apelin decreases blood pressure and increases the contractile force of the myocardium while decreasing pre- and post-load, actions opposing those of vasopressin and angiotensin II. These data confirm that this new circulating vasoactive (neuro)peptide could play a crucial role in maintaining water and electrolyte balance and cardiovascular functions (Galanth et al. 2012).

It is very difficult to mention a standard normal level of apelin due to the presence of various forms of apelin (apelin-12, apelin-13, apelin-18, apelin-36) in the circulation (Fig. 2).

Besides this factor, different kits for measurement and the cross-reaction between the types of apelin lead to various levels mentioned in the literature. Malyszko reported apelin-36 levels as 84.0 ± 9.26 pg/ml, whereas mean apelin-12 level was reported as 304 pg/ml, both in healthy populations (Karadag et al. 2014).

The angiotensin I receptor-related protein J (APJ) receptor was first identified as an orphan G-protein-coupled receptor, with the closest identity to the angiotensin II (Ang II) receptor, type AT1a. In the ensuing years, the receptor was de-orphanized when its cognate ligand, apelin, was isolated from bovine stomach extracts (Tatemoto et al. 1998).

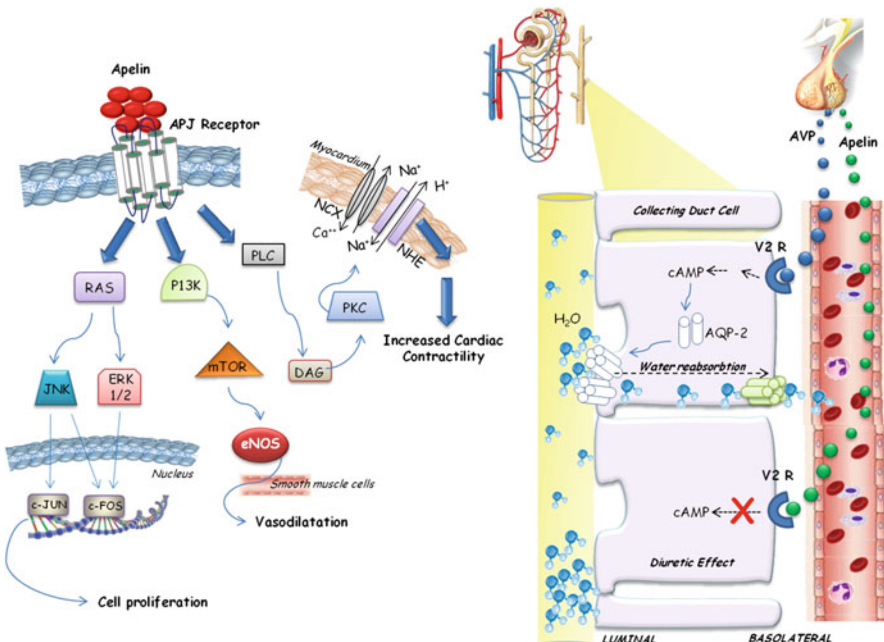

Fig. 3 Copeptin and apelin signaling pathways

The inner strip of the outer medulla in kidneys contains the highest level of APJ mRNA. Additionally, the collecting duct also contains apelin receptor, suggesting that apelin might act as an aquaretic peptide in this segment (Hus-Citharel et al. 2008).

Moreover, apelin and its receptor can be found extensively in the heart, vascular endothelium, lungs, mammary glands, and central nervous system (Kawamata et al. 2001) (Fig. 3).

Pathological Involvement of Copeptin and Apelin

Animal models and human studies strongly suggest a role for vasopressin, via V2 receptors, in the progression of various forms of CKD and in salt-sensitive hypertension. Recent insights also point to the possible role of vasopressin in albuminuria and diabetic nephropathy, via V2-receptor-mediated actions. These results raise the possibility that, in addition to blood-pressure lowering, renin–angiotensin system blockade, and other treatments, inhibiting the V2-receptor-mediated actions of vasopressin, might provide further therapeutic benefit for these patients. Whereas the effect of vasopressin in autosomal dominant polycystic kidney disease (ADPKD) is relatively straightforward and mainly dependent on V2 receptors, its role in other forms of CKD is more complex (Table 1).

Diabetes Mellitus and Kidney Dysfunction

Type 2 diabetes mellitus (DM) is a major cause of kidney and cardiovascular diseases, and screening for albuminuria and proteinuria is recommended for stratification of risk of these comorbidities.

Table 1 Apelin and copeptin roles in renal diseases

Disease	Apelin	Copeptin	Conclusion	Future prospects	References
Acute kidney disease	Not available data in humans	Serum ↑↑	In critically ill patients, an elevation of plasma copeptin is strongly associated with development of AKI	Early diagnostic marker	Meyer et al. (2008)
			Apelin-13 treatment protects against renal ischemia/reperfusion (I/R) injury in rats inhibiting inflammatory factors, TGF-β1, and apoptosis	Late prognostic factor	Chen et al. (2015)
				Further apelin analyses in human studies	
				Apelin as a potential therapy	
Chronic kidney disease	Serum ↑	Serum ↑↑	Copeptin levels are significantly high in the CKD group and closely related to GFR and atherosclerosis markers	Prognostic marker	Li et al. (2013)
			Elevated apelin in CKD patients may be due to impaired renal clearance and inflammation		Malyszko et al. (2008)
Diabetic nephropathy	Serum ↓↓	Serum ↑↑	High copeptin level is strongly associated with the risk of severe renal outcomes, with a direct effect on urinary albumin excretion	Prognostic marker	Velho et al. (2013)
			Patients with high apelin levels have better survival rates than patients with lower levels of apelin, with a decreased risk of cardiovascular mortality		Silva et al. (2013)

(*continued*)

Table 1 (continued)

Disease	Apelin	Copeptin	Conclusion	Future prospects	References
ADPKD	Serum ↓↓	Serum ↑↑	High copeptin levels are independently associated with an increase in TKV and a decrease in GFR	Diagnostic markers	Nakajima A et al. Clin Exp Nephrol. 2015
			Low serum apelin levels in ADPKD patients represent an independent predictor of renal disease progression and renal replacement therapy	Prognostic markers	Boertien et al. (2012)
				Marker of therapy response	Lacquaniti et al. (2013)
				Apelin as a potential therapy	
ESRD/ dialysis	Serum ↓↓	Serum ↑↑	AVP in ESRD patients with little or no residual urine output exerts its functions primarily via the V1a and V1b receptors	Prognostic marker	Fenske et al. (2011)
			There is a strong and independent association of increased copeptin levels with stroke, cardiovascular events, sudden death, and all-cause mortality	Further studies are required	Codognotto et al. (2007)

AKI acute kidney injury, *TFG* tumor growth factor, *CKD* chronic kidney disease, *GFR* glomerular filtration rate, *TKV* total kidney volume, *ADPKD* autosomal dominant polycystic kidney disease, *AVP* arginine vasopressin, *ESRD* end-stage renal disease

Despite improvements in glycemic and blood pressure control and the efficacy of RAS blockade for proteinuria reduction, diabetic nephropathy is the most frequent cause of end-stage renal disease in developed countries (Lacquaniti et al. 2013b).

However, as shown in recent randomized trials, even with optimal treatment, up to 20 % of patients with diabetes and proteinuria develop end-stage renal disease (ESRD) within a 3-year follow-up (Brenner et al. 2001).

In various experimental models, it has been shown that AVP infusion induces glomerular hyperfiltration and albuminuria (Bankir et al. 2001).

While high AVP levels may be beneficial in the short term by limiting the amount of water required for the excretion of the high osmolar load presented by glucosuria, in the long term, it might cause adverse outcomes by aggravating hyperglycemia and its sequelae, such as diabetic nephropathy. In this context, it is also of interest that copeptin concentrations are significantly associated with hypertension, abdominal obesity, inflammation, and the metabolic syndrome, suggesting that increased

activity of the AVP system may be “a unifying factor” in the metabolic syndrome (Enhörning et al. 2011).

It was in fact demonstrated that high copeptin concentration was strongly associated with the risk of severe renal outcomes (doubling of plasma creatinine concentration and/or end-stage renal disease) in DM patients, independently of relevant covariates such as age, duration of diabetes, blood pressure and baseline levels of glycated hemoglobin, urinary albumin excretion (UAE), and eGFR (Velho et al. 2013).

Moreover, baseline copeptin levels were significantly associated with high albumin-to-creatinine ratio (ACR) and low eGFR, but only in DM patients who were not treated with RAS inhibitors. A possible explanation is that the deleterious effect of AVP (measured as copeptin) is mediated at least in part via the renin–angiotensin system, explaining the significant interaction between RAS inhibitor use and copeptin for the association with eGFR (Boertien et al. 2013a).

The association between copeptin and UAE was independent of systemic blood pressure, indicating that other mechanisms of action may be important. Urinary osmolality was independently associated with UAE, but less strong than the association between copeptin and UAE, suggesting that besides the antidiuretic effect of vasopressin, other effects (e.g., pressor effects) may also be involved in the relationship of copeptin with UAE (Enhörning et al. 2013).

In a recent population study (Prevention of Renal and Vascular End-Stage Disease [PREVEND]), including adults with and without kidney dysfunction, the association between copeptin and microalbuminuria was confirmed, highlighting a direct effect of AVP on urinary albumin excretion. In particular, microalbuminuria was about two times more prevalent in individuals with copeptin plasma levels in the highest quintile (>10.5 pmol/L) compared with individuals with copeptin levels in the lowest two quintiles (<5.3 pmol/L).

In the same study, high levels of copeptin were associated with low renal function, hypothesizing that copeptin caused renal function decline or that subjects with low renal function were less sensitive to the actions of copeptin/vasopressin (Meijer et al. 2010).

Several studies have shown an association between apelin levels and overt diabetes.

It was demonstrated that apelin, as an endocrine or paracrine peptide, mediated angiogenesis, facilitates abnormal vessel formation, and increased permeability in diabetic glomeruli, assuming a crucial role in the pathogenesis and progression of diabetic nephropathy (Zhang et al. 2013).

Moreover, Erdem demonstrated that plasma apelin was lower in newly diagnosed and untreated DM patients than healthy controls (Erdem et al. 2008).

The role of apelin in diabetes pathogenesis was also confirmed by Soriguer who showed the association between apelin levels, glucose concentrations, and insulin sensitivity (Soriguer et al. 2009).

An association of apelin with mortality and hospitalization events, as well as its relationship with renal function and cardiovascular risk factors, was assessed in a

homogeneous population of DM patients with a diagnosis of mild-to-moderate CKD. In particular, patients with higher apelin levels tended to have better survival rates than patients with lower levels of apelin, with a decreased risk of cardiovascular mortality (Silva et al. 2013).

However, further studies with a larger sample size are needed to investigate the relationship between these biomarkers, DM, and its comorbidities.

Autosomal Dominant Polycystic Kidney Disease

GFR is recognized as a poor predictive marker of renal function decline in ADPKD patients, as glomerular hyperfiltration in functioning nephrons is able to compensate for the ongoing loss of renal tissue. In early stages, the change in total kidney volume (TKV) still appears to be the most sensitive marker of disease progression (Grantham et al. 2006).

AVP, having a detrimental role in the pathogenesis of ADPKD, is already elevated in the early stages of the disease, as a compensatory mechanism to maintain fluid balance and plasma osmolality within the normal range, in patients characterized by a urinary concentrating defect. In subjects with ADPKD, copeptin is associated with a decline in kidney function and need for renal replacement therapy, independently of confounding parameters, such as age, gender, or baseline GFR (Boertien et al. 2012).

Moreover, high baseline copeptin levels were independently associated with an increase in TKV and a decrease in GFR, representing a biomarker easy to measure that may help to predict outcome in ADPKD (Boertien et al. 2013b).

Furthermore, a cross-sectional study involving 102 ADPKD patients confirmed these data, revealing that copeptin levels were associated with various markers of disease severity, such as albuminuria, GFR, renal blood flow, and TKV (Meijer et al. 2011).

The rise in vasopressin (measured as copeptin) precedes a decline in GFR. Consequently, lowering vasopressin can lead to renoprotection. To lower vasopressin concentration, one of the options is to achieve ample hydration.

Plasma osmolality and plasma copeptin level decreased, in fact, after high water intake with a positive correlation between plasma copeptin and TKV slope, supposing that increase urine volume by drinking relatively solute-free water ameliorates cyst growth via AVP reduction (Higashihara et al. 2014).

Another way to suppress the effect of vasopressin is to block the V2 receptor in the kidney with medication. This option has been tested in animal experiments and humans, revealing that vasopressin antagonism through tolvaptan prevented cyst growth and kidney function decline (Torres et al. 2011, 2012).

Whereas several studies evaluated the role of copeptin in cystic kidney disease, only one prospective study demonstrated that low serum apelin levels in ADPKD patients represented an independent predictor of renal disease progression and renal replacement therapy (Lacquaniti et al. 2013c).

End-Stage Renal Disease

Dialysis patients are at a very high risk of death (about 20 % per year). Thus, the identification of new risk factors with the potential of applying intervention strategies to improve outcome in the future is of utmost importance (Buemi et al. 2008).

Several biomarkers have consistently been shown to be closely associated with patient mortality and improve risk prediction provided by clinical variables alone (Lacquaniti et al. 2009, 2011).

In this context, the search for new biomarkers reflecting different facts of cardiovascular risk is still ongoing.

AVP in ESRD patients with little or no residual urine output can probably not efficiently act via V2 receptors, but exerts its functions primarily via the V1a and V1b receptors. Thus, an increase in cardiovascular risk and all-cause mortality in ESRD patients might be partly linked to the predominant activation of V1a and V1b receptor function (Teitelbaum and McGuinness 1995). It was demonstrated a strong and independent association of increased copeptin levels with stroke, cardiovascular events, sudden death, and all-cause mortality in hemodialysis patients, but not with myocardial infarction or death caused by chronic heart failure (Fenske et al. 2011).

This discrepancy could be related to the cohort and statistical power since the study comprised 1,241 solely diabetic patients reaching the end point by 37–49 %.

The increased copeptin levels in hemodialysis patients most likely reflect accumulation, indicating an impaired clearance of this low molecular weight protein (Artunc et al. 2014).

On the other hand, modern dialysis practices such as high-flux dialyzers or hemodiafiltration can remove significant amounts of low molecular weight proteins, such as β2-microglobulin.

The literature data about apelin levels in ESRD is limited, reporting lower values in dialysis patients compared with the general population.

Apelin level was found to be lower in uremic patients with dilated cardiomyopathy than in non-uremic counterparts, demonstrating that uremia decreases apelin levels irrespective of the degree of heart failure (Codognotto et al. 2007).

There is no data about the clearance of apelin during hemodialysis (HD) or peritoneal dialysis (PD) in the literature, but, due to its molecular weight, it is expected to be filtered through the glomerulus but not through low-flux hemodialysis membranes; so dialysis clearance cannot be the reason of lower levels in HD patients, whereas it could happen in PD patients, due to larger pore size of the peritoneal membrane.

Moreover, AVP/apelin balance changes with plasmatic osmolality variations and a close relationship with arterial hypo- and hypertension induced by hemodialysis were also demonstrated (Cernaro et al. 2012).

Potential Applications to Prognosis, Other Diseases, or Conditions

Kidney disease detection, whether acute or chronic, is based mainly on serum creatinine, which is considered to delay prompt diagnosis and management, thus

increasing substantially patient morbidity and mortality and prolonged hospitalization. Several biomarkers have been evaluated as early prognostic markers, but they are still far from being implicated into clinical practice. Hence, more sensitive and specific biomarkers are needed to enable us recognize individuals at increased risk for progression of CKD to ESRD and occurrence of cardiovascular complications.

It has been demonstrated that apelin and copeptin could represent valid biomarkers for various types of kidney diseases, such as diabetic nephropathy or ADPKD, and may serve as a future target molecule potentially useful for understanding disease processes or innovative therapeutic strategies, due to their role in body fluid homeostasis and in the regulation of the cardiovascular system, reflecting the dialog between the heart and kidney (Table 2).

In fact, Voors demonstrated that copeptin was a strong marker for mortality and morbidity in patients with heart failure after acute myocardial infarction (MI) in a subset of 224 patients of the OPTIMAAL study (Optimal Trial in Myocardial Infarction with Angiotensin II Antagonist Losartan). In addition, the predictive value of copeptin was even stronger than brain natriuretic peptide (BNP) and NT-proBNP (Voors et al. 2009).

Atrium is known as one of the major sources of apelin production, and during heart failure the apelin level in atria falls. Thus, its level in plasma drops consequently (Foldes et al. 2003; Basile et al. 2014).

Kadoglou confirmed this trend in patients with unstable angina and acute MI, revealing significantly lower level of apelin compared to patients with asymptomatic coronary artery disease (Kadoglou et al. 2010).

In addition to the possibility of application of apelin as a heart failure biomarker, it also plays direct biological roles, including vasodilatory with hypotensive response and a positive cardiac inotropic action, opposing its effects to angiotensin II on vascular tone, blood pressure, and fluid homoeostasis (Brame et al. 2015).

It was in fact demonstrated, in vivo studies, that apelin injection resulted in an improvement in cardiac function, as well as a reduction in cardiac loading (Ashley et al. 2005; Chandrasekaran et al. 2008).

Starting from these assumptions, it will be plausible that an optimum dose of apelin will improve both systolic and diastolic functions of the heart and thus could have beneficial effects on the failing hearts in patients with chronic renal diseases (Berry et al. 2004).

Carcinogenesis could represent another potential field of application for apelin, due to its marked properties to regulate angiogenesis, both in vitro and in vivo, stimulating endothelial cell proliferation and migration.

Apelin is in fact upregulated during tumor angiogenesis, and its overexpression was demonstrated to increase in vivo tumor growth. The molecular mechanisms which regulate its expression are still largely unknown, but it has been reported that hypoxia-inducible factor (HIF)-1 regulates apelin synthesis (Bolignano et al. 2010).

It was demonstrated in humans that apelin represents a prognostic factor for survival and a predictive factor for cancer disease progression, becoming a biomarker of enhanced angiogenesis and closely related to increased metastatic capacity. The inhibition of apelin signaling may be a promising target of therapies for

Table 2 Apelin and copeptin in cardiac diseases

Disease	Apelin	Copeptin	Conclusion	Future prospects	References
Acute myocardial infarction	Serum ↓↓	Serum ↑↑	Copeptin is elevated in the early hours after the onset of an ST-elevation AMI when CK-MB and cTnT are still low	Precocious diagnostic marker	Gu et al. (2011)
			Apelin values are lower in patients with unstable angina and AMI than patients with asymptomatic coronary artery disease	Late prognostic factor	Kadoglou et al. (2010)
				Marker of therapy response	
				Apelin as a potential therapy	
Chronic heart failure	Serum ↓↓	Serum ↑↑	Copeptin is a strong marker for mortality and morbidity in patients with HF	Prognostic marker	Voors et al. (2009)
			The predictive value of copeptin is even stronger than BNP	Marker of therapy response	Chandrasekaran et al. (2008)
			Apelin is reduced in patients with heart failure and upregulated following favorable left ventricular remodeling	Apelin as a potential therapy	
Atrial fibrillation	Serum ↓	Serum ↑	High copeptin levels are associated with AF and a higher risk of CVE	Prognostic marker	Smith et al. (2010)
			Low plasma apelin levels before external electrical cardioversion are an independent prognostic factor for arrhythmia recurrence in patients with AF treated with anti-arrhythmic drugs	Marker of therapy response	Falcone et al. (2010)
				Further studies are required	

(*continued*)

Table 2 (continued)

Disease	Apelin	Copeptin	Conclusion	Future prospects	References
Heart transplant	Not available data	Serum ↑	Copeptin independently predicts outcomes of heart transplant patients, and it is closely related to kidney function and intraventricular septal thickness	Prognostic marker	Malyszko et al. (2010)
				Further studies are required, involving apelinergic system	Przybylowski et al. (2010)

AMI acute myocardial infarction, *CK-MB* creatine kinase–myocardial band, *cTnT* cardiac troponin T, *HF* heart failure, *BNP* brain natriuretic peptide, *AF* atrial fibrillation, *CVE* cardiovascular events

cancer treatment, with a new class of anti-angiogenesis drugs. Furthermore, considering its vasopressin antagonist role, apelin could be a new drug to treat the syndrome of inappropriate antidiuretic hormone (SIADH)-related hyponatremia, often involving oncologic patients (Lacquaniti et al. 2015).

Table 3 resumed the potential diagnostic and prognostic roles of copeptin and apelin in various diseases.

Conclusion

These findings are of great importance toward understanding the pathophysiological mechanisms involving several types of kidney diseases, as well as the role of vasopressin, copeptin, and apelin as diagnostic and prognostic biomarkers.

Copeptin and apelin represent in fact prognostic marker for these patients and predictive factors for progression to ESRD. Their measurement may be included in a "renal" work-up for patients at high risk, and their evaluation may be helpful also to predict a therapy response, especially in the immediate future, when the aquaretic tolvaptan, selective V2 receptor antagonist, will be available for treating patients with ADPKD.

However, further studies are needed in order to make these results more reliable and applicable in larger populations.

Summary Points

- Arginine vasopressin plays deleterious renal effects, inducing hypertension, albuminuria, and glomerulosclerosis, whereas its inhibition led to renoprotection.
- This chapter focuses on apelin and copeptin, two biomarkers closely related to vasopressin system.

Table 3 Apelin and copeptin in different disorders behind cardiorenal pathologies

Disease	Apelin	Copeptin	Conclusion	Future prospects	References
Sepsis and septic shock	Serum ↑	Serum ↑↑	Copeptin is positively correlated with APACHE II score and inflammatory markers in patients with sepsis, reflecting disease severity	Precocious diagnostic marker	Jochberger et al. (2009)
			Serum Apelin level was significantly high in adult patients and neonates with sepsis and septic shock	Late prognostic factor	Gad et al. (2014)
				Marker of therapy response	
Diabetes insipidus	Not available data	Serum ↑↑	Without prior thirsting, a single baseline copeptin level >21.4 pmol/L differentiated nephrogenic DI from other etiologies with 100 % sensitivity and specificity, rendering water deprivation testing unnecessary in such cases	Early diagnostic marker	Timper K et al. J Clin Endocrinol Metab. 2015 jc20144507
			In patients undergoing pituitary procedures, low copeptin levels reflect postoperative DI, while high levels virtually exclude it	Prognostic marker	Winzeler B et al. J Clin Endocrinol Metab. 2015 jc20144527
SIADH	Serum ↑	Serum ↑	High copeptin levels reflect the abnormal AVP secretion in the case of SIADH	Diagnostic markers	Fenske et al. (2014)
			High apelin concentrations and apelin-to-copeptin ratios characterize SIADH patients	Marker of therapy response	Blanchard et al. (2013)
				Further studies are required	

(*continued*)

Table 3 (continued)

Disease	Apelin	Copeptin	Conclusion	Future prospects	References
Liver cirrhosis	Serum and histology ↑	Serum ↑↑	Copeptin concentrations increased along with the severity of liver disease, predicting 1-year mortality or liver transplantation	Prognostic markers	Moreno et al. (2013)
			Apelin is expressed in the cirrhotic liver, and it is involved in the disease progression. It may be implicated in multiple facets in the fibrosis–carcinoma axis	Marker of therapy response Apelin as a potential therapy target	Farid et al. (2014)
Cancer	Serum ↑	Serum ↑	High apelin and copeptin levels could be found in oncologic patients because of their vasoactive properties	Precocious diagnostic biomarker	Belting et al. (2012)
			Apelin behaves as a potent activator of tumor neoangiogenesis, representing a strong and independent risk marker for cancer progression	Prognostic marker Apelin as a potential therapy target Further studies are required	Lacquaniti et al. (2015)

APACHE II Acute Physiology and Chronic Health Evaluation II, *DI* diabetes insipidus, *SIADH* syndrome of inappropriate antidiuretic hormone

- Copeptin represents the C-terminal portion of the precursor of vasopressin, it is stoichiometrically secreted with it, and it is detectable through a reliable and sensitive analytical method.
- Apelin, highly expressed in the supraoptic and paraventricular hypothalamic nuclei and co-localized with vasopressin in a subset of magnocellular neurons, exerts an active diuretic action, counteracting the effects of vasopressin.
- High copeptin levels, closely associated with marker of renal dysfunction, such as microalbuminuria and high albumin-to-creatinine ratio, were strongly associated with the risk of severe renal outcomes in diabetic patients.
- Low apelin values were related with mortality and hospitalization events in diabetic patients, with a strict relationship with renal function and cardiovascular risk factors.
- Vasopressin plays a critical role in the pathophysiology of the autosomal dominant polycystic kidney disease (ADPKD).

- In fact, copeptin levels are elevated in ADPKD patients, and it is associated with various markers of disease severity.
- Furthermore, apelin represents an independent predictor of renal disease progression in ADPKD patients, providing predictive information about the patient's risk for renal replacement therapy.
- In patients with end-stage renal disease, treated by hemodialysis or peritoneal dialysis, apelin and copeptin represent new risk factors, closely associated with cardiovascular events and patient mortality.
- Behind nephrologic application, apelin and copeptin play diagnostic and prognostic roles in cardiologic and oncologic pathologies, providing a target pathway potentially useful for understanding disease processes or innovative therapeutic strategies.
- However, further larger prospective studies are necessary to confirm these potential applications of copeptin and apelin and to ascertain their eventual relevance as a parameter for monitoring the development and the progression of renal diseases.

References

Artunc F, Nowak A, Mueller C, et al. Plasma concentrations of the vasoactive peptide fragments mid-regional pro-adrenomedullin, C-terminal pro-endothelin 1 and copeptin in hemodialysis patients: associated factors and prediction of mortality. PLoS One. 2014;9:e86148.

Ashley EA, Powers J, Chen M, et al. The endogenous peptide apelin potently improves cardiac contractility and reduces cardiac loading in vivo. Cardiovasc Res. 2005;65:73–82.

Balanescu S, Kopp P, Gaskill MB, et al. Correlation of plasma copeptin and vasopressin concentrations in hypo-, iso-, and hyperosmolar States. J Clin Endocrinol Metab. 2011;96:1046–52.

Bankir L, Bardoux P, Ahloulay M. Vasopressin and diabetes mellitus. Nephron. 2001;87:8–18.

Basile G, Crucitti A, Cucinotta MD, et al. Serum levels of Apelin-36 are decreased in older hospitalized patients with heart failure. Eur Geriatr Med. 2014;5:242–5.

Belting M, et al. Cancer Epidemiol Biomarkers Prev. 2012;21:513–22.

Berry MF, Pirolli TJ, Jayasankar V, et al. Apelin has in vivo inotropic effects on normal and failing hearts. Circulation. 2004;110:187–93.

Bhandari SS, Loke I, Davies JE, et al. Gender and renal function influence plasma levels of copeptin in healthy individuals. Clin Sci (Lond). 2009;116:257–63.

Birnbaumer M. Vasopressin receptors. Trends Endocrinol Metab. 2000;11:406–10.

Blanchard A, et al. J Clin Endocrinol Metab. 2013;98:2084–9.

Boertien WE, Meijer E, Zittema D, et al. Copeptin, a surrogate marker for vasopressin, is associated with kidney function decline in subjects with autosomal dominant polycystic kidney disease. Nephrol Dial Transplant. 2012;27:4131–7.

Boertien WE, Riphagen IJ, Drion I, et al. Copeptin, a surrogate marker for arginine vasopressin, is associated with declining glomerular filtration in patients with diabetes mellitus (ZODIAC-33). Diabetologia. 2013a;56:1680–8.

Boertien WE, Meijer E, Li J, et al. Relationship of copeptin, a surrogate marker for arginine vasopressin, with change in total kidney volume and GFR decline in autosomal dominant polycystic kidney disease: results from the CRISP cohort. Am J Kidney Dis. 2013b;61:420–9.

Bolignano D, Medici MA, Coppolino G, et al. Aquaretic inhibits renal cancer proliferation: role of vasopressin receptor-2 (V2-R). Urol Oncol. 2010;28:642–7.

Brame AL, Maguire JJ, Yang P, et al. Design, characterization, and first-in-human study of the vascular actions of a novel biased apelin receptor agonist. Hypertension. 2015;65:834–40.

Brenner BM, Cooper ME, de Zeeuw D, RENAAL Study Investigators, et al. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. N Engl J Med. 2001;345:861–9.
Buemi M, Lacquaniti A, Bolignano D, et al. Dialysis and the elderly: an underestimated problem. Kidney Blood Press Res. 2008;31:330–6.
Cernaro V, Lacquaniti A, Lorenzano G, et al. Apelin, plasmatic osmolality and hypotension in dialyzed patients. Blood Purif. 2012;33:317–23.
Chandrasekaran B, Dar O, McDonagh T. The role of apelin in cardiovascular function and heart failure. Eur J Heart Fail. 2008;10:725–32.
Chen H, et al. Biochim Biophys Acta. 2015;1852:1278–87.
Codognotto M, Piccoli A, Zaninotta M, et al. Evidence for decreased circulating apelin beyond heart involvement in uremic cardiomyopathy. Am J Nephrol. 2007;27:1–6.
Enhörning S, Struck J, Wirfält E, et al. Plasma copeptin, a unifying factor behind the metabolic syndrome. J Clin Endocrinol Metab. 2011;96:E1065–72.
Enhörning S, Bankir L, Bouby N, et al. Copeptin, a marker of vasopressin, in abdominal obesity, diabetes and microalbuminuria: the prospective Malmo Diet and Cancer Study cardiovascular cohort. Int J Obes (Lond). 2013;37:598–603.
Erdem G, Dogru T, Tasci I, Sonmez A, Tapan S. Low plasma apelin levels in newly diagnosed type 2 diabetes mellitus. Exp Clin Endocrinol Diabetes. 2008;116:289–92.
Falcone C, et al. Int J Immunopathol Pharmacol. 2010;23:917–25.
Farid RM, et al. Int J Clin Exp Pathol. 2014;7:2707–17.
Fenske W, Wanner C, Allolio B, et al. Copeptin levels associate with cardiovascular events in patients with ESRD and type 2 diabetes mellitus. J Am Soc Nephrol. 2011;22:782–90.
Fenske WK, et al. J Am Soc Nephrol. 2014;25:2376–83.
Foldes G, Horkay F, Szokodi I, et al. Circulating and cardiac levels of apelin, the novel ligand of the orphan receptor APJ, in patients with heart failure. Biochem Biophys Res Commun. 2003;308:480–5.
Gad GI, et al. J Neonatal-Perinatal Med. 2014;7:207–12.
Galanth C, Hus-Citharel A, Li B, et al. Apelin in the control of body fluid homeostasis and cardiovascular functions. Curr Pharm Des. 2012;18:789–98.
Grantham JJ, Torres VE, Chapman AB, et al. Volume progression in polycystic kidney disease. N Engl J Med. 2006;354:2122–30.
Gu JL, et al. Clin Res Cardiol. 2011;100:1069–76.
Higashihara E, Nutahara K, Tanbo M, et al. Does increased water intake prevent disease progression in autosomal dominant polycystic kidney disease? Nephrol Dial Transplant. 2014;29:1710–9.
Hus-Citharel A, Bouby N, Frugiere A, et al. Effect of apelin on glomerular hemodynamic function in the rat kidney. Kidney Int. 2008;74:486–94.
Imbert M, Chabardes D, Montegut M, et al. Vasopressin dependent adenylate cyclase in single segments of rabbit kidney tubule. Pflugers Arch. 1975;357:173–86.
Jard S. Vasopressin receptors. A historical survey. Adv Exp Med Biol. 1998;449:1–13.
Jochberger S, et al. Crit Care Med. 2009;37:476–82.
Kadoglou NP, Lampropoulos S, Kapelouzou A, et al. Serum levels of apelin and ghrelin in patients with acute coronary syndromes and established coronary artery disease- KOZANI STUDY. Transl Res. 2010;155:238–46.
Karadag S, Ozturk S, Gursu M, et al. The relationship between apelin and cardiac parameters in patients on peritoneal dialysis: is there a new cardiac marker? BMC Nephrol. 2014;15:18.
Kawamata Y, Habata Y, Fukusumi S, et al. Molecular properties of apelin: tissue distribution and receptor binding. Biochim Biophys Acta. 2001;1538:162–71.
Lacquaniti A, Bolignano D, Campo S, et al. Malnutrition in the elderly patient on dialysis. Ren Fail. 2009;31:239–45.
Lacquaniti A, Bolignano D, Donato V, et al. Obestatin: a new element for mineral metabolism and inflammation in patients on hemodialysis. Kidney Blood Press Res. 2011;34:104–10.

Lacquaniti A, Buemi F, Lupica R, et al. Can neutrophil gelatinase-associated lipocalin help depict early contrast material-induced nephropathy? Radiology. 2013a;267:86–93.
Lacquaniti A, Donato V, Pintaudi B, et al. "Normoalbuminuric" diabetic nephropathy: tubular damage and NGAL. Acta Diabetol. 2013b;50:935–42.
Lacquaniti A, Chirico V, Lupica R, et al. Apelin and copeptin: two opposite biomarkers associated with kidney function decline and cyst growth in autosomal dominant polycystic kidney disease. Peptides. 2013c;49:1–8.
Lacquaniti A, Altavilla G, Picone A, et al. Apelin beyond kidney failure and hyponatremia: a useful biomarker for cancer disease progression evaluation. Clin Exp Med. 2015;15:97–105.
Li X, et al. Chin Med J. 2013;126:823–7.
Llorens-Cortes C, Moos F. Apelin and vasopressin: two work better than one. J Neuroendocrinol. 2012;24:1085–6.
Malyszko J, et al. Adv Med Sci. 2008;53:32–6.
Malyszko J, et al. Transplant Proc. 2010;42(10):425. 9–62.
Meijer E, Bakker SJ, Halbesma N, et al. Copeptin, a surrogate marker of vasopressin, is associated with microalbuminuria in a large population cohort. Kidney Int. 2010;77:29–36.
Meijer E, Bakker SJ, van der Jagt EJ, et al. Copeptin, a surrogate marker of vasopressin, is associated with disease severity in autosomal dominant polycystic kidney disease. Clin J Am Soc Nephrol. 2011;6:361–8.
Meyer B, et al. Crit Care. 2008;12:P439.
Moreno JP, et al. Liver Int. 2013;33:843–51.
Okada H, Suzuki H, Kanno Y, et al. Effects of vasopressin V1 and V2 receptor antagonists on progressive renal failure in rats. Clin Sci (Lond). 1994;86:399–404.
Preibisz JJ, Sealey JE, Laragh JH, et al. Plasma and platelet vasopressin in essential hypertension and congestive heart failure. Hypertension. 1983;5:I129–38.
Przybylowski P, et al. Transplant Proc. 2010;42:1808–11.
Silva AP, Fragoso A, Silva C, et al. What is the role of apelin regarding cardiovascular risk and progression of renal disease in type 2 diabetic patients with diabetic nephropathy? Biomed Res Int. 2013;2013:247649.
Smith JG, et al. J Am Coll Cardiol. 2010;56:1712–9.
Soriguer F, Garrido-Sanchez L, Garcia-Serrano S, et al. Apelin levels are increased in morbidly obese subjects with type 2 diabetes mellitus. Obes Surg. 2009;19:1574–80.
Szinnai G, Morgenthaler NG, Berneis K, et al. Changes in plasma copeptin, the c-terminal portion of arginine vasopressin during water deprivation and excess in healthy subjects. J Clin Endocrinol Metab. 2007;92:3973–8.
Tangri N, Stevens LA, Schmid CH, et al. Changes in dietary protein intake has no effect on serum cystatin C levels independent of the glomerular filtration rate. Kidney Int. 2011;79:471–7.
Tatemoto K, Hosoya M, Habata Y, et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochem Biophys Res Commun. 1998;251:471–6.
Teitelbaum I, McGuinness S. Vasopressin resistance in chronic renal failure. Evidence for the role of decreased V2 receptor mRNA. J Clin Invest. 1995;96:378–85.
Torres VE, Meijer E, Bae KT, et al. Rationale and design of the TEMPO (Tolvaptan efficacy and safety in management of autosomal dominant polycystic kidney disease and its outcomes) 3–4 study. Am J Kidney Dis. 2011;57:692–9.
Torres VE, Chapman AB, Devuyst O, et al. Tolvaptan in patients with autosomal dominant polycystic kidney disease. N Engl J Med. 2012;367:2407–18.
Velho G, Bouby N, Hadjadj S, et al. Plasma copeptin and renal outcomes in patients with type 2 diabetes and albuminuria. Diabetes Care. 2013;36:3639–45.
Voors AA, von Haehling S, Anker SD, et al. C-terminal pro-vasopressin (copeptin) is a strong prognostic marker in patients with heart failure after an acute myocardial infarction: results from the OPTIMAAL study. Eur Heart J. 2009;30:1187–94.
Zhang BH, Wang W, Wang H, et al. Promoting effects of the adipokine, apelin, on diabetic nephropathy. PLoS One. 2013;8:e60457.

BLyS and APRIL Cytokines as Biomarkers of Kidney Diseases

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Abstract

Kidney damages mediated by the immune system have been reported for glomerular diseases, tubulointerstitial nephritis, and rejection of the allograft posttransplantation. B-lymphocytes have an important role in the pathogenesis for these kidney diseases. There are increasing evidences that TNF-like ligands which regulate B-cell homeostasis such as B-lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL) can be used to detect kidney diseases as well as acute rejection of the kidney allograft. This chapter will review these two ligands, BLyS and APRIL, and its promising prospect in detecting glomerular diseases and acute rejection of the kidney allograft.

BLyS and APRIL are found in the plasma and membrane proteins of macrophages, monocytes, and dendritic cells. Both can activate the proliferation of B-cells and plasma cells. They are expressed on tissues of the lymph node, bone marrow, synovium, and kidney. Even though both cytokines share a common receptor on the B-cells, however, their roles in B-cell activation are significantly different. When BLyS is activated, this will result in severe B-cell hyperplasia in BLyS-transgenic mice. In contrast, when APRIL is activated, there is no effect on the homeostasis of the lymphoid in APRIL-transgenic mice.

BLyS was initially discovered to be a potential therapeutic target for lupus patients. Later, it was shown that both BLyS and APRIL were somehow associated with lupus nephritis. Both cytokines are expressed on the infiltrating monocytes and/or macrophages of patients with lupus nephritis. It remains unknown whether the blockade of both cytokines can be used to treat the lupus disease. However, serum APRIL has been shown to be a useful biomarker in detecting lupus nephritis in patients with a poor prognosis.

In kidney transplantation, B-cell is an important factor for the long-term survival of the kidney allograft. High levels of BLyS or APRIL can activate alloreactive B-cells by inducing antibodies against the donor antigens (donor-specific antibody, DSA) and result in rejection of the allograft. Aside from that, BLyS is an important growth factor for IL-10-producing B-cells known as the

regulatory B-cells. It has been shown that BLyS from the mice can increase a proportion of regulatory B-cells ex vivo. These evidences indicate that BLyS plays a major role in the activation and regulation of the immune system affecting the success and/or outcome of the kidney transplantation.

Keywords
Biomarker • BLyS • BAFF • APRIL • Kidney disease • SLE • Kidney transplantation • B-cell

Abbreviations

ABMR	Antibody-mediated rejection
AKD	Acute kidney disease
APRIL	A proliferation-inducing ligand
BAFF	B-cell-activating factor
BAFF-R	B-cell-activating factor receptor
BCMA	B-cell maturation antigen
BCR	B-cell receptor
BLyS	B-lymphocyte stimulator
CD	Cluster of differentiation
CKD	Chronic kidney disease
CR	Complement receptor
Cr	Creatinine
DSA	Donor-specific antibody
GFR	Glomerular filtration rate
HLA	Human leukocyte antigen
IF/TA	Interstitial fibrosis/tubular atrophy
IL	Interleukin
NSTDA	National Science and Technology Development Agency
PLA2R	M-type phospholipase A2 receptor
SLE	Systemic lupus erythematosus
suPAR	Soluble urokinase-type plasminogen activator receptor
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TNF	Tumor necrosis factor

Key Facts

Key Facts of BLyS and APRIL

- BLyS is required for the early stages of B-cell development, whereas APRIL is needed much later for the survival of the plasma cell.
- BlyS and APRIL are mainly produced by innate immune cells such as macrophages, monocytes, and B-cells.

- BLyS and APRIL contribute to the development of the immune-mediated kidney disease (i.e., SLE) and rejection of the kidney allograft.
- BLyS and APRIL are new biomarkers that can be used to detect active B-cell-mediated kidney disease and antibody-mediated rejection in kidney transplantation.
- BLyS and APRIL are markers for B-cell homeostasis required by both the regulatory and allo-/autoreactive B-cells for their survival.
- Current B-cell target therapy such as belimumab, atacicept, or rituximab can affect the serum levels of BLyS and APRIL.

Key Facts of Lupus Nephritis

- An autoimmune kidney disease that typically affected young adult female.
- A leading cause of secondary glomerular diseases worldwide.
- Typical symptoms and signs are leg edema and foamy urine.
- Autoreactive B-lymphocyte cells play a pivotal role in the mechanism of the disease.

Key Facts of Kidney Transplantation

- Kidney transplantation is the best treatment of end-stage kidney disease.
- The important barrier of kidney transplantation is alloreactive cellular immunity.
- Biomarkers are required to monitor for an immune response to kidney allograft so-called “rejection.”

Definitions

Antibody-mediated rejection (ABMR) Allograft rejection due mainly to anti-HLA antibody.

Anti-HLA Alloantibody to HLA antigen. Patients sensitive to self-antigen will have high levels of anti-HLA.

Autoreactive B-cell B-cells that can differentiate into plasma cells and produce antibody to the self-antigen.

Donor-specific antibody (DSA) Anti-HLA that is specific to the donor HLA.

Glomerulonephritis Glomerular disease is caused by the inflammation of the glomeruli and has the following clinical presentations of proteinuria, red blood cells/casts in the urine, and elevated levels of the serum creatinine.

IL-10-producing B-cell Regulatory B-cell with immunoregulatory function can inhibit inflammation by controlling the production of IL-10. Regulatory B-cell plays an important role in the long-term survival of the allograft post-kidney transplantation.

Immune-mediated kidney disease Kidney disease caused by the immune system (e.g., lupus nephritis, membranoproliferative glomerulonephritis (MPGN), membranous nephropathy).

Immunoregulation The immune mechanism which regulatory cells counterbalance an inflammation. Those cells include regulatory T-lymphocyte and regulatory B-lymphocyte cells.

Renal vasculitis Kidney disease is caused by the inflammation of mainly small vessels (e.g., Wegener granulomatosis and microscopic polyangiitis).

Introduction

Unmet Needs for AKD and CKD

The kidney is one of the most vulnerable organs that can be damaged by several factors. The damages of the kidney function can appear early or much later during the course of the destruction of the kidney and tend to result in end-stage kidney disease. Kidney diseases are simply classified according to its severity which can be either acute or chronic. A 3-month observational period can distinguish between an acute kidney disease (AKD) and the chronic kidney disease (CKD). An impaired kidney function is defined as having a glomerular filtration rate (GFR) less than 60 mL/min/1.73 m^2. The gold standard for determining GFR is via the inulin clearance or radioisotope nuclear clearance (Stevens and Levey 2009). However, the most commonly used kidney function test is the serum creatinine or creatinine clearance. Both tests have long been used in the medical field despite the fact that they are not sensitive enough for detecting early stages of the kidney disease. Also, creatinine is not involved in any part of the processes that causes damage to the kidney so it is not recommended to solely use it to diagnose kidney injury (Shemesh et al. 1985; Yurasov et al. 2005; Meyer-Bahlburg et al. 2008). Thus a more accurate diagnostic test that can specifically detect each type of kidney disease is needed.

This is possible if mechanisms involved in various kidney diseases are targeted. It has been shown that the B-lymphocyte is a major player in causing immune-mediated kidney diseases such as glomerulonephritis, renal vasculitis, or rejection of the kidney allograft (Yurasov et al. 2005; Parsons et al. 2009; Redfield et al. 2011; Wilde et al. 2013). Therefore further investigations of the B-cells have dramatically improved the performances of the diagnostic and prognostic tests for immune-mediated kidney diseases as well as provide better targets for therapy. A perfect example of this can be seen with the treatment for systemic lupus erythematosus (SLE) and nephritis. Because the B-lymphocytes and plasma cells are involved in the

development of SLE and nephritis (Odendahl et al. 2000; Arce et al. 2001; Liu et al. 2011), hence, the only way to counteract that would be by blocking the specific autoreactive B-lymphocytes which have proved to be quite helpful (Sfikakis et al. 2005; Anolik et al. 2007). But it should be mentioned that none of the targeted B-cell treatments have been recognized or approved by the regulatory health agency to treat the disease. Many clinical trials failed to show the benefit of targeted B-cell therapy which can partly be explained by their poor inclusion criteria (Merrill et al. 2010; Furie et al. 2011; Rovin et al. 2012). This can be rectified by stratifying the SLE patients based on their B-cell markers. This technique will allow the detection of a subgroup of SLE patients that will benefit from the targeted therapy. Therefore it is recommended that serum B-cell cytokine levels such as B-lymphocyte stimulator (BLyS) or a proliferation-inducing ligand (APRIL) be used to identify groups of people who will benefit from the B-cell therapy.

Biology of BLyS and APRIL

BLyS and APRIL do not directly destroy the kidney but enhance the activities of the B-cells and plasma cells resulting in the immune-mediated kidney disease. The cytokines are associated with the development of autoreactive B-cell in autoimmune diseases. One of the autoreactive B-cells is the B2 cells which come from the bone marrow and are highly dependent on BLyS and APRIL to survive. The other autoreactive B-cell is the B1 cell or innate immune response B-cell which is less dependent on BLyS and APRIL because it is originated from the fetal liver, the peritoneum, and the mucosa. In general, harmful autoreactive B-cell is controlled by the bone marrow and spleen. During the development of the B-cell, these processes are neutralized by apoptosis or changes in the morphological structure of the B-cell receptor (BCR). Thus the affinity of BCR (BCR engagement) and availability of survival cytokines such as BLyS and APRIL are important for the survival of the B-cell.

It should be noted that APRIL plays a different role compared to that of BLyS during the autoimmune reactivation process (Fig. 1). While BLyS is required earlier during the development of the B-cell, in contrast, the APRIL cytokine is needed much later post-development for the survival of the plasma cell (Mackay and Schneider 2009). The plasma cells of APRIL-deficient mice mature normally but have a poorer survival rate and impaired switching of the isotype class (Dillon et al. 2006; Belnoue et al. 2008; Benson et al. 2008). The functions between BLyS and APRIL are different even though they use the same receptor. BLyS can bind to any of the three receptors at a time: B-cell-activating factor receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) receptor, and B-cell maturation antigen (BCMA) receptor. Among the three receptors, APRIL can bind to TACI and BCMA. The only receptor that is not shared between BLyS and APRIL is the BAFF-R which is expressed on the immature B-cell. BAFF-R is important for the survival of the B-cell and upon its activation produces a large volume of cells that can synthesize proteins and translate mRNAs but cannot undergo cell division (Mackay and Schneider 2009). Unlike BAFF-R, TACI and BCMA are expressed on mature

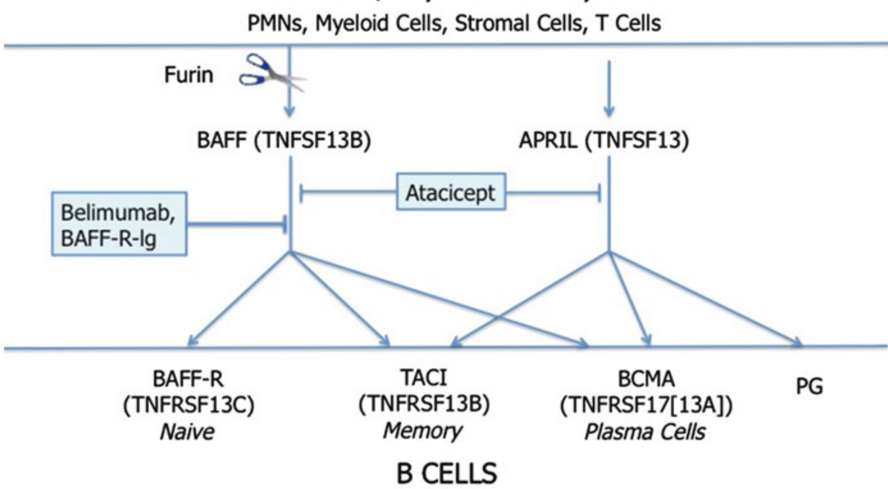

Fig. 1 The interactions between BAFF/BLyS-APRIL and cellular receptors. BAFF appears in the early stages of B-cell differentiation. The survival of the mature B-cell (plasma cell) is highly dependent on APRIL

B-cells and important for switching of the immunoglobulin isotype class as well as the survival of the plasma cell. When BLyS is attached to either TACI or BCMA, it will cause the B-cell to mature because its affinity for BAFF-R is stronger than TACI and BCMA. On the other hand, when APRIL is attached to TACI or BCMA, this will help increase the life-span of the plasma cell because its affinity for BCMA is very strong (Marsters et al. 2000; Day et al. 2005).

Notable characteristics and key features of BLyS and APRIL were derived from the mouse models so are worthy to be mentioned here (Fig. 1):

1. **Animal models for BAFF-R**
   In BAFF-R-deficient mice, BAFF-R is expressed initially on immature B-cells and strongly on follicular and marginal zone B-cells (Meyer-Bahlburg et al. 2008; Hsu et al. 2002; Stadanlick et al. 2008). The expression of BAFF-R increases when the BCR is activated. Upon the activation of BAFF-R, increased levels of CD21 (complement receptor 2, CR2) and an additional signal released by the C3d receptor will help activate B-cells (Gorelik et al. 2004). BAFF –/– mice (BAFF-deficient mice) and transgenic A/WySnJ BAFF-R mice (mutated BLyS rendered to be nonfunctional) have similar characteristics such as impaired maturation of the B-cell, low levels of immunoglobulin, and weaker B-cell immune responses (Mackay et al. 2003; Shulga-Morskaya et al. 2004; Mackay and Leung 2006). When there is an overexpression of BAFF-R activity, this will induce B-cell hyperplasia and phenotypic characteristics of the autoimmune diseases (i.e., glomerulonephritis and destruction of the salivary gland) (Mackay et al. 2005; Groom et al. 2007).

2. **Animal models for APRIL**
   APRIL –/– mice (APRIL-deficient mice) are able to form a normal germinal center formation, but its plasma cells have a shorter life-span (Dillon et al. 2006; Belnoue et al. 2008; Benson et al. 2008). On the other hand, the life-span of B1 cell in the transgenic APRIL mice shows a longer life-span and possibly develops a neoplasia (Dillon et al. 2006). These data support the importance of APRIL as an important survival factor.
3. **Animal models for BCMA and TACI**
   BCMA, a receptor for BLyS and APRIL, is expressed on B-cells of the germinal center, memory B-cells, and plasma cells (Gras et al. 1995; Darce et al. 2007; Hildebrand et al. 2010). BCMA –/– mice (BCMA-deficient mice) have a normal B-cell response and B-cell development, but its plasma cells have a shorter life-span (Xu and Lam 2001; O'Connor et al. 2004).

   TACI is expressed on both mature B-cells and plasma cells. TACI can be activated only by the oligomeric or membrane-bound form of either BLyS or APRIL (Bossen et al. 2008). TACI –/– mice (TACI-deficient mice) have excessive amount of B-cells, B-cell hyperplasias, overproduction of autoantibodies, and an extensive presence of B-cell lymphomas (Mackay and Schneider 2008).

Current Biomarkers Used to Diagnose and Prognose Kidney Diseases

Physicians or nephrologists usually require certain laboratory tests for diagnosing or prognosing the kidney diseases. For example, serum creatinine has been used to determine the kidney function but its interpretations are quite limiting. A patient with a subtle kidney disease or focal histological changes may have a normal level of the serum creatinine or kidney function. Hence, the measurement of serum creatinine level alone cannot detect any significant changes of the kidney function (Doolan et al. 1962; Levey 1990; van Acker et al. 1992).

Another biomarker used to diagnose glomerular disease is the use of albuminuria. One of its limitations is that it is unable to differentiate the scars of the kidney from the presence of active disease. High levels of albuminuria are associated with having an active disease which requires higher doses of immunosuppressant such as steroids (Yamagata et al. 1996). This can ultimately harm the patient unnecessarily if the wrong diagnosis is made.

Other biomarkers such as urine cells or sediments can also be used to detect glomerular diseases. These cells can be easily seen with a simple microscope but it requires a skilled medical technician. However, the red blood cells or white blood cells from the urine can appear during an infection of the urinary bladder, tumor, or inflammation making it difficult to correctly diagnose for glomerular diseases. In addition, the urine cells can also be found in the kidney stones, the ureter, or the area up to the kidney so it cannot be used to specifically detect for glomerular diseases.

However, the gold standard for diagnosing glomerular diseases is the kidney biopsy because histopathology can provide the basic pathology of the glomerular

disease and the area of the damaged tissues. Unfortunately its invasive procedure can result in various complications that can range from mild hematuria to serious bleeding. Yet like the other biomarkers previously discussed, one of its limitations is that it is a cross-sectional observation so it is difficult to predict the course of glomerular disease. Furthermore, if the condition of the patient becomes worse or resistant to treatment, another biopsy will be required. Many clinicians try to avoid repeating the procedure and most of the patients will also refuse to have it done again.

As a result of this, there is an urgent need for noninvasive techniques utilizing biomarkers that can clinically and accurately detect kidney diseases. An ideal biomarker should be accurate, reproducible, and not invasive. Such a biomarker has not been discovered but there are many promising biomarker candidates. The most interesting biomarkers are from soluble proteins that are involved in the mechanisms of the glomerular diseases such as lupus nephritis' anti-DNA antibody (Cortes-Hernandez et al. 2004; Forger et al. 2004; Alba et al. 2003), membranous nephropathy's anti-M-type phospholipase A2 receptor (anti-PLA2R) antibody (Beck et al. 2009), or focal segmental glomerulosclerosis' soluble urokinase-type plasminogen activator receptor (suPAR) (Wei et al. 2012; Li et al. 2014).

BLyS and APRIL as a Therapeutic Biomarker to Guide the Treatment for Kidney Diseases

The biomarkers that are of interest are BLyS and APRIL because both are involved in the mechanisms of SLE and lupus kidney disease. Serum BLyS level is associated with SLE disease activity and complement levels (Table 1, Fig. 2). Hence the blockage of BLyS can attenuate the disease activity (Morimoto et al. 2007; Vincent et al. 2014). In contrast, the level of serum APRIL is associated with lupus nephritis (Table 1, Fig. 2). When the cutoff level for serum APRIL was used at 3.6 ng/mL, it proved to be very useful in predicting resistance to treatment (Treamtrakanpon et al. 2012; Eilertsen and Nossent 2014) as well as difficult-to-treat lupus nephritis patients. High levels of serum APRIL are indicative of active nephritis and associated with resistance to steroids and cyclophosphamide therapy. The expression of APRIL in the kidney tissue can identify refractory nephritis (Treamtrakanpon et al. 2012). Based on these information, a new schema using BLyS and APRIL has been proposed to guide the treatment for kidney diseases (Fig. 3).

Other Roles of BLyS

One of the factors in having successful kidney transplantation is dependent on the use of immunosuppressant agents at the right dosage. Overdose of these agents can result in infections and cancers, whereas underusage can result in the loss of the allograft. Currently, therapeutic drug monitoring of the immunosuppressive drug is the standard of care for managing kidney transplantation. However, the pharmacokinetic monitoring of the drug cannot predict the future biological changes of the

Table 1 Serum levels of BLyS and APRIL in SLE patients

BLyS			APRIL		
	Spearman *r*	*p*-value		Spearman *r*	*p*-value
R_SLEDAI	0.255	0.057	Duration of disease	0.353	0.017*
Anti-dsDNA	−0.362	0.090	R_SLEDAI	0.219	0.148
C3	0.560	0.002*	Uprotein	0.438	0.002*
C4	0.621	0.002*	WBC	0.267	0.061
CH50	0.568	0.003*	PMN	0.262	0.006
WBC	−0.485	<0.001*	Activity score	0.335	0.017*
PMN	−0.402	<0.001*			
Lymphocyte	−0.417	0.002*			
Prednisolone (dose)	0.543	<0.001*			
Mycophenolate (dose)	0.369	0.007*			
ACEI (dose)	0.272	0.051			
BLyS at 6 months apart	0.580	0.005*			

Serum BLyS and APRIL levels are associated with different stages of systemic lupus erythematosus (SLE) and lupus nephritis. Serum APRIL is associated with kidney activity of lupus nephritis (i.e., urine protein, active score of renal pathology). In contrast, serum BAFF is associated with the activity indices of systemic lupus disease (i.e., serum complement levels, total leukocyte count, and renal systemic lupus erythematosus disease activity index (R_SLEDAI))

kidney allograft. Hence a better understanding of the allograft rejection and regulation of the immune system can provide newer methods to appropriately monitor the outcome of the kidney transplantation (Fig. 4).

It has been known for a long time that the immunosuppression of the T-cell contributes to the rejection of the allograft. This information is used to prevent the rejection of the kidney allograft. Over the years, there have been many new advances in the field of T-cell immunology and immunosuppression. Despite this, only short-term graft outcomes have improved. To date, long-term graft outcome and the condition of the patient have not changed (Kasiske et al. 2005). The reason for this may be due to the involvement of other parts of the immune system. Recent studies highlighted the role of the humoral response, such as the B-cells, plasma cells, and anti-HLA, as the key factor in providing a successful long-term outcome of the kidney allograft (Everly et al. 2013; Wiebe and Nickerson 2013). Previous murine cardiac allograft model showed that the BLyS-deficient mice have a longer allograft survival rate compared to the wild type (Ye et al. 2004). In humans, the presence of BLyS in the recipients' kidney allograft was associated with acute rejection. The interstitial fibrosis/tubular atrophy (IF/TA) of the graft can be detected when stained with C4d (Xu et al. 2009). These studies confirm the role of BLyS in transplantation. BLyS has been proposed to be used as a noninvasive monitoring biomarker for both pre- and post-kidney transplantation. Clinical trials manipulating the levels of BLyS by anti-BLyS agents are still ongoing.

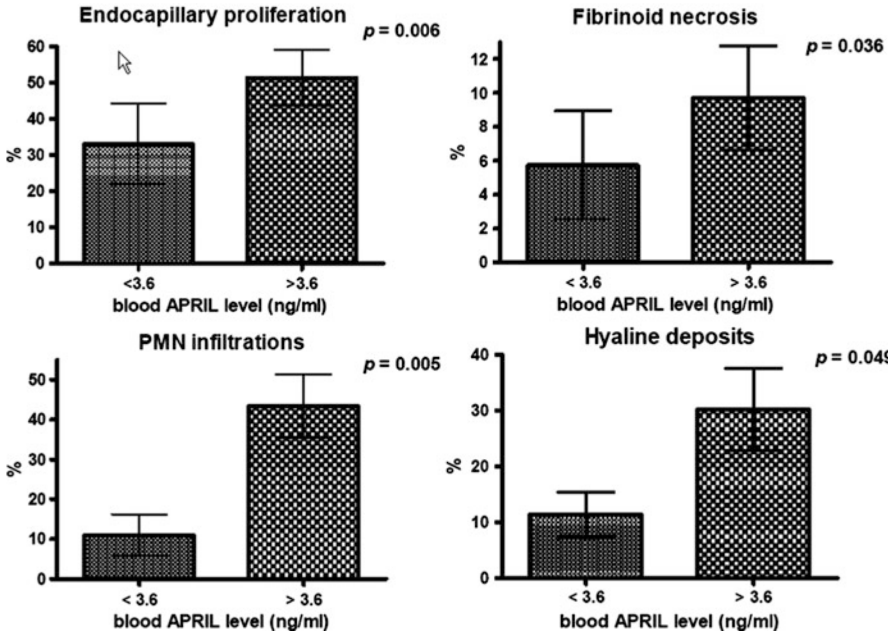

Fig. 2 An association of serum APRIL and pathology of the lupus nephritis. For patients with SLE, a level of APRIL more than 3.6 ng/mL is associated with active renal pathology which consists of endocapillary proliferation, fibrinoid necrosis, infiltrations of the polymorphonuclear (PMN) cells, and depositions of hyaline in the glomerular capillaries

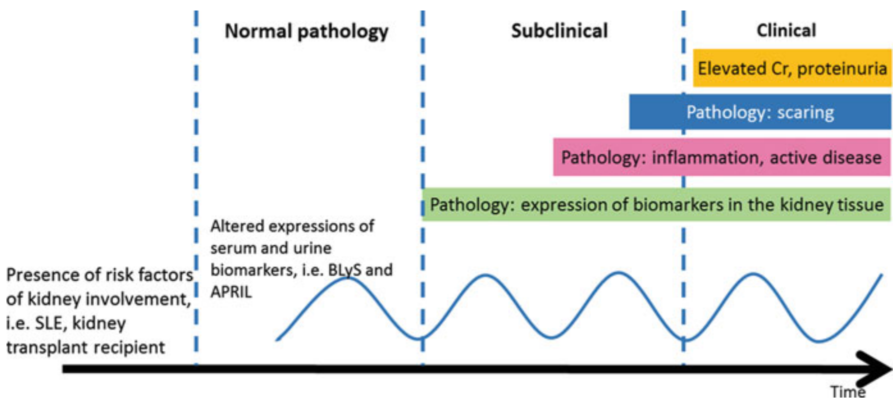

Fig. 3 Biomarker-guided strategy. Biomarker-guided strategy can provide early diagnosis and appropriate treatment for the kidney diseases. The early stages of the kidney diseases can be detected by serum levels of BLyS and APRIL or urine cytokines. However, the subclinical pathology occurs at the late stage so an invasive tissue biopsy is needed. An alternative noninvasive method currently utilizes the serum creatinine to diagnose the late stage of kidney disease. The prognosis of the kidney disease becomes more accurate when the biomarkers are used together to diagnose and prognose the disease

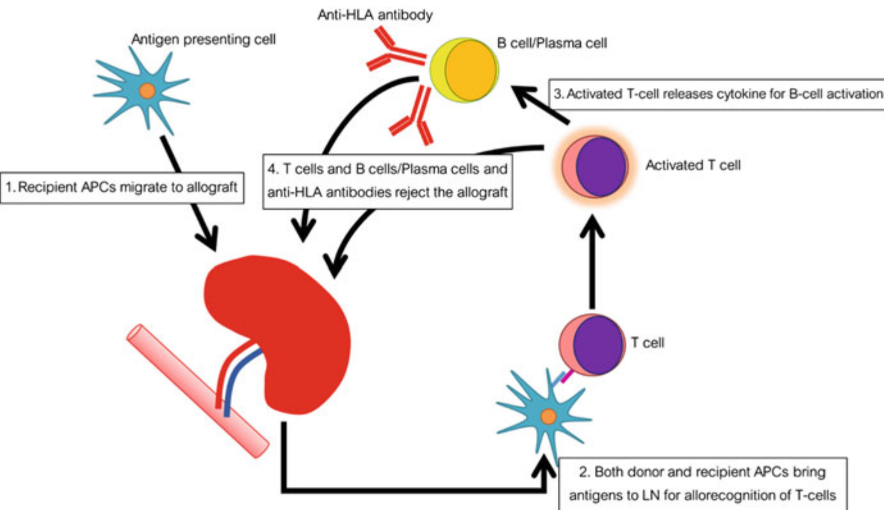

Fig. 4 Mechanism of antibody-mediated kidney allograft rejection. Recipient's antigen-presenting cells recognize donor antigen. The antigen-presenting cells bring antigens to regional lymph nodes. Alloreactive T-cells recognize donor antigen. The alloreactive immune response is activated. B-cells differentiate to plasma cells and produce antibodies against donor HLA. The anti-HLA antibodies, produced by plasma cells, continuously injure the kidney graft

Factors that Affect the BLyS Level During Kidney Transplantation

The production of BLyS by innate immune cells and consumption of BLyS by various B-cells at different stages of development can affect the levels of BLyS. High levels of BLyS indicate an increased production and/or decreased consumption of BLyS by B-cells (Fig. 5). Besides this, other important factors that affect the BLyS levels after a kidney transplantation are the characteristics of the recipients and the duration posttransplantation.

Levels of BLyS Pretransplantation

There are various degrees of sensitized patients that can affect the pretransplant BLyS level. Patients with high titer of anti-HLA antibodies also have high BLyS levels, but this has not been positively correlated with the rejection rate posttransplantation (Snanoudj et al. 2014). However, there are a subgroup of patients who have a higher pre-desensitization or pretransplant BLyS level which was positively correlated with the amount of posttransplant antibody-mediated rejection (ABMR) (Banham et al. 2013). These findings indicate that the recipient with high pretransplant BLyS levels will need a more intensive pretransplant desensitization protocol and immunosuppressive regimen. In addition, anti-BLyS treatment may be beneficial for these groups of kidney transplant recipients.

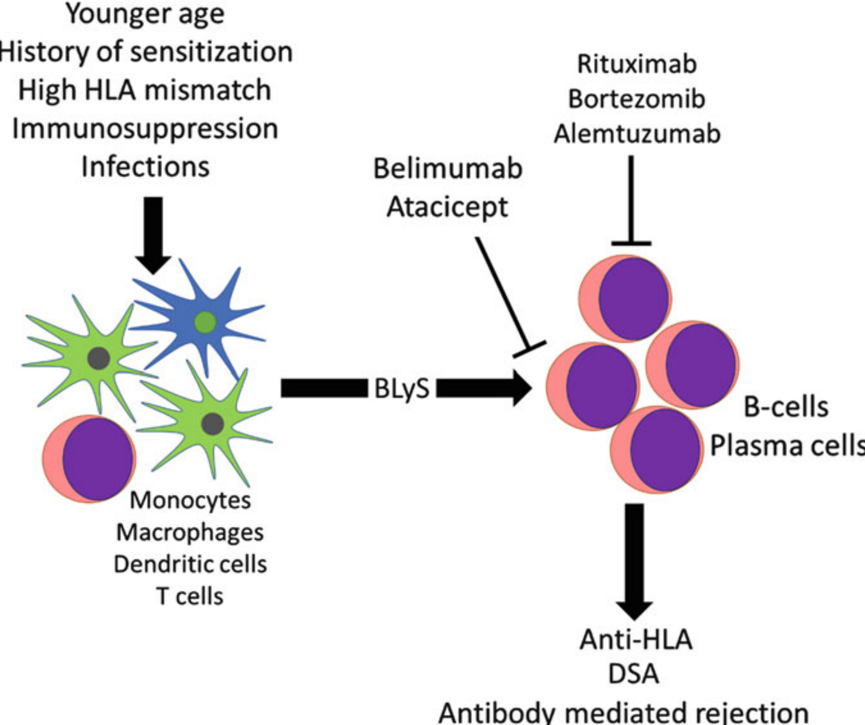

Fig. 5 Factors associated with B-lymphocyte stimulator (BLyS) factor. B-lymphocyte stimulator (BLyS) factor is the key factor for the development of plasma cells and production of antibodies. The innate immune responses enhance BLyS levels, whereas immunosuppressive drugs inhibit the production of BLyS. Immunosuppressive drugs currently used to inhibit the activity of BlyS are belimumab and atacicept. Other immunosuppressive drugs such as rituximab, bortezomib, and alemtuzumab are used to suppress the antibody production of B-cells/plasma cells

BLyS Levels Posttransplantation

Thibault-Espitia A et al. assessed the BLyS level in 143 recipients posttransplantation and showed that there was an association between the high levels of BLyS and the development of the donor-specific antibody (DSA) (Thibault-Espitia et al. 2012). However, the trial was a cross-sectional study and did not mention the use of any immunosuppressant agent such as rituximab which can also alter the BLyS level. Moreover, the study did not show the BLyS level prior to transplantation. In another study, recipients who received antirejection therapy such as rituximab had a significant peak of BLyS levels at 3 months posttreatment (Zarkhin et al. 2009). The high BLyS levels were positively correlated with more than 6 months of B-cell depletion after therapy. These results confirm that the B-cells can lower the levels of BLyS by consuming it. On the other hand, patients treated with a different immunosuppressant agent such as alemtuzumab had a higher rate of

ABMR and excessive amount of serum BLyS (Bloom et al. 2009). The BLyS levels from the alemtuzumab-treated patients did not correlate with the amount of CD20 B-cells. This can be explained by the effects of alemtuzumab which has an activity against B-cells as well as other innate immune cells. On the other hand, data from a well-designed, longitudinal study of BLyS levels in pediatric recipients found no correlation between BLyS levels and the development of de novo DSA. Therefore the monitoring of BLyS could not be used to predict the development of DSA and chronic ABMR in the pediatric recipients (Comoli et al. 2015). However, the recipients with and without de novo DSA have similar BLyS kinetics. The BLyS levels are seen to gradually increase in the first 12 months after transplantation and eventually reach a plateau indicating the presence of early pan-activated B-cells in both unsensitized and previously sensitized patients. Hence BLyS may contribute to the rejection or tolerance of the kidney allograft.

Immunoregulation of BLyS

The immune system has a mechanism to counterbalance all types of immune responses. When the immune system is activated, there are regulatory systems that counterbalance the inflammatory immune response. There are evidences that regulatory B-cells can influence the levels of BLyS. This regulatory function is of concern especially during the anti-BLyS therapy. ELISPOT data showed that the regulatory IL-10-producing B-cells were significantly abundant when B-cells were cocultured with BLyS. Mice treated with BLyS also had an increased amount of IL-10-producing B-cells (Yang et al. 2010). The regulatory role of BLyS was later confirmed in a placebo-controlled, double-blind phase 2 trial of atacicept, a BLyS signal inhibitor. This study was conducted in patients with multiple sclerosis whose clinical activity peaked and relapsed after receiving atacicept (Kappos et al. 2014). As a result of this, the manipulation of the BLyS system with an inhibitor is a double-edged sword that can affect both the activated B-cells and regulatory B-cells.

BLyS as a Biomarker for Transplantation

To date, BLyS can significantly impact the outcome of the kidney transplantation. The concerns to use BLyS as a biomarker for the kidney transplantation are discussed here. First, the production of BLyS from the innate immune system is counterbalanced by the B-cells' consumption of BLyS. To further complicate the matter, high levels of BLyS are not always associated with B-cell activation, but a direct inhibition of the B-cells can increase the BLyS level by reducing the consumption of BLyS. Second, there are evidences that BLyS can regulate the immune system. BLyS can activate B-cells, plasma cells, and regulatory B-cells of the immune system. Certain, favorable conditions of the BLyS level can induce tolerance posttransplantation.

Thus additional studies of the BLyS levels in different, specific conditions are needed to rule out the potential effects of the confounding factors and assess the

benefits of using this noninvasive method to monitor the outcome of the transplantation. Since there is no single best biomarker, hence, the monitoring of BLyS should be done with other biomarkers and interpreted by correlating the results to the clinical data. A longitudinal follow-up of the BLyS level may yield future potential benefits when monitoring the immune system posttransplantation.

Potential Applications to Prognosis, Other Diseases, or Conditions

BLyS and APRIL may in the future be used to diagnose various B-cell-/plasma cell-related diseases such as multiple myeloma, mixed cryoglobulinemia, and other autoimmune diseases including Sjögren's syndrome and multiple sclerosis. The level of serum BLyS may be useful in predicting the activity and severity of the disease. Additional studies looking at serial monitorings of BLyS are warranted for future treatment guidelines.

For example, the combined use of BLyS levels and donor-specific antibody (DSA) may enhance the accuracy to detect antibody-mediated rejection of the graft. DSA alone can only detect ABMR in one-third of the patients (Wu et al. 2013). So a combination of its use with BLyS may be useful for future diagnostic tests of solid organ transplantation.

As for APRIL, its other uses may be its ability to prognose various B-cell-mediated diseases. The reason for this is based on the data obtained from Treamtrakanpon et al. who showed that a high serum APRIL level was associated with treatment failure and early loss of kidney function (Treamtrakanpon et al. 2012). There are evidences that have shown APRIL's ability to accurately prognose lupus kidney disease. Hence APRIL is considered a good prognostic biomarker for lupus kidney disease. Thus the use of APRIL should also be beneficial for other diseases caused by memory and/or plasma cells.

Conclusions

Biomarkers to detect kidney diseases are an unmet medical need. Noninvasive tests can be repetitively used to safely monitor the injury of the kidney. Levels of B-cell-activating cytokines may be potential candidates for detecting immune-mediated kidney injury.

Summary Points

- BLyS and APRIL are key cytokines of B-cell growth and maturation.
- The effects of the innate immune system and homeostasis of the B-cells affect the serum levels of BLyS and APRIL.

- B-cell markers such as BLyS and APRIL cytokines are good examples of biomarkers that can be used to prognose lupus kidney disease and outcome of the kidney transplantation.
- Data interpretations of BLyS biomarkers should be done per patient together with clinical data and other biomarkers to increase the accuracy of the prognosis for patients with kidney diseases or those who have had a kidney transplantation.
- BLyS biomarkers can be used to diagnose, prognose, and help guide the treatment for B-cell-associated kidney disease particularly lupus nephritis and kidney transplantation.

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References

Alba P, Bento L, Cuadrado MJ, Karim Y, Tungekar MF, Abbs I, Khamashta MA, D'Cruz D, Hughes GR. Anti-dsDNA, anti-Sm antibodies, and the lupus anticoagulant: significant factors associated with lupus nephritis. Ann Rheum Dis. 2003;62(6):556–60.

Anolik JH, Barnard J, Owen T, Zheng B, Kemshetti S, Looney RJ, Sanz I. Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. Arthritis Rheum. 2007;56(9):3044–56.

Arce E, Jackson DG, Gill MA, Bennett LB, Banchereau J, Pascual V. Increased frequency of pre-germinal center B cells and plasma cell precursors in the blood of children with systemic lupus erythematosus. J Immunol. 2001;167(4):2361–9.

Banham G, Prezzi D, Harford S, Taylor CJ, Hamer R, Higgins R, Bradley JA, Clatworthy MR. Elevated pretransplantation soluble BAFF is associated with an increased risk of acute antibody-mediated rejection. Transplantation. 2013;96(4):413–20.

Beck Jr LH, Bonegio RG, Lambeau G, Beck DM, Powell DW, Cummins TD, Klein JB, Salant DJ. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med. 2009;361(1):11–21.

Belnoue E, Pihlgren M, McGaha TL, Tougne C, Rochat AF, Bossen C, Schneider P, Huard B, Lambert PH, Siegrist CA. APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells. Blood. 2008;111(5):2755–64.

Benson MJ, Dillon SR, Castigli E, Geha RS, Xu S, Lam KP, Noelle RJ. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J Immunol. 2008;180(6):3655–9.

Bloom D, Chang Z, Pauly K, Kwun J, Fechner J, Hayes C, Samaniego M, Knechtle S. BAFF is increased in renal transplant patients following treatment with alemtuzumab. Am J Transplant. 2009;9(8):1835–45.

Bossen C, Cachero TG, Tardivel A, Ingold K, Willen L, Dobles M, Scott ML, Maquelin A, Belnoue E, Siegrist CA, Chevrier S, Acha-Orbea H, Leung H, Mackay F, Tschopp J, Schneider P. TACI, unlike BAFF-R, is solely activated by oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts. Blood. 2008;111(3):1004–12.

Comoli P, Quartuccio G, Cioni M, Parodi A, Nocera A, Basso S, Fontana I, Magnasco A, Sioli V, Guido I, Klersy C, Zecca M, Cardillo M, Ghiggeri GM, Ginevri F. Posttransplant soluble B-cell activating factor kinetics in pediatric recipients of first kidney allograft. Transplantation. 2015;99(1):243–9.

Cortes-Hernandez J, Ordi-Ros J, Labrador M, Bujan S, Balada E, Segarra A, Vilardell-Tarres M. Antihistone and anti-double-stranded deoxyribonucleic acid antibodies are associated with renal disease in systemic lupus erythematosus. Am J Med. 2004;116(3):165–73.

Darce JR, Arendt BK, Wu X, Jelinek DF. Regulated expression of BAFF-binding receptors during human B cell differentiation. J Immunol. 2007;179(11):7276–86.

Day ES, Cachero TG, Qian F, Sun Y, Wen D, Pelletier M, Hsu YM, Whitty A. Selectivity of BAFF/BLyS and APRIL for binding to the TNF family receptors BAFFR/BR3 and BCMA. Biochemistry. 2005;44(6):1919–31.

Dillon SR, Gross JA, Ansell SM, Novak AJ. An APRIL to remember: novel TNF ligands as therapeutic targets. Nat Rev Drug Discov. 2006;5(3):235–46.

Doolan PD, Alpen EL, Theil GB. A clinical appraisal of the plasma concentration and endogenous clearance of creatinine. Am J Med. 1962;32:65–79.

Eilertsen GO, Nossent JC. APRIL levels strongly correlate with IL-17 in systemic lupus erythematosus. Lupus. 2014;23(13):1383–91.

Everly MJ, Rebellato LM, Haisch CE, Ozawa M, Parker K, Briley KP, Catrou PG, Bolin P, Kendrick WT, Kendrick SA, Harland RC, Terasaki PI. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. Transplantation. 2013;95(3):410–7.

Forger F, Matthias T, Oppermann M, Becker H, Helmke K. Clinical significance of anti-dsDNA antibody isotypes: IgG/IgM ratio of anti-dsDNA antibodies as a prognostic marker for lupus nephritis. Lupus. 2004;13(1):36–44.

Furie R, Petri M, Zamani O, Cervera R, Wallace DJ, Tegzova D, Sanchez-Guerrero J, Schwarting A, Merrill JT, Chatham WW, Stohl W, Ginzler EM, Hough DR, Zhong ZJ, Freimuth W, van Vollenhoven RF. A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. Arthritis Rheum. 2011;63(12):3918–30.

Gorelik L, Cutler AH, Thill G, Miklasz SD, Shea DE, Ambrose C, Bixler SA, Su L, Scott ML, Kalled SL. Cutting edge: BAFF regulates CD21/35 and CD23 expression independent of its B cell survival function. J Immunol. 2004;172(2):762–6.

Gras MP, Laabi Y, Linares-Cruz G, Blondel MO, Rigaut JP, Brouet JC, Leca G, Haguenauer-Tsapis R, Tsapis A. BCMAp: an integral membrane protein in the Golgi apparatus of human mature B lymphocytes. Int Immunol. 1995;7(7):1093–106.

Groom JR, Fletcher CA, Walters SN, Grey ST, Watt SV, Sweet MJ, Smyth MJ, Mackay CR, Mackay F. BAFF and MyD88 signals promote a lupuslike disease independent of T cells. J Exp Med. 2007;204(8):1959–71.

Hildebrand JM, Luo Z, Manske MK, Price-Troska T, Ziesmer SC, Lin W, Hostager BS, Slager SL, Witzig TE, Ansell SM, Cerhan JR, Bishop GA, Novak AJ. A BAFF-R mutation associated with non-Hodgkin lymphoma alters TRAF recruitment and reveals new insights into BAFF-R signaling. J Exp Med. 2010;207(12):2569–79.

Hsu BL, Harless SM, Lindsley RC, Hilbert DM, Cancro MP. Cutting edge: BLyS enables survival of transitional and mature B cells through distinct mediators. J Immunol. 2002;168(12):5993–6.

Kappos L, Hartung HP, Freedman MS, Boyko A, Radu EW, Mikol DD, Lamarine M, Hyvert Y, Freudensprung U, Plitz T, van Beek J. Atacicept in multiple sclerosis (ATAMS): a randomised, placebo-controlled, double-blind, phase 2 trial. Lancet Neurol. 2014;13(4):353–63.

Kasiske BL, Gaston RS, Gourishankar S, Halloran PF, Matas AJ, Jeffery J, Rush D. Long-term deterioration of kidney allograft function. Am J Transplant. 2005;5(6):1405–14.

Levey AS. Measurement of renal function in chronic renal disease. Kidney Int. 1990;38(1):167–84.

Li F, Zheng C, Zhong Y, Zeng C, Xu F, Yin R, Jiang Q, Zhou M, Liu Z. Relationship between serum soluble urokinase plasminogen activator receptor level and steroid responsiveness in FSGS. Clin J Am Soc Nephrol. 2014;9(11):1903–11.

Liu Z, Zou Y, Davidson A. Plasma cells in systemic lupus erythematosus: the long and short of it all. Eur J Immunol. 2011;41(3):588–91.

Mackay F, Leung H. The role of the BAFF/APRIL system on T cell function. Semin Immunol. 2006;18(5):284–9.

Mackay F, Schneider P. TACI, an enigmatic BAFF/APRIL receptor, with new unappreciated biochemical and biological properties. Cytokine Growth Factor Rev. 2008;19(3–4):263–76.

Mackay F, Schneider P. Cracking the BAFF code. Nat Rev Immunol. 2009;9(7):491–502.

Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. Annu Rev Immunol. 2003;21:231–64.

Mackay F, Sierro F, Grey ST, Gordon TP. The BAFF/APRIL system: an important player in systemic rheumatic diseases. Curr Dir Autoimmun. 2005;8:243–65.

Marsters SA, Yan M, Pitti RM, Haas PE, Dixit VM, Ashkenazi A. Interaction of the TNF homologues BLyS and APRIL with the TNF receptor homologues BCMA and TACI. Curr Biol. 2000;10(13):785–8.

Merrill JT, Burgos-Vargas R, Westhovens R, Chalmers A, D'Cruz D, Wallace DJ, Bae SC, Sigal L, Becker JC, Kelly S, Raghupathi K, Li T, Peng Y, Kinaszczuk M, Nash P. The efficacy and safety of abatacept in patients with non-life-threatening manifestations of systemic lupus erythematosus: results of a twelve-month, multicenter, exploratory, phase IIb, randomized, double-blind, placebo-controlled trial. Arthritis Rheum. 2010;62(10):3077–87.

Meyer-Bahlburg A, Andrews SF, Yu KO, Porcelli SA, Rawlings DJ. Characterization of a late transitional B cell population highly sensitive to BAFF-mediated homeostatic proliferation. J Exp Med. 2008;205(1):155–68.

Morimoto S, Nakano S, Watanabe T, Tamayama Y, Mitsuo A, Nakiri Y, Suzuki J, Nozawa K, Amano H, Tokano Y, Kobata T, Takasaki Y. Expression of B-cell activating factor of the tumour necrosis factor family (BAFF) in T cells in active systemic lupus erythematosus: the role of BAFF in T cell-dependent B cell pathogenic autoantibody production. Rheumatology (Oxford). 2007;46(7):1083–6.

O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, Lin LL, Mantchev GT, Bram RJ, Noelle RJ. BCMA is essential for the survival of long-lived bone marrow plasma cells. J Exp Med. 2004;199(1):91–8.

Odendahl M, Jacobi A, Hansen A, Feist E, Hiepe F, Burmester GR, Lipsky PE, Radbruch A, Dorner T. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. J Immunol. 2000;165(10):5970–9.

Parsons RF, Vivek K, Redfield RR, Migone TS, Cancro MP, Naji A, Noorchashm H. B-cell tolerance in transplantation: is repertoire remodeling the answer? Expert Rev Clin Immunol. 2009;5(6):703.

Redfield 3rd RR, Rodriguez E, Parsons R, Vivek K, Mustafa MM, Noorchashm H, Naji A. Essential role for B cells in transplantation tolerance. Curr Opin Immunol. 2011;23(5):685–91.

Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J, Maciuca R, Zhang D, Garg JP, Brunetta P, Appel G. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis Rheum. 2012;64(4):1215–26.

Sfikakis PP, Boletis JN, Lionaki S, Vigklis V, Fragiadaki KG, Iniotaki A, Moutsopoulos HM. Remission of proliferative lupus nephritis following B cell depletion therapy is preceded by down-regulation of the T cell costimulatory molecule CD40 ligand: an open-label trial. Arthritis Rheum. 2005;52(2):501–13.

Shemesh O, Golbetz H, Kriss JP, Myers BD. Limitations of creatinine as a filtration marker in glomerulopathic patients. Kidney Int. 1985;28(5):830–8.

Shulga-Morskaya S, Dobles M, Walsh ME, Ng LG, MacKay F, Rao SP, Kalled SL, Scott ML. B cell-activating factor belonging to the TNF family acts through separate receptors to support B cell survival and T cell-independent antibody formation. J Immunol. 2004;173(4):2331–41.

Snanoudj R, Candon S, Roelen DL, Jais JP, Claas FH, Legendre C, Chatenoud L. Peripheral B-cell phenotype and BAFF levels are associated with HLA immunization in patients awaiting kidney transplantation. Transplantation. 2014;97(9):917–24.

Stadanlick JE, Kaileh M, Karnell FG, Scholz JL, Miller JP, Quinn 3rd WJ, Brezski RJ, Treml LS, Jordan KA, Monroe JG, Sen R, Cancro MP. Tonic B cell antigen receptor signals supply an NF-kappaB substrate for prosurvival BLyS signaling. Nat Immunol. 2008;9(12):1379–87.

Stevens LA, Levey AS. Measured GFR as a confirmatory test for estimated GFR. J Am Soc Nephrol. 2009;20(11):2305–13.

Thibault-Espitia A, Foucher Y, Danger R, Migone T, Pallier A, Castagnet S, G-Gueguen C, Devys A, C-Gautier A, Giral M, Soulillou JP, Brouard S. BAFF and BAFF-R levels are associated with risk of long-term kidney graft dysfunction and development of donor-specific antibodies. Am J Transplant. 2012;12(10):2754–62.

Treamtrakanpon W, Tantivitayakul P, Benjachat T, Somparn P, Kittikowit W, Eiam-ong S, Leelahavanichkul A, Hirankarn N, Avihingsanon Y. APRIL, a proliferation-inducing ligand, as a potential marker of lupus nephritis. Arthritis Res Ther. 2012;14(6):R252.

van Acker BA, Koomen GC, Koopman MG, de Waart DR, Arisz L. Creatinine clearance during cimetidine administration for measurement of glomerular filtration rate. Lancet. 1992;340 (8831):1326–9.

Vincent FB, Morand EF, Schneider P, Mackay F. The BAFF/APRIL system in SLE pathogenesis. Nat Rev Rheumatol. 2014;10(6):365–73.

Wei C, Trachtman H, Li J, Dong C, Friedman AL, Gassman JJ, McMahan JL, Radeva M, Heil KM, Trautmann A, Anarat A, Emre S, Ghiggeri GM, Ozaltin F, Haffner D, Gipson DS, Kaskel F, Fischer DC, Schaefer F, Reiser J. Circulating suPAR in two cohorts of primary FSGS. J Am Soc Nephrol. 2012;23(12):2051–9.

Wiebe C, Nickerson P. Posttransplant monitoring of de novo human leukocyte antigen donor-specific antibodies in kidney transplantation. Curr Opin Organ Transplant. 2013;18(4):470–7.

Wilde B, Thewissen M, Damoiseaux J, Knippenberg S, Hilhorst M, van Paassen P, Witzke O, Cohen Tervaert JW. Regulatory B cells in ANCA-associated vasculitis. Ann Rheum Dis. 2013;72(8):1416–9.

Wu P, Everly MJ, Rebellato LM, Haisch CE, Briley KP, Bolin P, Kendrick WT, Kendrick SA, Morgan C, Harland RC, Terasaki PI. Trends and characteristics in early glomerular filtration rate decline after posttransplantation alloantibody appearance. Transplantation. 2013;96(10):919–25.

Xu S, Lam KP. B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses. Mol Cell Biol. 2001;21 (12):4067–74.

Xu H, He X, Sun J, Shi D, Zhu Y, Zhang X. The expression of B-cell activating factor belonging to tumor necrosis factor superfamily (BAFF) significantly correlated with C4D in kidney allograft rejection. Transplant Proc. 2009;41(1):112–6.

Yamagata K, Yamagata Y, Kobayashi M, Koyama A. A long-term follow-up study of asymptomatic hematuria and/or proteinuria in adults. Clin Nephrol. 1996;45(5):281–8.

Yang M, Sun L, Wang S, Ko KH, Xu H, Zheng BJ, Cao X, Lu L. Novel function of B cell-activating factor in the induction of IL-10-producing regulatory B cells. J Immunol. 2010;184(7):3321–5.

Ye Q, Wang L, Wells AD, Tao R, Han R, Davidson A, Scott ML, Hancock WW. BAFF binding to T cell-expressed BAFF-R costimulates T cell proliferation and alloresponses. Eur J Immunol. 2004;34(10):2750–9.

Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, Nussenzweig MC. Defective B cell tolerance checkpoints in systemic lupus erythematosus. J Exp Med. 2005;201(5):703–11.

Zarkhin V, Li L, Sarwal MM. BAFF may modulate the rate of B-cell repopulation after rituximab therapy for acute renal transplant rejection. Transplantation. 2009;88(10):1229–30.

Erythrocyte Glutathione Transferase as a Biomarker in Kidney Health and Disease

26

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Abstract

Erythrocyte glutathione transferase (e-GST), an enzyme involved in the detoxification of endogenous and exogenous toxic compounds, is overexpressed in chronic kidney disease (CKD) patients. e-GST transcription increases linearly along the stages of kidney disease progression, and a positive correlation with the extent of hyperhomocysteinemia, a characteristic defect of thiol metabolism in CKD, has consistently been demonstrated. e-GST expression varied in the comparison between patients treated with pure diffusive and convective dialytic procedures, and preliminary data demonstrate the persistence of increased e-GST levels in kidney transplanted patients.

Analogously with the use of glycated hemoglobin in the retrospective monitoring of blood glucose in diabetic patients, e-GST may serve as an indicator over a temporal span of a few weeks of the systemic exposure to uremic toxins, which could be particularly important in verifying the efficacy of depurative techniques and devices. Applications as prognostic marker of toxicological processes and comorbidity in CKD as well as in transplanted patients are worth of further clinical investigation.

Keywords

Biomarker • Erythrocyte glutathione transferase • Maintenance hemodialysis • Blood toxicity • Chronic kidney disease • Kidney transplantation

Abbreviations

ADQI	Acute dialysis quality initiative
AhR	Aryl hydrocarbon receptor
AKI	Acute kidney injury
ARE	Antioxidant-responsive element
ASK1	Apoptosis signal-regulating kinase 1
Bax	BCL-2-associated X protein
C/EBPβ	CAATT/enhancer-binding protein
CAR	Constitutive androstane receptor
CKD	Chronic kidney disease
e-CAT	Erythrocyte catalase
e-GST	Erythrocyte glutathione transferase
EpRE	Electrophile-responsive element
ESRD	End-stage renal disease
eGFR	Estimated glomerular filtration rate
GSH	Glutathione
GSTP1-1	Glutathione transferase class P1-1
Hcy	L-homocysteine
HD	Hemodialysis
HDF	Hemodiafiltration
IL-6, IL-8; IL-18	Interleukin-6, -8, -18
K/DOQI	Kidney disease outcomes quality initiative

KDIGO	Kidney disease improving global outcomes
KIM-1	Kidney injury molecules
L-FABP	LTYPE fatty acid-binding protein
MDRD	Modification of diet in renal disease
MHD	Maintenance hemodialysis
NGAL	Neutrophil gelatinase-associated lipocalin
NHE3	Sodium-hydrogen exchanger 3
Nrf2	NF-E2-related factor 2
PINI	Prognostic inflammatory and nutrition index
PPARγ	Peroxisome proliferator-activated receptor-γ
Prxd-6	Peroxiredoxin-6
PXR	Pregnane X receptor
RBC	Red blood cell
T2DM	Type 2 diabetes mellitus
TNF-α	Tumor necrosis factor-alpha
TRAF2	TNF-receptor associated factor 2

Key Facts

e-GST is involved in the cell detoxification against many toxic compounds.

e-GST can be used as a prognostic biomarker and monitoring tool of human conditions associated with increased exposure to endogeneous toxins or xenobiotics and oxidative stress.

e-GST overexpression is part of the stress adaption response of bone marrow and blood and thus is a natural defense mechanism against endogenous and exogenous stressors (such as uremic toxins, xenobiotics, pollutants, etc.).

e-GST activity may be determined by a rapid, easy, and unexpensive spectrophotometric procedure.

The evauation of e-GST overexpression can be used to assess systemic toxicity with analogy to glycated hemoglobin used in clinical monitoring of blood glucose.

Definitions

Biomarker Natural component (or substance) used to indicate biological events such as a specific disease or the response to a therapy.

Chronic kidney disease Known as chronic renal disease which consists in a progressive loss in renal function.

Dialysis Technology accomplished by a filtration device used in biology and medicine to depurate fluids and solutions such as the same human blood.

e-GST Enzyme expressed in red blood cells devoted to toxic compound detoxifications.

Glomerular filtration rate The glomerular filtration rate is a test to measure the level of kidney function and to determine the stage of kidney disease.

Glutathione Also referred as reduced glutathione (GSH) is a tripeptide containing glutamic acid, cysteine, and glycine, which serves as a reducing agent in many biochemical reactions.

Glycated hemoglobin Glycated hemoglobin is a particular form of hemoglobin synthesized via a nonenzymatic glycation pathway during its exposure to plasma glucose.

Homocysteine A modified amino acid that often increases above normal concentrations in chronic kidney disease patients.

Kt/Vurea A mathematical model that takes into account the urea clearance in a single hemodialysis session to quantify the dose and thus the adequacy of dialysis therapy.

Systemic sclerosis Also known as scleroderma is a chronic connective tissue disease generally classified as one of the autoimmune rheumatic diseases.

Introduction

Acute kidney injury (AKI) and chronic kidney disease (CKD) are conditions associated with poor prognosis and high impact on healthcare systems. Achieving timely and appropriate diagnosis and therapeutic prescription in these patient populations has major impact on morbidity and mortality indices.

Unluckily, available diagnostic and prognostic tools cannot fulfill these goals. Routine laboratory protocols identify macroscopic defects of glomerular filtration and tubular reabsorption, but cannot provide a timely appropriate diagnosis of the complex series of metabolic and toxicologial aspects of the renal disease, and consequently their prognostic value is limited or null. Actually, many of these protocols focus on water balance and small- to middle-size molecule removal, thus completely missing several of the toxicological aspects associated with formation and retention of uremic solutes. Compelling clinical research has revealed that biological and toxicological effects of uremic solutes strongly depend on their reactivity with components of cells and body fluids (Piroddi et al. 2013). Secondary products of this abnormal chemistry, such as lipid-derived reactive carbonyls and protein adducts, are bioactive compounds themselves that produce increasing waves of scavenging and inflammatory responses of tissues. During kidney disease progression, such an adverse chemistry sustains mechanisms of stress and toxicity that spread from renal tissue, in the

earliest phases of the disease, to a systemic level. The consequent chronic-degenerative lesions of tissues further sustain mechanisms of toxicity and the abnormal signaling of uremic tissues. All these aspects of the uremic stress response are disregarded in the clinical investigation and management of AKI and CKD patients.

Even worst the performance of current laboratory protocols in monitoring the efficacy of depurative dialysis procedures remains a simple weighing of solute and water exchanges throughout dialyzer membranes.

Biomarkers of Stress Response in Kidney Disease

Classically, the assessment of glomerular function is based on the measurement of creatinine clearance (Ronco et al. 2010). However, creatinine cannot be considered an ideal biomarker. This should be an endogenous substance that circulates at almost stable concentrations and completely filtered by the kidney and excreted in the urine without being reabsorbed or excreted from the renal tubule. In this respect, the creatinine clearance test has a number of limitations. In fact, creatinine is completely filtered by the glomerulus, but it is also in part reabsorbed and especially excreted by the renal tubule. Moreover, circulating levels of creatinine are influenced by age and body mass, and dietary habits.

Creatinine tests cannot be applied in the course of AKI, since increased values of creatinine occur in presence of a significant, often irreversible, tissue damage. Again, the large functional reserve of the kidney implies that serum creatinine significantly changes only when more than 50 % of the renal function is compromised (Ronco et al. 2008; Cruz et al. 2009). As a consequence biomarkers of kidney injury more specific and sensitive than creatinine are needed (Devarajan 2010a; Parikh et al. 2010).

For this purpose, other biomarkers have been proposed as clinically useful in diagnostic protocols of AKI syndrome, such as cystatin C, KIM-1 (kidney injury molecule-1), NGAL (neutrophil gelatinase-associated lipocalin), NHE3 (sodium-hydrogen exchanger 3), some cytokines (IL-6, IL-8; IL-18), the depolymerizing factor of the actin-actin complex (actin-actin-depolymerizing F lipocalin), L-FABP (LTYPE fatty-acid-binding protein), Netrin-1, and chemokine derived from keratin (keratin-derived chemokine) (Ronco et al. 2008, 2010). However, clinical validation in AKI has been provided and is available in the literature only for cystatin C and NGAL (Devarajan 2010b; Parikh et al. 2010), and reliable unbiased laboratory protocols are not available for the routine clinical assessment of other biomarkers. Finally, most recent guidelines published by the acute dialysis quality initiative (ADQI) (Ronco et al. 2010) reported that only NGAL and cystatin C could be integrated in clinical practice protocols in the near future.

However, cystatin C should be considered an index of kidney function rather than of tubular damage. Recent clinical trials suggest that NGAL is a reliable diagnostic and prognostic biomarker of AKI (Devarajan 2010b; Vaidya et al. 2008; Haase et al. 2009). ADQI guidelines recommend the use of NGAL assay in patients with suspected AKI syndrome, being this an early and reliable marker of renal damage, both in animal models and humans (Ronco et al. 2010).

However, increased circulating levels of NGAL may result from damage or impairment/activity of other organs or tissues, such as cells of the immune system.

As far as CKD concerns, the introduction in 2002 of a classification system based on estimated glomerular filtration rate (eGFR) produced a compelling need for accurate methods to estimate GFR. As a consequence, the Modification of Diet in Renal Disease (MDRD) estimating equation was adopted worldwide (K/DOQI 2004; Levey et al. 2006; KDIGO 2013; Fraser et al. 2015).

The more recently developed Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) serum creatinine equation has been shown to improve accuracy of eGFR estimation over the MDRD equation also improving prediction models for mortality risk and progression rate to end-stage renal disease (ESRD) (Levey et al. 2009; Matsushita et al. 2012). Subsequently, in 2012, Kidney Disease Improving Global Outcomes (KDIGO) recommendations advocate the routine use of the CKD-EPI equation for reporting eGFR levels (KDIGO 2013).

As previously stated, serum creatinine levels are influenced by factors such as diet and muscle mass, and, despite the use of weighting for age, gender, and race in the eGFR estimating equations, this can result in patient misclassification (Stevens et al. 2007, 2010). Serum cystatin C is less influenced by these factors, which suggests a valid alternative for estimating GFR (Tangri et al. 2011; Baxmann et al. 2008).

Moreover, no one of the proposed biomarkers may provide any level of information on extrarenal events of toxicity associated with the uremic condition and the onset and progression of different aspects of CKD comorbidity.

Therefore, the lack of specific biomarkers of the efficiency of the kidney organ as well as of dialysis therapy in depurating the organism from different types of circulating toxins is a major clinical limitation. The ideal biomarker should provide such clinical information in temporal spans not limited to a single day or dialysis session, thereby representing a sort of long-term and versatile biosensor of uremic toxicity. In this context, erythrocyte glutathione transferase (e-GST), an enzyme compartmentalized in the red cells, and then non-dialyzable, seems to be an interesting long-term probe either to assess kidney function or the adequacy of depurative therapies. GSTs represent a superfamily of ubiquitous enzymes devoted to cell protection by promoting the conjugation of glutathione with toxins of very different shapes and chemical nature (Armstrong 1997; Hayes et al. 2005; Awasthi et al. 1994). Furthermore, GSTs may act like ligandins by binding and sequestering a variety of small or large toxic compounds and peptides. Protein-protein interaction properties are also emerging as an important role of GSTP1-1, which appear to control signaling pathways and transcriptional responses of cells (Bartolini and Galli 2016). The apoptotic signaling of Jun-kinase (Wang et al. 2001) and Bax (Kampranis et al. 2000) is under the influence of this interaction. GSTs also modulate calcium channels decreasing the apoptotic mobilization of calcium ions (Dulhunty et al. 2001). Other relevant protein interactions of GSTP in the apoptotic pathway include TNF-α and TNF-receptor associated factor 2 (TRAF2) and downstream the apoptosis signal-regulating kinase 1 (ASK1) (Wu et al. 2006); also the activity of Prxd-6, a 1-Cys membrane peroxidase, is controlled in a redox-dependent manner by the interaction with GSTP protein, and recent evidence has been obtained on the

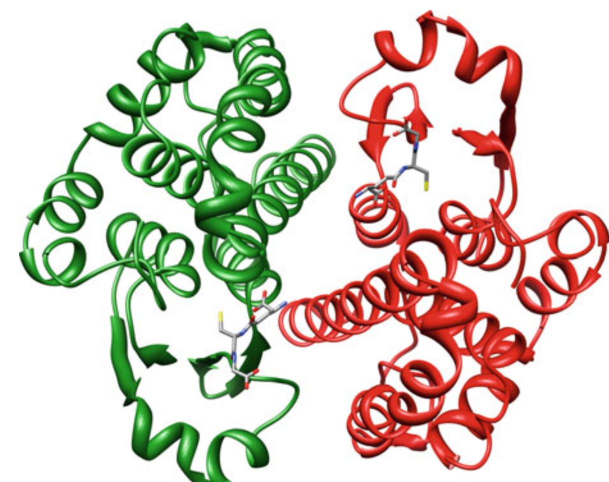

Fig. 1 X-ray crystallographic structure of e-GST (GSTP1-1). Three-dimensional structure of glutathione transferase P1-1 (i.e., erythrocyte glutathione transferase) from PDB code: 6gss (Oakley et al. 1997). The two monomers are in green and red ribbons. Glutathione molecule is also reported according to atom type (Picture was drawn by means of UCSF Chimera (Pettersen et al. 2004))

existence of a GSTP-dependent feedback of Nrf2 transcription factor activity that may share the same molecular mechanism (Bartolini et al. 2015). The interleukin 6 activated STAT3 transcription factor is another node of the regulatory interactome of GSTP (Bartolini and Galli 2016).

GSTs have been found to bind the dinitrosyl-glutathionyl-iron complex, a natural derivative of nitric oxide with toxic properties, which becomes harmless when bound to GST (De Maria et al. 2003; Bocedi et al. 2013). Other functions include selenium-independent peroxidase activity with some organic peroxides (Bartling et al. 1993).

Human cytosolic GSTs are dimeric proteins classified into seven different gene-independent classes termed alpha, mu, pi, theta, omega, sigma, and zeta. Human glutathione transferase P1-1 (*h*GSTP1-1) is an homodimeric intracellular protein of about 46 kDa expressed in different organs and cell types (Sheehan et al. 2001). The GSTP1-1 is the most abundant form of intra-erythrocyte transferase representing 95 % of an entire GST pool (Awasthi and Singh 1984). Its X-ray structure is reported in Fig. 1.

The expression of GSTP gene varies depending on the activity of different trascription factors (Bartolini and Galli 2016; Higgins and Hayes 2011). Besides classical transcriptional activators such as some anticancer drugs and other xenobiotics, GSTP gene is modulated by the levels of endogenous activators. These include toxins produced by the functional defect of depurative organs such as liver and kidney (Carmagnol et al. 1981). In CKD patients, this hyperactivity of GSTP gene was observed also in blood leukocytes and was proposed to represent an attempt of the cell detoxification machinery to set a defense response against the systemic toxicity of the uremic condition (Mimic-Oka et al. 1992; Galli et al. 2014). GSTP is, in fact, one of the most effective components within the family of phase II genes associated with the detoxification function and redox signaling of glutathione.

This chapter collects the available information in literature that suggests how e-GST investigation can represent a very simple and inexpensive laboratory tool in

clinical nephrology. This could monitor toxicological aspects of uremia associated with the transcriptional activation of detoxification and stress-response genes that share their regulation with that of e-GST. Similarly to glycated hemoglobin analysis in the long-term monitoring of blood glucose, the e-GST induction response provides a span coverage of several weeks in the retrospective evaluation of molecular effects of uremic toxicity, i.e., that deriving from red blood cell (RBC) life span.

Molecular and Kinetic Properties of e-GST

e-GST has been extensively characterized in terms of molecular and kinetic properties. It was identified as GSTP1-1, an acidic GST isoenzyme belonging to the P1 class GST that is also present in the human spleen, skin, and brain. It is a dimeric protein composed by two identical subunits of about 23 kDa (Armstrong 1997). Each subunit has an active site competent for glutathione (GSH) binding (G-site) and a second one able to accommodate different toxic compounds (H-site) which are conjugated to GSH (Armstrong 1997). e-GST, as well as other GST isoenzymes, may act also like a ligandin, binding toxic compounds and facilitating their elimination (Armstrong 1997). In blood, this enzyme is exclusively present in erythrocyte, where its concentration in healthy subjects is about 5.6 U/g Hb (Dessì et al. 2012). Serum does not contain detectable levels of e-GST. The presence of other GST isoenzymes (in particular of the alpha-class isoenzyme) in sufficient amount to be detected with a simple spectrophotometer has been reported in a single study, but recent reexamination established that this result is merely artifactual (Fabrini et al. 2012a).

In healthy subjects, e-GST activity levels remain virtually constant throughout childhood and adult life (Strange et al. 1980), but upon transcriptional stimulation, the enzyme protein can be up-regulated several folds over baseline non-stimulated levels. GSTs contain in the promoter region the consensus sequences “antioxidant-responsive element” (ARE) and “electrophile-responsive element” (EpRE), as well as other sequences with an equivalent or synergistic function downstream of transcriptional factors such as PXR, CAR, AhR, Nrf2, PPARγ, and C/EBP β (Higgins and Hayes 2011). Such a molecular redundancy and the presence of transregulation mechanism between the transcriptional factors that control GST expression demonstrate the importance of this family of genes in cellular homeostasis and particularly in the stress adaption response to electrophilic substances.

Activity Determination of e-GST and Its Correlation with the Protein Expression

e-GST activity can be easily determined using a conventional spectrophotometric apparatus. Using 1-chloro-2,4-dinitobenzene as co-substrate, the rate of conjugation with GSH can be followed at 340 nm (extinction coefficient of the GSH-conjugation

complex = 9,600 M^{-1} cm^{-1}) (Habig and Jakoby 1981). The pH optimum for this enzyme is 7.5, but the assay is usually performed at pH 6.5 because at higher pH values the spontaneous reaction becomes too fast. In early studies, e-GST activity was determined after erythrocytes purification (Carmagnol et al. 1981; Mimic-Oka et al. 1992; Galli et al. 1999). More recent papers described a simplified procedure which uses only four microliters of hemolyzed total blood (Dessì et al. 2012). This assay, which is accurate and specific for e-GST since this enzyme is not detectable in serum, is very simple, unexpensive, and rapid (about 1 min per sample). A major advancement for the application of this assay in high-throughput routine protocols has been the development of an automatic procedure that performs hundreds of determinations in a short time (Dessì et al. 2012).

However, accuracy of GST assay in blood and the relationship between the enzyme activity and protein expression investigated in some of the earliest studies on CKD patients (Galli et al. 1999) can be biased by the interference of some relevant factors. First, inactive forms of GSTP1-1 could be present in total blood being this a redox-sensitive form of GST. In fact the isoenzyme shows two reactive cysteines (Cys47 and Cys101) that can form an intramolecular disulfide bridge after exposure to conditions of oxidative stress to produce a completely inactive form of this enzyme protein (Ricci et al. 1991). However, the oxidized inactive form can be efficiently restored to active by the reducing activity of cellular thiols. Accordingly, the studies on purified e-GST of healthy or nephropathic patients suggested that, if present, inactive forms of e-GST in blood are negligible (Dessì et al. 2012), and immunoblotting experiments were in line with this finding (Galli et al. 1999). In conclusion, the increased e-GST activity of CKD patients is directly related with the levels of protein expressed in the circulating RBCs.

Other important aspects in assay performance deal with sample storage and processing. e-GST is a very stable enzyme when total blood samples are stored at 4 °C (100 % activity recovered after 7 days), but storage at temperatures $\leq$0 °C must be avoided since this may lead to irreversible inactivation (Dessì et al. 2012). Simple storage precautions are thus sufficient to handle samples in large multicenter trials or screening programs.

Overexpression of e-GST in Uremic Patients Under Hemodialysis

Early in the 1980s, Carmagnol and coworkers first reported of increased e-GST levels in pediatric hyperbilirubinemia and in CKD patients (Carmagnol et al. 1981). After a decade, Mimic-Oka and coworkers (Mimic-Oka et al. 1992) confirmed this finding reporting of increased GST and GSH levels in RBC and leukocytes of CKD patients either in pre-dialysis conservative therapy or hemodialysis treatment. Only a few years later, Galli and coworkers (Galli et al. 1999) first demonstrated that the increased e-GST activity of CKD patients is the consequence of an increased expression rather than a kinetic modulation of the enzyme protein. In this larger observational study carried out on 118 subjects, e-GST overexpression was found to be a highly prevalent trait in the different subpopulations of dialysis patients (72 % of

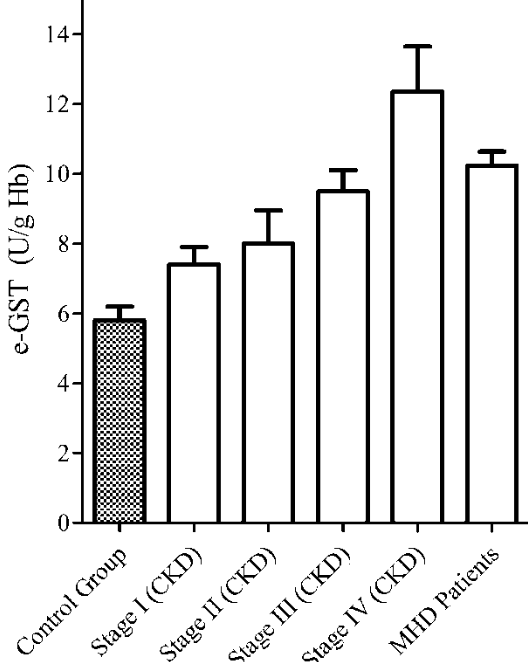

Fig. 2 e-GST activity in healthy subjects and pre-dialysis and dialysis patients. e-GST activity is reported as U/gHb. Healthy subjects, control group; pre-dialysis patients,CKD stage I–IV; dialysis patients, MHD patients (Modified from Dessì et al. 2012)

patients on standard three times/week bicarbonate hemodialysis and 57 % of peritoneal dialysis patients get over the mean + 2SD cutoff levels of healthy subjects). The study also suggested that e-GST overexpression is not associated with surrogate markers of oxidative stress and was not influenced by the increased content of RBC vitamin E produced after treatment with vitamin E-modified hemodialyzers. In the same manner, even the response to erythropoietin therapy apparently did not influence e-GST levels, and preliminary experiments in that study suggested that high-molecular-weight or protein-bound toxins could play some role in GST overexpression. In the same study, only a few subjects in pre-dialysis phase were assessed, and a lower prevalence of GST overexpression than in dialysis patients was reported (approx. 20 %).

A later study (Dessì et al. 2012), performed with an automated procedure of analysis on total blood of 62 maintenance hemodialysis patients and 80 healthy controls, further confirmed the increased levels of e-GST activity in MHD patients (5.8 ± 0.4 vs 10.2 ± 0.4 U/g Hb, respectively) (Fig. 2) and first described the linear correlation between enzyme activity and plasma levels of homocysteine (Hcy) ($r^2 = 0.64$, $P < 0.0001$) (Fig. 3), a modified amino acid that increases above normal concentrations in more than 90 % of CKD patients. No other significant correlations were found when e-GST activity was matched with hemoglobin, transferrin, blood iron, and markers of systemic inflammation and renal function such as alpha-1 acid glycoprotein and high-sensitive C-reactive protein, beta-2 microglobulin, and the malnutrition-inflammation index PINI. This automated procedure of analysis was

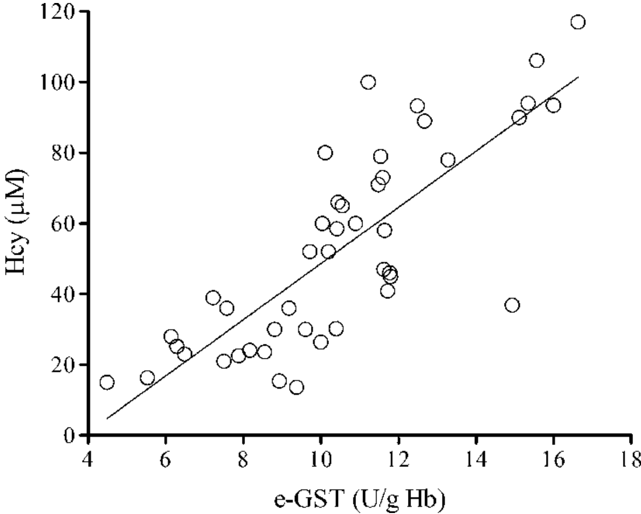

Fig. 3 Correlation between plasma homocysteine levels (mM) and e-GST in MHD patients. Correlation between plasma Hcy levels (μM) and e-GST activity (U/g Hb) (Modified from Dessì et al. 2012)

validated and is now available to further explore this phase-II-dependent response of the uremic syndrome at the clinical level (Dessì et al. 2012).

A later study (Noce et al. 2012) verified the potential of e-GST as a biomarker complementary to the Kt/Vurea parameter to assess the dose and adequacy of dialysis techniques, i.e., standard bicarbonate hemodialysis (HD) and online hemodiafiltration (HDF). In this respect, e-GST may assess adequacy not as a one-spot evaluation procedure performed during a single session of dialysis, as it occurs for Kt/Vurea, but rather as an indicator of depurative efficiency of a series of treatments accomplished during a span of few weeks. In this study the increased e-GST activity of CKD patients was confirmed in 103 patients that were compared with 82 healthy controls (9.0 ± 0.4 vs 5.8 ± 0.4 U/g Hb, respectively) (Fig. 4). Then of this patient population, 44 patients on HD and 59 patients on HDF were compared and e-GST activity was found to be significantly lower in HDF than HD subgroup (8.2 ± 0.4 vs. 10.0 ± 0.4 U/g Hb, respectively). Single-pool Kt/Vurea and total weekly Kt/Vurea were higher in HDF than in HD (1.5 ± 0.1 vs. 1.3 ± 0.1, and 4.6 ± 0.1 vs. 3.9 ± 0.2), but no significant correlation was found between e-GST activity and Kt/Vurea data (Noce et al. 2012). The findings in this study were consistent with the use of e-GST as a biomarker of long-term depurative efficacy of dialysis treatments, which is worth of further clinical investigation.

Finally a recent retrospective study (Galli et al. 2014) of a population of 98 HD patients investigated for plasma Hcy and blood thiol status between 1999 and 2004 demonstrated that a daily (2 h) dialysis schedule (daily HD or DHD) ($n = 28$) can lead to a better correction of the uremic retention solute Hcy than a standard three times/week protocol of HD (SHD) ($n = 70$). Levels of Cys and Cys-Gly measured

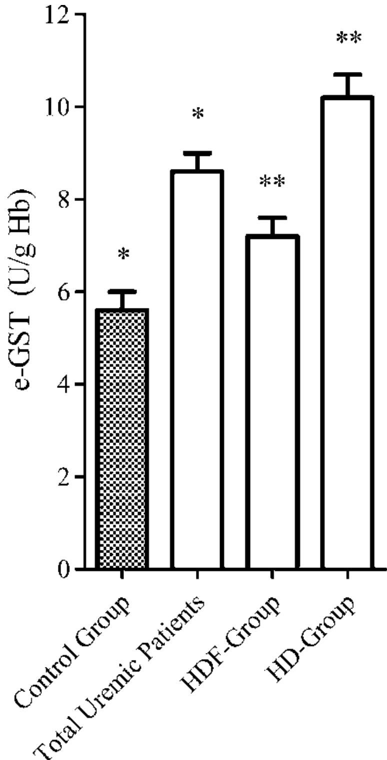

Fig. 4 e-GST activity in uremic patients under hemodialysis. e-GST activity in all the uremic patients in standard bicarbonate hemodialysis (HD group), in online hemodiafiltration therapy (HDF group), and in the control group. *$P <$ 0.0001 e-GST activity in total uremic patients vs the control group. **$P < 0.0001$ e-GST activity in the HD group *vs* the HDF group (Modified from Noce et al. 2012)

in plasma were also influenced, and according to past observation (Dessì et al. 2012), the levels of hyperhomocysteinemia correlated with e-GST levels as well as with plasma GSH. According with the findings obtained with the comparison between diffusive and convective methods (Noce et al. 2012), these findings point to a better depurative function of high-efficiency dialysis procedures, such as frequent dialysis. Uremic retention solutes could be among the toxic components that may interfere with thiol metabolism and redox balance of CKD patients, some of which may interfere with e-GST transcription in the bone marrow. These solutes await further investigation for molecular identification and better removal by more efficient dialysis therapies.

e-GST in CKD Patients Under Conservative (Pre-dialysis) Therapy

When e-GST activity was investigated for the first time in pre-dialysis patients (Mimic-Oka et al. 1992), a linear negative correlation was found between the enzyme activity and the levels of creatinine clearance, a measure of renal function directly reflecting GFR values. A more extensive and recent study (Dessì et al. 2012) examined e-GST activity in 72 pre-dialysis patients, and a significant increase of

e-GST activity was also reported with a positive correlation with disease severity weighted according to the four stages of "Kidney Disease Outcomes Quality Initiative" classification (7.4 ± 0.5, 8 ± 1, 9.5 ± 0.6, 12 ± 1 U/g Hb, respectively) (Fig. 2). In these patients, e-GST activity did not correlate with conventional markers of either acute phase or chronic inflammation and kidney disease, although these markers also increased according with the severity of the uremic disease (Table 1).

An extension of this study correlated the levels of e-GST, Hcy, and erythrocyte catalase (e-CAT) in 328 type-2 diabetes mellitus patients (T2DM), of which 61 were non-nephropathic patients and 267 were also CKD patients under conservative pre-dialysis therapy. Average e-GST activity was significantly higher in all T2DM patients compared to the control group (7.9 ± 0.3 vs. 5.8 ± 0.4 U/g Hb) (Fig. 5), and enhanced activity levels were also observed in all the four subgroups of CKD diabetic patients divided according with the K/DOQI guidelines (Noce et al. 2014). Mean Hcy levels in diabetic patients were higher than in healthy subjects (33.4 ± 1.2 vs. 13.6 ± 0.8 μM), and Hcy increased according with the stage of CKD. As expected from previous studies (Noce et al. 2012), a significant correlation was found between e-GST and Hcy levels (Noce et al. 2014). These findings demonstrated that e-GST transcriptional activation is a specific response to, and thus an early biomarker of the uremic condition.

Environmental and Endogenous Factors Affecting e-GST Levels in Healthy Subjects and in Non-uremic Patients

Two different studies showed that, besides CKD, e-GST overexpression can be the consequence of other conditions, such as the exposure to environmental pollutants or in the case of the autoimmune disease known as scleroderma or systemic sclerosis (SSc). Also in this latter disease the exposure to toxins of unknown origin and nature is proposed to play a major pathogenic role. SSc is characterized by endothelial dysfunction and fibrosis of the skin and internal organs (Fabrini et al. 2013), and a renal involvement is frequent in these patients. Interestingly, e-GST is highly overexpressed in SSc patients ($n = 102$) reaching a mean value of 13 U/g Hb, i.e., more than two times higher than normal healthy levels (5.8 U/g Hb) (Fig. 6a, b) (Fabrini et al. 2013). Enzyme levels in these patients correlated ($r^2 = 0.49$, $P < 0.0001$) with the Medsger DSS and DAI Valentini indices that quantify the severity and activity of the disease. Interestingly, e-GST levels of SSc patients were not influenced by the presence of kidney damage or by other defects of specific organs taken separately. e-GST hyper-expression in this condition appears thus to be linked with the exposure to putative toxins that cause the disease and not to the autoimmune disease per se, to the damage of specific organs, or to other consequences of the disease that may also include oxidative stress.

A study on about 500 healthy volunteers living in eight distinct areas at or near the Sacco river valley, a region of the Frosinone district (Lazio, Italy) well known for its environmental pollution (Table 2) (Fabrini et al. 2012b), proposed a role for e-GST evaluation as a biomarker of environmental pollution hazard. Subjects of six

Table 1 Main clinical features and laboratory findings in 72 pre-dialysis patients divided into four subgroups according to K/DOQI stage (stage I to IV) CKD, 62 ESRD patients on MHD, and 80 healthy subjects (control group) (Modified from Dessì et al. 2012)

	Control group	Stage I (CKD)	Stage II (CKD)	Stage III (CKD)	Stage IV (CKD)	ESRD on MHD
e-GST (U/g Hb)	5.8 ± 0.4	7.4 ± 0.5	8 ± 1	9.5 ± 0.6	12 ± 1	10.2 ± 0.4
hs-CRP (mg/l)	1.2 ± 0.7	3 ± 1	3.4 ± 0.4	4.0 ± 0.8	7 ± 2	7 ± 1
GFR (ml/min)[a]	118 ± 2	109 ± 3	77 ± 2	42 ± 2	20 ± 1	<4.7[b]
PINI	0.5 ± 0.1	0.6 ± 0.3	1.0 ± 0.6	0.5 ± 0.1	1.3 ± 0.4	2 ± 1
Alpha- 1 acid glycoprotein (g/l)	0.70 ± 0.03	0.90 ± 0.06	0.95 ± 0.04	0.92 ± 0.06	1.21 ± 0.09	1.24 ± 0.05
Beta-2 microglobulin (mg/l)	0.90 ± 0.08	1.84 ± 0.09	2.2 ± 0.1	5.6 ± 0.5	12 ± 1	37 ± 2
Mean age (years)	46.1	45.8	51.1	64.4	62.3	58.0

Data are expressed as mean ± SEM

[a]GFR was calculated on the basis of MDRD equation

[b]Only two MHD patients showed a residual renal function while 60 were totally anuretic

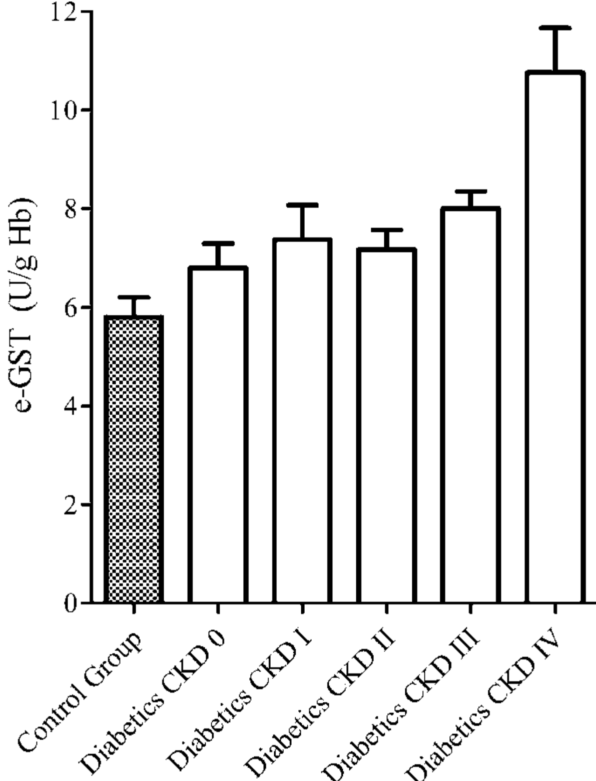

Fig. 5 E-GST activity in diabetic CKD patients. e-GST activity in diabetic patients non-CKD group (CKD 0) and all CKD stages (CKD I–IV) compared to the control group (healthy subjects) (Modified from Noce et al. 2014)

different areas of that region showed 18–44 % increased levels of e-GST when compared to 400 volunteers living in the Rome hinterland, and the highest GST levels were observed in the areas of higher risk of pollution (Fig. 7). Oxidation-dependent changes of GST activity were not observed in the blood specimens of the exposed populations (Fabrini et al. 2012b).

Overexpression of e-GST in Kidney-Transplanted Patients

Preliminary data about e-GST activity in renal-transplanted patients are available (Bocedi and Ricci *unpublished data*) and surprisingly demonstrated a remarkable enzyme overexpression in these patients (n = 80) (Fig. 8a) with levels of activity similar to those found in stage IV CKD patients. Only slightly different e-GST levels were observed between patients receiving organs from living donors and cadavers. No correlation has been found between e-GST levels and the clinical parameters used to assess kidney function in these patients, except for eGFR. Also different immunosuppressive therapies seem to not influence the e-GST expression. These results, if further confirmed, may suggest the use of e-GST as an indicator of the

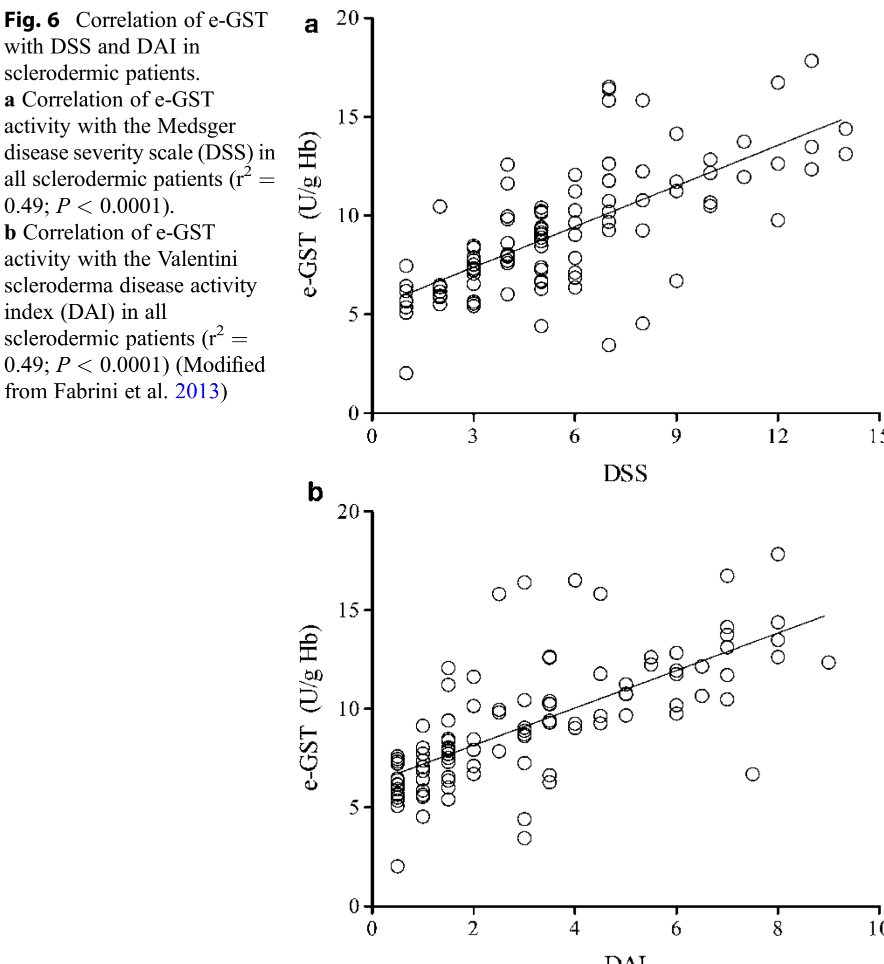

Fig. 6 Correlation of e-GST with DSS and DAI in sclerodermic patients. **a** Correlation of e-GST activity with the Medsger disease severity scale (DSS) in all sclerodermic patients ($r^2 = 0.49$; $P < 0.0001$). **b** Correlation of e-GST activity with the Valentini scleroderma disease activity index (DAI) in all sclerodermic patients ($r^2 = 0.49$; $P < 0.0001$) (Modified from Fabrini et al. 2013)

subclinical impairment of the transplanted kidneys. Interestingly, oxidized albumin, used as a long-term biomarker of oxidative stress, demonstrated the presence of an oxidative challenge in the transplanted patients and particularly in those receiving postmortem-explanted kidneys (Fig. 8b).

Future Perspectives

The use of e-GST as a CKD biomarker is promising and worth of further clinical validation. Reference values of e-GST levels in different ethnic groups and populations as well as in different regions and habitats (i.e., urban, mountain, country, coastal, etc.) need to be determined.

Table 2 Geographic features of selected areas (Modified from Fabrini et al. 2012b)

Selected areas in the Frosinone district	Territorial extension (Km^2)	Geographic features
Area 1	25	Nearby confluence of the Sacco and Liri rivers
Area 2	10	Close to the Liri river
Area 3	90	After confluence of the Sacco and Liri rivers
Area 4	40	Near the Sacco river – presence of industrial site
Area 5	40	Liri river flows through the area – presence of regularized landfill and compost sites
Area 6	30	Close to important industrial site
Area 7	60	Sacco river flows through the area
Area 8	40	Close to Sacco and Liri rivers – presence of incineration plant

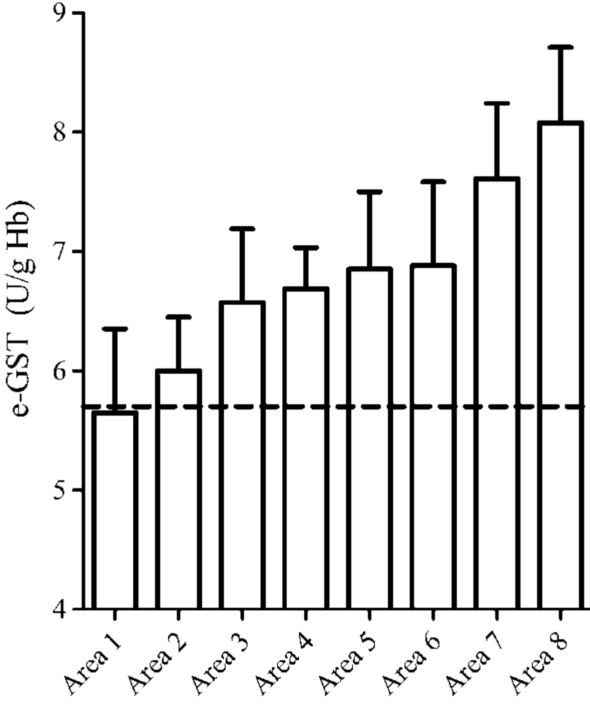

Fig. 7 e-GST activity in the Sacco river valley and its relative increase compared to the Rome area. Cumulative (men and women) e-GST activity. Continuous *dotted line* is the reference value for the Rome area (Modified from Fabrini et al. 2012b)

e-GST will become an even more promising biomarker of kidney disease if personal reference values could be obtained along the different ages and situations of life, which may be attained by screening protocols in childhood and youth. Only a few microliters of total blood obtainable by means of a simple pinprick on a finger are needed to perform an e-GST assay according with the procedure described in this chapter, and the development of an automatic analysis procedure makes this a relatively feasible and cheap task even for large populations of subjects.

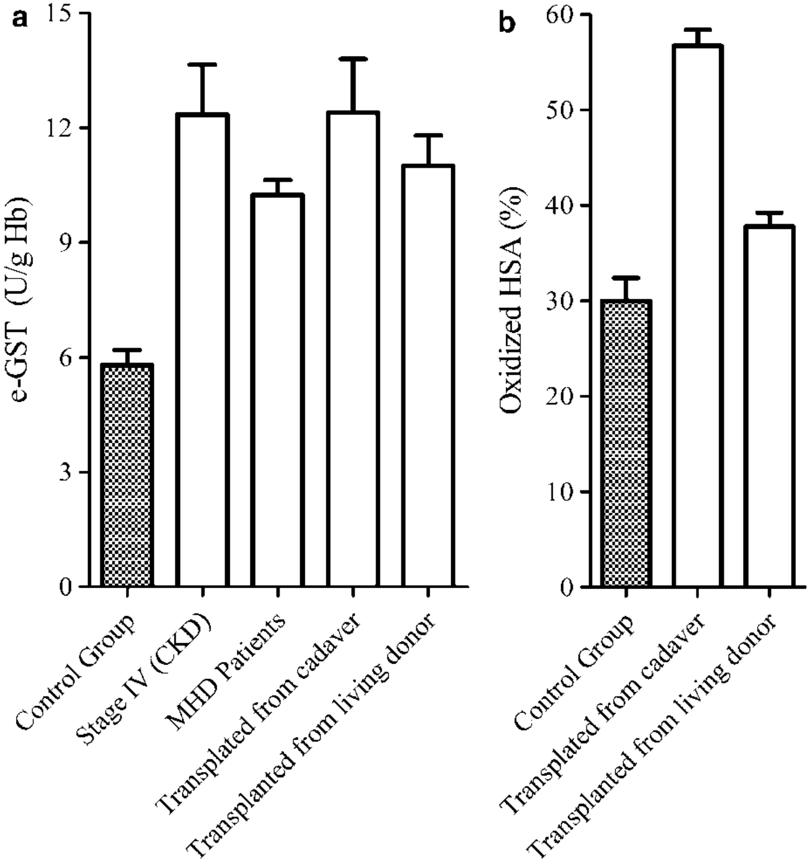

Fig. 8 e-GST activity in transplanted patients and oxidized albumin in serum of transplanted patients. **a** Preliminary e-GST levels in 80 kidney-transplanted patients compared to healthy subjects and CKD (stage IV) and MHD patients. **b** Oxidized serum albumin in kidney-transplanted patients compared to healthy subjects (Bocedi and Ricci *unpublished results*)

The study of e-GST levels in the different moments and context of life of a single subject and the comparison with other subjects of the same area will eliminate major biases for diagnostic and prognostic application of this biomarker.

Potential Applications to Prognosis, Other Diseases, or Conditions

e-GST analysis represents a reliable diagnostic and prognostic tool of nephropathic diseases. Other potential applications in other uncommon situations associated with increased exposure to endogenous or environmental stressors or toxins have been identified, such as the chronic exposition to environmental pollutants in urban areas

or working places. Given that all mammalians express e-GST with very similar molecular properties, enzyme investigation could be used also to broader protocols of environmental, veterinary and, food safety monitoring.

Summary Points

- e-GST is an inducible enzyme involved in cellular detoxification and redox homeostasis of blood and systemic compartments.
- e-GST overexpression is highly prevalent in chronic kidney disease and is also observed in other human conditions such as hyperbilirubinemia and systemic sclerosis.
- e-GST overexpression appears very early in CKD and the extent of this transcriptional response follows that of disease severity, thus pointing to a role of this enzyme as a reliable and sensitive biomarker of uremic toxicity.
- e-GST overexpression in CKD can be used to monitor the depurative efficiency and the dose of dialysis methods and devices. High-efficiency dialysis therapies can decrease, at least in part, e-GST levels in maintenance HD patients.
- e-GST is also overexpressed in kidney-transplanted subjects that may further demonstrate the sensitivity of this gene to even a partial (subclinical) defect of kidney function.
- e-GST expression represents a circulating biosensor useful in the retrospective assessment of the systemic exposure to electrophilic toxicants and also to other cellular stressors. Retrospection spans through the 3 months of the erythrocyte life span.

References

Armstrong RN. Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem Res Toxicol. 1997;10:2–18.

Awasthi YC, Singh SV. Purification and characterization of a new form of glutathione S-transferase from human erythrocytes. Biochem Biophys Res Commun. 1984;125:1053–60.

Awasthi YC, Sharma R, Singhal SS. Human glutathione S-transferase. Int J Biochem. 1994;26:295–308.

Bartling D, Radzio R, Steiner U, Weiler EW. A glutathione S-transferase with glutathione-peroxidase activity from *Arabidopsis thaliana*. Molecular cloning and functional characterization. Eur J Biochem. 1993;216:579–86.

Bartolini D, Commodi J, Piroddi M, Incipini L, Sancineto L, Santi C, Galli F. Glutathione S-transferase pi expression regulates the Nrf2-dependent response to hormetic diselenides. Free Radic Biol Med. 2015;88:466–80. doi: 10.1016 j.freeradbiomed.2015.06.039.

Bartolini D, Galli F. The functional interactome of GSTP: a regulatory biomolecular network at the interface with the Nrf2 adaption response to oxidative stress. J Chromat B. 2016. doi:10.1016/j.jchromb.2016.02.002

Baxmann AC, Ahmed MS, Marques NC, Menon VB, Pereira AB, Kirsztajn GM, Heilberg IP. Influence of muscle mass and physical activity on serum and urinary creatinine and serum cystatin C. Clin J Am Soc Nephrol. 2008;3:348–54.

Bocedi A, Fabrini R, Farrotti A, Stella L, Ketterman AJ, Pedersen JZ, Allocati N, Lau PC, Grosse S, Eltis LD, Ruzzini A, Edwards TE, Morici L, Del Grosso E, Guidoni L, Bovi D, Lo Bello M,

Federici G, Parker MW, Board PG, Ricci G. The impact of nitric oxide toxicity on the evolution of the glutathione transferase superfamily: a proposal for an evolutionary driving force. J Biol Chem. 2013;288:24936–47.
Carmagnol F, Sinet PM, Rapin J, Jerome H. Glutathione-S-transferase of human red blood cells; assay, values in normal subjects and in two pathological circumstances: hyperbilirubinemia and impaired renal function. Clin Chim Acta. 1981;117:209–17.
Cruz DN, Ricci Z, Ronco C. Clinical review: RIFLE and AKIN – time for reappraisal. Crit Care. 2009;13:211.
De Maria F, Pedersen JZ, Caccuri AM, Antonini G, Turella P, Stella L, Lo Bello M, Federici G, Ricci G. The specific interaction of dinitrosyl-diglutathionyl-iron complex, a natural NO carrier, with the glutathione transferase superfamily: suggestion for an evolutionary pressure in the direction of the storage of nitric oxide. J Biol Chem. 2003;278:42283–93.
Dessì M, Noce A, Dawood KF, Galli F, Taccone-Gallucci M, Fabrini R, Bocedi A, Massoud R, Fucci G, Pastore A, Manca di Villahermosa S, Zingaretti V, Federici G, Ricci G. Erythrocyte glutathione transferase: a potential new biomarker in chronic kidney diseases which correlates with plasma homocysteine. Amino Acids. 2012;43:347–54.
Devarajan P. Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury. Biomark Med. 2010a;4:265–80.
Devarajan P. Review: neutrophil gelatinase-associated lipocalin: a troponin-like biomarker for human acute kidney injury. Nephrology. 2010b;15:419–28.
Dulhunty A, Gage P, Curtis S, Chelvanayagam G, Board P. The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. J Biol Chem. 2001;276:3319–23.
Fabrini R, Bocedi A, Massoud R, Federici G, Ricci G. Spectrophotometric assay for serum glutathione transferase: a re-examination. Clin Biochem. 2012a;45:668–71.
Fabrini R, Bocedi A, Del Grosso E, Morici L, Federici G, Palleschi A, Ricci G. Erythrocyte glutathione transferase: a novel biomarker to check environmental pollution hazardous for humans. Biochem Biophys Res Commun. 2012b;426:71–5.
Fabrini R, Rosato E, Gigante A, Bocedi A, Cianci R, Barbano B, Del Grosso E, Ricci F, Zingaretti V, Salsano F, Ricci G. Erythrocyte glutathione transferase: a non-antibody biomarker for systemic sclerosis, which correlates with severity and activity of the disease. Cell Death Dis. 2013;4:e736.
Fraser SD, Aitken G, Taal MW, Mindell JS, Moon G, Day J, O'Donoghue D, Roderick PJ. Exploration of chronic kidney disease prevalence estimates using new measures of kidney function in the health survey for England. PLoS ONE. 2015;10:e0118676.
Galli F, Rovidati S, Benedetti S, Buoncristiani U, Covarelli C, Floridi A, Canestrari F. Overexpression of erythrocyte glutathione-S-transferase in uremia and dialysis. Clin Chem. 1999;45:1781–8.
Galli F, Piroddi M, Bartolini D, Ciffolilli S, Buoncristiani E, Ricci G, Buoncristiani U. Blood thiol status and erythrocyte glutathione-S-transferase in chronic kidney disease patients on treatment with frequent (daily) hemodialysis. Free Radic Res. 2013;48:273–81.
Haase M, Bellomo R, Devarajan P, Schlattmann P, Haase-Fielitz A, NGAL Meta-analysis Investigator Group. Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. Am J Kidney Dis. 2009;54:1012–24.
Habig WH, Jakoby WB. Assays for differentiation of glutathione S-transferases. Methods Enzymol. 1981;77:398–405.
Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Ann Rev Pharmacol Toxicol. 2005;45:51–88.
Higgins LG, Hayes JD. Mechanisms of induction of cytosolic and microsomal glutathione transferase (GST) genes by xenobiotics and pro-inflammatory agents. Drug Metab Rev. 2011;43:92–137.

Kampranis SC, Damianova R, Atallah M, Toby G, Kondi G, Tsichlis PN, Makris AM. A novel plant glutathione S-transferase/peroxidase suppresses Bax lethality in yeast. J Biol Chem. 2000;275:29207–16.
KDIGO. KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. Kidney Int Suppl. 2013;3:1–150.
Kidney Disease Outcomes Quality Initiative (K/DOQI). K/DOQI clinical practice guidelines on hypertension and antihypertensive agents in chronic kidney disease. Am J Kidney Dis. 2004;43 (5 Suppl 1):S1–290.
Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, Kusek JW, Van Lente F, Chronic Kidney Disease Epidemiology Collaboration. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. Ann Intern Med. 2006;145:247–54.
Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro 3rd AF, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J, CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration). A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009;150:604–12.
Matsushita K, Mahmoodi BK, Woodward M, Emberson JR, Jafar TH, Jee SH, Polkinghorne KR, Shankar A, Smith DH, Tonelli M, Warnock DG, Wen CP, Coresh J, Gansevoort RT, Hemmelgarn BR, Levey AS, Chronic Kidney Disease Prognosis Consortium. Comparison of risk prediction using the CKD-EPI equation and the MDRD study equation for estimated glomerular filtration rate. JAMA. 2012;307:1941–51.
Mimic-Oka J, Simic T, Djukanovic L, Stefanovski J, Ramic Z. Glutathione and its associated enzymes in peripheral blood cells in different stages of chronic renal insufficiency. Amino Acids. 1992;2:215–24.
Noce A, Ferrannini M, Fabrini R, Bocedi A, Dessì M, Galli F, Federici G, Palumbo R, Di Daniele N, Ricci G. Erythrocyte glutathione transferase: a new biomarker for hemodialysis adequacy, overcoming the Kt/V(urea) dogma? Cell Death Dis. 2012;3:e377.
Noce A, Fabrini R, Dessì M, Bocedi A, Santini S, Rovella V, Pastore A, Tesauro M, Bernardini S, Di Daniele N, Ricci G. Erythrocyte glutathione transferase activity: a possible early biomarker for blood toxicity in uremic diabetic patients. Acta Diabetol. 2014;51:219–24.
Oakley AJ, Lo Bello M, Battistoni A, Ricci G, Rossjohn J, Villar HO, Parker MW. The structures of human glutathione transferase P1-1 in complex with glutathione and various inhibitors at high resolution. J Mol Biol. 1997;274:84–100.
Parikh CR, Lu JC, Coca SG, Devarajan P. Tubular proteinuria in acute kidney injury: a critical evaluation of current status and future promise. Ann Clin Biochem. 2010;47:301–12.
Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera – a visualization system for exploratory research and analysis. J Comput Chem. 2004;25:1605–12.
Piroddi M, Bartolini D, Ciffolilli S, Galli F. Nondialyzable uremic toxins. Blood Purif. 2013;35 Suppl 2:30–41.
Ricci G, Del Boccio G, Pennelli A, Lo Bello M, Petruzzelli R, Caccuri AM, Barra D, Federici G. Redox forms of human placenta glutathione transferase. J Biol Chem. 1991;266:21409–15.
Ronco C, Haapio M, House AA, Anavekar N, Bellomo R. Cardiorenal syndrome. J Am Coll Cardiol. 2008;52:1527–39.
Ronco C, McCullough P, Anker SD, Anand I, Aspromonte N, Bagshaw SM, Bellomo R, Berl T, Bobek I, Cruz DN, Daliento L, Davenport A, Haapio M, Hillege H, House AA, Katz N, Maisel A, Mankad S, Zanco P, Mebazaa A, Palazzuoli A, Ronco F, Shaw A, Sheinfeld G, Soni S, Vescovo G, Zamperetti N, Ponikowski P, Acute Dialysis Quality Initiative (ADQI) consensus group. Cardio-renal syndromes: report from the consensus conference of the acute dialysis quality initiative. Eur Heart J. 2010;31:703–11.
Sheehan D, Meade G, Foley VM, Dowd CA. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem J. 2001;360:1–16.

Stevens LA, Coresh J, Feldman HI, Greene T, Lash JP, Nelson RG, Rahman M, Deysher AE, Zhang YL, Schmid CH, Levey AS. Evaluation of the modification of diet in renal disease study equation in a large diverse population. J Am Soc Nephrol. 2007;18:2749–57.

Stevens LA, Schmid CH, Greene T, Zhang YL, Beck GJ, Froissart M, Hamm LL, Lewis JB, Mauer M, Navis GJ, Steffes MW, Eggers PW, Coresh J, Levey AS. Comparative performance of the CKD Epidemiology Collaboration (CKD-EPI) and the Modification of Diet in Renal Disease (MDRD) Study equations for estimating GFR levels above 60 mL/min/1.73 m^2. Am J Kidney Dis. 2010;56:486–95.

Strange RC, Johnston JD, Coghill DR, Hume R. A comparison of erythrocyte glutathione-S-transferase activity from human foetuses and adults. Biochem J. 1980;188:475–9.

Tangri N, Stevens LA, Schmid CH, Zhang YL, Beck GJ, Greene T, Coresh J, Levey AS. Changes in dietary protein intake has no effect on serum cystatin C levels independent of the glomerular filtration rate. Kidney Int. 2011;79:471–7.

Vaidya VS, Ferguson MA, Bonventre JV. Biomarkers of acute kidney injury. Ann Rev Pharmacol Toxicol. 2008;48:463–93.

Wang T, Arifoglu P, Ronai Z, Tew KD. Glutathione S-transferase P1-1 (GSTP1-1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. J Biol Chem. 2001;276:20999–1003.

Wu Y, Fan Y, Xue B, Luo L, Shen J, Zhang S, Jiang Y, Yin Z. Human glutathione S-transferase P1-1 interacts with TRAF2 and regulates TRAF2-ASK1 signals. Oncogene. 2006;25:5787–800.

Plasma Growth Arrest-Specific Protein 6 (Gas6) as a Biomarker of Renal Diseases

27

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Abstract

Gas6 (growth arrest-specific 6) is a unique protein belonging to the family of plasma vitamin K-dependent proteins and a ligand for the tyrosine kinase family (Tyro3, Axl, Mer). Gas6 bears a great structural resemblance to the anticoagulant

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protein S. Interestingly, in spite of the presence of a g-carboxyglutamic acid domain in its structure, Gas6 has not been discored to show any function in the coagulation cascade. Gas6 has been reported to participate in cell proliferation, apoptosis, chemotaxis, leukocyte migration, sequestration, platelet aggregation, vascular homeostasis, and inflammation. In addition, it plays a role in the activation of various kinds of cells, including platelets and endothelial and vascular smooth-muscle cells (VSMC). After it was demonstrated that Gas6 induces proliferation of VSMC and serum-starved NIH 3T3 (mouse embryonic fibroblast cell lines) fibroblasts, mitogenic effects of Gas6 were found in cultured mesangial cells. As a result, it has been reported to take part in various pathophysiological processes, for instance, atherosclerosis, renal diseases, cancer, and thrombosis. The main subject of the present review is the role Gas6 plays in renal diseases.

Keywords
Chronic allograft injury • Diabetic nephropathy • Gas6 • Gas6 (growth arrest-specific 6) • Acute and chronic renal nephritis induction • Cellular actions • Human chronic renal failure • Human inflammatory renal diseases • IgA nephropathy • Mesangial-cell proliferation stimulation • Receptor interrelations • Renal cell carcinoma • Renal damage • Renin-angiotensin-aldosterone system • TAM system in vasculature • Mitogenic activity

Abbreviations

Ang	Angiotensin
CKD	Chronic kidney disease
CR	Chronic rejection
DN	Diabetic nephropathy
DOCA	Deoxycorticosterone acetate
EC	Endothelial cells
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
Gas6	Growth arrest-specific gene protein 6
GFR	Glomerular filtration rate
Gla	g-Carboxyglutamic acid
GN	Glomerulonephritis
GO	Glycoxidized
HD	Hemodialysis
IgAN	IgA nephropathy
IMT	Intima-media thickening
LDL	Low-density lipoprotein
MHC	Myosin heavy chain
MMCs	Mouse mesangial cells
MR	Mineralocorticoid receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NIH 3T3	Mouse embryonic fibroblast cell lines
NK	Natural killer
NTN	Nephrotoxic nephritis

NTS	Nephrotoxic serum
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-3-kinase
RAAS	Renin-angiotensin-aldosterone system
RCC	Renal cell carcinoma
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SHGB	Sex hormone-binding globulin
STAT	Signal transducers and activators of transcription
STZ	Streptozotocin
TAM receptors	Tyro3, Axl, and Mer receptors
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
VKD	Vitamin K-dependent
VSMC	Vascular smooth-muscle cells

Key Facts of Enzyme-Linked Immunosorbent Assay (ELISA)

- The enzyme-linked immunosorbent assay (ELISA) is a technique that usually uses antibodies and color change to determine an antigen or uses antigens and color change to identify an antibody in a liquid sample.
- If an antigen is a subject for detection, sample is applied to a solid phase which is pre-coated microstrip plates with specific capture antibodies and antigens are attached to capture antibodies. After incubation, the plates are washed with a mild detergent solution to remove any nonspecifically bound or unbound material. Then, a further specific detection antibody is applied over the solid phase so it can bind to the antigen which is binded to capture antibody. This detection antibody is linked to an enzyme, and, after an incubation, second washing step proceeds. Finally the enzyme's substrate is added. The further reaction causes producing of a detectable signal, generally a color change in the substrate. After stop solution is added, the optical density is read with an ELISA plate reader (Fig. 3).
- Capture antibody, substance which is wanted to detect and detecting antibody binds each other, respectively, and is called "sandwich" ELISA because of the sandwich-like formation and it is the most common form.
- Unlike other spectrophotometric lab assay formats where the same reaction well (e.g., a cuvette) can be reused after washing, ELISA plates are not reusable because they are coated with antibody or antigen.
- Enzyme/substrate reaction is short term so microstrip plate must be read as soon as possible (up to 20 min).
- Eligible for analyzing a wide variety of test parameters, and quick (approximately 86 tests in 3–4 h application are the advantages of ELISA method. And also antigens of very low concentration level able to be detected if capture antibody is very specific to antigen.

Definitions

Angiotensin II It causes blood vessels to constrict (vasoconstriction) and high blood pressure. Also it triggers the release of the water-retaining hormone vasopressin from the pituitary gland and afterward secretion of aldosterone, adrenaline, and noradrenaline in the adrenal gland.

Chronic kidney disease Chronic kidney disease comprises conditions that damage kidneys and reduce their capacity to keep our body healthy, being present for at least 3 months. Both lower GFR and greater levels of albuminuria are independently related to cardiovascular events, mortality, and the rate of end-stage renal disease. If kidney disease gets worse, wastes can accumulate and become high levels in blood and some complications may develop like high blood pressure, anemia (low blood count), poor nutritional health, weak bones, and nerve damage.

Glomerular filtration rate (GFR) GFR is the gold test to determine level of kidney function which describes the flow rate of filtered fluid through the kidney and identifies the stage of renal disease. In clinical practice, serum creatinine level-based estimates of creatinine clearance are used to quantify GFR. A widespread used formula for estimate of creatinine clearance is the Cockcroft-Gault formula. It can be calculated from the blood creatinine test and patient's age, gender, and body size. GFR indicates patient's stage of kidney disease and is used while planning treatment.

Renin-angiotensin-aldosterone system RAAS is a hormone signaling system responsible for the regulation of fluid balance and blood pressure in our body. When blood pressure is low or certain nerve impulses occur by stressful situations, the kidneys release an enzyme into the circulation called renin. Plasma renin then triggers the conversion of angiotensinogen (released from the liver) to angiotensin I. This is converted by another enzyme, the angiotensin-converting enzyme (ACE) (found in the lungs), into angiotensin II.

Reverse transcription polymerase chain reaction (RT-PCR) RT-PCR is a kind of the polymerase chain reaction (PCR). This assay is usually used in molecular biology and genetics to determine RNA expression. RT-PCR enables creation of complementary DNA (cDNA) transcripts from RNA and identifies gene expression.

Vitamin K-dependent (VKD) proteins VKD proteins require the presence of vitamin K to become biologically active via carboxylation. Commonly known VKD proteins are the factors II, VII, IX, and X in coagulation system. Other well-known VKD proteins in anticoagulation system are protein C and protein S. Osteocalcin, Gas6, matrix Gla protein, proline-rich Gla proteins 1,2, and transmembrane Gla proteins 3,4 are the VKD proteins which are less popular.

Introduction

Expansion of mesangial cells is shown in many kinds of glomerular disease and usually is linked with matrix enlargement, contributing to progression to glomerulosclerosis (Doi 2001).

It has been pointed out that several growth factors and cytokines cause mesangial-cell proliferation: platelet-derived growth factor, basic fibroblast growth factor, transforming growth factor beta 1, angiotensin (Ang) II, vascular endothelial growth factor, interleukin-6 (Donate-Correa et al. 2015), Smad1 (Mima et al. 2008), and growth arrest-specific 6 (Gas6) (Yanagita 2004). Gas6, a newly discovered vitamin K-dependent (VKD) protein, is a ligand for TAM receptors Tyro3, Axl, and Mer. Gas6/TAM signaling plays a part in chemotaxis and phagocytosis (Fridell et al. 1998); cell migration, proliferation, and adhesion (McCloskey 1997); cell survival and mitogenic activities (Goruppi et al. 1996); and hematopoiesis and inflammation (Angelillo-Scherrer et al. 2005; Tjwa et al. 2008).

In 1988, Gas6 was determined by screening genes whose expression was upregulated in embryonic mouse NIH 3T3 fibroblasts. The name of the protein was derived from the property of cells whose growth was arrested (Schneider et al. 1988). The gene was sequenced 5 years later (Manfioletti et al. 1993) and named growth arrest-specific gene 6; it has been shown to share 44 % similarity with protein S, a further VKD protein encoded by the PROS1 gene in humans. The best determined role of protein S is its function in the anticoagulation pathway, where it acts as a cofactor to protein C in the inhibition of factors Va and VIIIa. To the best of our knowledge, Gas6 functions are limited to TAM-receptor activation.

Both Gas6 and its receptor Axl are expressed in the kidneys, principally in intraglomerular mesangial cells. Mesangial-cell proliferation, a hallmark of glomerular sclerosis, has been stimulated by Gas6/Axl. Put-down of mesangial-cell proliferation with either warfarin, an inhibitor of gamma-carboxylation, or an extracellular portion of Axl suggests that Gas6 plays a role in glomerular disease (Yanagita 2004).

Gas6 appears to exhibit an important role in a wide variety of cell types and generally is associated with specifications of injury, inflammation, and repair. This chapter focuses on the role of Gas6 in kidney diseases.

Gas6 Structure

Gas6 is a 75 kDa molecular weight multimodular protein and containing several posttranscriptional modifications. The main one is gamma (γ)-carboxylation of the N-terminal domain (the Gla module). The Gla module contains 11–12 γ-carboxylated glutamyl residues. Glutamyl residues are modified to γ-carboxylated glutamyl residues by VKD carboxylase enzyme posttranslationally. Carboxylation allows VKD clotting factors to bind to membrane phospholipids on platelets that induce the coagulation pathway. In the absence of carboxylation, the coagulation process becomes defective, yet it has not been shown that Gas6 has a direct effect on the coagulation cascade (Huang et al. 2003).

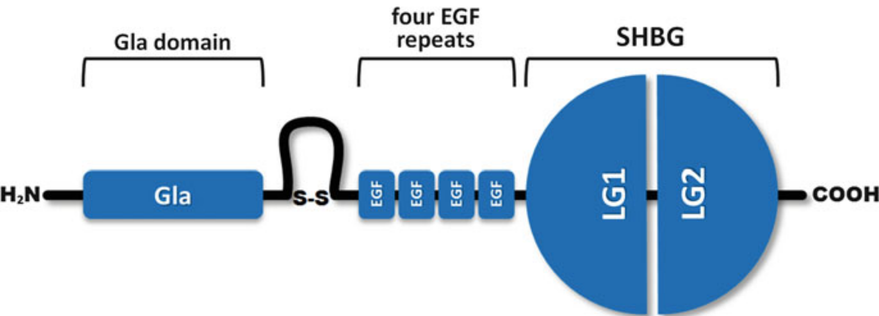

Fig. 1 Structure of Gas6. Gas6 consists of N-terminal Gla domain, a loop region with a disulfide bridge, four EGF-like domains, and 2 C-terminal LamG-like subdomains containing the SHBG domain. *EGF* epidermal growth factor, *SHBG* sex hormone-binding globulin

The Gla domain contributes to VKD proteins the capability of binding anionic phospholipids at the cell surface. In the remaining cells, situated in the inner leaflet of the plasma membrane are anionic phospholipids. At the cell surface, the demonstration of anionic phospholipids is a characteristic of cell injury, activation, and apoptosis. In situations similar to these, such lipids maintain a docking surface on which VKD proteins bind and their activity takes place. As a result, Gla domains aim Gas6 to apoptotic or activated cells that occur in a wide range of pathologies (Laurance et al. 2012).

Provided by a disulfide bridge, a loop follows the Gla domain. The Gas6 loop does not appear to be susceptible to the performance of serine proteases, in contrast with the protein S loop. In the event of protein S, this loop is necessary since it interacts with activated protein C and is inhibited by thrombin (He et al. 1998). Four epidermal growth-factor modules, two of which including calcium-binding consensus sequences, followed by a sex hormone-binding globulin (SHBG)-like module, compose the principal framework of Gas6 after the loop. This module includes two submodules which have a framework similar to the globular modules of laminin A (LamG). It is likely to observe such kind of globular structure in proteins since they interact with steroids, heparin sulfates, or integrins (Fig. 1).

Although there is remarkable structural similarity between protein S and Gas6, Gas6 tissue expressions for Gas6 and PROS 1 definitely are different. Most plasma VKD factors have a gene expression restricted to the liver, especially factor IX and factor X. Expression of PROS1 is not so specific to the liver, and comparable levels of transcript can be found in the kidney, lungs, gonads, and endothelial cells. Gas6 is unique among the genes of the family, because Gas6 expression in the liver is minor compared with expression in other tissues (Nakano et al. 1995). Gas6 is substantially expressed and has been shown in the lungs, heart, kidneys, intestines, and terminally differentiated cells, as well as in endothelial cells within the capillaries, vascular smooth-muscle cells (VSMCs), bone marrow, and monocytes (Avanzi et al. 1997).

Several enzyme-linked immunosorbent assay (ELISA) methods have been developed to quantify Gas6, and its presence in plasma has been demonstrated

(Balogh et al. 2005; Borgel et al. 2006; Clauser 2007; Alciato et al. 2008; Ekman et al. 2010; Erek-Toprak et al. 2014). Gas6 has been found in human plasma in concentrations (15–63 ng/ml) that are much lower than the other VKD proteins of plasma.

Gas6 in Cellular Actions

The function of Gas6 has been debated since its discovery. In reality, Gas6 was identified as a growth factor in several varieties of cells, particularly cultured cells derived from serum (Nakano et al. 1995; Li et al. 1996; Goruppi et al. 1996). Growth factors are primary influences on cell adhesion, differentiation, migration, duplication, and survival. These influences are mediated by interaction with specific cell-membrane receptors, most of them with intrinsic protein-tyrosine kinase activity; thus they are known as receptor tyrosine kinases (RTKs) (Schlessinger and UIlrich 1992).

Binding the extracellular portion of its cognate polypeptide ligand causes dimerization or oligomerization of the receptor, transducing the signal to the intracellular portion of the molecule and triggering tyrosine kinase activity, which results in tyrosine phosphorylation of the receptor. RTKs are classified according to their extracellular domain, which generally is composed of a modular structure. Members of RTK subgroups often bind common or similar ligands.

A crucial discovery in the understanding of Gas6 biology was the discovery that it was a ligand of a previously orphan RTK, Axl. Axl is part of a three-member family of receptors comprising Axl, Tyro3, and MerTK, which had been cloned by various groups and given various names: Axl (ARK, Tyro7, Ufo), Tyro3 (Sky, Rse, Brt, Dtk, Tif, Etk2, Rek), and MerTK (Mer, c-Eyk, Nyk, Tyro12). The architecture of these receptors is preserved, and it is constituted by two immunoglobulin domains sequenced by two fibronectin type III (Fig. 2). This RTK family is named TAM after the first letter of the names of its three components (Tyro3, Axl, and MerTK).

Beginning with the first studies of Gas6 action on cultured cells, the antiapoptotic effect of Gas6 has been found in many cells, including NIH 3T3 fibroblasts, VSMCs, chondrocytes, endothelial cells, neurons, oligodendrocytes, epithelial cells, and several types of cancer cells. Investigations have shown that serum deprivation, TNF-α, changes in pH, oxidative stress, soluble phospholipase, and the amyloid peptide cause Gas6's antiapoptotic effects (Bellido Martin and Frutos 2008).

Mitogenic activity is another research subject that shows the cellular effects of Gas6. Mitogenic effects have been described in VSMCs, cardiac fibroblasts, Schwann cells, and mesangial cells (Nakano et al. 1995; Stenhoff et al. 2004; Li et al. 1996; Yanagita et al. 2001b).

Another cellular action of Gas6 is induction of phagocytosis by macrophages and other phagocytic cells. This induction has been shown to be specific to apoptotic cell removal or phosphatidylserine-containing liposomes, imitating the situation in the cellular membranes of apoptotic cells (Ishimoto et al. 2000; Hall et al. 2001; Wu et al. 2005).

Some studies have demonstrated Gas6-induced chemotaxis of VSMCs (Fridell et al. 1998). In certain tumor types, migration and invasiveness correlate with

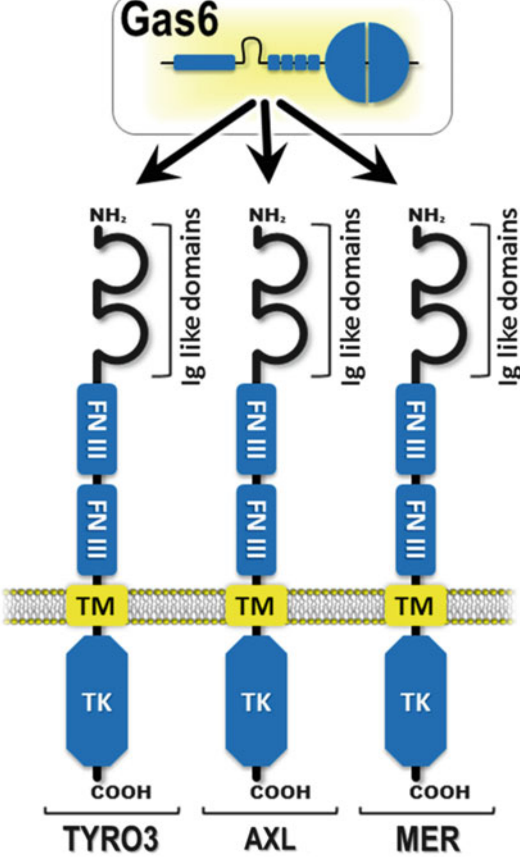

Fig. 2 Structure of TAM receptors. TAM family receptors consist of 2 Ig-like modules, 2 FN III domains, a TM, and a TK domain. *FN III* fibronectin type III-like, *TAM* Tyro3, Axl, and Mer, *TK* tyrosine kinase, *TM* transmembrane domain

expression of Gas6 receptors, especially in Axl (Shieh et al. 2005), and Gas6 subsidiary signaling via Axl leads to invasiveness of glioma cells in animal models (Vajkoczy et al. 2006).

A further cellular action of Gas6 is cell activation and differentiation. It has been reported that natural killer (NK) cells express Axl, Mer, and Tyro3, and all three TAM receptors are essential for normal differentiation and maturation of NK cells (Caraux et al. 2006). In addition, it has been demonstrated that Gas6 stimulation downregulates expression of inflammatory cytokines (Correll et al. 2004).

Furthermore, induction of differentiation by Gas6 has been reported in the maturation process of adipocytes (Maquoi et al. 2005). Collett et al. (2003) have shown that Gas6 activity modulates differentiation models on vessel-wall cells, including inhibiting osteogenic differentiation of pericytes. Ming et al. (2001) have shown the differentiation of VSMCs into foam cells. These properties could play a role in cellular homeostasis of vasculature after injury (Ruan and Kazlauskas 2012).

Gas6-Receptor Interrelations

As mentioned, Gas6 is the ligand for the TAM-receptor family, which consists of Axl, Tyro3, and Mer. The affinity sequence for Gas6 is Axl, Tyro3, and Mer, from highest to lowest . The extracellular domain of these receptors contains two Ig-like domains, which are features of adhesion molecules, sequenced by two fibronectin type III-like figures. The cytoplasmic last segment contains a tyrosine kinase domain (Fig. 2). Discovered to a product of a transforming gene in T-cell leukemia cells, this gene was given the name "Axl," which originates from the Greek "anexelekto," meaning uncontrolled, on the basis of initial observations of its function. Axl is a 140-kDa protein expressed omnipresent (Laurance et al. 2012; Nagata et al. 1996).

With its ability to transform, Gas6's intracellular domain can activate tumors in mice independently of its ligand. Furthermore, it seems to be overexpressed in many human cancers (Wu et al. 2014). In addition to the role it plays in the development of cancer, Axl takes part in cell adhesion due to its extracellular domain, free from its tyrosine kinase domain (Bellosta et al. 1995).

Tyro3 and Mer were determined by a number of researchers in 1994. Tyro3 is expressed mainly in the brain and central nervous system, whereas Mer is expressed in monocytic-cell generation. In contrast to Axl, there is not so much data concerning possible roles of Tyeo3 and Mer in cancer. However, recently it has been found that knocking down Gas6 and Mer restricts the proliferation of myeloma cell lines, while downregulation of Axl or Tyro has no effect (Waizenegger et al. 2015).

From the first studies on Gas6, numerous researchers have pointed out that following Gas6/Axl binding, phosphatidylinositol-3-kinases (PI3K) is activated in many cell types, including endothelial cells (EC), VSMC, chrondrocytes, fibroblasts, neurons, oligodendrocytes, and a number of cancer cells (Goruppi et al. 1999; Allen et al. 1999; Hasanbasic et al. 2004; Wu et al. 2014). PI3K/Akt activation is essential for Gas6's antiapoptotic role. The cell-survival effect is blocked by pharmacological inhibitors of the PI3K pathway (Goruppi et al. 1999). Activation of Akt causes to inactivation of Bad, a proapoptotic mediator, and to an enhancement in the antiapoptotic protein Bcl-2 by an NFkB-dependent mechanism (Hasanbasic et al. 2004). Several isoforms of PI3K have been shown to interact with Axl and potentially could cause discrete signaling pathways from the receptor (Hafizi et al. 2002). In this context, it is interesting to note that C1-TEN, a protein phosphatase similar to the PI3K/Akt/PKB inhibitor PTEN, has been found to interact with Axl, although the potential regulatory role of the Axl/C1-TEN interaction has not been elucidated (Hafizi et al. 2002).

Gas6/TAM System in Vasculature

Evidence from several in vivo and in vitro models has suggested that Gas6 plays an important role in vascular biology. In early studies, Gas6 was demonstrated in cloning a growth-inducing factor of VSMCs, and this was the initial relationship between Gas6 and the biology of vascular vessels (Nakano et al. 1995). Shortly

thereafter, it was shown that Gas6 and Axl proteins were increased by vascular injury in rat carotid arteries. In VSMC culture, Axl expression and stimulation were potentialized by thrombin and Ang II (Melaragno et al. 1998). In addition, it was shown that Axl is a redox-sensitive receptor activated by H2O2 in cultured VSMC and intact aorta VSMC (Konishi et al. 2004). More recently, a redox-induced interaction between Axl and glutathiolated nonmuscle myosin heavy chain (MHC)-IIB in VSMCs has been found. MHC-IIB is involved in directed cell migration. The Axl-MHC-IIB relationship occurs via inducing VSMCs with both reactive oxygen species and Gas6 (Cavet et al. 2010).

Another study has reported the effect of glucose levels on Axl signal transduction in VSMCs. It demonstrated Gas6-Axl signal alteration by glucose by modulating interaction of Axl with special signaling proteins. Gas6-Axl stimulation increased ERK1/2 and PI3K-Akt-mTOR activation in high and low glucose concentrations, respectively, showing that 140-kDa Axl could be responsible for ERK1/2 activation, whereas the 114-kDa Axl is responsible for Akt activation (Cavet et al. 2008).

In addition, a function for Gas6/Axl has been described in a reproducible mouse model of flow-dependent vascular remodeling that resembles human intima-media thickening (IMT). It found that in Axl knockout mice, IMT of the carotid was decreased by rising cell apoptosis and changing vascular inflammation. A major finding of this research was that the Gas6/Axl pathway adjusted the performance of some types of cells in the vessel wall and seemed to behave in an autocrine/paracrine manner. Another finding was that remodeled carotids from Axl−/− mice had importantly changed inflammatory responses in comparison with Axl+/+ mice. Specific changes, such as relatively more monocytes than VSMC, diminished macrophages and neutrophils in the intima and augmented neutrophils in the adventitia. There was an important retardation of inflammatory response in Axl−/− mice during vascular remodeling, which could be attributed to apoptosis and/or a failure to complete phagocytosis brought about by impaired macrophage function (Korshunov et al. 2006). A similar situation was demonstrated in atherosclerosis of Gas6 knockout animals, which had a lower content of lymphocytes and macrophages (Lutgens et al. 2000).

Furthermore, it has been shown that Gas6/Axl interactions play a role in vascular calcifications. Regulation of Axl signaling is a crucial event in matrix mineralization and pericyte differentiation. After it had been demonstrated that Axl and its ligand, Gas6, are expressed in bovine retinal pericytes, research found that expression was downregulated in post-confluent cultures containing mineralized nodules. Research suggested that the ability to modulate the Axl signaling pathway in vivo may lead to a novel therapeutic target to stop or prevent ectopic calcification (Collet et al. 2003). This effect is supported by several reports that implicate the Gas6/TAM system in osteoclast function (Kawaguchi et al. 2004). More recently, it has been shown that Axl inhibits induction of calcification of cultured VSMCs (Collett et al. 2007).

In another study, statins were shown to prevent apoptosis of aortic smooth-muscle cells by renovating the Gas6/Axl pathway, eventually inhibiting calcification (Son et al. 2006). In this context, the link between genetic variants in Gas6 and stroke

could be due to the influence of Gas6 on vascular calcification, a process obviously associated with risk of stroke (Munoz et al. 2007).

A different effect of Gas6 on vascular biology is its role on the endothelium, which has been suggested from its antiapoptotic effect in culture (Goruppi 1997; Hasanbasic et al. 2004). Furthermore, addition of Gas6 has been shown to inhibit adhesion of granulocytes to endothelial cells (Avanzi et al. 1998).

Platelets are leading members of the vascular system, mainly providing primary hemostasis, and mediating inflammatory responses and cellular interactivities. The first proof suggesting a function for TAM receptors in platelet physiology originated from researches of Gas6-deleted mice. It was shown that Gas6−/− mice avoided thrombosis and presented defective platelet aggregation. Moreover, reverse transcription polymerase chain reaction (RT-PCR) study represented that platelets express Tyro3, Axl, and Mer (Angelillo-Scherrer et al. 2001). A follow-up study showed that all three receptors are necessary for normal platelet aggregation (Angelillo-Scherrer et al. 2005). Furthermore, soluble Mer (Mer-Fc) inhibits platelet aggregation in vitro and protects against collagen/epinephrine-stimulated thrombosis in vivo (Sather et al. 2007). In additionally, mice with two or three TAM receptors deleted exhibited more critical impairment of platelet function than single-deleted mice (Wang et al. 2007). More recently, it was found that Gas6 promotes and increases sequestration of circulatory platelets and leukocytes in a P-selectin-dependent fashion (Tjwa et al. 2008).

Even though it has been reported that Gas6 is present in rat (İshimato et al. 2000) and mouse platelets, Balogh et al. (2005) reported that using immunoblotting, combined IP immunoblotting and a highly sensitive and specific ELISA for Gas6 did not enable detection of Gas6 in washed human platelets. The previously proposed role of Gas6 in platelet aggregation may be due to Gas6 coming from the circulation.

Gas6 in Renal Damage

In many types of glomerular disease, too much proliferation of mesangial cells greatly affects glomerular structure and function. Regardless of the source of injury, an impairment in the control of mesangial-cell proliferation seems to play a vital role in developing progressive renal injury and glomerulosclerosis (Doi 2001).

Gas6 Stimulates Mesangial-Cell Proliferation

Gas6 contributes to renal diseases mainly by stimulating mesangial-cell proliferation. After it was shown that Gas6 induces proliferation of VSMC and serum-starved NIH 3T3 fibroblasts (Nakano et al. 1995; Goruppi et al. 1999), the mitogenic effect of Gas6 was found in mesangial-cell culture (Yanagita et al. 1999). Additionally, Yanagita et al. exhibited that the conditioned medium of serum-starved mesangial cells prepared in the presence of warfarin did not stimulate proliferation of mesangial

cells. It was concluded that Gas6 is an autocrine growth factor for mesangial cells, and warfarin restrains mesangial-cell proliferation by decreasing production of active Gas6 as a consequence of inhibiting the VKD γ-carboxylation of its Gla domain.

Additional studies have pointed out that STAT3, a member of the signal transducer and activator of transcription (STAT) protein family, is a main signaling molecule involved in Gas6-mediated mesangial-cell proliferation in vitro and in vivo. STAT proteins, which are latent transcription factors, are activated via phosphorylation (Yanagita et al. 2001b). Activated STAT proteins cause translocation to the nucleus and induce STAT-specific transcription. Activation of STAT proteins is linked to regulation of cell differentiation and growth. It has been shown that phosphorylation of STAT proteins is directly proportional to increasing proliferation of mesangial cells. In addition, administering warfarin or Axl-Fc restricts the Gas6-specific pathway by phosphorylating STAT3 in glomeruli and by mesangial-cell proliferation (Yanagita et al. 2001b).

Gas6 Induces Acute and Chronic Renal Nephritis

Another effect of Gas6 related to renal damage was shown in an acute rat model of glomerulonephritis (GN) stimulated by injecting anti-Thy1.1 antibody. Expression of Gas6 and Axl was increased significantly in Thy1 GN in correlation with the degree of mesangial-cell proliferation. Double immunostaining indicated that most parts of Gas6 and Axl were localized together in mesangial cells. Thus, it seems logical that Gas6 is secreted from mesangial cells in vivo to induce the mesangial-cell surface receptor, Axl. Treating rats with Thy1 GN with low doses of warfarin and Axl-ECD can greatly inhibit mesangial-cell proliferation and extracellular matrix accumulation and can reduce the degree of glomerular injury in vivo in a dose-related way. Furthermore, it has been reported that blocking the Gas6/Axl pathway with warfarin or Axl-Fc reduced expression of platelet-derived growth factor B (PDGF-B) in Thy1 GN, indicating that the Gas6/Axl pathway can affect PDGF-B production in vivo. These findings show that the Gas6/Axl pathway plays an important role in progression of glomerular diseases by modulating expression of other growth factors in vivo (Yanagita et al. 2001a).

Investigating the function of Gas6 in stimulated nephrotoxic nephritis (NTN) contributes further insight into understanding whether Gas6 is involved in chronic renal injury. Early studies with Thy1 GN were important as a means of demonstrating the role of Gas6 in human diseases, but they did not provide sufficient proof about progressive kidney diseases leading to chronic renal failure in human subjects, because Thy1 GN is self-limited and calms spontaneously. NTN is a model of a progressive form of glomerulonephritis in which glomerular hypercellularity, crescent lesion formation, and glomerular sclerosis occur via inflammatory-cell infiltration and glomerular proliferation (Topham et al. 1999). NTN is stimulated by injecting preimmunized mice with heterologous nephrotoxic serum (NTS, a sheep anti-rat glomerular antiserum) that has reactivity to different glomerular cell and

basal membrane antigens. Gas6−/− mice have been used to research the role of Gas6 in NTN. In Gas6−/− mice, decreased phosphorylation of STAT3, remarkable reduction of proteinuria, less glomerular cell proliferation, less crescent formation, and decreased mortality were found compared with Gas6+/+ mice. Furthermore, administering recombinant Gas6 to NTS-treated Gas6−/− mice induced massive proteinuria, glomerular cell proliferation, and glomerulosclerosis compared to the responses observed in wild-type NTN mice. These data indicate that Gas6 is involved in progression of chronic kidney damage (Yanagita et al. 2002).

Apart from receptor Axl, another member of TAM family, Mer, has been examined in experiments on glomerulonephritis. The crucial role of Mer in clearing apoptotic cells in the immune system and its function in lessening immune responses by modulating cytokine production has caused a great deal of interest in this molecule in the field of autoimmunity. Mer-deficient mice develop a lupus-like autoimmune syndrome, possibly resulting from Mer-mediated inflammatory response with impaired clearing of apoptotic cells (Lemke and Rothlin 2008). More recently, Shao et al. (2010) used NTS-mediated kidney disease, which is a mouse model of GN. In this model, the severity of injury correlates directly with the dose of antibody injected. Histological changes stimulated by inflammatory injury exhibit apoptosis linked subtly with degrees of abnormalities. The study detected high levels of Mer protein in the kidney and more precise localized expression of renal Mer in mesangial and endothelial cells within the glomerulus, and the study found that Mer was upregulated during experimental GN. Interestingly, Mer knockout mice were much more prone to NTS-nephritis than were the wild type. Severe renal damage was observed within 3 days of NTS injection in Mer knockout mice but not in wild-type controls. In addition, it was found that early-onset renal damage in Mer−/− mice was associated with increased inflammatory cytokines, massive amounts of apoptotic cells, and abundant infiltration of neutrophils. It seems Mer plays a protective role in development of NTS-mediated nephritis, in contrast with the reported role of Axl in worsening renal disease.

Thus, Mer and Axl, two receptor kinases that belong to the same subfamily, possibly play contrasting roles in glomerular inflammation. Their opposing roles in vivo may depend on their relative expression, which is far greater for Mer in the basal state and in availability of ligands. Further studies will be required to clarify how capillary-rich organs, such as the kidneys, are protected via Mer and to clarify the obviously complicated relationship between Axl and Mer in renal pathology.

Gas6 in Chronic Allograft Injury

Chronic allograft injury still is a contributory factor to late kidney-graft loss, despite improvements in immunosuppressive drugs and a reduction in acute T-cell-mediated rejection (Zhang et al. 2015). Chronic rejection is responsible for more than half of graft failures in surviving recipients after renal transplantation (Paul 1995). Typical pathological features of chronic rejection are cellular proliferation and fibrosis in glomeruli, tubules, and the media layer of arterioles. Immunological and

nonimmunological mechanisms may play roles in tissue remodeling by helping local production of growth factors that affect cell migration and proliferation.

Yin et al. (2001) investigated expression of Gas6 in a rat model of chronic rejection (CR) of kidney transplant and found that Gas6 expression accelerates significantly above already-high basal levels during development of CR. The Gas6 response has a significant peak at 4 weeks – meaning during the initial stages of CR – in the allograft compared with expression in the isograft. Transforming growth factor beta (TGF-β) remains elevated at all time points tested up to 24 weeks post transplant. This suggests that Gas6 plays a role in the initial stages of CR development. Moreover, the same researchers later investigated expression of Gas6 and its receptors in human renal allografts that were demonstrating either acute rejection, chronic rejection, acute tubular necrosis, or calcineurin inhibitor toxicity. They found that expression of Gas6 increased in the acute tubular necrosis and CR groups, whereas Axl expression was upregulated in acute tubular necrosis. This shows that Gas6 expression positively correlates with expression of a CR marker, α-smooth muscle actin. These findings suggest the possibility that Gas6 plays a part not only in the process of kidney allograft dysfunction but also in CR (Yin et al. 2002).

Gas6 in Diabetic Nephropathy

More than 387 million people worldwide suffer from diabetes mellitus, and it is the main factor in chronic kidney disease, responsible for up to 45 % of end-stage kidney disease (www.idf.org/diabetesatlas/6e/Update20124). Given this, lightening the molecular mechanism of diabetic nephropathy (DN) is crucial.

The main characteristics of the initial phase of DN are mesangial-cell expansion, glomerular hypertrophy, increasing glomerular filtration rate (GFR), accumulation of extracellular matrix (ECM), and albuminuria, which frequently progress to glomerulosclerosis and impairment of renal function if untreated. Much comprehensive research has been done to identify molecules involved in mesangial broadening and glomerular hypertrophy.

Nagai et al. (2003) used streptozotocin (STZ)-induced diabetic rats and reported 12 weeks after injection that they exhibited characteristics of the initial phase of DN, such as glomerular hypertrophy, increased glomerular filtration rate, and albuminuria. Gas6 and Axl expression in the glomeruli of these rats was augmented, and application of low-dose warfarin to STZ-induced rats diminished GFR increase, albuminuria, and glomerular hypertrophy. In addition, in STZ-treated Gas6−/− mice, less mesangial and glomerular hypertrophy was found compared to identically treated Gas6+/+ mice. These findings showed that the Gas6/Axl pathway may play a crucial part in the initial phase of DN in vivo.

To research the signaling molecules that contribute to mesangial hypertrophy and find how Gas6 is involved in DN, Nagai et al. (2005) studied the Akt/mTOR pathway involved in mesangial-cell hypertrophy and examined a major mechanism responsible for glomerular hypertrophy in the pathway's downstream signaling

molecules, p70 S6 kinase and 4EBP-1, in vivo and in vitro. The in vivo part of the study analyzed the glomeruli of STZ-treated Gas6 knockout mice and found activation of Akt and 4E-BP-1, induction of p27, and localization of phosphorylated Akt in mesangial cells of diabetic glomeruli. Furthermore, STZ-treated Gas6−/− mice showed less phosphorylated Akt-positive areas than did STZ-treated, +/+ mice. The in vitro part of the study examined the influence of high glucose on Gas6/Axl, the role of Gas6/Axl in high glucose-stimulated mesangial hypertrophy, and the role of the Akt/mTOR pathway in Gas6-stimulated mesangial hypertrophy. It concluded that Gas6 can be stimulated by high glucose and is able to stimulate mesangial hypertrophy in an Akt-dependent fashion. The Akt/mTOR pathway is induced primarily in mesangial cells when mesangial and glomerular hypertrophy are determined in the initial phase of diabetic DN in vivo. Gas6 may be one of the molecules contributing to activation of this pathway.

Low-density lipoprotein (LDL) has been associated with diabetic microvascular complications and is modified by enhanced oxidation, glycation, or glycoxidation. Modified LDL levels are increased abundantly in diabetic patients, despite good glycemic control (Lyons 1993). Modified LDL leads to development of glomerular injury in diabetes by increasing transforming growth factor (TGF)-b1 expression, which plays a key role in proliferation of mesangial cells in diabetes. More recently, Kim et al. (2015) examined the relationship between glycoxidized (GO)-LDL and Axl/Gas6 signaling pathways in DN and studied glomerular mouse mesangial cells (MMCs) in vitro to show a GO-LDL-induced gene expression profile. They reported that Axl gene and protein expression was significantly increased by GO-LDL in MMCs. In addition, they found that Axl expression was increased in MMCs cultured under diabetic conditions. Furthermore, Gas6 stimulated TGF-b1 secretion, and this TGF-b1secretion stimulated Axl expression. It seems GO-LDL can increase Axl expression by Gas6-induced TGF-b1 upregulation. The study concluded that Axl/Gas6 signaling may be a new therapeutic aim for renal diseases induced by GO-LDL.

There are not many studies investigating Gas6 effects in human DN. The first, by Erek-Toprak et al. (2014), showed that in patients with type 2 diabetes, plasma Gas6 levels were significantly higher in patients with albuminuria than in patients with normoalbuminuria. The plasma Gas6 levels between subgroups of type 2 diabetes were checked and found to be lower in the group with microalbuminuria than in the group with macroalbuminuria. There was a significant correlation between albuminuria and plasma Gas6 levels and between Gas6 and HbA_{1c}. The study concluded that there seems to be an association between plasma Gas6 levels and albuminuria in patients with type 2 diabetes.

In this context, more recently, Erkoc et al. (2015) suggested that the Gas6 intron 8 c.834+7G>A gene polymorphism might be a risk factor for DN in type 2 diabetic individuals and compared frequency of Gas6 intron 8 c.834+7G>A gene polymorphism between type 2 diabetic patients with DN and non-diabetic individuals. No correlation between Gas6 intron 8 c.834+7G>A gene polymorphism and DN in type 2 diabetic individuals was found. Interestingly, the study found that Gas6 intron 8 c.834+7G>A polymorphism was associated with diabetic retinopathy among DN

patients. Further studies with more subjects are needed to investigate the genetic basis of type 2 DN and its relationship to the Gas6 gene.

Gas6 in Human Chronic Renal Failure

In the only research on human chronic kidney disease (CKD), Lee et al. (2012) studied all stages of human CKD in hemodialysis (HD) patients (23 % of them having diabetes) and reported increased levels of Gas6 in HD and CKD patients compared with control subjects. In addition, this study found that Gas6 increased significantly as the stages of CKD advanced. Furthermore, patients who had been on dialysis longer had higher Gas6 levels. The study concluded that dysregulation of the Gas6 protein could represent a novel inflammatory pathway contributing to human vascular disease in renal failure.

Gas6 in Human Inflammatory Renal Diseases

One of the first studies to investigate Gas6 effects in human renal diseases was conducted by Fiebeler et al. (2004). They gathered human renal specimens from 26 patients with IgA nephritis, lupus nephritis, diffuse immune complex glomerulonephritis, antineutrophil cytoplasmic antibody-associated glomerulonephritis, and acute rejection and examined whether Axl/Gas6 expression is influenced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in vitro. The study found that Gas6 and Axl immunofluorescence were barely detectable in normal kidneys, whereas in specimens with diseases, Axl was abundantly expressed in the small vessel media, distal tubules, glomeruli, and collecting ducts. Similarly, Gas6 was upregulated in all segments of the renal tubules, small vessel intima and media, and glomeruli. Gas6 and Axl upregulation was a noticeable but nonspecific finding in these renal diseases. Cultured rat VSMCs and immortalized human mesangial cells were induced with Ang II for 6 or 18 h. Western blot and confocal microscopy showed Ang II-dependent Gas6 and Axl expression. The study reported that the Ang II-induced Gas6 and Axl expression may be dependent on NADPH-oxidase and concluded that Gas6 and Axl are important molecules in human renal diseases and seem to be potential targets for therapy.

Gas6 in IgA Nephropathy

Human IgA nephropathy (IgAN) is a form of glomerulonephritis which is the most common worldwide. However, pathological demonstrations of IgAN are extensive and can range from mild mesangial hypercellularity to a rapidly progressive glomerulonephritis with fulminant crescents and endocapillary proliferation. The results of IgAN vary hugely (Tumlin et al. 2007). Therefore, IgAN might be divided into different subgroups according to etiology, histopathology, or clinical manifestations.

In this respect, expression of Gas6 and its receptors were investigated by using biopsy-proven IgAN cases to see whether Gas6 and its receptors were participated in the progression of IgAN by analyzing the link between expression of Gas6 and clinicopathological features (Nagai et al. 2013). In this study, Gas6 was upregulated in either podocytes or endothelial/mesangial cells. In IgAN, Axl was in endothelial/ mesangial cells while Tyro3 was the receptor for Gas6 in podocytes. The study reported that Gas6 expression in podocytes correlated with several prognostic factors, for instance, mesangial proliferation and proteinuria, and was inversely associated with p27 expression. Gas6 was involved in human IgAN, via Tyro3 and Axl in podocytes and endothelial/mesangial cells, respectively (Yanagita et al. 2001a). However, in human IgAN, Gas6 upregulation was observed mainly in podocytes, while endothelial/mesangial-dominant expression was seen in few patients. The study concluded that the expression pattern can be a marker to classify IgAN and therapeutic effects and prognosis should reevaluated according to the pattern.

Gas6 Effect on Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system (RAAS) is a widely known regulator of blood pressure (BP) and plays a main role in tissue perfusion, extracellular volume and determining target-organ damage. It controls fluid and electrolyte balance via regulated influences on the heart, blood vessels, and kidneys. RAAS activation can cause hypertension, atherosclerosis, and cardiac and renal failure, which can be prevented by blocking Ang II action. Ang II exerts its vasoconstrictor effect mainly on the postglomerular arterioles by accelerating glomerular hydraulic pressure and ultrafiltration of plasma proteins, influences that may be involved in onset and progression of chronic kidney damage. In addition, Ang II may directly contribute to accelerating renal damage by maintaining cell growth, fibrosis, and inflammation. Ang II is an effective, direct stimulus of aldosterone (Ald) synthesis. Ald signaling is mediated via the mineralocorticoid receptor (MR), and MR blocking has a renoprotective function and may slow or even stop progression of chronic nephropathies and reduce cardiovascular and renal morbidity (Atlas 2007). Nevertheless, MR-regulated pathways and signal molecules are incompletely understood.

To explore whether Gas6 plays a role in RAAS, Park et al. used an Ald-induced target-organ damage experimental model. They reported that Gas6 expression was upregulated in a highly activated RAAS rat model. MR blocking decreased this upregulation and prevented target-organ damage. In addition, VSMCs were tested in vitro and it was found that Ald induced Gas6 expression without the presence of Ang II. Furthermore, the effects of deoxycorticosterone acetate (DOCA) on Gas6−/− mice were explored, and it was observed that the gene-deleted mice were protected from cardiac hypertrophy, inflammation, and fibrosis and had improved renal function with reduced albuminuria, renal fibrosis, and fibronectin deposition compared to wild-type mice. This protection was independent of BP reduction. The study concluded that Gas6 seems to play a role in mineralocorticoid receptor-mediated target-organ damage.

More recently, Batchu et al. (2013) reported the differences in immune-specific mechanisms regulated by Axl during initial versus late phases of salt-dependent hypertension. They found that expression of Axl in hematopoietic cells is crucial to kidney pathology in the initial phase of salt-dependent hypertension. In addition, in both hematopoietic and nonhematopoietic cell lines, Axl contributes to the late phase of hypertension.

Gas6 in Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the most common cancer of the adult kidney and represents 2–3 % of all human cancers. Incidence of RCC peaks at 60–70 years of age. Unfortunately, the outcome of metastatic RCC has a dismal prognosis, and mortality remains very high (Jonasch et al. 2014).

Because no markers are currently recommended to assess the risk of progressive disease, there have been great efforts to find novel markers. Taking into account that Axl is overexpressed in several cancers, Gustafsson et al. (2009a) conducted a study to determine expression of Axl and its ligand, Gas6, in various RCC types in 308 patients. They reported that Axl and Gas6 expression in RCC are associated with tumor advancement and patient survival. In particular, low-tumor Axl mRNA levels independently correlated with improved survival.

Another study carried out by the same authors and published the same year used a cell-based RCC model system to explore the complex role of Gas6 and Axl in RCC. This study demonstrated inhibition of migration and survival in RCC 786-O cells as a result of Gas6 signaling through the Axl RTK (Gustafsson et al. 2009b). To date, Axl has been reported as increasing oncogenic effects in most cancers in which it has been studied (Wu et al. 2014). However, in line with the results of Gustaffson et al., Gas6 has been reported to have contrasting effects, inhibiting vascular endothelial growth factor A (VEGFR-A)-driven migration, specifically through activating Axl RTK (Gallicchio et al. 2005). So, the literature implies that the biology of Gas6 and Axl is complex and probably context-specific. Perhaps in RCC, where high Gas6 expression correlates with improved prognosis (Gustafsson et al. 2009a), Gas6 can be protective and can retard cancer progression by mechanisms such as decreasing migratory potential and decreased survival.

Conclusion

The Gas6 signaling pathway is highly regulated in several pathological conditions. Gas6/Axl-dependent signaling plays a crucial role in cell survival, aggregation, migration, and growth through multiple downstream pathways.

Further studies are needed to identify the molecules participating in the Gas6/Axl pathway. Understanding the potential role and effects of the Gas6/Axl signaling pathway may help affect the course of the future therapies by aiming this pathway in patients with renal diseases.

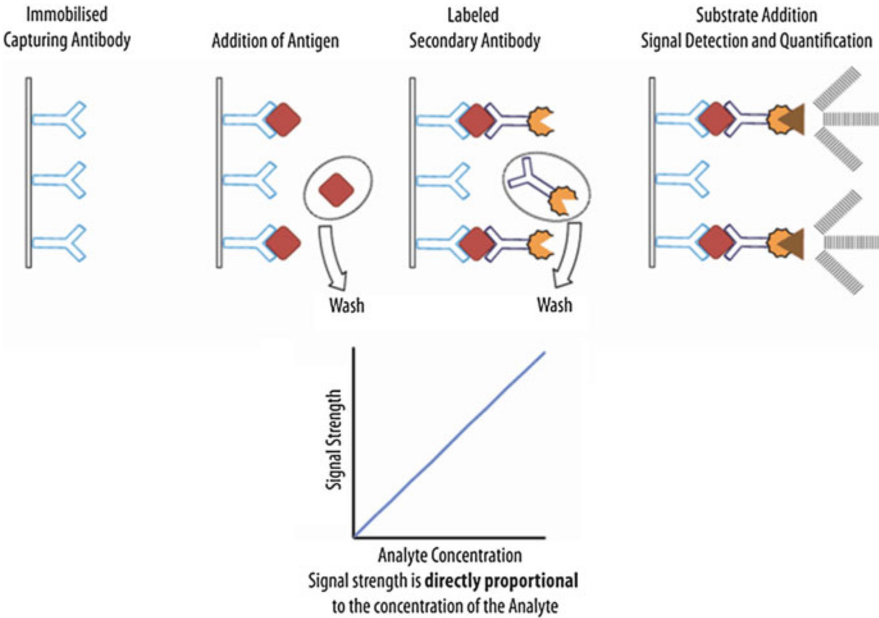

Fig. 3 ELISA for the quantitation of an antigen in a biological sample

Potential Applications to Prognosis, Other Diseases or Conditions

The levels of Gas6 in circulation have been studied in a variety of renal diseases, and generally it has been detected increased comparing with controls. The serum or plasma concentrations or expression of Gas6 is also determined in diabetes (Folli et al. 2011), obesity, insulin resistance, atherosclerosis, acute coronary syndrome, cardiovascular diseases, hypertension, coronary artery bypass grafting, stroke, rheumatoid arthritis (Kim et al. 2014), systemic lupus erythematosus, Behcet disease, multiple sclerosis, sepsis, preeclampsia, and psoriasis. All studies demonstrate that Gas6/Axl signaling alters and may be associated with prognosis and treatment of the disease.

In different cancer types including ovarian, endometrial, gastric, thyroid, renal clear cell carcinoma (Gustafsson et al. 2009a), and glioblastoma tumors, Gas6 expression is accelerated. Also in several primary human cancers, including lung adenocarcinoma (Shieh et al. 2005), brain cell tumor (Vajkoczy et al. 2006), ocular melanoma (Van Ginkel et al. 2004), pancreatic cancer (Wu et al. 2014) leukemia, gastric cancer, colon cancer, breast cancer, ovarian cancer, and glioblastoma, Axl is found overexpressed (Laurance et al. 2012). Furthermore AXL and GAS6 activated in cancers have been correlated with short survival time, poor prognosis, stimulation of accelerated invasiveness/metastasis, and drug resistance.

The literature supports GAS6 /AXL as a new candidate of treatment target in a variety of cancers as well as in different renal diseases and several other diseases. However most of the researches was in vitro and the number of in vivo studies is not so much especially in human. Future perspectives of more comprehensive researches will highlight in detail the role of Gas6 in diagnosis, follow-up, and treatment of diseases (Fig. 3).

Summary Points

- Gas6 (growth arrest-specific gene protein 6) is structurally linked to proteins from the family of plasma vitamin K-dependent ones.
- Gas6 is a ligand for Tyro3, Axl, and Mer (TAM receptors), and these receptors are members of a large family, receptor tyrosine kinases.
- Gas6/TAM signaling plays a role in chemotaxis; phagocytosis; cell migration, proliferation, adhesion, survival, and mitogenic activities; hematopoiesis; and inflammation.
- Gas6 plays a crucial role in several renal diseases.
- Uncovering the possible function and effects of the Gas6/Axl pathway may contribute forthcoming therapies which intend this pathway in patients with renal diseases.

References

Allen MP, Zeng C, Schneider K, et al. Growth arrest-specific gene 6 (Gas6)/adhesion related kinase (Ark) signaling promotes gonadotropin-releasing hormone neuronal survival via extracellular signal-regulated kinase (ERK) and Akt. Mol Endocrinol. 1999;13:191–201.

Angelillo-Scherrer A, García de Frutos P, Aparicio C, et al. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med. 2001;7:215–21.

Angelillo-Scherrer A, Burnier L, Flores N, et al. Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. J Clin Invest. 2005;115:237–46.

Atlas SA. The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. J Manag Care Pharm. 2007;13:9–20.

Avanzi GC, Gallicchio M, Cavalloni G, et al. GAS6, the ligand of Axl and Rse receptors, is expressed in hematopoietic tissue but lacks mitogenic activity. Exp Hematol. 1997;25:1219–26.

Avanzi GC, Gallicchio M, Bottarel F, et al. GAS6 inhibits granulocyte adhesion to endothelial cells. Blood. 1998;91:2334–40.

Balogh I, Hafizi S, Stenhoff J, et al. Analysis of Gas6 in human platelets and plasma. Arterioscler Thromb Vasc Biol. 2005;25:1280–6.

Batchu SN, Hughso A, Gerloff J, et al. Role of Axl in early kidney inflammation and progression of salt-dependent hypertension. Hypertension. 2013;62:302–9.

Bellido-Martín L, Frutos PG. Vitamin K-dependent actions of Gas6. Vitam Horm. 2008;78:185–209.

Bellosta P, Costa M, Lin DA, et al. The receptor tyrosine kinase ARK mediates cell aggregation by homophilic binding. Mol Cell Biol. 1995;15:614–25.

Borgel D, Clauser S, Bornstain C, et al. Elevated growth-arrest-specific protein 6 plasma levels in patients with severe sepsis. Crit Care Med. 2006;34:219–22.

Caraux A, Lu Q, Fernandez N, et al. Natural killer cell differentiation driven by Tyro3 receptor tyrosine kinases. Nat Immunol. 2006;7:747–54.
Cavet ME, Smolock EM, Ozturk OH, et al. Gas6-axl receptor signaling is regulated by glucose in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2008;28:886–91.
Cavet ME, Smolock EM, Menon P, et al. Gas6-Axl pathway: the role of redox-dependent association of Axl with nonmuscle myosin IIB. Hypertension. 2010;56:105–11.
Clauser S, Bachelot-Lozat C, Fontana P, et al. Physiological plasma Gas6 levels do not influence platelet aggregation. Arterioscler Thromb Vasc Biol. 2006;26:e22.
Collett G, Wood A, Alexander MY, et al. Receptor tyrosine kinase Axl modulates the osteogenic differentiation of pericytes. Circ Res. 2003;92:1123–9.
Collett GD, Sage AP, Kirton JP, et al. Axl/phosphatidylinositol 3-kinase signaling inhibits mineral deposition by vascular smooth muscle cells. Circ Res. 2007;100:502–9.
Correll PH, Morrison AC, Lutz MA, et al. Receptor tyrosine kinases and the regulation of macrophage activation. J Leukoc Biol. 2004;75:731–7.
Couchie D, Lafdil F, Martin-Garcia N, et al. Expression and role of Gas6 protein and of its receptor Axl in hepatic regeneration from oval cells in the rat. Gastroenterology. 2005;129:1633–42.
Dahlbäck B, Villoutreix BO. Regulation of blood coagulation by the protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition. Arterioscler Thromb Vasc Biol. 2005;25:1311–20.
Demarchi F, Verardo R, Varnum B, et al. Gas6 anti-apoptotic signaling requires NF-kappa B activation. J Biol Chem. 2001;276:31738–44.
Doi T. The contribution of mesangial cell proliferation to progressive glomerular injury. J Med Investig. 2001;48:1–4.
Donate-Correa J, Martín-Núñez E, Muros-de-Fuentes M, et al. Inflammatory cytokines in diabetic nephropathy. J Diabetes Res. 2015;2015:948417.
Erek-Toprak A, Bingol-Ozakpinar O, Karaca Z, et al. Association of plasma growth arrest-specific protein 6 (Gas6) concentrations with albuminuria in patients with type 2 diabetes. Ren Fail. 2014;36:737–42.
Erkoc R, Cikrikcioglu MA, Aintab E, et al. GAS6 intron 8 c.834+7G>A gene polymorphism in diabetic nephropathy, renal failure. Accepted 18 Mar 2015 ID:LRNF 2014 0756R1.
Folli F, Okada T, Perego C, et al. Altered insulin receptor signalling and β-cell cycle dynamics in type 2 diabetes mellitus. PLoS One. 2011;6(11):e28050.
Fridell YW, Villa Jr J, Attar EC, et al. GAS6 induces Axl-mediated chemotaxis of vascular smooth cells. J Biol Chem. 1998;273:7123–6.
Gallicchio M, Mitola S, Valdembri D, et al. Inhibition of vascular endothelial growth factor receptor 2-mediated endothelial cell activation by Axl tyrosine kinase receptor. Blood. 2005;105:1970–6.
Goruppi S, Ruaro E, Schneider C. Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts. Oncogene. 1996;12:471–80.
Goruppi S, Ruaro E, Varnum B. Gas6-mediated survival in NIH3T3 cells activates stress signalling cascade and is independent of Ras. Oncogene. 1999;18:4224–36.
Gould WR, Baxi SM, Schroeder R, et al. Gas6 receptors Axl, Sky and Mer enhance platelet activation and regulate thrombotic responses. J Thromb Haemost. 2005;3:733–41.
Gustafsson A, Boström AK, Ljungberg B, et al. Gas6 and the receptor tyrosine kinase Axl in clear cell renal cell carcinoma. PLoS One. 2009a;4:e7575.
Gustafsson A, Martuszewska D, Johansson M, et al. Differential expression of Axl and Gas6 in renal cell carcinoma reflecting tumor advancement and survival. Clin Cancer Res. 2009b;15:4742–9.
Guttridge KL, Luft JC, Dawson TL, et al. Mer receptor tyrosine kinase signaling: prevention of apoptosis and alteration of cytoskeletal architecture without stimulation or proliferation. J Biol Chem. 2002;277:24057–66.
Hafizi S, Alindri F, Karlsson R, et al. Interaction of Axl receptor tyrosine kinase with C1-TEN, a novel C1 domain-containing protein with homology to tensin. Biochem Biophys Res Commun. 2002;299:793–800.

Hall MO, Prieto AL, Obin MS, et al. Outer segment phagocytosis by cultured retinal pigment epithelial cells requires Gas6. Exp Eye Res. 2001;73:509–20.

Hansson K, Stenflo J. Post-translational modifications in proteins involved in blood coagulation. J Thromb Haemost. 2005;3:2633–48.

Hasanbasic I, Cuerquis J, Varnum B, et al. Intracellular signaling pathways involved in Gas6-Axl-mediated survival of endothelial cells. Am J Physiol Heart Circ Physiol. 2004;287:1207–13.

He X, Shen L, Villoutreix BO, et al. Amino acid residues in thrombin-sensitive region and first epidermal growth factor domain of vitamin K-dependent protein S determining specificity of the activated protein C cofactor function. J Biol Chem. 1998;273:27449–58.

Huang M, Rigby AC, Morelli X, et al. Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins. Nat Struct Biol. 2003;10:751–6.

International Diabetes Federation Diabetes Atlas, 6th ed. http://www.idf.org/diabetesatlas/6e/Update20124. Accessed March 2015.

Ishimoto Y, Nakano T. Release of a product of growth arrest-specific gene 6 from rat platelets. FEBS Lett. 2000;466:197–9.

Ishimoto Y, Ohashi K, Mizuno K, et al. Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. J Biochem. 2000;127:411–7.

Jonasch E, Gao J, Rathmell WK. Renal cell carcinoma. BMJ. 2014;349:g4797.

Kawaguchi H, Katagir M, Chikazu D. Osteoclastic bone resorption through receptor tyrosine kinase and extracellular signal-regulated kinase signaling in mature osteoclasts. Mod Rheumatol. 2004;14:1–5.

Kim HA, Nam JY, Jung JY, et al. Serum growth arrest-specific protein 6 levels are elevated in adult-onset Still's disease. Clin Rheumatol. 2014;33:865–8.

Konishi A, Aizawa T, Mohan A, et al. Hydrogen peroxide activates the Gas6-Axl pathway in vascular smooth muscle cells. J Biol Chem. 2004;279:28766–70.

Korshunov VA, Mohan AM, Georger MA, et al. Axl, a receptor tyrosine kinase, mediates flow-induced vascular remodeling. Circ Res. 2006;98:1446–52.

Laurance S, Lemarié CA, Blostein MD, et al. Growth Arrest-Specific Gene 6 (gas6) and Vascular Hemostasis. Adv Nutr. 2012;3:196–203.

Lee IJ, Hilliard B, Swami A, et al. Growth arrest-specific gene 6 (Gas6) levels are elevated in patients with chronic renal failure. Nephrol Dial Transplant. 2012;27:4166–72.

Lemke G, Rothlin CV. Immunobiology of the TAM receptors. Nat Rev Immunol. 2008;8:327–36.

Li R, Chen J, Hammonds G, et al. Identification of Gas6 as a growth factor for human Schwann cells. J Neurosci. 1996;16:2012–9.

Lutgens E, García de Frutos P, Aparicio C. Gas6−/−/ApoE−/− mice develop a collagen-rich, disorganized plaque phenotype, prone to intra-plaque hemorrhage. Circulation. 2000;102:38.

Lyons TJ. Glycation and oxidation: a role in the pathogenesis of atherosclerosis. Am J Cardiol. 1993;71:26B–31.

Manfioletti G, Brancolini C, Avanzi G, et al. The protein encoded by a growth arrest-specific gene (Gas6) is a new member of the vitamin-K-dependent proteins related to protein-S, a negative coregulator in the blood-coagulation cascade. Mol Cell Biol. 1993;13:4976–85.

Maquoi E, Voros G, Carmeliet P, et al. Role of Gas-6 in adipogenesis and nutritionally induced adipose tissue development in mice. Arterioscler Thromb Vasc Biol. 2005;25:1002–7.

Melaragno MG, Wuthrich DA, Poppa V, et al. Increased expression of Axl tyrosine kinase after vascular injury and regulation by G protein-coupled receptor agonists in rats. Circ Res. 1998;83:697–704.

Mima A, Arai H, Matsubara T, et al. Urinary Smad1 is a novel marker to predict later onset of mesangial matrix expansion in diabetic nephropathy. Diabetes. 2008;57:1712–22.

Muñoz X, Obach V, Hurtado B, et al. Association of specific haplotypes of GAS6 gene with stroke. Thromb Haemost. 2007;98:406–12.

Murao K, Imachi H, Sayo Y, et al. A product of growth arrest-specific gene 6 modulates scavenger receptor expression in human vascular smooth muscle cells. FEBS Lett. 1999;459:363–6.

Nagai K, Arai H, Yanagita M, et al. Growth arrest-specific gene 6 is involved in glomerular hypertrophy in the early stage of diabetic nephropathy. J Biol Chem. 2003;278:18229–34.

Nagai K, Matsubara T, Mima A, et al. Gas6 induces Akt/mTOR-mediated mesangial hypertrophy in diabetic nephropathy. Kidney Int. 2005;68:552–61.
Nagai K, Miyoshi M, Kake T, et al. Dual involvement of growth arrest-specific gene 6 in the early phase of human IgA nephropathy. PLoS One. 2013;8:e66759.
Nagata K, Ohashi K, Nakano T, et al. Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. J Biol Chem. 1996;271:30022–7.
Nakano T, Higashino K, Kikuchi N, et al. Vascular smooth muscle cell-derived, Gla-containing growth-potentiating factor for Ca(2+)-mobilizing growth factors. J Biol Chem. 1995;270:5702–5.
Paul LC. Chronic renal transplant loss. Kidney Int. 1995;47:1491–9.
Sather S, Kenyon KD, Lefkowitz JB, et al. A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. Blood. 2007;109:1026–33.
Schlessinger J, Ullrich A. Growth factor signaling by receptor tyrosine kinases. Neuron. 1992;9:383–91.
Schneider C, King RM, Philipson L. Genes specifically expressed at growth arrest of mammalian cells. Cell. 1988;54:787–93.
Sen P, Wallet MA, Yi Z, et al. Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. Blood. 2007;109:653–60.
Shao WH, Zhen Y, Rosenbaum J, et al. A protective role of Mer receptor tyrosine kinase in nephrotoxic serum-induced nephritis. Clin Immunol. 2010;136:236–44.
Shieh YS, Lai CY, Kao YR, et al. Expression of axl in lung adenocarcinoma and correlation with tumor progression. Neoplasia. 2005;7:1058–64.
Son BK, Kozaki K, Iijima K, et al. Statins protect human aortic smooth muscle cells from inorganic phosphate-induced calcification by restoring Gas6-Axl survival pathway. Circ Res. 2006;98:1024–31.
Son BK, Kozaki K, Iijima K, et al. Gas6/Axl-PI3K/Akt pathway plays a central role in the effect of statins on inorganic phosphate-induced calcification of vascular smooth muscle cells. Eur J Pharmacol. 2007;556:1–8.
Stenhoff J, Dahlbäck B, Hafizi S. Vitamin K-dependent Gas6 activates ERK kinase and stimulates growth of cardiac fibroblasts. Biochem Biophys Res Commun. 2004;319:871–8.
Su AI, Cooke MP, Ching KA, et al. Large-scale analysis of the human and mouse transcriptomes. Proc Natl Acad Sci. 2002;99:4465–70.
Tjwa M, Bellido-Martin L, Lin Y, et al. Gas6 promotes inflammation by enhancing interactions between endothelial cells, platelets, and leukocytes. Blood. 2008;111:4096–105.
Topham PS, Csizmadia V, Soler D, et al. Lack of chemokine receptor CCR1 enhances Th1 responses and glomerular injury during nephrotoxic nephritis. J Clin Invest. 1999; 104:1549–57.
Tumlin JA, Madaio MP, Hennigar R. Idiopathic IgA nephropathy: pathogenesis, histopathology, and therapeutic options. Clin J Am Soc Nephrol. 2007;2:1054–61.
Vajkoczy P, Knyazev P, Kunkel A, et al. Dominant-negative inhibition of the Axl receptor tyrosine kinase suppresses brain tumor cell growth and invasion and prolongs survival. Proc Natl Acad Sci. 2006;103:5799–804.
Valverde P. Effects of Gas6 and hydrogen peroxide in Axl ubiquitination and downregulation. Biochem Biophys Res Commun. 2005;333:180–5.
Van Ginkel PR, Gee RL, Shearer RL, et al. Expression of the receptor tyrosine kinase Axl promotes ocular melanoma cell survival. Cancer Res. 2004;64:128–34.
Vandivier RW, Henson PM, Douglas IS. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. Chest. 2006;129:1673–82.
Villoutreix BO, de Frutos G, Lovenklev P, et al. SHBG region of the anticoagulant cofactor protein S: secondary structure prediction, circular dichroism spectroscopy, and analysis of naturally occurring mutations. Proteins. 1997;29:478–91.

Waizenegger JS, Ben-Batalla I, Weinhold N, et al. Role of growth arrest-specific gene 6-Mer axis in multiple myeloma. Leukemia. 2015;29:696–704.
Walzer T, Vivier E. NK cell development: gas matters. Nat Immunol. 2006;7:702–4.
Wang H, Chen S, Chen Y, et al. The role of Tyro 3 subfamily receptors in the regulation of hemostasis and megakaryocytopoiesis. Haematologica. 2007;92:643–50.
Wu Y, Singh S, Georgescu MM, et al. A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. J Cell Sci. 2005;118:539–53.
Wu X, Liu X, Koul S, et al. AXL kinase as a novel target for cancer therapy. Oncotarget. 2014;5:9546–63.
Yanagita M. Gas6, warfarin, and kidney diseases. Clin Exp Nephrol. 2004;8:304–9.
Yanagita M, Arai H, Ishii K, et al. Gas6 regulates mesangial cell proliferation through Axl in experimental glomerulonephritis. Am J Pathol. 2001a;158:1423–32.
Yanagita M, Arai H, Nakano T, et al. Gas6 induces mesangial cell proliferation via latent transcription factor STAT3. J Biol Chem. 2001b;276:42364–9.
Yanagita M, Ishimoto Y, Arai H, et al. Essential role of Gas6 for glomerular injury in nephrotoxic nephritis. J Clin Invest. 2002;110:239–46.
Zhang ZX, Huang X, Jiang J,et al. Natural killer cells mediate long-term kidney allograft ınjury. transplantation. 2015 Feb 25. [Epub ahead of print].

Soluble Urokinase Receptor as a Biomarker in Kidney Disease 28

Takehiko Wada

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Abstract

Urokinase receptor (uPAR), a glycosylphosphatidylinositol (GPI)-anchored protein, engages in multiple protein-protein interactions and various biological functions. Its soluble form, soluble uPAR (suPAR), has been linked to various

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diseases and conditions including focal segmental glomerulosclerosis (FSGS). It has been long speculated that a circulating permeability factor should be implicated in the pathogenesis of the disease because a substantial portion of the patients experience recurrence shortly after renal transplantation. In 2011, Reiser and colleagues suggested that suPAR might be not only a potential pathogenic permeability factor but also a diagnostic biomarker. Following this report, renal researchers worldwide have analyzed the validity of suPAR as a diagnostic biomarker for primary FSGS and posttransplant FSGS. Furthermore, the utility of suPAR as a biomarker in other renal diseases including diabetic nephropathy and immunoglobulin A (IgA) nephrosis has been also suggested.

In this chapter, a comprehensive review of suPAR as a diagnostic biomarker and as a predictive biomarker in kidney diseases is provided.

Keywords

Antineutrophil cytoplasmic antibody-associated glomerulonephritis (ANCA-GN) • Biomarker suPAR (*see* soluble urokinase receptor (suPAR)) • Cardiotrophin-like cytokine 1 (CLC-1) • C-reactive protein (CRP) • Cytokine receptor-like factor 1 (CRLF-1) • Diabetic nephropathy • Enzyme-linked immunosorbent assay (ELISA) • Focal segmental glomerulosclerosis (FSGS) • Circulating permeability factors • End-stage renal disease • *In vitro* experiments • Posttransplant recurrence • Primary nephrotic syndrome • ROC analysis • Immunoglobulin A nephropathy (IgAN) • Lupus nephritis • Minimal change disease (MCD) • Nephrotic Syndrome Study Network (NEPTUNE) • Receiver operating characteristic (ROC) analysis • Soluble urokinase receptor (suPAR) • Diabetic nephropathy • Systemic lupus erythematosus (SLE) • Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) • Urokinase receptor (uPAR) • Monoclonal blocking antibody

Key Facts of Focal Segmental Glomerulosclerosis

- Focal segmental glomerulosclerosis (FSGS) was first described in 1957.
- FSGS was originally a histopathological term that describes scarring which occurred only in segments in some of glomeruli.
- FSGS is one of the major glomerular diseases that cause nephrotic syndrome.
- A variety of underlying mechanism of FSGS have been suggested, and podocyte injury is considered to be a common cause of FSGS.
- A large proportion of cases show resistance to treatment and the renal prognosis is generally poor.
- Renal transplantation is a treatment option for FSGS patients developing renal failure; however, the rate of posttransplant recurrence is substantially high (20–50 %).

Definitions

Enzyme-linked immunosorbent assay (ELISA) ELISA is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies, etc. In quantitative sandwich immunoassay technique employed in the assay for uPAR, an antibody specific for uPAR has been precoated onto a microplate. Samples and controls are pipetted into the wells, and uPAR is bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for uPAR is added to the wells. Following a washing process, a substrate solution is added, and the intensity of the developed color is measured.

Galactose affinity chromatography Affinity chromatography is a separation method based on a specific interaction between an immobilized ligand and its binding partner. In search for a circulating permeability factor, Savin and colleagues postulated that the factor might have a lectin-like interaction with sugar of the podocyte glycocalyx. Therefore, they used galactose-agarose beads to isolate a substance which could interact with galactose.

Glycosylphosphatidylinositol (GPI) anchor GPI anchor is a posttranslational modification that links modified protein in the outer leaflet of the plasma membrane.

Podocyte The podocyte is one of the resident cells in the glomerulus. Podocytes are located at the outer aspect of capillary tuft. A podocyte is considered to be a terminally differentiated cell, and it comprises a large cell body, major processes, and foot processes. The cells play an important role in filtration barrier system.

Receiver operating characteristic (ROC) analysis An ROC graph is a technique for visualizing, organizing, and selecting classifiers based on their performance. The curve is created by plotting the true positive rate against the false-positive rate at various threshold settings. An analysis using ROC curve (ROC analysis), which is useful to select possibly optimal models, has been extended for use in visualizing and analyzing the behavior of diagnostic systems.

Introduction

The urokinase receptor (also known as urokinase-type plasminogen activator receptor: uPAR) is a critical regulator of extracellular matrix (ECM) proteolysis, cell-ECM interactions, and cell signaling. The human uPAR cDNA encodes a polypeptide of 335 amino acids, including an N-terminal 22-residue secretion signal peptide and a C-terminal segment of 30 amino acids that is removable with the attachment of a glycosylphosphatidylinositol (GPI) anchor.

uPAR is a protein with three Ly-6 and uPAR (LU) domains (D1, D2, and D3) that is a proteinase receptor for urokinase. It is also involved in non-proteolytic pathways

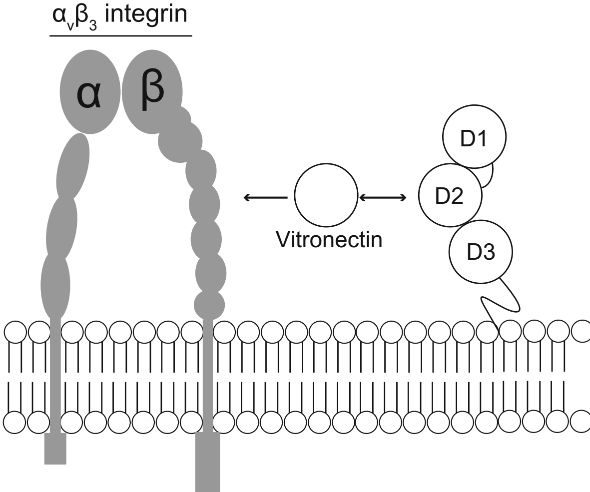


Fig. 1 Urokinase receptor and β_3-integrin signaling. Urokinase receptor (uPAR) activates β_3-integrin outside-in signaling. Vitronectin, which binds to uPAR and β_3-integrins, is required for the activity of this pathway. The activation of β_3-integrin signaling stimulates cell motility and invasion in murine podocytes

by forming signaling complexes with other transmembrane proteins, including integrins, caveolin, and G-protein-coupled receptors. uPAR is expressed in monocytes (Estreicher et al. 1990), neutrophils (Plesner et al. 1994), activated T cells (Nykjaer et al. 1994), endothelial cells (Barnathan et al. 1990), keratinocytes (Grondahl-Hansen et al. 1988), fibroblasts (Plow et al. 1986), smooth muscle cells (Reuning et al. 1994), megakaryocytes (Wohn et al. 1997), and tumor cells (Thuno et al. 2009). In the kidney, its expression has been detected in tubular epithelial cells (Florquin et al. 2001) and podocytes (Wei et al. 2008). The coordination of extracellular matrix proteolysis and cell signaling by uPAR underlies its biological function in cell migration, proliferation, and survival (Smith and Marshall 2010). Because uPAR is attached to the plasma membrane with a GPI anchor and lacks both transmembrane and intracellular domains, it requires transmembrane co-receptors such as integrins and vitronectin (Fig. 1). Importantly, uPAR can be released from the plasma membrane by the cleavage of the GPI anchor to become soluble. This form is called suPAR (soluble urokinase receptor, or soluble urokinase-type plasminogen activator receptor, Fig. 2). Recently, suPAR has attracted much attention from nephrologists. Here, suPAR as a biomarker of kidney diseases, mainly focal segmental glomerulosclerosis (FSGS), will be reviewed.

Potential Implication of Circulating Permeability Factors in FSGS

FSGS is a group of clinicopathological syndromes sharing a common glomerular lesion. It is estimated that primary FSGS accounts for approximately 40 % of primary nephrotic syndrome cases in adults worldwide, and the estimated incidence

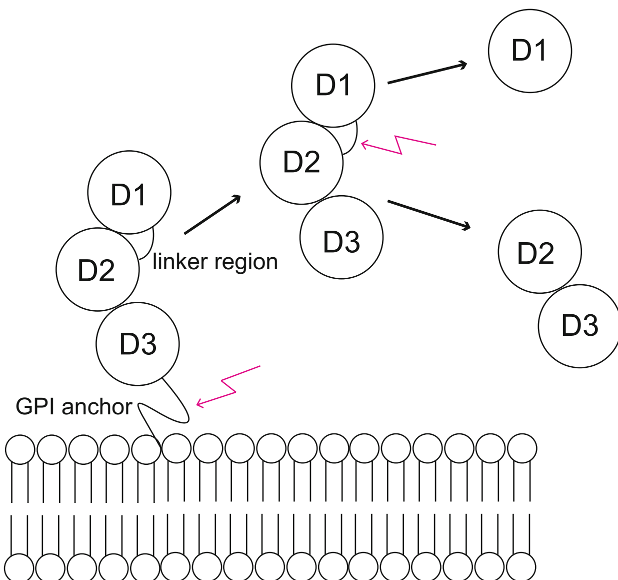


Fig. 2 The structure of urokinase receptor (uPAR) and the mechanism of soluble uPAR (suPAR) formation. Urokinase receptor (uPAR) is expressed on various cell types and is attached to the plasma membrane with a glycosylphosphatidylinositol (GPI) anchor. The cleavage of a GPI anchor results in the release of its soluble form, soluble urokinase receptor (suPAR). Both suPAR and uPAR contain three Ly-6 and uPAR (LU) domains, designated D1–D3, connected by short linker regions. The cleavage of the linker regions results in the shorter suPAR fragments

is approximately seven per one million (Kitiyakara et al. 2003). The patients with primary FSGS typically show a similar clinical presentation as minimal change disease (MCD), including heavy proteinuria with abrupt onset, severe hypoalbuminemia, and marked peripheral edema. Many cases are refractory to treatment by steroids and/or immunosuppressants, resulting in progressive renal impairment. This disorder is one of the most common primary glomerular diseases that cause end-stage kidney disease (ESRD) and has a prevalence of approximately 4 % in the USA. Although various factors including viruses, drugs, and genetic mutations have been identified as causes of FSGS, approximately 80 % of cases are idiopathic (D'Agati et al. 2011).

Primary FSGS has long been attributed to a putative circulating permeability factor for several reasons. First, disease recurrence after initial renal transplantation occurs in 20–50 % of recipients with primary FSGS. The recurrence rate may exceed 80 % in patients with a history of allograft loss due to recurrence (Vincenti and Ghiggeri 2005). Some recipients experience disease recurrence within hours after transplantation. Moreover, several lines of evidence have shown that patients with recurrent primary FSGS might have a substantial reduction in proteinuria after plasmapheresis (Davenport 2001; Matalon et al. 2001). Second, the plasma or plasma fractions from patients with FSGS can cause proteinuria in rats (Zimmerman 1984; Sharma et al. 2002; Avila-Casado Mdel et al. 2004). Third, sera from some

FSGS patients increased the permeability to albumin in glomeruli isolated from rats (Savin et al. 1996). Fourth, an infant born to a mother with FSGS had heavy, transient proteinuria, suggesting that a circulating permeability factor may be transmitted from the mother to her infant and may be responsible for the development of proteinuria (Kemper et al. 2001).

Furthermore, an interesting case of renal retransplantation was reported in 2012 (Gallon et al. 2012). A 27-year-old patient with ESRD due to primary FSGS received a kidney transplant from his healthy 24-year-old sister. Despite repeated plasmapheresis during his perioperative period, heavy proteinuria developed on the second post-operation day, and his renal function progressively declined. Allograft biopsy on day 6 revealed disease recurrence. On posttransplantation day 14, the allograft was removed and retransplanted to another patient, who was a 66-year-old man with ESRD due to type 2 diabetes mellitus. Immediately after the retransplantation, the allograft regained function and proteinuria improved from 25 to 1.2 g/24 h. Moreover, allograft biopsy on the post-retransplantation days 8 and 25 showed a reversal of the histopathologic lesions. In addition, the authors reported that the recipient continued to have excellent allograft function and mild proteinuria (0.27 g per 24 h) at 8 months after the retransplantation. This clinical course of “serial renal transplantation” strongly suggests that a circulating permeability factor is involved in the pathogenesis of the primary FSGS of the first recipient.

Searching for a Circulating Permeability Factor

Based on the observations described above, identification of a circulating permeability factor in FSGS has been a top priority for renal researchers. Thus far, cardiotrophin-like cytokine 1 (CLC-1) and anti-CD40 antibody have been suggested as potential circulating factors that can impair the glomerular filtration barrier and cause FSGS. CLC-1 is a member of interleukin-6 family and was found in serum from patients with active FSGS. In addition, several lines of evidence have suggested that CLC-1 might be a circulating factor associated with primary FSGS. Virginia Savin and her colleagues found CLC-1 in the active fraction from galactose affinity chromatography. They found that CLC-1 mimicked the effects of FSGS plasma on the permeability to albumin and decreased nephrin expression in glomeruli and cultured podocytes. A monoclonal antibody against CLC-1 blocked the effect of FSGS sera on albumin permeability (McCarthy et al. 2010), and recombinant human CLC-1 increased the albumin permeability of isolated rat glomeruli (Sharma et al. 2015). This effect was inhibited by a heterodimer composed of CLC-1 and co-secreted molecule cytokine receptor-like factor 1 (CRLF-1) (Sharma et al. 2015). It was suggested that Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling might be involved in the effects of CLC-1. However, the precise roles of CLC-1 in primary FSGS remain to be determined.

Recently, Delville et al. described a potential circulating antibody that can contribute to FSGS disease pathogenesis (Delville et al. 2014). They evaluated pathogenic antibodies in posttransplant recurrent FSGS utilizing serum samples

from 64 patients with and without recurrent FSGS and 34 non-FSGS control patients. They screened approximately 9,000 antigens in pre-transplant sera and selected ten antibodies targeting glomerular antigens. Among these, anti-CD40 antibody had the best correlation with the risk of posttransplant recurrent FSGS. Interestingly, although the binding capacity of anti-CD40 antibody to its antigen was low in the enzyme-linked immunosorbent assay (ELISA), a peptide microarray scan revealed that the immunogenicity of the CD40 protein in the two β-strand regions was specifically altered in the recurrent FSGS sera, suggesting that a perturbation in the conformation of the CD40 protein might cause posttransplant recurrence of FSGS. Furthermore, anti-CD40 antibodies purified from recurrent FSGS patients caused injury in human cultured podocytes, and this injury was ameliorated with a monoclonal blocking antibody against uPAR or with cycloRGDfv, a small molecule that blocks αvβ3 integrin activity. The injection of anti-CD40 antibodies purified from recurrent FSGS sera into wild-type mice caused a mild but significant increase in albuminuria during the first 8 days after injection, and albuminuria was markedly enhanced in the presence of suPAR. In contrast, no effect was observed in CD40-deficient mice or wild-type mice injected with blocking antibody to CD40. Based on these results, the authors suggested that the combination of anti-CD40 antibody and suPAR might contribute to glomerular injury in mice.

Taken together, CLC-1 and anti-CD40 antibody are potential candidates that functionally cause proteinuria and glomerulosclerosis through podocyte injury. However, to date, no data are available on the potential application of these molecules as diagnostic biomarkers for FSGS.

suPAR as a Potential Circulating Permeability Factor in FSGS

After a long search by many researchers, Wei et al. published an article reporting that suPAR might be a potential circulating permeability factor (Wei et al. 2011). Prior to this report, they had suggested that uPAR expressed in podocytes might have a deleterious effect on podocyte integrity. Utilizing uPAR-deficient mice and cultured cells, they demonstrated that uPAR activation led to foot process effacement and proteinuria through a mechanism that included the activation of αvβ3 integrin and the small GTPases cdc42 and Rac1 (Wei et al. 2008).

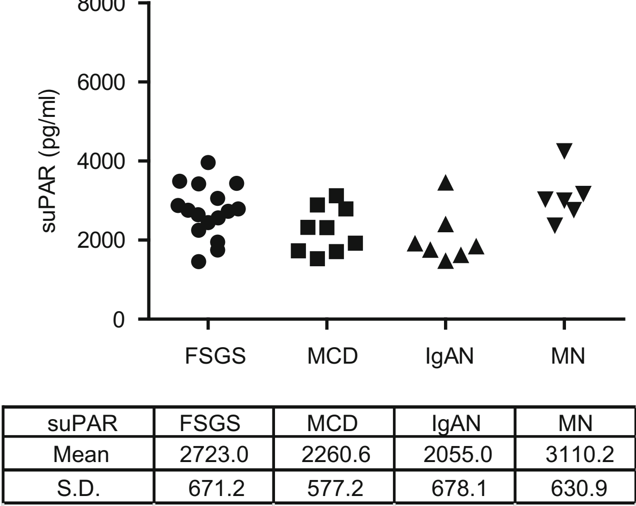
Following the study on uPAR, they focused on its soluble form, suPAR. They reported that the suPAR serum levels were elevated in 70 % of FSGS patients, and the suPAR levels in FSGS patients were significantly higher than those in patients with MCD (either in relapse or in remission), membranous nephropathy (MN), and preeclampsia or in healthy control subjects. They also reported that serum suPAR levels in patients with recurrent FSGS were significantly higher than those in patients with primary FSGS or nonrecurrent FSGS (Wei et al. 2011). In posttransplant recipients 1 year after transplantation, the patients who developed recurrent FSGS had had significantly higher levels of suPAR compared with those who did not (Wei et al. 2011).

This study also demonstrated that circulating suPAR activated podocyte β3 integrin and suggested that suPAR might play a causal role in primary FSGS. This indicates that suPAR might not only be a biomarker but also a pathogenic permeability factor for primary FSGS. This article caused considerable excitement in the field. Moreover, this group reported elevated serum suPAR levels in two different FSGS cohorts compared with healthy control subjects (Wei et al. 2012). They measured the serum suPAR concentration in 70 patients from the North America-based FSGS clinical trial (CT) and 94 patients from PodoNet, the Europe-based consortium studying steroid-resistant nephrotic syndrome. While only 6 % of age- and gender-matched control subjects showed serum suPAR levels higher than 3,000 pg/mL, elevated suPAR levels were observed in 84.3 % and 55.3 % of patients with FSGS patients in the CT and PodoNet cohorts, respectively. Their multiple regression analysis demonstrated that lower suPAR levels were associated with higher estimated glomerular filtration rate (eGFR), male gender, and treatment with mycophenolate mofetil. Huang et al. measured plasma suPAR in 74 patients with primary FSGS and demonstrated that their plasma suPAR levels were significantly higher than those of patients with MCD and MN and normal subjects, although they could not discriminate secondary FSGS from primary FSGS (Huang et al. 2013). On the basis of these promising data, renal researchers directed their attention to serum/plasma suPAR levels as a potential diagnostic biomarker for FSGS.

Is suPAR a Valid Diagnostic Biomarker for Primary FSGS?

Following the promising data reported by Wei and colleagues, several clinical studies were conducted worldwide to validate serum suPAR levels for the diagnosis of primary FSGS. However, data obtained from those different cohorts called the exciting hypothesis in question.

Maas and colleagues were the first to refute this hypothesis (Maas et al. 2012, 2013) by showing no difference in serum suPAR concentrations between idiopathic FSGS, secondary FSGS, and MCD in their small cohort. Later, this group reported the suPAR levels in 54 patients with biopsy-proven idiopathic FSGS as well as 476 non-FSGS patients (Meijers et al. 2014). In this study, they found that the serum suPAR levels and eGFR were negatively correlated and that the suPAR levels in idiopathic FSGS overlapped with those in non-FSGS controls. Taken together, they concluded that suPAR is not a clinical biomarker for FSGS (Meijers et al. 2014). A Japanese group performed a multicenter cross-sectional cohort study for patients with primary glomerular diseases, including FSGS (Wada et al. 2014). Serum suPAR concentrations in 69 patients with biopsy-proven primary glomerular diseases (38 patients with primary FSGS, 11 with MCD, 11 with IgA nephropathy, nine with MN) were measured. A reverse relationship between renal function and suPAR levels for the entire group of patients was detected (Fig. 3). Among the patients with normal renal function (eGFR >60 mL/min/1.73 m^2), the suPAR levels could not discriminate primary FSGS from other glomerular diseases or even healthy controls (Fig. 4).



suPAR	FSGS	MCD	IgAN	MN
Mean	2723.0	2260.6	2055.0	3110.2
S.D.	671.2	577.2	678.1	630.9

Fig. 3 Serum levels of the soluble urokinase receptor (suPAR) in patients with primary glomerular diseases without impaired renal function. Japanese patients with primary glomerular diseases whose estimated glomerular filtration rate (eGFR) was 60 ml/min/1.73 m^2 or higher were analyzed for their serum suPAR levels. One-way analysis of variance (ANOVA) revealed that there were significant differences in suPAR levels among the disease groups. However, no significant difference was detected in a multiple comparison (From Wada et al. 2014). Abbreviations: *FSGS* focal segmental glomerulonephritis, *MCD* minimal change disease, *IgAN* immunoglobulin A nephrosis, *MN* membranous nephropathy. The unit for suPAR concentration is pg/mL

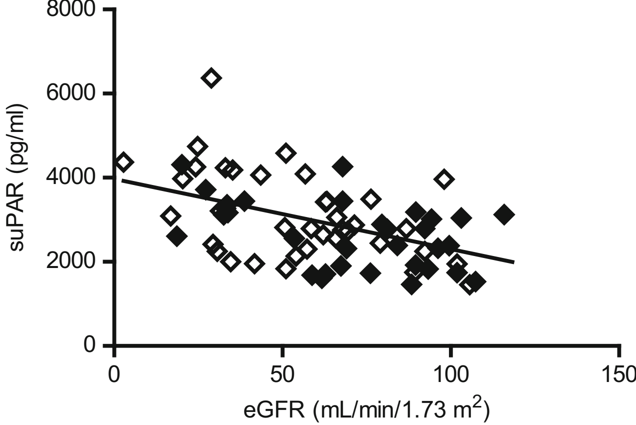


Fig. 4 The correlation between soluble urokinase receptor (suPAR) and the estimated glomerular filtration rate (eGFR). Serum suPAR levels were significantly and inversely correlated with the eGFR (Pearson's correlation coefficient test, $R^2 = 0.242$, $P < 0.0001$) in a cohort of Japanese patients with primary glomerular diseases. *Open diamonds* indicate focal segmental glomerulosclerosis (FSGS) patients. *Filled diamonds* indicate patients with minimal change disease, IgA nephropathy, or membranous nephropathy (From Wada et al. 2014)

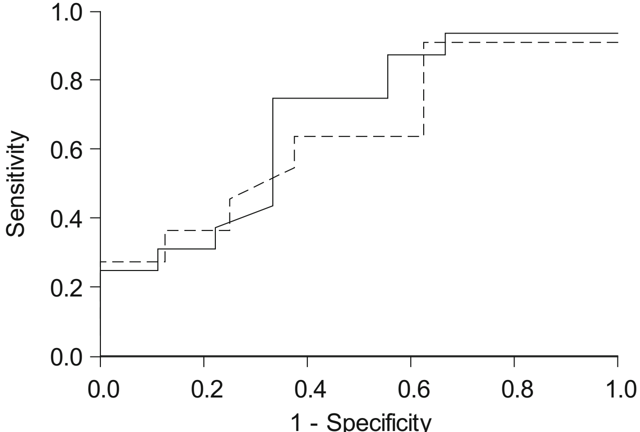


Fig. 5 Receiver operating characteristic (ROC) analysis of the serum soluble urokinase receptor (suPAR) in Japanese patients with focal segmental glomerulosclerosis (FSGS). ROC analysis in patients with FSGS or minimal change disease (MCD) from Wada et al. (2014). *Solid line*, ROC curve for patients with FSGS or MCD whose estimated glomerular filtration rate (eGFR) was 60 ml/min/1.73 m^2 or higher. The area under the ROC curve (AUC-ROC) was 0.684 ± 0.114 (95 % confidence interval (CI): 0.461–0.907, $P = 0.13$). *Dotted line*, ROC curve for the nephrotic patients with FSGS or MCD whose eGFR was 60 ml/min/1.73 m^2 or higher. The AUC-ROC was 0.621 ± 0.093 (95 % CI: 0.438–0.803, $p = 0.21$)

In this study, receiver operating characteristic (ROC) analysis was performed to determine whether the serum suPAR level is a valid diagnostic biomarker that can discriminate FSGS from MCD. As a result, the area under the ROC curve (AUC-ROC) was only 0.684 ± 0.114 (95 % confidence interval: 0.461–0.907, $p = 0.13$), which suggests that suPAR cannot be used to differentiate FSGS from MCD (Fig. 5).

There are also several reports on pediatric cohorts. Bock and colleagues described that suPAR levels were higher in children with nonglomerular kidney diseases compared with FSGS (Bock et al. 2013). Interestingly, female patients with heavy proteinuria had lower suPAR levels than those without proteinuria. Moreover, posttransplantation patients with either FSGS or non-FSGS had similar suPAR levels as before transplantation, independent of proteinuria, race, or sex. On the basis of those data, they also concluded that serum suPAR is unlikely the leading cause of childhood idiopathic FSGS (Bock et al. 2013). Sinha et al. measured serum suPAR levels prospectively in an Indian cohort of 469 children with renal disease, including steroid-resistant ($n = 237$), steroid-sensitive ($n = 138$), and congenital nephrotic syndrome ($n = 9$) and other proteinuric kidney diseases ($n = 85$), with samples from control children ($n = 85$) (Sinha et al. 2014). Similar percentages of patients in each group had elevated serum suPAR levels (>3,000 pg/mL). While approximately half of the children with proteinuric renal disease had elevated suPAR levels, there were no significant differences between the different histopathological disease groups. The serum suPAR levels were inversely correlated with eGFR as in the

studies described above and directly correlated with C-reactive protein (CRP). Furthermore, suPAR levels did not change significantly after therapy or during remission (Sinha et al. 2014). Harita et al. evaluated serum suPAR levels in Japanese pediatric patients with FSGS ($n = 20$), steroid-sensitive nephrotic syndrome (SSNS: $n = 26$), chronic glomerulonephritis (CGN: $n = 24$), and nonglomerular kidney disease ($n = 24$) (Harita et al. 2014). They reported that serum suPAR levels were significantly higher in patients with FSGS than in patients with SSNS or CGN but not higher compared with patients with nonglomerular kidney diseases. Notably, FSGS patients had lower eGFR compared with patients with SSNS or CGN. Therefore, higher suPAR levels in FSGS patients could be attributable to lower renal function. In this study, serum suPAR levels were negatively correlated with eGFR. Interestingly, serum suPAR levels in four patients who underwent renal transplantation decreased after transplantation. However, the same tendency was observed in three transplant recipients with nonglomerular kidney diseases, indicating that the decrease in suPAR levels after kidney transplantation is not disease-specific and that the reduction in suPAR might be due to improved renal function. The authors also observed that suPAR levels were not significantly high at the acute phase of posttransplant FSGS recurrence, even in patients who responded well to plasmapheresis. These results suggest that elevated suPAR levels are attributed mainly to impaired renal function.

Association Between Serum suPAR and Renal Function

As described above, most of the studies for both adult and pediatric cohorts showed that the suPAR levels in serum or plasma negatively correlated with renal function. In addition to those studies, several studies that tested a relatively large number of patients have been reported. Taniguchi et al. reported that this was the case in a Japanese cohort of 476 patients with chronic kidney disease (CKD), irrespective of underlying kidney diseases (Taniguchi et al. 2014). They also found that suPAR levels were associated with the decline rate of renal function. A recent report on 241 patients from the prospective, longitudinal, multicenter observational cohort of the Nephrotic Syndrome Study Network (NEPTUNE) described that serum suPAR levels at baseline were inversely correlated with eGFR (Spinale et al. 2015). In contrast, the initial report by Wei et al. (2011) did not provide information on the renal function of the patients studied. In their subsequent study on the CT cohort, they reported data from a multiple regression analysis showing that suPAR was negatively associated with eGFR at baseline (Wei et al. 2012). Although Li et al. reported that serum suPAR levels in FSGS patients were significantly higher than those in patients with MCD or MN, renal function in FSGS patients was significantly lower than in the other disease groups and the control subject group. Therefore, we cannot exclude the possibility that there were some biases in terms of renal function in this study (Li et al. 2014).

These results confirmed that serum suPAR levels are inversely associated with renal function. Although the behavior of suPAR in the kidney is currently unclear, given that its molecular size is small (20–50 kDa, depending on the degree of glycosylation and proteolytic cleavage), the molecule is likely to be filtered through the glomerular slit diaphragm. Therefore, it is possible that the decline in suPAR excretion with decreased GFR may cause increased serum levels.

suPAR as a Biomarker Predicting Clinical Course

As described above, most of the clinical studies on suPAR and primary glomerular diseases concluded that serum suPAR levels are not a valid diagnostic biomarker for FSGS. However, several lines of evidence have suggested that suPAR might be valid as a biomarker predicting clinical course.

Wei et al. reported in their article on CT and PodoNet cohorts that there was a positive association between the relative reduction of suPAR after 26 weeks of treatment and the reduction of proteinuria, with higher odds for complete remission in the CT cohort (Wei et al. 2012). Huang et al. described the relationship between the change in plasma suPAR level and clinical outcome during follow-up with a mean duration of 78.0 (IQR 22.0–119.4) weeks. Although plasma suPAR levels did not differ among the patient groups with treatment response (complete remission, partial remission, and treatment failure), the suPAR level of patients with complete remission decreased significantly, whereas patients with partial remission showed no significant change. Furthermore, the treatment failure group showed a significant increase in plasma suPAR levels (Huang et al. 2013).

Fujimoto et al. investigated the change in suPAR levels at baseline and at 2 months after treatment induction with the responsiveness to treatment. The serum suPAR levels in MCD patients significantly decreased during the 2-month treatment period, while those in the patients with FSGS or MN did not change significantly. As for suPAR levels in the non-intractable nephrotic syndrome group and intractable nephrotic syndrome group, they found significant suPAR decreases in patients with non-intractable nephrotic syndrome, whereas patients with intractable nephrotic syndrome showed significant suPAR increases. These results suggest that the change in suPAR during the first 2 months of treatment might be associated with clinical outcome (Fujimoto et al. 2015). Taniguchi et al. reported that serum suPAR levels at baseline were significantly associated with the decline rate of renal function during the first year and during the first 2 years of follow-up in their CKD cohort (Taniguchi et al. 2014). Li et al. performed a prospective study utilizing the Nanjing cohort to evaluate the relationship between serum suPAR levels and steroid responsiveness. They found that patients who were sensitive to steroids had significantly higher suPAR levels than patients who were resistant to steroids. For evaluation of the predictive value of baseline suPAR concentration on steroid responsiveness, they defined a cutoff value (3,400 pg/mL) based on their ROC analysis. As a result, at the 6-month follow-up in 84 patients with FSGS, suPAR levels were significantly decreased in those with high suPAR ($\geqq$3,400 pg/mL), while

suPAR levels did not change in the low-suPAR group even though they exhibited reduced proteinuria. In addition, a higher proportion of patients with higher suPAR were more likely to reach complete remission compared with patients with low suPAR concentrations (Li et al. 2014). In contrast, Meijers et al. reported that there was no difference in serum suPAR levels between steroid-resistant FSGS patients ($n = 15$) and steroid-sensitive FSGS patients ($n = 23$) in their Nijmegen cohort (Meijers et al. 2014). This discrepancy might be explained by the exclusion of patients with low eGFR (<40 ml/min/1.73 m^2) in the study by Li et al. Based on these studies, suPAR might be useful as a diagnostic marker in patients with reasonably preserved kidney function (Meijers and Sprangers 2014). Further studies will be needed to settle this controversy (Table 1).

It is also controversial whether serum suPAR levels are associated with the risk of posttransplant recurrence of FSGS. Wei et al. reported that subjects with recurrent FSGS sustained higher serum suPAR levels over the course of 1 year compared to those in which no posttransplant recurrence occurred, although no difference in posttransplant eGFR was observed between subjects with and without recurrent FSGS. Moreover, plasmapheresis significantly reduced serum suPAR levels in FSGS patients, and β_3-integrin activity evaluated with AP5 staining was lower in human cultured podocytes incubated with pheresed sera. They also reported the effects of plasmapheresis on clinical outcome in patients with posttransplant recurrent FSGS. While the serum suPAR levels in two patients who reached a clinical remission fell below 2,000 pg/mL, those in two patients who failed to reach a remission despite plasmapheresis remained elevated (Wei et al. 2011). In contrast, Harita et al. demonstrated that serum or plasma suPAR levels at posttransplant recurrence in the acute phase were not significantly high in their pediatric cohort, suggesting that posttransplant recurrence of FSGS was not caused by elevated suPAR levels (Harita et al. 2014). Their data also suggested that serum or plasma suPAR levels did not have predictive value for recurrent FSGS.

Issues Concerning the Measurement of suPAR Levels

The current ELISA system provided by R&D systems, which has been employed in virtually all of the studies examining the potential ability of suPAR as a diagnostic biomarker, measures all of its fragments. As discussed above, suPAR consists of three LU domains and can be cleaved to smaller fragments, a D1 fragment and a D2-D3 fragment (Fig. 2). Reiser's group suggested that the D2-D3 fragment of suPAR, which was specifically detected in sera from patients with FSGS, might contribute to FSGS through the activation of $\alpha_v\beta_3$-integrin signaling (unpublished data). However, to date, a system that can measure a specific suPAR fragment has not been developed. That type of ELISA system would facilitate the evaluation of the potential role of suPAR as a diagnostic biomarker. Moreover, because the glycosylation of suPAR molecules might be associated with their activity, the relationship between the glycosylation and activity of suPAR should be investigated.

Table 1 List of the studies investigating the validity of serum/plasma suPAR levels as a diagnostic biomarker for primary focal segmental glomerulosclerosis (FSGS)

n (FSGS)	Age	suPAR in FSGS (pg/mL)	Diseases for comparison (n)	Correlation with renal function	Reference	Notes
23	19 (12–44)	Unknown	MCD (25) MN (16) preeclampsia (7)	Unknown	Wei et al. 2011	Sera from 54 transplant FSGS patients were also evaluated
70	Unknown	4,588 ± 203	C (150)	Yes (multiple regression analysis)	Wei et al. 2012	CT cohort
94	Unknown	3,497 ± 195	C (150)	Unknown	Wei et al. 2012	PodoNet cohort
11	Unknown	2,392 (median)	MCD (7) secondary FSGS (5)	Yes	Maas et al. 2012	
74	29 (13–84)	2,923 (2,205–4,360)	MCD (14) MN (29) secondary FSGS (56) C (56)	Yes (only in FSGS patients)	Huang et al. 2013	
20	12.1 ± 5.0	2,487 (2,191–3,351)	Non-FSGS glomerular diseases (24), nonglomerular CKD (26), C (29)	Yes	Bock et al. 2013	
44	47 (33–60)	3,772 (2,622–4,422)	FSGS remission (10) non-FSGS CKD (476)	Yes	Meijers et al. 2014	
126	9.4 ± 4.8	3,316.1 ± 101.2	Steroid-resistant MCD (142) steroid-sensitive nephrotic syndrome (151) proteinuric CKD (85)	Yes	Sinha et al. 2014	eGFR >30 mL/min/1.73 m^2 only
16	55.6 ± 16.3	2,723 ± 6,71.2	MCD (11) MN (9) IgAN (11) C (17)	Yes	Wada et al. 2014	eGFR >60 mL/min/1.73 m^2 only

20	13.1 ± 5.2	2,837 ± 1,266	SSNS (26) CGN (24) nonglomerular kidney disease (24)	Yes	Harita et al. 2014	
109	28 ± 14	3,512 (2,232–4,231)	MCD (20) MN (22)	Yes (univariate model only)	Li et al. 2014	
95	36 (17–52)	3,178 (2,681–3,763)	MCD (62) MN (52) IgAN (32)	Yes	Spinale et al. 2015	NEPTUNE cohort
8	48.0 (29.0–68.0)	3,212 (2,769–4,196)	MCD (12) MN (15) MPGN (2) ANCA-GN (13)	Yes	Fujimoto et al. 2015	

FSGS focal segmental glomerulosclerosis, *MCD* minimal change disease, *MN* membranous nephropathy, *C* control, *CKD* chronic kidney disease, *MPGN* membranoproliferative glomerulonephritis, *ANCA-GN* antineutrophil cytoplasmic antibodies-associated glomerulonephritis, *IgAN* immunoglobulin A nephropathy, *SSNS* steroid-sensitive nephrotic syndrome, *CGN* chronic glomerulonephritis

Continuous variables are presented as median (interquartile range) or mean ± standard deviation

Urinary suPAR as a Biomarker for FSGS

As described above, the results from most of the clinical studies conducted so far suggested that the serum suPAR concentration was not a valid diagnostic biomarker for FSGS. In contrast, urinary suPAR levels might be a more promising parameter as a biomarker, and several clinical studies have been conducted to evaluate the value of urinary suPAR as a diagnostic tool. Several study groups have described positive correlations between serum or plasma suPAR and urinary suPAR. Huang et al. analyzed urinary suPAR levels in patients with several different glomerular diseases in a Chinese cohort. In their study, urinary suPAR levels were significantly correlated with plasma suPAR levels in primary FSGS patients but not in MCD or MN patients (Huang et al. 2014). Fujimoto et al. showed that urinary suPAR levels correlated with serum suPAR levels in patients with primary nephrotic syndrome due to FSGS, MCD, MN, or membranoproliferative glomerulonephritis (MPGN) in a Japanese cohort (Fujimoto et al. 2015). The significant positive correlation was also observed in a NEPTUNE cohort (Spinale et al. 2015). However, in an Indian pediatric cohort, urinary suPAR levels were not significantly correlated with serum suPAR levels (Sinha et al. 2014).

Urinary suPAR levels consistently correlated with urinary protein excretion, although the patients with glomerular diseases other than primary FSGS failed to show positive associations in a Chinese cohort. Huang et al. found that urinary suPAR levels positively correlated with urinary protein excretion in primary FSGS patients but not in MCD, MN, or secondary FSGS patients. The urinary suPAR levels in primary FSGS patients were significantly higher than those in patients with MCD, MN, and secondary FSGS and normal subjects (Huang et al. 2014).

suPAR as a Causative Factor for Primary FSGS

Aside from the role of suPAR as a diagnostic biomarker, suPAR in FSGS patient sera could play a causative role in primary FSGS. Wei et al. used immunoprecipitation to demonstrate that suPAR could interact with β_3-integrin. Their *in vitro* experiments revealed that sera from recurrent FSGS patients with high levels of suPAR strongly induced the AP5 signal, indicating that β_3-integrin signaling was activated. Activation of podocyte β_3-integrin signaling was also shown in human biopsy specimens from patients with primary or recurrent FSGS. Furthermore, they demonstrated that suPAR caused proteinuria and FSGS utilizing three different mouse models, including uPAR-knockout mice injected with recombinant suPAR, hybrid-transplant mice modeling endogenous suPAR release, and genetically engineered wild-type mice that drive the expression of a suPAR plasmid in the skin (Wei et al. 2011).

In contrast, Spinale et al. could not reproduce the pathogenic effects of suPAR in their recent study (Spinale et al. 2015). They utilized wild-type mice injected with uPAR/Fc chimera as an acute model. However, they did not observe an increase in urinary protein excretion at 12 or 24 h after the injection, although serum levels of suPAR showed a 12-fold increase at 4 h, and a nearly 6-fold increase in suPAR

persisted at 24 h. As a chronic model, they generated an inducible transgenic mouse that can express suPAR in its liver. Although serum suPAR concentration increased approximately twofold by day 13 and nearly threefold at day 44, urinary protein excretion was not detected when the experiment was terminated. These results suggest that the upregulation of suPAR in the circulation might not be pathogenic. In addition, Cathelin et al. also investigated the effects of forced increases in suPAR levels in mice. In that experiment, they failed to induce podocyte injury or proteinuria by the injection of monomeric mouse uPAR produced in eukaryotic S2 cells or uPAR/Fc chimera, even though glomerular suPAR deposits were observed (Cathelin et al. 2014). As discussed above, the studies on the pathogenesis of suPAR used different uPAR/suPAR molecules. Monomeric three-domain mouse suPAR was produced in *Drosophila melanogaster* Schneider 2 cells and used in the study by Cathelin and colleagues (2014). This monomeric mouse suPAR was structurally well characterized (Gardsvoll and Ploug 2007). The recombinant uPAR/Fc chimera protein produced in mouse NS0 cells can be structurally considered a circulating molecule, and elevated levels of suPAR were detected by ELISA assay in the injected mice (Spinale et al. 2015). The administration of this molecule induced significant proteinuria in Plaur-deficient mice (Wei et al. 2011) but not in wild-type mice (Spinale et al. 2015; Cathelin et al. 2014). Delville et al. used recombinant human suPAR in their study. Their animal study revealed that proteinuria induced by anti-CD40 antibody was exacerbated in the presence of this human suPAR.

Wei et al. used a mouse suPAR cDNA clone (IMAGE cDNA clone 3158012) to evaluate the effects of chronic suPAR overexpression. This clone, which contains the coding sequence for the D1 and D2 domains, was delivered into mouse skin by *in vivo* electroporation. This clone contains a retained intron 4 that results in a frameshift mutation at the site corresponding to amino acid residue 133 within the second uPAR domain and premature termination of translation within the uPAR D2 domain (Fig. 2). On the basis of its predicted structure, some have questioned whether this cDNA could produce a properly folded and stable protein. As discussed, different experimental settings have yielded conflicting results, and the causative roles of suPAR in proteinuria remain elusive.

suPAR and Antineutrophil Cytoplasmic Antibodies-Associated Glomerulonephritis

As described above, it remains controversial whether serum suPAR levels are a valid diagnostic biomarker for FSGS. Among the other renal diseases, antineutrophil cytoplasmic antibodies-associated glomerulonephritis (ANCA-GN) has been linked to suPAR. Wada et al. compared serum suPAR concentrations in five patients with ANCA-GN with those in five patients with primary glomerular diseases matched for age and eGFR. The suPAR concentrations in the ANCA-GN group were significantly higher than in the control group (6,791.3 ± 1,513.0 pg/mL vs. 3,727.5 ± 8,182 pg/mL, $P = 0.01$). Fujimoto et al. also measured serum suPAR levels in myeloperoxidase (MPO)-ANCA-GN patients ($n = 13$). In that study, they found

that serum suPAR levels before immunosuppressive therapy were significantly correlated with clinical severity determined by age, serum creatinine level, serum CRP level, and coexistence of pulmonary lesions (Fujimoto et al. 2015). They also reported that serum suPAR and CRP levels were significantly correlated in the ANCA-GN group. Given that previous studies suggested that elevated serum suPAR levels were associated with nonspecific inflammation (Backes et al. 2012; Koch et al. 2011; Yilmaz et al. 2011), the high levels of suPAR observed in ANCA-GN patients might be attributable to inflammation in this disease setting.

suPAR and Lupus Nephritis

A Chinese group recently reported plasma suPAR levels in patients with lupus nephritis (Qin et al. 2015). They compared plasma suPAR levels among the lupus nephritis group, the nonrenal systemic lupus erythematosus (SLE) group (SLE patients without renal involvement), and the control group. The plasma suPAR levels in the lupus nephritis group were significantly higher than those in the nonrenal SLE group or in the control group. When they compared the plasma suPAR levels longitudinally in 14 patients with lupus nephritis between the active and remission phases, the average plasma suPAR levels in remission decreased significantly compared with the self-controls in the active phases. As with FSGS described above, plasma suPAR levels in patients with lupus nephritis negatively correlated with eGFR. When the subjects were limited to the lupus nephritis patients whose eGFR was normal (>60 mL/min/1.73 m^2), plasma suPAR levels in the patients with lupus nephritis were still higher than those in the nonrenal SLE patients or in the control subjects. Interestingly, plasma suPAR levels positively correlated with the clinical disease activity assessed by Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). In terms of histopathological findings, within lupus nephritis, no significant difference in plasma suPAR levels was observed among the histological classes according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification. However, they described significant correlations between plasma suPAR levels and renal activity indices scores, endocapillary hypercellularity, cellular crescents, interstitial inflammation, renal chronic indices scores, tubular atrophy, and interstitial fibrosis. In their longitudinal study, patients with lupus nephritis were followed up for an average duration of 38 months. Their univariate analysis of renal prognosis showed that plasma suPAR level was a significant risk factor ($p = 0.01$, HR 6.326, 95 % CI 1.466–27.298), while their multivariate analysis failed to detect a significant association ($p = 0.61$, HR 1.091, 95 % CI 0.790–1.507). They additionally described a group of patients with higher suPAR levels (>3,443.4 pg/mL) that presented with worse renal outcome, although they did not show clinical and histopathological profiles in these two groups, and it is unclear if there were any confounders (Qin et al. 2015).

A Hungarian group reported that SLE patients had significantly higher levels of serum suPAR ($n = 89$, median 4,580 pg/mL, IQR: 3,720–6,300) compared with healthy controls ($n = 29$, median 2,800, IQR: 2,060–3,420). Their ROC analysis

revealed that the cutoff value of suPAR to discriminate between patients with high disease activity (SLEDAI >8) and those with moderate disease activity or in remission (SLEDAI ≦8) was 5,700 pg/mL. However, they did not show the data of the suPAR levels specifically in the patients with lupus nephritis (Toldi et al. 2012).

Enocsson et al. recently described the association of suPAR levels with organ damage in patients with SLE (Enocsson et al. 2013). In their cohort, serum creatinine, CRP, erythrocyte sediment rate (ESR), C4, and urinary albumin as well as leukocyte, erythrocyte, and platelet counts were significantly associated with serum suPAR levels. No significant association between suPAR levels and the disease activity defined as either the SLE disease activity index 2000 (SLEDAI-2K) or the physician's global assessment (PGA) was detected. However, a significant positive association was found between suPAR levels and organ damage. The group found that renal, ocular, neuropsychiatric, skin, and peripheral vascular organ damages had a significant positive association with serum suPAR levels. Renal injury had the most pronounced impact on suPAR levels.

suPAR and Diabetic Nephropathy

Several lines of evidence have indicated that serum or plasma suPAR levels may be associated with diabetic complications. To date, most of the evidence is on diabetic macrovascular complications such as atherosclerosis (Olson et al. 2010), cardiovascular disease (Persson et al. 2012, 2014; Sehestedt et al. 2011), and stroke (Persson et al. 2014). Microinflammation in atherosclerosis might be associated with elevated suPAR levels. Recently, Theilade et al. described the results from their single-center cross-sectional study on type 1 diabetes patients (Theilade et al. 2015). In terms of renal complications, they found a stepwise increase in median suPAR levels in patients with normo-, micro-, and macroalbuminuria. Using ROC analyses, the authors found that suPAR was a better predictor of albuminuria than age, systolic blood pressure, or CRP. However, further large-scale prospective studies will be needed to determine if serum suPAR concentration is a biomarker of diabetic nephropathy.

suPAR and IgA Nephropathy

Immunoglobulin A nephropathy (IgAN), characterized by IgA deposition in the glomerular mesangium, is the most common form of idiopathic glomerulonephritis leading to chronic kidney disease. Segmental glomerulosclerosis, one of the variables in the Oxford classification of IgAN, is similar to the histopathological feature observed in FSGS and is associated with poor prognosis. A group from China recently described their data on plasma suPAR in 569 IgAN patients (Zhao et al. 2015). Plasma suPAR levels were similar between patients with and without segmental glomerulosclerosis. However, suPAR plasma levels positively correlated with proteinuria and negatively correlated with eGFR. Even after the data were adjusted for eGFR, plasma suPAR remained positively correlated with proteinuria. The plasma suPAR levels in IgAN

patients and in primary FSGS patients with nephrotic syndrome were not significantly different. In that study, plasma suPAR levels in patients with extensive effacement of podocyte foot processes were significantly higher than those with segmental effacement on the basis of comparable eGFR (Zhao et al. 2015). Wada et al. also reported that the serum suPAR levels in IgAN patients were not different from those in patients with other glomerulopathies in the cohort of patients with normal renal function (Fig. 3, Wada et al. 2014). Taken together, in IgAN patients, plasma or serum suPAR levels are again associated with renal function, and they are likely to be dependent upon the severity of glomerular damage.

Potential Applications to Prognosis, Other Disease, or Conditions

As discussed in the section "suPAR as a Biomarker Predicting Clinical Course," the change in serum/plasma suPAR levels might be associated with treatment response in patients with nephrotic syndrome; however, conflicting results have been reported, and the predictive value for treatment response remains controversial. A biomarker that can predict posttransplant FSGS recurrence would be extremely valuable for FSGS patients. However, it is still undetermined if suPAR levels are predictive in posttransplant recurrence. Taken together, the value of suPAR levels as a prognostic biomarker in renal diseases remains elusive.

Meanwhile, the elevated serum levels of suPAR have been reported under several different disease conditions such as sepsis (Backes et al. 2012), liver cirrhosis (Berres et al. 2012), rheumatic arthritis (Slot et al. 1999), and malignancies (Mazar et al. 1999). However, suPAR does not appear to be superior to other biomarkers (e.g., CRP and procalcitonin for sepsis) (Donadello et al. 2012). Recently, the value of suPAR as a biomarker for cardiovascular disease (CVD) has gotten substantial attention (Hodges et al. 2015). In the Danish MONICA cohort, elevated levels of suPAR were associated with the incidence of CVD, type 2 diabetes mellitus, cancer, and mortality (Eugen-Olsen et al. 2010). In this population, suPAR predicted CVD with HR 1.7 (95 % CI 1.1–2.8, $p = 0.027$) for women and HR 2.1 (95 % CI 1.4–3.2, $p < 0.001$) for men (Lyngbæk et al. 2013). Meijers et al. conducted a prospective observational study of 476 patients with mild-to-moderate CKD (mean follow up period; 57 months). Higher suPAR levels were significantly associated with overall mortality (univariate HR 5.35) and cardiovascular events (univariate HR 5.06), and the multivariate analysis also demonstrated that higher suPAR levels were associated with cardiovascular events (Meijers et al. 2015).

Conclusion

As discussed above, although suPAR was once viewed as a promising diagnostic biomarker for primary FSGS, studies from several different groups have questioned its validity. Most clinical studies have revealed that serum or plasma suPAR levels are negatively correlated with renal function. Serum or plasma suPAR levels

measured with the currently available ELISA kit are likely to reflect the patients' renal function, inflammation, and some other clinical conditions and are not valid diagnostic biomarkers for primary glomerular diseases. Further research will be necessary to determine if a certain form of suPAR (i.e., shorter fragments of suPAR or hypoglycosylated suPAR) could be a valid biomarker. It also remains unclear whether suPAR could be a pathogenic factor to induce podocyte injury, resulting in glomerulosclerosis.

Summary Points

- The discovery of a diagnostic biomarker for focal segmental glomerulosclerosis (FSGS) has been awaited because differentiating between FSGS and minimal change disease (MCD) is difficult, especially in the early phase of the disease.
- Clinical findings have suggested that circulating permeability factor(s) might be implicated in the pathogenesis of idiopathic or recurrent FSGS.
- Soluble urokinase receptor (suPAR) was described as a potential diagnostic biomarker for primary FSGS and posttransplant recurrent FSGS and as a pathogenic factor that causes podocyte injury through the activation of β3-integrin signaling.
- Most studies have not supported the initial data showing that suPAR could be used as a diagnostic biomarker for FSGS and reported that serum/plasma suPAR levels were inversely correlated with renal function.
- Serum/plasma suPAR levels might be a valid biomarker to predict the patients' clinical course.
- The ELISA system currently used in virtually all of the studies on suPAR levels measured all forms of suPAR, which might mask the differences in shorter fragments or glycosylation of suPAR.
- Several studies have suggested that the suPAR urinary level might be a better biomarker than the serum/plasma levels.
- Data from animal experiments have also questioned the causative roles of suPAR in FSGS.
- Clinical data on the validity of suPAR as a diagnostic marker for other renal diseases have yielded some positive data. However, we cannot exclude the possibility that there might be confounding factors such as renal dysfunction and inflammation.

References

Avila-Casado Mdel C, Perez-Torres I, Auron A, et al. Proteinuria in rats induced by serum from patients with collapsing glomerulopathy. Kidney Int. 2004;66:133–43.

Backes Y, van der Sluijs KF, Mackie DP, et al. Usefulness of suPAR as a biological marker in patients with systemic inflammation or infection: a systematic review. Intensive Care Med. 2012;38:1418–28.

Barnathan ES, Kuo A, Kariko K, et al. Characterization of human endothelial cell urokinase-type plasminogen activator receptor protein and messenger RNA. Blood. 1990;76:1795–806.
Berres ML, Schlosser B, Berg T, et al. Soluble urokinase plasminogen activator receptor is associated with progressive liver fibrosis in hepatitis C infection. J Clin Gastroenterol. 2012;46:334–8.
Bock ME, Price HE, Gallon L, et al. Serum soluble urokinase-type plasminogen activator receptor levels and idiopathic FSGS in children: a single-center report. Clin J Am Soc Nephrol. 2013;8:1304–11.
Cathelin D, Placier S, Ploug M, et al. Administration of recombinant soluble urokinase receptor per se is not sufficient to induce podocyte alterations and proteinuria in mice. J Am Soc Nephrol. 2014;25:1662–8.
D'Agati VD, Kaskel FJ, Falk RJ. Focal segmental glomerulosclerosis. N Engl J Med. 2011;365:2398–411.
Davenport RD. Apheresis treatment of recurrent focal segmental glomerulosclerosis after kidney transplantation: re-analysis of published case-reports and case-series. J Clin Apher. 2001;16:175–8.
Delville M, Sigdel TK, Wei C, et al. A circulating antibody panel for pretransplant prediction of FSGS recurrence after kidney transplantation. Sci Transl Med. 2014;6:256ra136.
Donadello K, Scolletta S, Covajes C, et al. suPAR as a prognostic biomarker in sepsis. BMC Med. 2012;10:2.
Enocsson H, Wettero J, Skogh T, et al. Soluble urokinase plasminogen activator receptor levels reflect organ damage in systemic lupus erythematosus. Transl Res. 2013;162:287–96.
Estreicher A, Muhlhauser J, Carpentier JL, et al. The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. J Cell Biol. 1990;111:783–92.
Eugen-Olsen J, Andersen O, Linneberg A, et al. Circulating soluble urokinase plasminogen activator receptor predicts cancer, cardiovascular disease, diabetes and mortality in the general population. J Intern Med. 2010;268:296–308.
Florquin S, van den Berg JG, Olszyna DP, et al. Release of urokinase plasminogen activator receptor during urosepsis and endotoxemia. Kidney Int. 2001;59:2054–61.
Fujimoto K, Imura J, Atsumi H, et al. Clinical significance of serum and urinary soluble urokinase receptor (suPAR) in primary nephrotic syndrome and MPO-ANCA-associated glomerulonephritis in Japanese. Clin Exp Nephrol. 2015;19:804–14.
Gallon L, Leventhal J, Skaro A, et al. Resolution of recurrent focal segmental glomerulosclerosis after retransplantation. N Engl J Med. 2012;366:1648–9.
Gardsvoll H, Ploug M. Mapping of the vitronectin-binding site on the urokinase receptor: involvement of a coherent receptor interface consisting of residues from both domain I and the flanking interdomain linker region. J Biol Chem. 2007;282:13561–72.
Grondahl-Hansen J, Lund LR, Ralfkiaer E, et al. Urokinase- and tissue-type plasminogen activators in keratinocytes during wound reepithelialization in vivo. J Invest Dermatol. 1988;90:790–5.
Harita Y, Ishizuka K, Tanego A, et al. Decreased glomerular filtration as the primary factor of elevated circulating suPAR levels in focal segmental glomerulosclerosis. Pediatr Nephrol. 2014;29:1553–60.
Hodges GW, Bang CN, Wachtell K, et al. suPAR: a new biomarker for cardiovascular disease? Can J Cardiol. 2015;31:1293–302.
Huang J, Liu G, Zhang YM, et al. Plasma soluble urokinase receptor levels are increased but do not distinguish primary from secondary focal segmental glomerulosclerosis. Kidney Int. 2013;84:366–72.
Huang J, Liu G, Zhang YM, et al. Urinary soluble urokinase receptor levels are elevated and pathogenic in patients with primary focal segmental glomerulosclerosis. BMC Med. 2014;12 (81). 7015-12-81.
Kemper MJ, Wolf G, Muller-Wiefel DE. Transmission of glomerular permeability factor from a mother to her child. N Engl J Med. 2001;344:386–7.

Kitiyakara C, Kopp JB, Eggers P. Trends in the epidemiology of focal segmental glomerulosclerosis. Semin Nephrol. 2003;23:172–82.

Koch A, Voigt S, Kruschinski C, et al. Circulating soluble urokinase plasminogen activator receptor is stably elevated during the first week of treatment in the intensive care unit and predicts mortality in critically ill patients. Crit Care. 2011;15:R63.

Li F, Zheng C, Zhong Y, et al. Relationship between serum soluble urokinase plasminogen activator receptor level and steroid responsiveness in FSGS. Clin J Am Soc Nephrol. 2014;9:1903–11.

Lyngbæk S, Marottt JL, Sehestedt T, et al. Cardiovascular risk prediction in the general population with use of suPAR, CRP, and Framingham risk score. Int J Cardiol. 2013;167:2904–11.

Maas RJ, Deegens JK, Wetzels JF. Serum suPAR in patients with FSGS: trash or treasure? Pediatr Nephrol. 2013;28:1041–8.

Maas RJ, Wetzels JF, Deegens JK. Serum-soluble urokinase receptor concentration in primary FSGS. Kidney Int. 2012;81:1043–4.

Matalon A, Markowitz GS, Joseph RE, et al. Plasmapheresis treatment of recurrent FSGS in adult renal transplant recipients. Clin Nephrol. 2001;56:271–8.

Mazar AP, Henkin J, Goldfarb RH. The urokinase plasminogen activator system in cancer: implications for tumor angiogenesis and metastasis. Angiogenesis. 1999;3:15–32.

McCarthy ET, Sharma M, Savin VJ. Circulating permeability factors in idiopathic nephrotic syndrome and focal segmental glomerulosclerosis. Clin J Am Soc Nephrol. 2010;5:2115–21.

Meijers B, Maas RJ, Sprangers B, et al. The soluble urokinase receptor is not a clinical marker for focal segmental glomerulosclerosis. Kidney Int. 2014;85:636–40.

Meijers B, Sprangers B. The hype cycle for soluble urokinase receptor in FSGS: passing the trough of disillusionment? Clin J Am Soc Nephrol. 2014;9:1835–6.

Meijers B, Poesen R, Claes K, et al. Soluble urokinase receptor is a biomarker of cardiovascular disease in chronic kidney disease. Kidney Int. 2015;87:210–6.

Nykjaer A, Moller B, Todd 3rd RF, et al. Urokinase receptor. An activation antigen in human T lymphocytes. J Immunol. 1994;152:505–16.

Olson FJ, Thurison T, Ryndel M, et al. Soluble urokinase-type plasminogen activator receptor forms in plasma as markers of atherosclerotic plaque vulnerability. Clin Biochem. 2010;43:124–30.

Persson M, Engstrom G, Bjorkbacka H, et al. Soluble urokinase plasminogen activator receptor in plasma is associated with incidence of CVD. Results from the Malmo Diet and Cancer Study. Atherosclerosis. 2012;220:502–5.

Persson M, Ostling G, Smith G, et al. Soluble urokinase plasminogen activator receptor: a risk factor for carotid plaque, stroke, and coronary artery disease. Stroke. 2014;45:18–23.

Plesner T, Ploug M, Ellis V, et al. The receptor for urokinase-type plasminogen activator and urokinase is translocated from two distinct intracellular compartments to the plasma membrane on stimulation of human neutrophils. Blood. 1994;83:808–15.

Plow EF, Freaney DE, Plescia J, et al. The plasminogen system and cell surfaces: evidence for plasminogen and urokinase receptors on the same cell type. J Cell Biol. 1986;103:2411–20.

Qin DD, Song D, Huang J, et al. Plasma-soluble urokinase-type plasminogen activator receptor levels are associated with clinical and pathological activities in lupus nephritis: a large cohort study from China. Lupus. 2015;24:546–57.

Reuning U, Little SP, Dixon EP, et al. Effect of thrombin, the thrombin receptor activation peptide, and other mitogens on vascular smooth muscle cell urokinase receptor mRNA levels. Blood. 1994;84:3700–8.

Savin VJ, Sharma R, Sharma M, et al. Circulating factor associated with increased glomerular permeability to albumin in recurrent focal segmental glomerulosclerosis. N Engl J Med. 1996;334:878–83.

Sehestedt T, Lyngbaek S, Eugen-Olsen J, et al. Soluble urokinase plasminogen activator receptor is associated with subclinical organ damage and cardiovascular events. Atherosclerosis. 2011;216:237–43.

Sharma M, Sharma R, Reddy SR, et al. Proteinuria after injection of human focal segmental glomerulosclerosis factor. Transplantation. 2002;73:366–72.

Sharma M, Zhou J, Gauchat JF, et al. Janus kinase 2/signal transducer and activator of transcription 3 inhibitors attenuate the effect of cardiotrophin-like cytokine factor 1 and human focal segmental glomerulosclerosis serum on glomerular filtration barrier. Transl Res. 2015;166:384–98.

Sinha A, Bajpai J, Saini S, et al. Serum-soluble urokinase receptor levels do not distinguish focal segmental glomerulosclerosis from other causes of nephrotic syndrome in children. Kidney Int. 2014;85:649–58.

Slot O, Brunner N, Locht H, et al. Soluble urokinase plasminogen activator receptor in plasma of patients with inflammatory rheumatic disorders: increased concentrations in rheumatoid arthritis. Ann Rheum Dis. 1999;58:488–92.

Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. Nat Rev Mol Cell Biol. 2010;11:23–36.

Spinale JM, Mariani LH, Kapoor S, et al. A reassessment of soluble urokinase-type plasminogen activator receptor in glomerular disease. Kidney Int. 2015;87:564–74.

Taniguchi Y, Shimamura Y, Horino T, et al. Serum levels of soluble urokinase plasminogen activator receptor in Japanese patients with chronic kidney disease. Kidney Int. 2014;86:209–10.

Theilade S, Lyngbaek S, Hansen TW, et al. Soluble urokinase plasminogen activator receptor levels are elevated and associated with complications in patients with type 1 diabetes. J Intern Med. 2015;277:362–71.

Thuno M, Macho B, Eugen-Olsen J. suPAR: the molecular crystal ball. Dis Markers. 2009;27:157–72.

Toldi G, Szalay B, Beko G, et al. Plasma soluble urokinase plasminogen activator receptor (suPAR) levels in systemic lupus erythematosus. Biomarkers. 2012;17:758–63.

Vincenti F, Ghiggeri GM. New insights into the pathogenesis and the therapy of recurrent focal glomerulosclerosis. Am J Transplant. 2005;5:1179–85.

Wada T, Nangaku M, Maruyama S, et al. A multicenter cross-sectional study of circulating soluble urokinase receptor in Japanese patients with glomerular disease. Kidney Int. 2014;85:641–8.

Wei C, El Hindi S, Li J, et al. Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. Nat Med. 2011;17:952–60.

Wei C, Moller CC, Altintas MM, et al. Modification of kidney barrier function by the urokinase receptor. Nat Med. 2008;14:55–63.

Wei C, Trachtman H, Li J, et al. Circulating suPAR in two cohorts of primary FSGS. J Am Soc Nephrol. 2012;23:2051–9.

Wohn KD, Kanse SM, Deutsch V, et al. The urokinase-receptor (CD87) is expressed in cells of the megakaryoblastic lineage. Thromb Haemost. 1997;77:540–7.

Yilmaz G, Koksal I, Karahan SC, et al. The diagnostic and prognostic significance of soluble urokinase plasminogen activator receptor in systemic inflammatory response syndrome. Clin Biochem. 2011;44:1227–30.

Zhao Y, Liu L, Huang J, et al. Plasma soluble urokinase receptor level is correlated with podocytes damage in patients with IgA nephropathy. PLoS One. 2015;10:e0132869.

Zimmerman SW. Increased urinary protein excretion in the rat produced by serum from a patient with recurrent focal glomerular sclerosis after renal transplantation. Clin Nephrol. 1984;22:32–8.

Salivary Urea Nitrogen as a Biomarker for Renal Dysfunction

29

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Abstract

Kidney disease is highly prevalent all over the world in acute and chronic forms. Patients with kidney dysfunction have a higher morbidity and mortality especially due to cardiovascular events. Biomarkers nowadays available for the assessment of renal function such as serum creatinine, cystatin C, and blood urea nitrogen are mostly laboratory dependent. Many studies have been conducted following the aim to determine biomarkers of kidney disease allowing early diagnosis, faster treatment initiation, and consequent better outcomes. However, in many places

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worldwide there are a large number of patients affected by acute and chronic kidney disease without receiving adequate diagnosis or treatment. The presence of urea in the saliva was firstly described in 1841 and has since then been repeatedly studied as an indicator of renal function. While it can be measured by laboratory methods, a real advantage is found in it as a bedside methodology (e.g., as a dipstick), which allows early and cost-effective diagnosis in areas with limited resources. The aim is to review current evidence of the saliva as a general biomarker and more specifically salivary urea nitrogen as a noninvasive and useful biomarker of decreased kidney function.

Keywords

Kidney disease • Renal biomarkers • Salivary renal biomarkers • Blood urea nitrogen • Saliva • Salivary urea nitrogen and renal disease

Abbreviations

AKI	Acute kidney injury
AUC	Area under the curve
BUN	Blood urea nitrogen
Ca^{+2}	Calcium
CKD	Chronic kidney disease
Cl^{-}	Chloride
$CO(NH_2)^2$	Urea
CO_2	Carbon dioxide
DHEA	Dehydroepiandrosterone
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
GFR	Glomerular filtration rate
H_2O	Water
HCO_3^{-}	Bicarbonate
HIV	Human immunodeficiency virus
IL-18	Interleukin-18
K^{+}	Potassium
KIM -1	Kidney injury molecule -1
MDRD	Modification of diet in renal disease
Mg^{+2}	Magnesium
MUC5B	Mucin 5B
MUC7	Mucin 7
Na^{+}	Sodium
NGAL	Neutrophil gelatinase-associated lipocalin
NH_3^{-}	Ammonia
NH_4^{-}	Ammonium ion
OH^{-}	Hydroxyl ion
ROC	Receiver operating characteristic
RRT	Renal replacement therapy
SCr	Serum creatinine

SUN	Salivary urea nitrogen
UN	Urea nitrogen

Key Facts of Saliva

- Saliva is an important fluid to maintain the mouth homeostasis with many diagnosis properties and has been widely studied since many years ago because of the many potential biomarkers it contains.
- The oral fluid composition varies in relation to the type of gland where it is produced. Mainly (90 %) of this fluid originates from three pairs of major salivary glands (parotid, sublingual, and submandibular) and from a larger number of minor salivary glands.
- There are many medications and systemic disease that can interfere in the saliva production causing dry mouth, which is known as the medical term xerostomy.
- Many substances measured in the blood can be assessed in the saliva such as urea nitrogen, which can, to some extent, be seen as a biomarker relating to kidney function.
- In 1841, Wright et al. first described the presence of urea in the saliva. At that time it was thought the saliva could be used to increase the excretion of urea in patients with kidney disease.

Definitions

Blood urea nitrogen (BUN) It is produced by the liver and is a waste product of protein digestion. It is an indicator of renal function. The normal ranges are 6–20 mg/dL. High-protein intake, liver diseases, dehydration, kidney disease, and gastrointestinal bleeding can cause an increase in BUN levels.

Creatinine It is an end product of muscle catabolism, derived from the phosphocreatine in the muscle. Its generation can be increased by creatine intake in meat and supplements. Creatinine is freely filtered, secreted by tubular cells, and eliminated via extrarenal. It is easily measured in the blood and urine at a low cost.

Electrolytes These are substances that ionize when diluted in some specific solvents. Salts, acids, bases, and gases can be dissolved and became some specific ions that are very important to the organism homeostasis. Some examples of electrolytes are the ions: sodium, chloride, potassium, magnesium, calcium, phosphate, hydrogen, ammonium, and many others.

IL-18 Interleukin-18 is a cytokine that is produced by macrophages and other cells. It can be measured in the urine due to its expression in the proximal tubule promoting tubule cell apoptosis and necrosis after an ischemic kidney injury. It has been proposed as an early biomarker of kidney dysfunction.

Kidney disease Some degree of impairment in renal function that can happen suddenly (acute kidney disease) or after many years of permanent damage (chronic kidney disease). It is diagnosed through the assessment of glomerular filtration rate and presence of proteins in the urine without other causes, with or without structural kidney damage detected by biopsy.

KIM-1 The kidney injury molecule-1 is a type 1 transmembrane protein, with an immunoglobulin and mucin domain. Its expression is upregulated in the proximal tubule in the post-ischemic kidney event, because it has been proposed as an early biomarker measured in the urine in patients with acute renal failure.

Negative predictive value It is a method that evaluates, according the prevalence of a specific disease or condition, the diagnostic performance of a test or statistical measurement. It represents the event when the test makes a negative prediction and the subject has a negative result under the gold standard method.

NGAL The neutrophil gelatinase-associated lipocalin is a protein identified as a biomarker for early diagnosis of kidney injury and can be measured in the plasma and urine.

Positive predictive value It is a method that evaluates, according the prevalence of a specific disease or condition, the diagnostic performance of a test or statistical measurement. It represents the event when the test makes a positive prediction and the subject has a positive result under the gold standard method.

Receiver operating characteristic It is a graphical representation of the true positive rate (sensitivity) and false negative rate (one-specificity) for a specific diagnostic test.

Renal replacement therapy It is the treatment proposed to substitute the blood filtration when the kidneys do not have the capability to do this anymore. The options are hemodialysis (HD) and its variations (daily HD, home HD, continuous HD, and others), peritoneal dialysis, and kidney transplantation. Each modality has specific indication and contraindication, and the moment to start the therapy varies from different literatures with a consensus to start as soon as the glomerular filtration rate is lower than 10–15 ml/min/1.73 m^2 or when the patients have uremic symptoms or uncontrolled hypervolemia.

Saliva urea nitrogen It is the blood urea nitrogen that achieves the salivary fluid through diffusion mechanisms and can be measured by laboratory devices and salivary strips.

Sensitivity It is a statistical measure that considers the proportion of positive results which are correctly identified as positive events, for example, when an individual has a

positive test for some disease that he/she is a carrier. It is calculated by the number of true positive tests divided by the sum of true positive and false negative tests.

Spearman's rank correlation It is a nonparametric statistical method to assess the relationship between two variables.

Specificity It is a statistical measure that considers the proportion of negative results which are correctly identified as negative events, for example, when an individual has a negative test for some disease that he/she is not a carrier. It is calculated by the number of true negative tests divided by the sum of true negative and false positive tests.

Introduction

Renal disease is highly prevalent worldwide (KDIGO 2012, 2013). Kidney dysfunction can happen suddenly, named a pathophysiologic process termed acute kidney injury (AKI) that often occurs as a consequence of comorbidities such as sepsis, hypovolemic shock, drug toxicity, and urinary tract obstruction, among others (KDIGO 2012). Alternatively renal function can also decrease in a slower fashion due to long-standing kidney injury as a consequence of chronic conditions such as diabetes mellitus, arterial hypertension, autoimmune glomerular diseases, or also prolonged AKI. The latter is named chronic kidney disease (CKD) (KDIGO 2013).

Both entities, acute and chronic kidney disease, are classified according to the degree of kidney dysfunction ranging from mild to severe impairment (KDIGO 2012, 2013). Over the decades many biomarkers have been proposed for an accurate and reliable assessment of kidney function. At this point glomerular filtration rate (GFR) is considered the most accurate measure of renal function. This parameter is determined by the clearance of a specific substance over a 24-h period and requires urine output collection over the entire time. Normalization to body surface area has been proposed to improve the accuracy of the estimation (Inker and Levey 2014). Most commonly used clearances are those of creatinine (90–120 ml/min/1.73 m^2), urea (40–65 ml/min/1.73 m^2), and inulin (100–130 ml/min/1.73 m^2) (Inker and Perrone 2014).

Creatinine is the waste product of muscle catabolism, derived from the metabolism of phosphocreatine in the muscle. It is primarily excreted by the kidneys, where it is freely filtered, secreted by tubular cells, and in addition eliminated via extrarenal such as via intestinal after degradation by intestinal flora (Levey et al. 1988). Creatinine generation can be increased by creatine intake in meat or dietary supplements; it is easily measured in the blood and urine at a considerably low cost; however, it is quantified through laboratory-dependent technique (Inker and Levey 2014).

As mentioned above urea nitrogen (UN) is another marker utilized to assess renal function. It is produced by the liver and is a waste product of protein digestion and cellular decomposition. It is freely filtered by the glomerulus and passively

reabsorbed in the proximal and distal tubules. Besides renal dysfunction, high-protein intake, volume depletion, and gastrointestinal bleeding can also cause an increase in UN levels (Dossetor 1966). Creatinine and urea were widely used jointly to quantify renal function, once creatinine could overestimate and urea, on the other hand, could underestimate GFR due to the mechanisms of secretion and reabsorption; both together could estimate GFR more accurately (Inker and Levey 2014).

Despite the low applicability in clinical practices, inulin clearance is considered the reference method to quantify kidney function due to its biochemical properties. This polysaccharide is metabolized by the bacterial flora in the colon and is completely excreted by the kidneys. It is not secreted or reabsorbed in any portion of the nephron allowing GFR to be calculated. It is determined by continuous intravenous infusion with blood and urine sample collections (Robson et al. 1949).

In clinical routine where time and cost effectiveness are of greatest importance, kidney function is often evaluated by the estimation of the GFR (eGFR) using the serum concentration of endogenous filtration biomarkers such as creatinine, urea, and cystatin C (Earley et al. 2012). Demographic characteristics are included in these estimating equations to provide more reliable results (Cockcroft and Gault 1976; Levey et al. 1999; Schwartz and Work 2009). One example of these equations widely used to estimate eGRF is the Modification of Diet in Renal Disease (MDRD) study equation with standardized creatinine expressed as per below (Levey et al. 1999):

$$\mathrm{eGFR}\left(\mathrm{mL/min/1.73\ m^2}\right) = 175 \times \mathrm{SCr}^{-1.154} \times \mathrm{age}^{-0.203} \times 0.742[\text{if female}] \times 1.210\ [\text{if black}]$$

New biomarkers for kidney damage have been proposed in the last years such as KIM-1, NGAL, and IL-18, with the aim of providing early diagnosis of tubular injury due to acute hemodynamic changes leading to acute kidney injury (Wasung et al. 2014; Devarajan and Murray 2014). Despite their excellent diagnostic performance, they are very costly, laboratory dependent and are not useful to evaluate chronic kidney damage.

Renal disease often goes undiagnosed, and many patients are not even aware of the risk factor causing it (Sumaili et al. 2009). Partly as a consequence, the incidence and prevalence of CKD and AKI are increasing in both developed and developing countries. According to the scarce data available in developing countries, renal disease most often is a de novo occurrence affecting young adults in their productive age (Tao Li et al. 2013). In contrast, in the developed world, AKI and CKD mainly affect older patients and those with many comorbidities. Nevertheless, in both worlds kidney diseases are responsible for a large number of deaths (Sumaili et al. 2009; Tao Li et al. 2013; El Nahas 2005).

The lack of medical infrastructure, particularly of laboratories, is one of the most important reasons for the limited capacity of health workers to diagnose CKD and AKI in many countries. Other contributory factors include late referral to a nephrologist or hospital, limited renal replacement therapy, especially in developing countries, where in addition to a lower level of awareness, the

scarcity of financial resources may affect prevention, diagnosis, and treatment (El Nahas 2005; Barsoum 2006). It was hypothesized that a cheap diagnostic tool used in the most remote areas would allow to increase diagnosis and, if an adequate treatment is available, could affect outcomes in these patients.

For a long time, saliva has been proposed as a fluid with diagnostic qualities for various purposes including diagnosis and monitoring of infectious diseases (HIV, hepatitis C, and others) and other systemic diseases such as neoplasms and renal diseases, assessment of hormonal dysfunctions, performance of genetic tests, measurement of pharmaceutical drug levels, and for the purpose of forensic analyses (Nagler et al. 2002; Malamud 2011; Vining and McGinley 1982; Aps and Martens 2005). Saliva allows the measurement of many different components present in the blood. These include potassium, sodium, uric acid, phosphate, and many others (Nagler et al. 2002; Lima et al. 2010; Chiappin et al. 2007) (Table 1).

Most interestingly for the herein reviewed purpose, UN can also be assessed through saliva, making this fluid possibly a valuable biomarker for the evaluation of kidney function. This suggests that the use of saliva for diagnosis of oral and systemic diseases could be the marker called for above, which allows the diagnosis of a reduction kidney function and may aid to improve outcomes of kidney injury in the developing world and areas with limited medical resources.

Saliva Physiology, Properties, and Function

Salivary glands are developed through a sensible embryonic process. The parotid gland originates from the oral ectoderm and mesenchyme. The embryogenic origin of the submandibular and sublingual glands is similar (Carlson 2000). Innervation of salivary glands by the parasympathetic and sympathetic branches of the autonomic nervous system is essential to secretion and tissue homeostasis of these glands (Martin and Burgen 1962). According to the stimuli the saliva secreted will have different compositions in the different glands (Aps and Martens 2005; Humphrey and Williamson 2001; Proctor and Carpenter 2007) (Table 2).

Salivary glands are composed of two main cell types: the acini, which produce the saliva, and the ductal cells (intercalated, striated, and excretory), which modify and transport the saliva to the mouth (Malamud 2011). The secretion of saliva involves the active secretion of sodium and chloride ions by the acinar cells into the ductal lumen of the gland after appropriate neural stimulation. Water derived from the blood system passes around via tight junctions and through/via aquaporin channels to the acinar cells to form saliva, which is isotonic with respect to the serum. In the parotid and submandibular glands, the sodium is mostly recovered by the striated ducts, which are impermeable to water (Amerongen 2008; Mandel 1989). This process changes the saliva from isotonic, as is secreted by the acini, to a hypotonic fluid, which allows salt detection in much lower levels. This fluid composition can change from resting to stimulated saliva and according the time of the day due to the circadian rhythm of these glands (Lima et al. 2010; Humphrey and Williamson 2001; Tiwari 2011).

Table 1 Substances found in the saliva and blood

Inorganic compounds (mmol/l) (Aps and Martens 2005; Chiappin et al. 2007)	Blood values	Whole unstimulated salivary range
Na^+ mmol/L	145	5
K^+ mmol/L	4	22
Cl^- mmol/L	120	15
Ca^{+2} mmol/L	2.2	1–4
HCO_3^- mmol/L	25	5
Mg^{+2} mmol/L	1.2	0.2
NH_3 mmol/L	0.03	6
Organic compounds (Nagler et al. 2002)		
Uric acid mg/dL	4.4 ± 0.83	2.7 ± 1.67
Bilirubin mg/dL	0.5 ± 0.24	0.03 ± 0.07
BUN mg/dL	18.08 ± 8.5	20.7 ± 11.8
Creatinine mg/dL	1.0 ± 0.10	0.07 ± 0.03
Glucose mg/dL	97.33 ± 10.4	0.67 ± 0.14
Cholesterol mg/dL	193.5 ± 27.9	0.42 ± 1.04
Hormones (Chiappin et al. 2007; Patel et al. 2004; Hansen et al. 2003; Lu et al. 1999; Chatterton et al. 2005; Low 2011)		
Cortisol	140–700 mmol/l	3.6–35.1 nmol/l
Testosterone	300–1,000 ng/dL (men)	140.3 ± 154.15 pg/ml
DHEA-s	20–640 μg/dL (according age and gender)	291.21 ± 294.81 pg/ml
Progesterone	5–20 ng/ml (women mid-cycle)	Luteal phase: 436 ± 34 pmol/l
	<1 ng/ml (women pre-ovulation)	Follicular phase: 22.1 ± 2.7 pmol/l
Estradiol	30–400 pg/ml (premenopausal)	Luteal phase: 20.6 ± 0.4 pmol/l
Aldosterone	<20 ng/ml (adults)	138–475 pmol/l

This table shows some examples of substances present in the blood and saliva and their respective values in each fluid according the reference used
Na^+ sodium, *K^+* potassium, *Cl^-* chloride, *Ca^{+2}* calcium, *$HCO3^-$* bicarbonate, *Mg^{+2}* magnesium, *NH_3* ammonia, *BUN* blood urea nitrogen, *DHEA* dehydroepiandrosterone

Saliva has several functions: digestion (presence of α-amylase), facilitation of taste perception, buffer and lubrication capacity, defense against microorganisms, and assists in teeth mineralization (Amerongen 2008; Tiwari 2011; Carpenter 2013). The oral fluid composition varies in relationship to the serous or mucous gland component, and it mainly (90 %) originates from three pairs of major salivary glands (parotid, sublingual, and submandibular) and from a larger number of minor salivary glands (Von Ebner and Blandin-Nühm glands) (Amerongen 2008). The major

Table 2 Characteristic differences in the saliva produced according to different nervous stimuli and consequent saliva aspect

Parameter	Beta-adrenergic stimulation	Alpha-adrenergic stimulation	Cholinergic stimulation
Volume	Low	Low	High
Viscosity	High	Low	Low
Protein concentration	High	High	Low
Mucin concentration	Very high	Low	Very low
Consequence	Viscous saliva rich in proteins and mucins with foamy appearance	Low-volume saliva, not foamy or viscous, rich in proteins	Watery and high-volume saliva rich in electrolytes

The characteristics of the saliva produced by each gland vary according to the central stimulation these glands receive. In this table the fluid characteristics are shown in agreement with the type of stimuli. It is important to be aware that the salivary glands receive more than one type of central stimuli (beta, alpha-adrenergic, and/or cholinergic) and can produce different types of fluid each time according to the more prominent stimuli (Adapted from Aps and Martens (2005)- with the permission of Elsevier)

contributions for the whole saliva production during resting conditions are submandibular, sublingual, and parotid glands, respectively. Under mechanical and chemical stimulation, parotid and submandibular glands are the most important ones to the saliva secretion. Non-glandular saliva sources are responsible for the other 10 % of the saliva composition and are represented by the gingival crevicular fluid, oral epithelial cells, nasopharyngeal discharge, food debris, bacteria, and their products (Nagler et al. 2002; Malamud 2011).

The principal compounder of saliva is water (99 %), while others are inorganic, organic nonprotein, protein, hormone, and lipid molecules (Table 1). The parotid gland is essentially a serous gland since it does not produce mucin. The parotid gland fluid composes of water, bicarbonate, and a large number of proteins. The main proteins secreted by the parotid glands are amylase (20 %), phosphoproteins (statherin – 7 %), and proline-rich proteins (60 %); these two last groups of proteins are responsible for the constitution of the protein pellicle on dental surfaces which keep the saliva supersaturated in calcium and phosphate which helps in tooth mineralization. On the other hand, submandibular and sublingual glands are mixed serous-mucous glands and are responsible, associated with the minor glands, for salivary mucin secretions (MUC5B and MUC7) that have important protective functions such as avoiding infection, inflammation, and mechanical injuries in both hard and soft tissues (Humphrey and Williamson 2001; Carpenter 2013; Tabak 1995). An amount of 0.5–1.5 ml of the whole saliva is produced per day. The normal pH of saliva ranges between 5.3 (low flow) and 7.8 (peak flow). The major glands contribute most of the volume secretion and electrolyte contents, and the minor glands contribute little volume secretion and most of the blood group components (Mandel 1989; Garrett et al. 1998).

The saliva and plasma exchange substances through a thin layer of epithelial cells separating the salivary ducts from the systemic circulation. The clearance of these compounds from the plasma into the saliva may involve several processes such as ultrafiltration (through gap junctions between cells of secretory units involving molecules with <1,900 Da: water, ions, and hormones such as catecholamine and steroids), transudation of plasma (e.g., albumin and other proteins), and selective transport through cellular membranes by passive diffusion of lipophilic molecules or by active transport through protein channels (Chiappin et al. 2007; Garrett et al. 1998; Marini and Cabassi 2002).

While these complex relationships and dynamics suggest many factors involved in the composition of the saliva, it has been, as mentioned above, repeatedly proposed as a body fluid with diagnostic qualities, which is easily accessible.

Urea Measurements in the Saliva

Generally the accuracy with which any random biomarker can be measured in the saliva mainly depends on the biochemical nature of the marker, the source and type of sample being taken, and the mechanism by which the marker enters the oral cavity (Humphrey and Williamson 2001). Saliva collection is an easy, noninvasive method, not expensive and already in use in many areas, particularly genetics and forensics (Vining and McGinley 1982; Aps and Martens 2005).

Salivary urea nitrogen (SUN) was first described by Wright in 1841 in a case study of a patient who developed a uterine tumor, consequently ascites and a decline in urine output. He believed that once the patient could not eliminate the urea through the urine, her organism developed compensatory mechanisms to eliminate urea through ascites and the saliva avoiding the neurological manifestations of this excretion product accumulation (Wright 1841).

Urea reaches the saliva by diffusion transports and, associated with bicarbonate, is responsible for the buffer capacity of this fluid (Humphrey and Williamson 2001; Amerongen 2008; Mandel 1989). In 1922 Hench and colleagues described a positive relationship between the salivary and blood urea levels, especially in patients with urea retention. The determination of urea for this study was made by the urease method (Hench and Aldrich 1922). Hench also found, in patients with urea retention, that the combined urea and ammonia nitrogen in the saliva was in average 80 % of the blood urea nitrogen, and that was always increased with an increase in BUN levels. The authors suggested that salivary urea nitrogen could precede or replace BUN determination. Salivary stimulation was proposed as a treatment for urea retention when patients had a low urine output and could not eliminate BUN through urine anymore (Hench and Aldrich 1922).

Later in 1923, Hench proposed a salivary urea nitrogen index and showed a distinct and continuous parallelism of the SUN and BUN values. After salivary stimulation with paraffin, two samples were collected, and SUN was determined using a mercury titration method modified from the one used by Friedlander in 1921

to measure urea in the urine. In this study Hench showed correlation between BUN and SUN in healthy and kidney disease patients. The salivary urea index, defined as the number of milliliters (ml) of 5 % solution of mercuric chloride used for each 100 ml of saliva, increased directly with the degree of urea retention as indicated by BUN. The SUN index had a sensitivity of 91 % for urea retention detection. He concluded that the SUN index could be a substitute for BUN determination (Hench and Aldrich 1923).

Barnett et al. in 1929 studied the influence of saliva stimulation in the SUN results. Many previous studies had shown a wide variation in the SUN results with stimulation by chewing paraffin, ranging from 50 % to 130 % in different publications (Hench and Aldrich 1923; Barnett and 1929). In this study, samples were collected with and without stimulation; the whole saliva and parotid saliva were used to perform the measurements. Stimulation was conducted using solutions of tartaric acid (0.05–0.5 %). The results were consistent with the previous studies, showing SUN and BUN correlating significantly when SUN was measured with and without stimulation. SUN measured in the unstimulated saliva had a better agreement with BUN when measured in both whole saliva and parotid salivary samples. On average SUN was 7.2 mg lower when the saliva flow was increased by stimulation (Barnett and Bramkamp 1929).

Later, studies were conducted to analyze SUN in specific salivary glands such as the parotid (Shannon and Prigmore 1961a, b; Shannon et al. 1977). Mean parotid urea concentrations were reported to range from 73 % to 95 % of serum levels. Forland et al. in 1964 proposed that SUN measured in the saliva collected from parotid glands could be used to monitoring hemodialysis patients (Forland et al. 1964). In Forland's study Urea was determined in both fluids with an auto analyzer with the use of a previously described modification of the Ormsby method (Ormsy 1942). Saliva was stimulated by a chewing gum or with a hard candy. The correlation coefficient of mean values for BUN and SUN monitored during hemodialysis was 0.99 despite rapid changes in BUN and SUN levels during treatment (Forland et al. 1964).

The first study using a test-strip method to measure SUN was done by Akai et al. in 1983 when a dipstick was created to assess it. The urease-containing (urease-bromocresol-green method) test strip and an automatic reflectance spectrometer were used to conduct these analyses. Forty-four CKD patients at different disease stages and 12 healthy patients serving as a control group were studied. SUN and BUN results changed according the temperature and the reaction time of 15 min, increasing their values with higher temperatures and prolonged time exposure. A saliva/serum ratio of approximately 1.03 was found with this method, and the degree of correlation was similar in both populations (Akai et al. 1983; Okuda et al. 1980).

Some years later, 1987, Sein et al. published another study where significant correlation coefficients ($r = 0.74$ and $r = 0.99$) between SUN and BUN in unstimulated whole saliva were found in 56 healthy subjects and in 50 patients undergoing hemodialysis, respectively. Serum and saliva urea nitrogen were determined by the urease/glutamate method using Roche reagent kits in a Cobas-Bio centrifugal analyzer (Sein and Arumainayagam 1987; Shull and Cerins-Overbey 1982).

Table 3 Outline of the saliva urea nitrogen chemical reaction

Salivary urea nitrogen cleavage reaction
$\mathbf{CO(NH_2)^2}$ (**Urea**) $+ \mathbf{H_2O} \xrightarrow{\text{Urease}} \mathbf{2\ NH_3 + CO_2}$
$\mathbf{NH_3 + H_2O \rightarrow NH_4^+ + OH^-}$
pH Indicator (Yellow) $+ \mathbf{OH^-} \longrightarrow$ **pH Indicator (Green/Blue)**

In the urea cleavage-process urea in contact with water is cleaved by urease enzyme and becomes ammonia and carbon dioxide. Ammonia in contact with water becomes the ion ammonium and hydroxyl. The release of hydroxyl ions will alter the pH which leads to changes in the test-pad color reflecting the salivary urea nitrogen result.
$CO(NH_2)^2$ urea, H_2O water, NH_3 ammonia, NH_4^+ ion ammonium, OH^- ion hydroxyl

In 2007 a dipstick was developed to measure SUN in the bedside setting. This tool is the first non-laboratory-dependent resource that has been applied in research settings in patients with acute and chronic renal disease and presented a good agreement with BUN levels (AUC ROC of 0.90 (95 % CI 0.85–0.95) for CKD – 68 patients and AUC ROC of 0.91; (95 % CI 0.80–1.0) for AKI – 44 patients) (Raimann et al. 2011; Calice-Silva et al. 2014).

Another study performed by Cardoso et al. in 2009 found a significant correlation between SUN and BUN ($r = 0.91$, $p < 0.001$) in both 78 healthy subjects and 154 CKD patients. The SUN had a sensitivity and specificity of 100 % in both groups in this study. The unstimulated whole saliva collected in the morning in fasting conditions was evaluated. A serum enzymatic colorimetric assay (urea color 2R, Wiener Laboratory, Argentina) was adapted for the SUN measurements (Cardoso et al. 2009).

In the last few years, other studies in this field were performed analyzing different techniques to measure SUN, comparing saliva stimulation versus non-stimulation and whole saliva versus saliva collected from specific glands, in healthy and CKD patients, but all of them utilized laboratory-dependent methods (Peng et al. 2013; Zuniga et al. 2012).

Dipstick Methods to Measure Saliva Urea Nitrogen

The aforementioned work by Akai in 1983 studied a dry strip method using a chromatographic method which required an automatic reflectance spectrometer (Akai et al. 1983). A more recently developed dipstick method developed by Integrated Biomedical Technology, IN, USA, allows the assessment of SUN, without additional devices.

For the employment of this method, 50 μL of liquid saliva are transferred to the test pad of the colorimetric SUN dipstick (Integrated Biomedical Technology, IN). The SUN in the saliva is cleaved by urease present in the test pad into ammonia and hydroxyl ions which change the pH and consequently the test-pad color (Table 3).

Table 4 Saliva urea nitrogen test-pad number and respective SUN ranges

SUN test-pad number (SUN range, mg/dL)	Color of the test pad
1 (5–14)	
2 (15–24)	
3 (25–34)	
4 (35–54)	
5 (55–74)	
6 (≥75)	

The SUN (saliva urea nitrogen) results are divided in six ranges from the lower to the higher levels and are represented by the colors and test-pad numbers showed above. Test-pad result 1 refers to SUN between 5 and 14 mg/dL, test-pad 2 (15–24 mg/dL), test-pad 3 (25–34 mg/dL), test-pad 4 (35–54 mg/dL), test-pad 5 (55–74 mg/dL), and test pad 6 (≥75 mg/dL). The greater the test-pad number the worse the kidney function

In 1 min the result can be read and the test-pad color compared to six standardized color fields indicating SUN concentrations of 5–14 (color pad #1), 15–24 (#2), 25–34 (#3), 35–54 (#4), 55–74 (#5), and ≥75 (#6) mg/dL, respectively (Table 4). This strip showed a good agreement between SUN and BUN at different concentrations.

Raimann et al. (2011) published a study where 68 CKD patients in the stages 1 to 5D were studied. The elevated BUN was diagnosed using this SUN dipstick; unstimulated whole saliva was collected to perform the measurements. SUN and BUN were correlated significantly (RS = 0.63; $p < 0.01$). Elevated BUN was diagnosed by SUN determination with an AUC ROC curve of 0.90 (95 % CI 0.85–0.95) and an interobserver coefficient of variation of 4.9 % at SUN levels >50 mg/dL and within-sample reproducibility of 90 % (Raimann et al. 2011).

The same tool was also applied in the AKI setting. Forty-four patients suffering from acute renal disease were analyzed in this cross-sectional study performed by Calice-Silva et al. (2014). In this group of patients, the SUN dipstick had a sensitivity of 0.91 and specificity of 0.79 to diagnose AKIN 3 (AUC ROC 0.91–95 % CI 0.80–1.0), the worse degree of AKI, and when the most part of the patients will need some kind of renal replacement therapy (RRT – hemodialysis or peritoneal dialysis). These findings make this tool useful to screen patients with suspected renal disease in areas with limited resources (Calice-Silva et al. 2014).

Despite promising results SUN and BUN do not have a good agreement at the lower levels of BUN. The delicate balance of strip buffer composition relative to the saliva pH and buffer capacity has been put forward as possible causes of inaccurate measurements at low levels. Further developments to increase accuracy at lower levels of BUN are under investigation at this time. Furthermore, more studies are needed to better understand the applicability of this tool in more diverse population, such as healthy patients, children, patients using multiple medications, and those suffering from others disorders potentially interfering with the accuracy of the method.

Limitations of SUN Assessments

Some biological factors may alter the BUN and SUN relationship, in particular urease-producing oral bacterial flora, which may give rise to falsely low SUN values (Burne and Chen 2000); this, however, does not affect the agreement at higher levels of SUN. Another important limitation is the circadian rhythm that salivary glands have, potentially leading a different secretion rate of some saliva compounder according the time of the measurement in relation to the time of the day. This suggests that the agreement between SUN and BUN may differ at different times of the day (Dawes 1972; Dawes and Ong 1973; Ferguson et al. 1973).

Many are the mechanisms that lead to a diminished saliva production. Both systemic diseases such as Sjögren syndrome and the use of multiple medications such as antihypertensive, antihistaminic, antidepressive, anticonvulsants, analgesics, and many others can interfere in the salivary flow and buffer capacity and cause changes in the salivary pH that possibly could result in inaccurate SUN measurements (Marques et al. 2014; Thomson et al. 1999). Regardless, this method has very important strengths: most importantly, the tool enables to assess a marker to a certain extent relating to kidney function quickly, noninvasively, and independently of a laboratory directly at bedside (Raimann et al. 2011; Calice-Silva et al. 2014).

New developments of the dipsticks are currently being investigated and promise to increase the precision of SUN strip. The main improvement is an additional control pad which contains the same compounds as the main test pad excluding the enzyme urease in order to evaluate the pH and buffer capacity of saliva, which, as mentioned above, may be one of the causes of inaccuracies.

This auxiliary test pad will show darker greenish/bluish color if there is an elevated pH (higher than 7.0) or some alteration in the buffer capacity in this fluid. Consideration of such a discoloration when comparing the color of the main test pad against the color code on the container allows an adjustment of the measurement or even the replication of this to confirmate it. This improvement aims to analyze SUN more accurately, particularly at lower levels where false negative results have been observed and also will help to increase the usefulness of the dipstick in cases where the SUN may be possibly low (e.g., for the screening of patients with only mild impairments of kidney function).

Potential Applications to Prognosis, Other Diseases, or Conditions

Renal disease leads to a high burden on society and health systems. Diagnosis and even more treatment are expensive. In many areas people do not have access to either due to high costs, available resources, and insurance coverage in different healthcare systems (Grassmann et al. 2005; Jha et al. 2013). Depending on the underlying cause, the disease can, despite often treatable and reversible when diagnosed early, be fatal in a short period of time. For those suffering from slow-progressing renal disease, early diagnosis may also be of benefit and allow, at the very least, dietary interventions or other affordable therapeutic options (Tao Li et al. 2013).

Despite a remarkable scarcity of data, it may be assumed that particularly in remote areas with limited medical infrastructure, there are still a large number of patients suffering from CKD or AKI without access to renal replacement therapy resulting in a fatal outcome as a consequence of uremia, hyperkalemia, or fluid overload. Even with the increment in dialysis facilities numbers and healthcare coverage all over the world, early diagnosis remains a challenge. As one of the most important examples, it needs mentioning that in developing countries many children die daily due to AKI secondary to infectious diseases (Rosa-Diez et al. 2014; De Vecchi et al. 1999; Schieppati et al. 2014; Cerda et al. 2008). It is of note that such an occurrence may be entirely avoided by adequate treatment, in the best case only requiring adequate volume replacement or a short regimen of peritoneal dialysis (Callegari et al. 2013).

For those with chronic disease, the situation is more complex since maintenance RRT requires more resources and is more difficult to successfully implement. However, early diagnosis may be of help to diagnose the condition early and allow preventive measures to slow the disease progression. Global screening programs for renal disease, as already proposed in the past, early detection, and prevention of progressive renal disease may be seen as the keys for a successful improvement in patients care and survival (Sumaili et al. 2009; Barsoum 2006).

Another important application could be in the setting of disasters where quick decisions based on easy-to-use methods are the key to improve outcomes and curtail fatalities. This has been repeatedly observed, hypothesized, and even shown in vivo during latest events (Vanholder et al. 2010; Naicker 2010).

SUN could also be an important biomarker for the monitoring of the treatment regimen for those maintained in home dialysis therapies such as home hemodialysis and peritoneal dialysis. It could be aid physicians, family members, and the patient himself or herself to control their treatment. This however needs to first be studied in a real-life setting.

Overall, early diagnosis may not only save lives but may also preserve years of productivity for those affected (Tao Li et al. 2013; Cerda et al. 2008). Regular screening of the population may allow the initiation of prevention programs and the early diagnosis for many suffering from undetected and undiagnosed renal disease. The combination of these with patients education in terms of diet and risk factors may possible an important improvement of public health (El Nahas 2005). Measurement of SUN with low-cost, noninvasively, and accurately may be of benefit to improve overall outcomes and should thus be considered as a marker for the diagnosis of deteriorating renal function.

Summary Points

- Renal disease can be manifested acutely or chronically and is responsible for an increased mortality risk in patients suffering from this illness. According to the degree of dysfunction, the patient may need renal replacement therapy represented mainly by hemodialysis, peritoneal dialysis, and kidney transplantation. Around 8–10 % of the worldwide population has some degree of renal

disease. Renal disease has a high morbidity and mortality, especially due to cardiovascular disease.

- Glomerular filtration rate is the principal method used to assess kidney function. Another form to assess kidney function is measuring some specific substances such as creatinine, urea, and cystatin C in the blood and urine.
- Saliva is also a fluid with diagnostic properties that has been studied since many years. Many substances measured in the blood can be assessed in the saliva such as urea nitrogen, which can, to some extent, be seen as a biomarker relating to kidney function. Salivary urea nitrogen (SUN) was first described in 1841 by Wright et al. A good agreement between the values of urea nitrogen in the blood and saliva has been shown in several studies.
- A recently developed salivary dipstick allows the measurement of SUN giving physicians the opportunity to diagnose renal disease at bedside. This may result in earlier treatment initiation and possible improved outcomes. Particularly for developing countries and medically isolated areas, this tool could be of great benefit. It may also be used for triage in the setting of natural disaster with the need of quick diagnosis and early treatment.
- Salivary urea nitrogen dipstick is a promising noninvasive, bedside tool to screen patients with suspected renal disease, in areas with limited health resources. SUN dipstick has higher diagnosis applicability in those patients with higher levels of BUN.

References

Akai T, Naka K, Yoshikawa C, et al. Salivary urea nitrogen as an index to renal function: a test-strip method. Clin Chem. 1983;29(10):1825–7.

Amerongen AV. Saliva: properties and functions. In: Wong DT, editor. Salivary diagnostics. 2nd ed. New Delhi: Wiley-Blackwell; 2008. p. 27–36.

Aps JK, Martens LC. Review: the physiology of saliva and transfer of drugs into saliva. Forensic Sci Int. 2005;150(2–3):119–31.

Barnett GD, Bramkamp RG. Influence of rate of secretion on urea. Proc Soc Exper Biol Med. 1929;27:118–120.

Barsoum RS. Chronic kidney disease in the developing world. N Engl J Med. 2006;354(10):997–9.

Burne RA, Chen YY. Bacterial ureases in infectious diseases. Microbes Infect/Inst Pasteur. 2000;2(5):533–42.

Calice-Silva V, Vieira MA, Raimann JG, et al. Saliva urea nitrogen dipstick – a novel bedside diagnostic tool for acute kidney injury. Clin Nephrol. 2014;82(12):358–66.

Callegari J, Antwi S, Wystrychowski G, Zukowska-Szczechowska E, Levin NW, Carter M. Peritoneal dialysis as a mode of treatment for acute kidney injury in sub-Saharan Africa. Blood Purif. 2013;36(3–4):226–30.

Cardoso EM, Arregger AL, Tumilasci OR, Elbert A, Contreras LN. Assessment of salivary urea as a less invasive alternative to serum determinations. Scand J Clin Lab Invest. 2009;69(3):330–4.

Carlson GW. The salivary glands. Embryology, anatomy, and surgical applications. Surg Clin N Am. 2000;80(1):261–73. xii.

Carpenter GH. The secretion, components, and properties of saliva. Ann Rev Food Sci Technol. 2013;4:267–76.

Cerda J, Bagga A, Kher V, Chakravarthi RM. The contrasting characteristics of acute kidney injury in developed and developing countries. Nat Clin Pract Nephrol. 2008;4(3):138–53.

Chatterton RT, Mateo ET, Hou N, et al. Characteristics of salivary profiles of estradiol and progesterone in premenopausal women. J Endocrinol. 2005;86:77–84.
Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. Clin Chim Acta Int J Clin Chem. 2007;383(1–2):30–40.
Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron. 1976;16(1):31–41.
Dawes C. Circadian rhythms in human salivary flow rate and composition. J Physiol. 1972;220(3):529–45.
Dawes C, Ong BY. Circadian rhythms in the concentrations of protein and the main electrolytes in human unstimulated parotid saliva. Arch Oral Biol. 1973;18(10):1233–42.
De Vecchi AF, Dratwa M, Wiedemann ME. Healthcare systems and end-stage renal disease (ESRD) therapies – an international review: costs and reimbursement/funding of ESRD therapies. Nephrol Dial Transplant Off Publ Eur Dial Transplant Assoc Eur Ren Assoc. 1999;14 Suppl 6:31–41.
Devarajan P, Murray P. Biomarkers in acute kidney injury: are we ready for prime time? Nephrol Clin Pract. 2014;127(1–4):176–9.
Dossetor JB. Creatininemia versus uremia. The relative significance of blood urea nitrogen and serum creatinine concentrations in azotemia. Ann Intern Med. 1966;65(6):1287–99.
Earley A, Miskulin D, Lamb EJ, Levey AS, Uhlig K. Estimating equations for glomerular filtration rate in the era of creatinine standardization: a systematic review. Ann Intern Med. 2012;156(11):785–95. W-270, W-271, W-272, W-273, W-274, W-275, W-276, W-277, W-278.
El Nahas M. The global challenge of chronic kidney disease. Kidney Int. 2005;68(6):2918–29.
Ferguson DB, Fort A, Elliott AL, Potts AJ. Circadian rhythms in human parotid saliva flow rate and composition. Arch Oral Biol. 1973;18(9):1155–73.
Forland M, Shannon IL, Katz FH. Parotid-fluid urea nitrogen for the monitoring of hemodialysis. N Engl J Med. 1964;271:37–8.
Garrett JR, Ekström J, Anderson LC. Glandular mechanism of salivary secretion, vol. 10. Basel: Karger; 1998.
Grassmann A, Gioberge S, Moeller S, Brown G. ESRD patients in 2004: global overview of patient numbers, treatment modalities and associated trends. Nephrol Dial Transplant Off Publ Eur Dial Transplant Assoc Eur Ren Assoc. 2005;20(12):2587–93.
Hansen AM, Garde AH, Christensen JM, Eller NH, Netterstrom B. Evaluation of a radioimmunoassay and establishment of a reference interval for salivary cortisol in healthy subjects in Denmark. Scand J Clin Lab Invest. 2003;63(4):303–10.
Hench PS, Aldrich M. The concentration of urea in saliva. JAMA Intern Med. 1922;79:1409–12.
Hench PS, Aldrich M. Salivary index to renal function. JAMA Intern Med. 1923;81:1997–2003.
Humphrey SP, Williamson RT. A review of saliva: normal composition, flow, and function. J Prosthet Dent. 2001;85(2):162–9.
Inker LA, Levey AS. National kidney foundation's primer on kidney diseases. In: Gilbert SJ, Weiner DE, editors. National kidney foundation's primer on kidney diseases. 6th ed. Philadelphia: Elsevier Saunders; 2014.
Inker LA, Perrone RD. Assessment of kidney function. 2014. http://www.uptodate.com/contents/assessment-of-kidney-function?source=search_result&search=Assessment+of+kidney+function&selectedTitle=1%7E150. Accessed Nov 2014.
Jha V, Garcia-Garcia G, Iseki K, et al. Chronic kidney disease: global dimension and perspectives. Lancet. 2013;382(9888):260–72.
KDIGO. 2012 clinical practice guideline for the evaluation and management of acute kidney injury. Kidney Int Suppl. 2012;2:19–36.
KDIGO. 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. Kidney Int Suppl. 2013;2:19–62.
Levey AS, Perrone RD, Madias NE. Serum creatinine and renal function. Annu Rev Med. 1988;39:465–90.

Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med. 1999;130(6):461–70.

Lima DP, Diniz DG, Moimaz SA, Sumida DH, Okamoto AC. Saliva: reflection of the body. Int J Infect Dis IJID Off Publ Int Soc Infect Dis. 2010;14(3):e184–8.

Low MJ. Neuroendocrinology. In: Melmed S, Polonsky KS, Larsen PR, editors. Williams textobook of endocrinology, vol. 7. 12th ed. Philadelphia, PA: Elsevier Saunders; 2011. p. 103–74.

Lu Y, Bentley GR, Gann PH, Hodges KR, Chatterton RT. Salivary estradiol and progesterone levels in conception and nonconception cycles in women: evaluation of a new assay for salivary estradiol. Fertil Steril. 1999;71(5):863–8.

Malamud D. Saliva as a diagnostic fluid. Dent Clin N Am. 2011;55(1):159–78.

Mandel ID. The role of saliva in maintaining oral homeostasis. J Am Dent Assoc. 1989;119(2):298–304.

Marini A, Cabassi E. La saliva: approccio complementare nella diagnostica clinica e nella ricerca biologica. Ann Fac Med Vet Parma. 2002;22:295–311.

Marques PL, Liborio AB, de Lima Saintrain MV. Hemodialysis-specific factors associated with salivary flow rates. Artif Organs. 2015 Feb;39(2):181–6.

Martin K, Burgen AS. Changes in the permeability of the salivary gland caused by sympathetic stimulation and by catecholamines. J Gen Physiol. 1962;46(2):225–43.

Nagler RM, Hershkovich O, Lischinsky S, Diamond E, Reznick AZ. Saliva analysis in the clinical setting: revisiting an underused diagnostic tool. J Invest Med Off Publ Am Fed Clin Res. 2002;50(3):214–25.

Naicker S. Challenges for nephrology practice in Sub-Saharan Africa. Nephrol Dial Transplant Off Publ Eur Dial Transplant Assoc Eur Ren Assoc. 2010;25(3):649–50.

Okuda K, Akai T, Tani A. The relationship of urea concentration to blood and to saliva. J Clin Chem Clin Biochem Z Klin Chem Klin Biochem. 1980;9:411–9.

Ormsy AA. Direct colorimetric method for determination of urea in blood and urine. J Biol Chem. 1942;146:595–604.

Patel RS, Shaw SR, Macintyre H, McGarry GW, Wallace AM. Production of gender-specific morning salivary cortisol reference intervals using internationally accepted procedures. Clin Chem Lab Med CCLM/FESCC. 2004;42(12):1424–9.

Peng CH, Xia YC, Wu Y, Zhou ZF, Cheng P, Xiao P. Influencing factors for saliva urea and its application in chronic kidney disease. Clin Biochem. 2013;46(3):275–7.

Proctor GB, Carpenter GH. Regulation of salivary gland function by autonomic nerves. Auton Neurosci Basic Clin. 2007;133(1):3–18.

Raimann JG, Kirisits W, Gebetsroither E, et al. Saliva urea dipstick test: application in chronic kidney disease. Clin Nephrol. 2011;76(1):23–8.

Robson JS, Ferguson MH, et al. The determination of the renal clearance of inulin in man. Q J Exp Physiol. 1949;35(2):111–34.

Rosa-Diez G, Gonzalez-Bedat M, Pecoits-Filho R, et al. Renal replacement therapy in Latin American end-stage renal disease. Clin Kidney J. 2014;7(4):431–6.

Schieppati A, Perico N, Remuzzi G. Eliminating treatable deaths due to acute kidney injury in resource-poor settings. Semin Dial. 2014.

Schwartz GJ, Work DF. Measurement and estimation of GFR in children and adolescents. Clin J Am Soc Nephrol CJASN. 2009;4(11):1832–43.

Sein KT, Arumainayagam G. Correlation between serum urea and salivary urea. Clin Chem. 1987;33(12):2303–4.

Shannon IL, Prigmore JR. Effects of urea dosage on urea correlations in human parotid fluid and blood serum. Arch Oral Biol. 1961;5:161–7.

Shannon IL, Prigmore JR. Serum and parotid fluid urea levels in urea-loaded healthy young adults. Sch Aviat Med. 1961;31–33.

Shannon IL, Feller RP, Eknoyan G, Suddick RP. Human parotid saliva urea in renal failure and during dialysis. Arch Oral Biol. 1977;22(2):83–6.

Shull BC, Cerins-Overbey N. Enzymatic assays for urea nitrogen and uric acid on the Cobas Bio centrifugal analyzer. Clin Chem. 1982;28(9):1992–3.
Sumaili EK, Cohen EP, Zinga CV, Krzesinski JM, Pakasa NM, Nseka NM. High prevalence of undiagnosed chronic kidney disease among at-risk population in Kinshasa, the Democratic Republic of Congo. BMC Nephrol. 2009;10:18.
Tabak LA. In defense of the oral cavity: structure, biosynthesis, and function of salivary mucins. Annu Rev Physiol. 1995;57:547–64.
Tao Li PK, Burdmann EA, Mehta RL. Acute kidney injury: global health alert. Int J Organ Transplant Med. 2013;4(1):1–8.
Thomson WM, Chalmers JM, Spencer AJ, Williams SM. The xerostomia inventory: a multi-item approach to measuring dry mouth. Community Dent Health. 1999;16(1):12–7.
Tiwari M. Science behind human saliva. J Nat Sci Biol Med. 2011;2(1):53–8.
Vanholder R, Gibney N, Luyckx VA, Sever MS. Renal disaster relief task F. renal disaster relief task force in Haiti earthquake. Lancet. 2010;375(9721):1162–3.
Vining RF, McGinley R. Transport of steroids from blood to saliva. Cardiff: Alpha Omega Publishing Limited; 1982. p. 56–63.
Wasung ME, Chawla LS, Madero M. Biomarkers of renal function, which and when? Clin Chim Acta Int J Clin Chem. 2014;438C:350–7.
Wright S. Case of ascites in which, during spontaneous ptyalism that occurred after tapping, urea was detected in saliva. Lancet. 1841;1:753–8.
Zuniga ME, Estremadoyro LO, Leon CP, Huapaya JA, Cieza JA. Validation of the salivary urea test as a method to diagnose chronic kidney disease. J Nephrol. 2012;25(3):431–6.

Part III

Specific Diseases and Conditions

Chronic Allograft Damage Index (CADI) as a Biomarker in Kidney Transplantation

30

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Abstract

Although the outcome of patients after kidney transplantation has improved markedly during the past decades, chronic scarring and loss of function of the allograft along time are a common problem. Histopathological changes precede

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the loss of function in the transplant, and chronic allograft damage index (CADI) was introduced in the early 1990, with the purpose of numerically classifying pathological lesions in transplanted kidneys. CADI is a sum score of six histopathological lesions commonly seen in biopsies taken from transplanted kidneys that correlate with the function and outcome of the graft. CADI includes interstitial inflammation, tubular atrophy, interstitial fibrosis, arterial fibrointimal thickening, glomerular mesangial matrix increase, and the percentage of globally sclerosed glomeruli. CADI score, analyzed from either donor biopsies taken before transplantation or from protocol biopsies taken after transplantation, correlates with graft function and later outcome and can be used as a surrogate marker in clinical trials. CADI has also been extensively studied in experimental models of kidney transplantation. In addition, CADI can be useful in clinical decision-making, as it gives a simple numeric score of the extent of chronic injury in the kidney allograft.

Keywords
Kidney transplantation • Pathology • Protocol biopsy • Donor biopsy • Outcome

Abbreviations

CADI	Chronic allograft damage index
CAN	Chronic allograft nephropathy
CsA	Cyclosporine A
MP	Methylprednisolone
MMF	Mycophenolate mofetil

Key Facts of Kidney Transplantation

- Kidney transplantation is the treatment of choice in patients with end-stage renal disease, and compared to maintenance dialysis treatment, it is very cost-effective and improves quality of life and survival.
- Annually more than 70,000 kidney transplantations are performed globally, of which approximately 40 % are from living donors and 60 % from donors after brain death or cardiac death.
- The average survival of a transplanted kidney is approximately between 10 and 20 years, depending on the patient and donor characteristics.
- Life-long immunosuppressive treatment is needed to prevent rejection of the transplant, and maintenance immunosuppression is usually a combination of two to three drugs. Immunosuppression is associated with increased risk of infections and malignancies, and in addition current drugs are associated with several adverse metabolic effects.
- The most common reason for the loss of a kidney transplant is premature death of the patient with a functioning graft, mostly to cardiovascular causes of malignancies.
- Chronic scarring of the transplanted kidney is an unsolved problem, leading to gradual loss of the transplant function and return to dialysis.

- Multiple causes lead to similar histopathological picture seen in kidney transplant biopsies, consisting of scarring and chronic inflammation.
- Histopathological changes precede loss of function in the transplants, and histopathological scoring of the lesions of well-functioning grafts gives estimates of the prognosis of the allograft and can be used as surrogate markers in clinical studies.

Definitions

Chronic allograft nephropathy A term referring to chronic scarring of the kidney allograft. It was originally introduced to include the multifactorial etiology of long-term allograft injury, including immunological and nonimmunological causes, but has later been abandoned from classifications due to the nondiagnostic and very unspecific nature of the term.

Chronic rejection Chronic immunological injury to the transplanted kidney. During the time chronic allograft damage index was developed, chronic rejection was used commonly to describe the unspecific long-term scarring of the kidney allograft, but later histopathological classifications have specified chronic rejection as its own entity, defined as evidence of certain characteristics of chronic inflammation in the biopsies.

Donor biopsy A core needle biopsy taken from a transplanted organ before the transplant operation, e.g., taken from a brain-dead donor during the explantation operation of the organ.

Protocol biopsy A core needle biopsy taken from the (kidney) allograft at a predefined time point after transplantation during stable graft function. The opposite of a protocol biopsy is an indication biopsy, which is taken due to graft dysfunction or signs of injury in the graft.

Introduction

Kidney transplantation is an excellent treatment for end-stage renal disease for patients who are evaluated suitable both for the operation and for long-term exposure to immunosuppressive drugs. The most common reason for the loss of a kidney transplant is premature death of the patient with a functioning graft, mostly due to cardiovascular causes or malignancies. However, chronic scarring of the transplanted kidney is an unsolved problem, leading to gradual loss of the transplant function and return to dialysis. Multiple causes of injury to the transplanted kidney all lead to similar histopathological picture seen in kidney transplant biopsies, consisting of scarring and chronic inflammation.

Despite advances in molecular diagnostics and development of several potential biomarkers from blood or urine, histopathological evaluation of the kidney from a core needle biopsy remains the gold standard for diagnosing kidney diseases.

In addition to native kidneys, biopsies are very important in assessing the well-being of a transplanted kidney, especially as it is well known that histopathological changes precede the loss of function in the transplants. The procedure of taking a biopsy from a kidney transplant with an automated biopsy device under ultrasound guidance is very safe, and serious complications occur very rarely. Two main studies provided knowledge about the safety on kidney graft biopsies performed either by indication or by protocol. The first study reported an incidence of 0.4 % for major complications after reviewing 2127 protocol biopsies (Furness et al. 2003a). In the second study including 1171 protocol biopsies and 499 indication biopsies, only 1 % of major complications were reported in either group (Schwarz et al. 2005). Therefore, many transplant centers have adopted the policy of taking protocol biopsies from the stable well-functioning transplanted kidney at a predefined time point after transplantation. Protocol biopsies are taken on one hand to detect underlying pathology despite normal graft function (most importantly subclinical rejection) (Rush et al. 1998), but on the other hand histopathology provides important information about the prognosis of the transplanted kidney. Protocol biopsies are also increasingly used as surrogate markers of graft prognosis in the context of randomized controlled trials, but a concrete benefit in the routine follow-up of kidney transplant patients is yet to be fully demonstrated. Therefore, graft function is usually monitored by serum creatinine, estimated glomerular filtration rate, and proteinuria, whereas a kidney biopsy is indicated to ascertain the cause of graft dysfunction. The potential benefit of recognizing unexpected pathological patterns in well-functioning kidneys early enough may have an impact on graft survival (Bosmans et al. 2008).

The most commonly used classification of renal allograft histopathology is the Banff classification. The Banff classification was created in a meeting held in Banff, Canada, in August 1991 and published in 1993 (Solez et al. 1993). The Banff Working Classification of Renal Allograft Pathology is a schema developed to standardize the interpretation of kidney allograft pathology across frontiers. Subsequent meetings have been held every other year to refine the classification. The modifications to the Banff schema made in 1997 synthesized two different classification systems: Banff '95 and the classification developed by CCTT (Collaborative Clinical Trials in Transplantation) (Racusen et al. 1999). In Banff classification, individual histopathological lesions are scored separately semiquantitatively (from 0 to 3), and certain diagnostic categories are defined. Banff '97 is still the most commonly used classification system in the literature of kidney transplant pathology. Details of the Banff '97 classification are shown in Table 1. The earliest versions of the Banff classification emphasized the diagnosis of acute rejection, mainly because this was the most common problem in those days. Chronic allograft nephropathy (CAN) was graded into three categories – mild, moderate, or severe – without an attempt to determine the pathophysiological mechanisms involved in this entity. The improvements in immunosuppressive regimens dramatically reduced the incidence of acute rejection, exposing the problem of chronic allograft damage. The elucidation of the role of donor-specific antibodies, suggested by C4d staining, caused a revolutionary modification of the Banff classification and led to the omission of the

Table 1 Description of the Banff 1997 classification for the pathology of a kidney allograft (Racusen et al. 1999)

1. Normal	
2. Antibody-mediated rejection, demonstrated to be due, at least in part, to antidonor antibody	
A. Immediate (hyperacute)	
B. Delayed (accelerated acute)	
3. Borderline changes suspicious for acute rejection. This category is used when no intimal arteritis is present, but there are foci of mild tubulitis (1–4 mononuclear cells/tubular cross section) and at least i1	
4. Acute/active rejection	
Type (grade)	Histopathological findings
I A	Cases with significant interstitial infiltration (>25 % of parenchyma affected) and foci of moderate tubulitis (>4 mononuclear cells/tubular cross section or group of 10 tubular cells)
I B	Cases with significant interstitial infiltration (>25 % of parenchyma affected) and foci of severe tubulitis (>10 mononuclear cells/tubular cross section or group of 10 tubular cells)
II A	Cases with mild to moderate intimal arteritis (v1)
II B	Cases with severe intimal arteritis comprising >25 % of the luminal area (v2)
III	Cases with transmural arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells (v3 with accompanying lymphocytic inflammation)
5. Chronic/sclerosing allograft nephropathy	
Grade	Histopathological findings
I (Mild)	Mild interstitial fibrosis and tubular atrophy without (a) or with (b) specific changes suggesting chronic rejection
II (Moderate)	Moderate interstitial fibrosis and tubular atrophy (a) or (b)
III (Severe)	Severe interstitial fibrosis and tubular atrophy and tubular loss (a) or (b)
6. Other	Changes considered not to be due to rejection

general term “chronic allograft nephropathy” (Solez et al. 2007) (Table 2). In an attempt to discriminate the underlying processes, the present recommendation distinguishes acute and chronic antibody-mediated rejection from acute and chronic T-cell-mediated rejection. Unspecific interstitial fibrosis and tubular atrophy (IFTA) are now a separate category, graded from mild to moderate and severe.

Although the Banff classification is an excellent diagnostic tool for pathologists for assessment of histopathological lesions and helps clinicians in interpreting the findings, there are limitations for the use of Banff classification in research and correlation of histopathological findings to later outcomes, which derive especially from the fact that every lesion is assessed separately. There is a real need for “score” of different histopathological lesions to evaluate the total damage in the allograft for both research purposes and for clinicians.

Chronic allograft damage index (CADI) was developed in the early 1990s to provide better tools for both research and clinical use in evaluating the severity of chronic changes in the kidney allograft. The terminology has changed many times

Table 2 Update of the Banff classification in 2007, which includes the addition of antibody-mediated rejection and new concepts of chronic active T-cell-mediated rejection (Solez et al. 2007)

1. Normal
2. Antibody-mediated rejection
Due to documented anti-donor antibody
Acute antibody-mediated rejection
Type (grade)
I. ATN-like – C4d+, minimal inflammation
II. Capillary margination and/or thromboses, C4d+
III. Arterial – v3, C4d+
Chronic active antibody-mediated rejection
Glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries, C4d+
3. Borderline changes: “suspicious” for acute T-cell-mediated rejection
No intimal arteritis is present, but there are foci of tubulitis (t1, t2, or t3 with i0 or i1) although the i2 t2 threshold for rejection diagnosis is not met
4. T-cell-mediated rejection
Acute T-cell-mediated rejection
Type (grade)
IA. Cases with significant interstitial infiltration (>25 % of parenchyma affected, i2 or i3) and foci of moderate tubulitis (t2)
IB. Cases with significant interstitial infiltration (>25 % of parenchyma affected, i2 or i3) and foci of severe tubulitis (t3)
IIA. Cases with mild to moderate intimal arteritis (v1)
IIB. Cases with severe intimal arteritis comprising >25 % of the luminal area (v2)
III. Cases with “transmural” arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3)
Chronic active T-cell-mediated rejection
“Chronic allograft arteriopathy” (arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neointima)
5. Interstitial fibrosis and tubular atrophy, no evidence of any specific etiology
Grade
I. Mild interstitial fibrosis and tubular atrophy (<25 % of cortical area)
II. Moderate interstitial fibrosis and tubular atrophy (26–50 % of cortical area)
III. Severe interstitial fibrosis and tubular atrophy/loss (>50 % of cortical area)
6. Other:
Changes not considered to be due to rejection – acute and/or chronic

during the past decades, and in the 1990s “chronic rejection” was the most widely used term for chronic failure of kidney allografts (Hayry et al. 1993). In the early 2000s “chronic allograft nephropathy” dominated the literature as the description of the unspecific scarring and failing of allografts (Paul 1999; Nankivell et al. 2003). In recent literature, progress has been made to try to define the specific cause of chronic scarring in the transplanted kidney and to try to avoid the use of nonspecific nondiagnostic terms such as chronic allograft nephropathy (Solez et al. 2007). Chronic rejection still exists in the current classification, but is only one subgroup

of reasons for scarring of the graft, and should include signs of continuous immunological damage to the graft. CADI was developed before Banff classification was presented, but these classification systems are not competing, rather more complementing each other. The purpose of this review is to describe the development of CADI and to describe how CADI can be employed both for clinical and research use.

Development of Chronic Allograft Damage Index

The development of CADI dates to the late 1980s to the early 1990s, when the concept of protocol biopsies was getting more popular within the transplant community. Research group in Helsinki lead by Prof. Pekka Häyry was interested in finding out whether histopathological changes in well-functioning kidney allografts may predict later outcome and to what extent they correlate to graft function and later chronic rejection. Key focus was on chronic rejection, as defined by that time (Häyry et al. 1993). The original paper in which CADI was introduced described a patient population participating in randomized controlled trial of immunosuppressive drugs. In this trial, triple-drug therapy with cyclosporine (CsA), methylprednisolone (MP), and azathioprine (AZA) was compared to any combination of two of these drugs. In this study, a protocol core needle biopsy was taken from all patients at 2 years after transplantation, and the patient population consisted of 89 patients with adequate biopsy material. In the first paper, 35 individual histopathological parameters were scored semiquantitatively from 0 to 3 (Isoniemi et al. 1992). From these parameters, the most common findings were correlated with graft function and donor age. The individual parameters, which were the best predictors of graft function, were selected to the CADI scoring system (Tables 3 and 4). In CADI, all individual parameters are scored from 0 to 3. A confirmation of the usefulness of CADI was published 2 years later, when from the same initial patient population, CADI score at 2 years was correlated to graft function and outcome at 6 years. CADI correlated well with graft function 4 years after the biopsy, and CADI also predicted graft failure (chronic rejection) at 6 years (Isoniemi et al. 1994).

Description of CADI

CADI results from the sum of the following six histopathological parameter scores: interstitial inflammation, tubular atrophy, interstitial fibrosis, arterial fibrointimal thickening, glomerular mesangial matrix increase, and the percentage of globally sclerosed glomeruli. The CADI score ranges from 0 to a maximum of 18. Details on individual scoring are depicted in Table 3 and Fig. 1. In the modern application of CADI, the individual parameters scored from 0 to 3 according to Banff '97 classification, except for the percentage of globally sclerosed glomeruli, which is not included in the Banff classification (0, no globally sclerosed glomeruli; 1, <15 %; 2, 16–50 %; and 3, >50 % globally sclerosed glomeruli). In the original CADI

Table 3 Description of the chronic allograft damage index, which is a sum score of six parameters of chronic lesions in kidney allograft pathology

Inflammation	(Focal/diffuse)
0	No or trivial inflammation
1	Up to 25 % of parenchyma inflamed
2	26–50 % of parenchyma inflamed
3	>50 % of parenchyma inflamed
Interstitial fibrosis	(Focal/diffuse/subcapsular)
0	No fibrosis
1	Up to 25 % of the interstitium affected
2	26–50 % of the interstitium affected
3	>50 % of the interstitium affected
Tubular atrophy	
0	No tubular atrophy
1	Tubular atrophy up to 15 % of proximal tubules
2	Tubular atrophy in 16–30 % of proximal tubules
3	Tubular atrophy in more than 31 % of proximal tubules
Mesangial matrix increase	
0	No mesangial matrix increase
1	Up to 25 % of non-sclerotic glomeruli affected (at least moderate)
2	26–50 % of non-sclerotic glomeruli affected (at least moderate)
3	>50 % of non-sclerotic glomeruli affected (at least moderate)
Intimal proliferation	Changes at least seen in 1 artery or 3 arterioles
0	No intimal thickness
1	Intimal thickness up to 25 % of the remaining lumen
2	Intimal thickness 26–50 % of the remaining lumen
3	Intimal thickness >50 % of the remaining lumen
Sclerosis	(Increase in extracellular matrix, sclerotic areas positive stained with PAS)
0	No changes
1	<15 % of glomeruli affected
2	16–50 % of glomeruli affected
3	>50 % of glomeruli affected

papers, the Banff '97 classification was not available, and some of the individual lesion scores differed slightly. Minor differences in the grading of chronic lesions between CADI and Banff are detailed in Table 5.

Correlation of CADI with Prognosis and Use of CADI as Surrogate Marker in Clinical Trials

Already the first studies presenting CADI described one of the key strengths of CADI, which is correlation of CADI to later graft function and outcome (Isoniemi

Table 4 Parameters evaluated in the histological specimens of renal grafts in the development phase of chronic allograft damage index (CADI)

Interstitium	
Lymphocytes	Hemorrhage
Macrophages	**Fibrosis**
Pyroninophilic cells	**Inflammation**
Neutrophils	Edema duplication
Eosinophils	Fibrin deposits
Glomeruli	
Mesangial cell proliferation	Number of glomeruli
Capillary basement membrane thickening	**Mesangial matrix increase**
Capillary thrombosis	Capillary basement membrane
Glomerular inflammation	Bowman capsular thickening
Glomerular necrosis	**Glomerular sclerosis**
Tubuli	
Epithelial vacuolation isometric	Epithelial swelling
Epithelial atrophy	Epithelial vacuolation anisometric
Casts	Necrosis
Dilatation	Inflammation
	Basement membrane thickening
Vessels	
Endothelial proliferation	Endothelial swelling
Inflammation	**Intimal proliferation**
Obliteration	Sclerosis

In **red,** the components selected for CADI

et al. 1994). After that, several other studies have correlated CADI with later outcome with similar results (Yilmaz et al. 2003). In two multicenter randomized controlled trials of mycophenolate in kidney transplantation, US trial and Tricontinental trial (Sollinger 1995; The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group 1996; Mathew 1998; US Renal Transplant Mycophenolate Mofetil Study Group 1999), protocol core needle biopsies were performed in certain centers participating in the studies, and the biopsy slides were sent for centralized blinded reading using CADI score. Altogether 621 protocol biopsies were analyzed from these studies and scored with CADI. Of the biopsies, 111 were taken at baseline, 302 at 12 months, and 206 at 36 months after transplantation. In multivariable models CADI score at 1 year was significantly and independently associated with 3-year graft survival (OR 1.6). For every unit increase in CADI score, the odds ratio of a graft loss or death increased by almost 50 % (Yilmaz et al. 2003). Clinical factors that independently correlated with elevated CADI at 1 year were donor age, acute rejection during the first posttransplant year, and CMV infections. In addition to the linear association of CADI and graft survival, CADI can be categorized as low (<2), elevated (2–4), and high (>4). In this study, none of the patients with low CADI lost their graft, whereas 4.6 % of patients with elevated CADI lost a graft and 16.7 % of patients with high CADI lost their graft

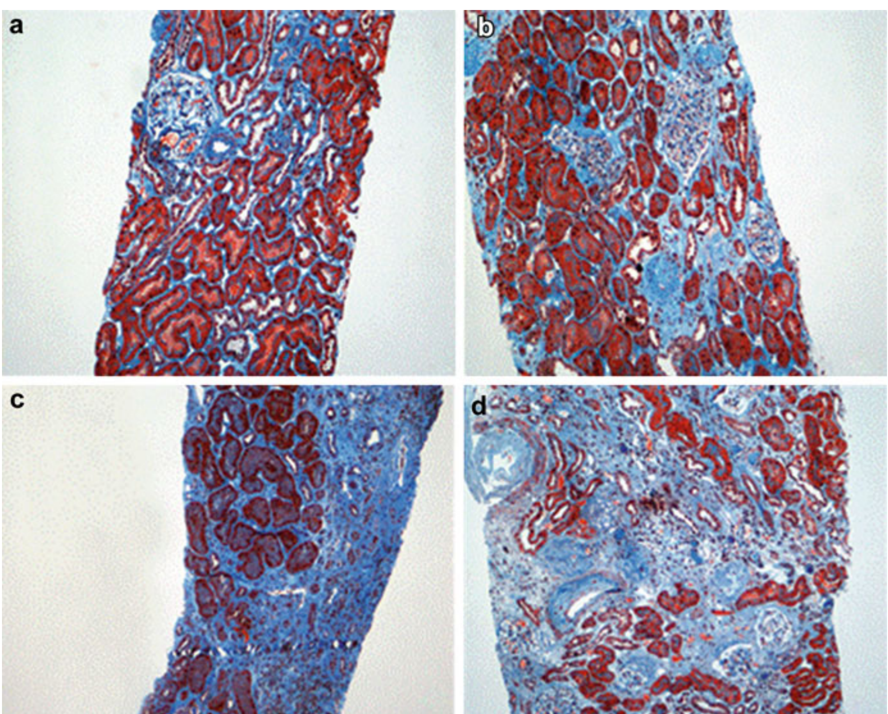

Fig. 1 Examples of rising CADI score in kidney transplant biopsies. Photomicrographs of four kidney biopsies showing a rising CADI score. (**a**) A 6-month protocol biopsy with normal histology, score: 0. (**b**) A 6-month protocol biopsy, score: 2, comprised of chronic vasculopathy (cv 1, not shown in the photomicrograph) and glomerulosclerosis (*gs 1*). (**c**) A 6-month protocol biopsy, score: 4, comprised of interstitial fibrosis (ci 1), tubular atrophy (ct 1), mesangial matrix increase (mm 1), and glomerulosclerosis (gs 1). (**d**) A 12-month protocol biopsy, score: 8, comprised of interstitial fibrosis (ci 1), tubular atrophy (ct 1), inflammation (i 1), glomerulosclerosis (gs 2), and severe chronic vasculopathy (cv 3). Masson's trichrome. Magnification × 100

(Yilmaz et al. 2003). As the authors of this study conclude, the good predictive value of CADI suggests that CADI can be used not only as surrogate marker in prevention trials but can also be used to identify the patient cohort at risk for intervention trials. After this study, several other studies have also described the predictive power of CADI in predicting later graft function or outcome (Helanterä et al. 2007), and also later randomized controlled trials have used CADI as a surrogate end point (Mota et al. 2004). Previously acute rejection and graft survival were mostly used as end points in clinical trials of immunosuppressive drugs. However, in the current era, in which graft survival is on average between 10 and 20 years and acute rejection is seen in only approximately 10 % of patients, reliable surrogate markers are crucial for clinical trials to get results in a timely manner and to avoid very expensive and long trials. Therefore, CADI provides an excellent biomarker and surrogate marker for clinical trials for immunosuppressive drugs. In addition, CADI as a linear

Table 5 Minor differences in the grading of chronic lesions between CADI and Banff (Racusen et al. 1999)

	CADI	Banff
Inflammation	Same grading	
Interstitial fibrosis	(Focal/diffuse/subcapsular)	
0	No fibrosis	Up to 5 %
1	Up to 25 % affected	6–25 %
2	Same grading	
3	Same grading	
Tubular atrophy		
0	Same grading	
1	Tubular atrophy up to 15 % of proximal tubules	Up to 25 %
2	Tubular atrophy in 16–30 % of proximal tubules	26–50 %
3	Tubular atrophy in more than 31 % of proximal tubules	Over
Mesangial matrix increase	Same grading	
Intimal proliferation	Same grading	
Sclerosis	(Increase in extracellular matrix, sclerotic areas positive stained with PAS)	Not graded
0	No changes	
1	<15 % of glomeruli affected	
2	16–50 % of glomeruli affected	
3	>50 % of glomeruli affected	

numeric score is very practical in different types of studies analyzing correlation of different variables with graft histopathology (Ortiz et al. 2005; Helanterä et al. 2007, 2010).

CADI as a Surrogate of Graft Function in Basic Science

The use of CADI expands to animal experimentation. Using a rat model for studying chronic rejection, CADI was useful to assess immunosuppressive drugs that may prevent the development of theses lesions (Malmstrom et al. 2008; Rintala et al. 2008; Palin et al. 2013). Similarly CADI was used to show the association between cytomegalovirus infections and chronic kidney transplant rejection (Lautenschlager et al. 1997; Kloover et al. 2000).

In human trials the detection of plasminogen activator inhibitor-1 in the serum of renal transplanted patients showed the potential to predict CADI scores (Chang et al. 2009). Also in a study focused on microarrays, the CADI score combined to certain biomarkers from the NLR family predicted the graft function at 1 year posttransplantation (Perco et al. 2009). In another research focused on the development of chronic changes possibly attributed to CsA toxicity, CADI score was not

correlated to transforming growth factor-β in protocol biopsies from patients under low-calcineurin inhibitor regimen (Ortiz et al. 2013), suggesting only a minor role for CsA nephrotoxicity in the development of chronic changes in the kidney graft.

Comparison of CADI to Individual Histopathological Lesions

There are several studies analyzing the association of single histopathological lesions with outcome. Both chronic scarring (interstitial fibrosis and tubular atrophy) and inflammation have been associated with inferior outcome (Seron et al. 1997; Mengel et al. 2007), and also several studies show an association with transplant glomerulopathy (duplication of glomerular capillary basement membranes) and inferior outcome (Husain and Sis 2013). However, whether single lesions are more powerful predictors of outcome compared to composite scores has not been studied. A few studies have addressed this question indirectly. In one analysis (Yilmaz et al. 2007), the CADI score or individual histopathological lesions from protocol biopsies taken at 1 or 3 years after transplantation were correlated with clinical parameters. Donor age, acute rejection, recipient age, and cold ischemia time correlated better with CADI and to lesser extent with individual histopathological lesions. Only about 60 % of the variation in histopathological score was explained by clinical factors, supporting the use of CADI score in risk evaluation in protocol biopsies taken after transplantation. In an analysis of donor biopsies (Anglicheau et al. 2008), CADI score was compared with clinical risk factors and a clinicopathological composite scoring in an attempt to find the best predictor of later graft function in marginal donors. In this study, CADI analyzed from donor biopsies was associated with low renal function after transplantation, as was also glomerulosclerosis or arteriolar hyalinosis as single lesions. However, best prediction of low renal function after transplantation was achieved with a composite score of serum creatinine, donor hypertension, and glomerulosclerosis.

CADI from Donor Biopsies

The mere use of clinical scores while assessing kidney quality before transplantation has not been predictors of subsequent graft function. Thus, the inclusion of histological analysis in the donor evaluation has become crucial, especially in the last years when marginal donors are more frequently accepted. CADI has been first utilized to evaluate donor biopsies in 1999 (Lehtonen et al. 1999). The authors detailed the nature of the lesions observed in implant biopsies and correlated the scores with subsequent graft function. The mean CADI score was 0.74, pointing the good quality of the kidneys. Although histopathological changes were uncommon, organs from donors older than 40 years of age had more frequent anisometric vacuolization, interstitial fibrosis, vascular hyalinosis, glomerular sclerosis, and

tubular basement membrane thickening. CADI was useful to quantitate the lesions in implant biopsies, but these abnormalities did not show an impact on the incidence of delayed graft function.

More recently, a study focused on marginal donors (Anglicheau et al. 2008) described that the factors associated with low glomerular filtration rate at 1 year posttransplant were glomerulosclerosis, arteriolar hyalinosis, the Pirani clinical score, and CADI. Other authors have also proposed the use of composite risk scores when evaluating donor kidney quality. Kahu et al. published the results of the follow-up of 481 donor biopsies and the paired recipients. They observed that >3 CADI score in donor biopsies was associated with worse graft function and survival. In this study CADI was used also in follow-up biopsies to assess the development of histological lesions in the first year after transplantation and its impact on prognosis (Kahu et al. 2011).

Progression of CADI as a Biomarker of Graft Prognosis

As previously described, histological lesions could be already observed in implant biopsies. It is of outstanding importance to follow up both the progression of these lesions and the generation of new pathological findings across time. CADI was used for this purpose in a study involving 83 kidney transplant patients (Ortiz et al. 2005). The authors concluded that delta CADI (defined as the difference in CADI scores at 6 months and implant biopsies) was affected by glomerulosclerosis in donor biopsies and serum creatinine concentration at hospital discharge. The delta-CADI concept allowed to investigate the factors related to this progression. In this study the risk for renal allograft damage progression was increased not only by donor histology but also by nonimmunologic factors, such as hypertension and dyslipidemia. In a similar fashion Yango et al. studied the progression of histological lesions in the graft from implantation to 6 months after transplantation, focusing on the impact of immunosuppression regimen on the progression. The authors found the use of CADI score as a suitable tool to evaluate the progression, even when the graft function was stable (Yango et al. 2008). In a similar fashion Nainani et al. used CADI to quantify the progression of the histological lesions in sequential biopsies obtained from patients under different immunosuppressive regimens and interestingly concluded that steroid discontinuation did not affect the progression in CADI score in patients free of acute rejection (Nainani et al. 2012).

Histological lesions increase with time. CADI allows also the quantification of theses lesions in later biopsies and the possible effects of changes in immunosuppression made later on. A few studies have focused on the switch from calcineurin inhibitor to sirolimus, and the effects were evaluated in follow-up biopsies scored with CADI (Mota et al. 2004; Ruiz et al. 2003). In both investigations the effect of the change in the immunosuppressive regimen was properly evaluated with CADI, resulting in lower scores in biopsies from patients on sirolimus.

CADI in Pediatric Kidney Transplantation

The progression of histopathological lesions in children after kidney transplantation has also been studied using CADI and serial protocol biopsies taken 1.5, 3, 5, and 7 years after transplantation, from 51 children transplanted under the age of 5 years (Qvist et al. 2000). However, in this study a modified version of CADI was used. Due mostly to very mild histopathological changes, all the individual parameters were scored with a scale of 0–6, and a sum of these scores was calculated (CADI range 0–36). This taken into account, the CADI scores were overall very low; at 1.5 years, mean CADI was 2.5 and at 7 years only 3.5 (Qvist et al. 2000), suggesting that the progression of histopathological lesions in small children with well-functioning grafts may be slower compared to adults. Concerning the individual parameters, interstitial inflammation tended to decrease with time, whereas interstitial fibrosis tended to increase over time. The most prominent finding was the progression of glomerular sclerosis. At 1.5 years, 3 % of the glomeruli were totally sclerosed, whereas 7 years after transplantation 36 % of the glomeruli were totally sclerosed. CADI correlated with graft function at the time of biopsy and also predicted later graft function, whereas the individual histological parameters did not predict graft function (Qvist et al. 2000). Especially in this pediatric material with very mild histopathological lesions, CADI may provide better prognostic tools compared to individual histopathological lesions. Clinical parameters, which were associated with higher CADI, were previous acute rejection episodes and kidney from a deceased donor (vs. living donor) (Qvist et al. 2000).

Reproducibility of CADI

A common problem with histopathological classification systems is the poor interobserver reproducibility, especially in quantifying the severity of lesions. This holds true also for kidney transplant pathology. There are several studies showing that quantifying lesions in the kidney allograft biopsy based on Banff classification differs widely between pathologists (Furness and Taub 2001, Furness et al. 2003b). When kappa values were calculated for the level of interobserver agreement (0 = no agreement; 1 = perfect agreement), all evaluated lesions had very poor reproducibility with kappa values differing between 0.1 and 0.4 (Furness and Taub 2001, Furness et al. 2003b). Even feedback systems applied in these multicenter studies did not significantly improve the interobserver variability (Furness et al. 2003b). These same limitations apply for when individual histopathological parameters are scored for CADI. However, in the pivotal studies of CADI score, an excellent interobserver agreement was reached with the two pathologists who were involved in the development of CADI; the correlation coefficient between the two pathologists was as high as 0.85 (Yilmaz et al. 2003). This suggests that reproducibility is possible with CADI, especially with devoted pathologists working in the same center. However,

due to the large interobserver variability, comparison of CADI between different centers must be interpreted with caution.

CADI in Helping Clinical Decision-Making

Although CADI was developed primarily as a tool for research, it may also be very useful in helping clinical practice. All kidney transplant biopsies in the authors' institution have been classified according to CADI already for the past 10 years. A numeric score of all the individual parameters and the sum score (CADI) are provided by local pathologists from all transplant biopsies and also from donor biopsies. The numeric table gives a quick overview of the severity of chronic changes and makes comparison of the change in the parameters compared to previous biopsies very easy. Also CADI score can be very illustrative and useful when the findings of the biopsy are interpreted to the patient. We recommend taking CADI as a part of routine clinical practice when interpreting kidney transplant biopsies.

Limitations of CADI

In addition to the general problem of reproducibility of pathological lesions in kidney transplant biopsies, several limitations exist in the CADI scoring system, and critique has been pointed for certain characteristics of CADI. One point of criticism is that CADI mixes chronic (fibrosis, tubular atrophy, glomerular sclerosis) and active lesions (inflammation). It is, however, well known that various degrees of inflammation are commonly seen in biopsies of stable kidney allografts and that this inflammation is one of the most important predictors of graft outcome (Mengel et al. 2007) regardless of the degree of inflammation. Therefore, strong arguments exist for including inflammation in CADI.

Another point is that mesangial matrix increase included in the CADI is not very strongly correlated with outcome and is not linked to any particular pathogenic process. Also, this variable is not very reproducible.

Another problem with CADI is that it is simply a sum score of all the parameters, whereas ideally based on their predictive value each parameter should be given individual weight in the score. In addition, some of the components are strongly correlated with each other (such as tubular atrophy and interstitial fibrosis) and are not very independent variables. The weight of this component is thus doubled in the current score.

All of these points of criticism are indeed adequate. However, one of the advantages of CADI is its simplicity, and calculating a weighted score would probably increase the predictive power in clinical trials, but would on the other hand complicate the interpretation of this score in clinical use. The current advantages and applications of CADI score are summarized in Figure 2.

Evaluation of the change in histopathological changes between different time-points (delta- CADI)

Evaluation of kidney transplant donor biopsies and correlation to graft outcome

Evaluation of protocol biopsies after transplantation and correlation to graft outcome

Chronic Allograft Damage Index (CADI)

Surrogate marker in clinical trials

Practical tool for clinicians in evaluating the level of injury in the kidney transplant

Fig. 2 Applications for chronic allograft damage index (*CADI*). Summary of the applications of chronic allograft damage index (*CADI*) as a biomarker in kidney transplantation

Potential Applications to Prognosis, Other Diseases, or Conditions

As CADI is developed specifically for kidney transplant pathology, it cannot therefore be applied directly to other transplanted organs or diseases. The chronic scarring in different transplanted organs differs in the cell types and tissues involved, and therefore same principles are not applicable in other organs. However, composite scoring systems could be helpful in quantifying the extent of chronic changes also in other transplanted organs, but must be developed specifically to each organ system.

As described above, one of the main advantages of CADI as a biomarker in kidney transplantation is its excellent correlation with graft prognosis, and it is also one of the key reasons why this composite scoring system was in fact developed. Linear correlation of CADI with graft function and prognosis makes it an excellent surrogate marker for clinical and experimental studies.

Summary Points

- Chronic allograft damage index (CADI) was introduced in the early 1990s, with the purpose of numerically classifying pathological lesions in transplanted kidneys.
- CADI is a sum score of six histopathological lesions commonly seen in biopsies taken from transplanted kidneys that correlate with the function and outcome of the graft.

- CADI includes interstitial inflammation, tubular atrophy, interstitial fibrosis, arterial fibrointimal thickening, glomerular mesangial matrix increase, and the percentage of globally sclerosed glomeruli.
- CADI score, analyzed from either donor biopsies taken before transplantation or from protocol biopsies taken after transplantation, correlates with graft function and later outcome and can be used as a surrogate marker in clinical trials.
- CADI can also be useful in clinical decision-making, as it gives a simple numeric score of the extent of chronic injury in the kidney allograft.

References

Anglicheau D, Loupy A, Lefaucheur C, et al. A simple clinico-histopathological composite scoring system is highly predictive of graft outcomes in marginal donors. Am J Transplant. 2008;8:2325–34.

Bosmans JL, Ysebaert DK, Verpooten GA. Chronic allograft nephropathy: what have we learned from protocol biopsies? Transplantation. 2008;85:S38–41.

Chang HR, Yang SF, Lian JD, et al. Prediction of chronic allograft damage index of renal allografts using serum level of plasminogen activator inhibitor-1. Clin Transpl. 2009;23:206–12.

Furness PN, Taub N. Convergence of European Renal Transplant Pathology Assessment Procedures (CERTPAP) Project. International variation in the interpretation of renal transplant biopsies: report of the CERTPAP Project. Kidney Int. 2001;60:1998–2012.

Furness PN, Philpott CM, Chorbadjian MT, et al. Protocol biopsy of the stable renal transplant: a multicenter study of methods and complication rates. Transplantation. 2003a;76:969–73.

Furness PN, Taub N, Assmann KJ, et al. International variation in histologic grading is large, and persistent feedback does not improve reproducibility. Am J Surg Pathol. 2003b;27:805–10.

Hayry P, Isoniemi H, Yilmaz S, et al. Chronic allograft rejection. Immunol Rev. 1993;134:33–81.

Helanterä I, Ortiz F, Helin H, Raisanen-Sokolowski A, Honkanen E, Koskinen P. Timing and value of protocol biopsies in well-matched kidney transplant recipients – a clinical and histopathologic analysis. Transpl Int. 2007;20:982–90.

Helanterä I, Ortiz F, Räisänen-Sokolowski A, Koskinen P. Impact of glucose metabolism abnormalities on histopathological changes in kidney transplant protocol biopsies. Transpl Int. 2010;23:374–81.

Husain S, ja Sis B. Advances in the understanding of transplant glomerulopathy. Am J Kidney Dis. 2013;62:352–63.

Isoniemi HM, Krogerus L, von Willebrand E, Taskinen E, Ahonen J, Häyry P. Histopathological findings in well-functioning, long-term renal allografts. Kidney Int. 1992;41:155–1160.

Isoniemi H, Taskinen E, Häyry P. Histological chronic allograft damage index accurately predicts chronic renal allograft rejection. Transplantation. 1994;58:1195–8.

Kahu J, Kyllönen L, Räisänen-Sokolowski A, Salmela K. Donor risk score and baseline biopsy CADI value predict kidney graft outcome. Clin Transpl. 2011;25:E276–83.

Kloover JS, Soots AP, Krogerus LA, et al. Rat cytomegalovirus infection in kidney allograft recipients is associated with increased expression of intracellular adhesion molecule-1 vascular adhesion molecule-1, and their ligands leukocyte function antigen-1 and very late antigen-4 in the graft. Transplantation. 2000;69:2641–7.

Lautenschlager I, Soots A, Krogerus L, et al. Effect of cytomegalovirus on an experimental model of chronic renal allograft rejection under triple-drug treatment in the rat. Transplantation. 1997;64:391–8.

Lehtonen SR, Taskinen EI, Isoniemi HM. Histopathological findings in renal allografts at time of transplantation and correlation with onset of graft function. APMIS. 1999;107:945–50.

Malmström NK, Kallio EA, Rintala JM, et al. Vascular endothelial growth factor in chronic rat allograft nephropathy. Transpl Immunol. 2008;19:136–44.
Mathew TH. A blinded, long-term, randomized multicenter study of mycophenolate mofetil in cadaveric renal transplantation: results at three years. Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group. Transplantation. 1998;65:1450–4.
Mengel M, Gwinner W, Schwarz A, et al. Infiltrates in protocol biopsies from renal allografts. Am J Transplant. 2007;7:356–65.
Mota A, Arias M, Taskinen EI, et al. Sirolimus-based therapy following early cyclosporine withdrawal provides significantly improved renal histology and function at 3 years. Am J Transplant. 2004;4:953–61.
Nainani N, Patel N, Tahir N, et al. Effect of steroid-free low concentration calcineurin inhibitor maintenance immunosuppression regimen on renal allograft histopathology and function. Nephrol Dial Transplant. 2012;27:2077–83.
Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. N Engl J Med. 2003;349:2326–33.
Ortiz F, Paavonen T, Tornroth T, et al. Predictors of renal allograft histologic damage progression. J Am Soc Nephrol. 2005;16:817–24.
Ortiz F, Helanterä IT, Räisänen-Sokolowski A, Honkanen EO, Koskinen PK. TGF-beta1 expression in kidney allograft protocol biopsies during cyclosporine A therapy. Int J Artif Organs. 2013;36:56–62.
Palin NK, Savikko J, Koskinen PK. Sirolimus inhibits lymphangiogenesis in rat renal allografts, a novel mechanism to prevent chronic kidney allograft injury. Transpl Int. 2013;26:195–205.
Paul LC. Chronic allograft nephropathy: an update. Kidney Int. 1999;56:783–93.
Perco P, Kainz A, Wilflingseder J, Soleiman A, Mayer B, Oberbauer R. Histogenomics: association of gene expression patterns with histological parameters in kidney biopsies. Transplantation. 2009;87:290–5.
Qvist E, Krogerus L, Ronnholm K, Laine J, Jalanko H, Holmberg C. Course of renal allograft histopathology after transplantation in early childhood. Transplantation. 2000;70:480–7.
Racusen LC, Solez K, Colvin RB, et al. The Banff 97 working classification of renal allograft pathology. Kidney Int. 1999;55:713–23.
Rintala JM, Savikko J, Rintala SE, von Willebrand E. FK778 ameliorates post-transplant expression of fibrogenic growth factors and development of chronic rejection changes in rat kidney allografts. Nephrol Dial Transplant. 2008;23:3446–55.
Ruiz JC, Campistol JM, Mota A, et al. Early elimination of cyclosporine in kidney transplant recipients receiving sirolimus prevents progression of chronic pathologic allograft lesions. Transplant Proc. 2003;35:1669–70.
Rush D, Nickerson P, Gough J, et al. Beneficial effects of treatment of early subclinical rejection: a randomized study. J Am Soc Nephrol. 1998;9:2129–34.
Schwarz A, Gwinner W, Hiss M, Radermacher J, Mengel M, Haller H. Safety and adequacy of renal transplant protocol biopsies. Am J Transplant. 2005;5:1992–6.
Seron D, Moreso F, Bover J, et al. Early protocol renal allograft biopsies and graft outcome. Kidney Int. 1997;51:310–6.
Solez K, Axelsen RA, Benediktsson H, et al. International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. Kidney Int. 1993;44:411–22.
Solez K, Colvin RB, Racusen LC, et al. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant. 2007;7:518–26.
Sollinger HW. Mycophenolate mofetil for the prevention of acute rejection in primary cadaveric renal allograft recipients. U.S. Renal Transplant Mycophenolate Mofetil Study Group. Transplantation. 1995;60:225–32.

The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group. A blinded, randomized clinical trial of mycophenolate mofetil for the prevention of acute rejection in cadaveric renal transplantation. Transplantation. 1996;61:1029–37.
US Renal Transplant Mycophenolate Mofetil Study Group. Mycophenolate mofetil in cadaveric renal transplantation. Am J Kidney Dis. 1999;34:296–303.
Yango A, Gohh R, Wang LJ, et al. The utility of 6-month protocol renal biopsy under modern immunosuppression. Clin Nephrol. 2008;70:490–5.
Yilmaz S, Tomlanovich S, Mathew T, et al. Protocol core needle biopsy and histologic Chronic Allograft Damage Index (CADI) as surrogate end point for long-term graft survival in multicenter studies. J Am Soc Nephrol. 2003;14:773–9.
Yilmaz S, McLaughlin K, Paavonen T, et al. Clinical predictors of renal allograft histopathology: a comparative study of single-lesion histology versus a composite, quantitative scoring system. Transplantation. 2007;83:671–6.

Biomarkers for Kidney Injury in Cystic Fibrosis

31

Kevin J. Downes and Stuart L. Goldstein

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Abstract

Cystic fibrosis (CF) is an autosomal recessive disease affecting sodium and chloride transport predominantly in the lungs and digestive system. Patients with this disease develop recurrent and chronic respiratory tract infections often necessitating repeated and aggressive antimicrobial therapy. Patients also commonly develop pancreatic insufficiency, diabetes, malabsorption, and liver problems. The role of the CF gene mutation in the kidney is not well described. However, patients with CF are at risk for development of acute kidney injury (AKI) due to receipt of nephrotoxic medications, particularly antibiotics, as well as long-term renal damage from long-standing diabetes and repeated nephrotoxin exposures. The traditional marker of kidney injury and function, serum creatinine, is an unreliable marker of kidney function and injury in patients with CF and low muscle mass: it often overestimates true kidney function and does not detect kidney injury until a significant number of nephrons have been affected. Novel biomarkers, such as cystatin C (CysC), retinol-binding protein (RBP), kidney injury molecule-1 (KIM-1), and neutrophil gelatinase-associated lipocalin (NGAL) have been studied preliminarily in this population but hold significant promise for early detection of AKI, risk stratification, and prognosis. With advances in medical therapies leading to substantial improvement in lifetime survival among CF patients, the long-term ramifications of the disease and its treatments on the kidney are becoming apparent. Methods to improve detection of kidney injury and risk of development of chronic kidney disease in patients with CF are paramount. This chapter reviews the available literature on the causes and impact of AKI in patients with CF, as well as the strengths, limitations, and potential uses of traditional and novel biomarkers of kidney injury in this population.

Keywords

Acute kidney injury • Aminoglycosides • Chronic kidney disease • Cystic fibrosis • Urinary biomarkers

Abbreviations

AG	Aminoglycoside
AKI	Acute kidney injury
B_2M	Beta-2-microglobulin
BUN	Blood urea nitrogen
CCl	Creatinine clearance
CF	Cystic fibrosis
CFRD	Cystic fibrosis-related diabetes

CKD	Chronic kidney disease
CysC	Cystatin C
eCCl	Estimated creatinine clearance
eGFR	Estimated glomerular filtration rate
GFR	Glomerular filtration rate
IL-18	Interleukin-18
IV	Intravenous
KIM-1	Kidney injury molecule-1
L-FABP	Liver-type fatty acid-binding protein
NAG	*N*-Acetyl-β-D-glucosaminidase
NGAL	Neutrophil gelatinase-associated lipocalin
NSAIDs	Nonsteroidal anti-inflammatory drugs
RBP	Retinol-binding protein
SCr	Serum creatinine
TDM	Therapeutic drug monitoring
UCr	Urine creatinine

Key Facts of Cystic Fibrosis and Kidney Injury

- Cystic fibrosis (CF) is an autosomal recessive genetic disease.
- A defect in the cystic fibrosis transmembrane conductance receptor (CFTR) leads to impaired sodium and chloride transport across epithelial cells.
- As a result of abnormal electrolyte transport, patients develop thick mucus which predisposes them to chronic and recurrent pulmonary infections, intestinal malabsorption, and pancreatic insufficiency.
- The impact of CFTR on kidney disease in CF has not been fully elucidated.
- The majority of kidney injury in this population stems from secondary insults from nephrotoxin administration (antibiotics, immunosuppressive medications), formation of renal stones, and CF-related diabetes (CFRD).
- As survival for patients with CF increases, so do opportunities for kidney injury and the likelihood of development of chronic kidney disease (CKD).
- The traditional marker of kidney injury and function, serum creatinine, is often inaccurate in patients with CF and decreased muscle mass.
- Novel serum and urine biomarkers hold great promise for the early detection and prognostication of acute kidney injury in patients with CF and other high-risk populations.

Definitions

Acute kidney injury (AKI) An acute, reversible decline in kidney function manifested by an increase in serum creatinine (SCr) combined with an inability of the kidney to regulate fluid and electrolytes appropriately.

Albuminuria The presence of albumin, a plasma protein, in the urine; this often reflects damage to the glomerulus (the filter) of the kidney.

Aminoglycoside A class of antibiotic used to treat certain bacterial infections.

Biomarker A molecule (protein, enzyme, etc.) found in the body (blood, urine, tissue, etc.) that signifies the presence of a disease.

Chronic kidney disease (CKD) A progressive and often irreversible loss of kidney function.

Creatinine A by-product of muscle breakdown; its concentration in the serum is often reflective of kidney function because it is nearly exclusively cleared by the kidney. Elevated serum creatinine concentrations reflect impaired kidney function.

Creatinine clearance (CCl) Describes how much creatinine is removed from the body by the kidney; used as a proxy for kidney function and glomerular filtration rate.

Drug clearance A measure of the amount of drug eliminated from the serum in a given amount of time.

Glomerular filtration rate (GFR) A measure of kidney function describing the rate of fluid filtered by the kidney.

Nephrotoxin A drug or medically administered substance (i.e., intravenous contrast) which can have injurious effects on the kidney.

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease that predisposes individuals to chronic and recurrent pulmonary infections, intestinal malabsorption, and pancreatic insufficiency. A defect in the cystic fibrosis transmembrane conductance receptor (CFTR) leads to impaired sodium and chloride transport across epithelial cells and is the underlying cause of the clinical manifestations of CF. In the human kidney, CFTR is present predominantly in the proximal and distal tubules (Crawford et al. 1991), but the impact of CFTR on kidney disease in CF has not been fully elucidated. Despite the presence of CFTR in the kidney, primary kidney diseases are relatively rare in CF patients. Thus, the majority of kidney injury in this population stems from secondary insults from nephrotoxin administration, formation of renal stones, and CF-related diabetes (CFRD). As survival for patients with CF increases, so do opportunities for kidney injury and the likelihood of development of chronic kidney disease (CKD). Improved methods for early detection of kidney injury are needed.

This chapter reviews the causes and methods of detection of acute kidney injury (AKI) in CF patients, with a focus on nephrotoxin-associated kidney injury, which is the most prevalent and potentially modifiable cause of kidney injury in this population. The impact of AKI in CF patients and the potential roles of novel urinary and serum kidney injury biomarkers in AKI detection are also discussed. Laboratory methods of biomarker measurement are not reviewed and can be found elsewhere in this book.

Kidney Injury Definitions

Acute kidney injury, formerly denoted acute renal failure, is defined as an acute, reversible increase in serum creatinine (SCr) and nitrogenous waste products combined with an inability of the kidney to regulate fluid and electrolytes appropriately (Andreoli 2009). Traditionally, the method for monitoring kidney injury (acute or chronic) has been through surveillance of SCr and blood urea nitrogen (BUN) measurements and monitoring urine output. Serum creatinine can be monitored directly or used to estimate the glomerular filtration rate (GFR) or creatinine clearance (CCl) through a variety of derived formulae. A number of AKI definitions have been developed based on the magnitude of SCr or estimated creatinine clearance (eCCl) changes and/or a reduction in urine output. Figure 1 displays the AKI

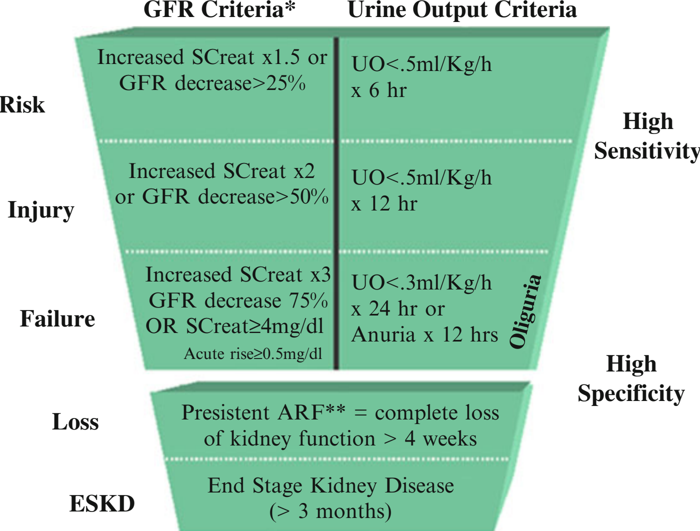

Fig. 1 RIFLE classification scheme for acute renal failure (*ARF*). The classification system includes separate criteria for creatinine and urine output (*UO*). A patient can fulfill the criteria through changes in serum creatinine (*SCreat*) or changes in UO or both. *Abbreviations*: *ARF* acute renal failure, *GFR* glomerular filtration rate (©2004; licensee BioMed Central Ltd. This is an Open Access article. http://ccforum.com/content/8/4/R204. Reproduced with permission from Bellomo et al. 2004)

classification scheme of the RIFLE criteria (Bellomo et al. 2004), which is one of the most frequently employed AKI definitions for adults. This classification system demonstrates how SCr-, GFR-, and urine output-based criteria relate and can be applied to identify patients with AKI. Most AKI definitions have been studied and validated in specific patient populations. However, there is no universally accepted and validated definition for AKI specific to patients with CF. As will be discussed below, SCr-based definitions of AKI may be inadequate in patients with CF due to the limitations and inaccuracies of SCr in this population.

Similar to AKI, chronic kidney disease (CKD) encompasses a number of stages which reflect the degree of kidney dysfunction. Chronic kidney disease typically reflects sustained ($\geq$3 months) and significant (GFR <60 mL/min/1.73 m^2) impairment (K/DOQI 2002). A reduction in kidney function to this degree, referred to as stage 3 CKD, generally equates to a loss of half of normal adult kidney function. Persistent kidney damage, manifest as structural or function abnormalities, can also meet the CKD definition.

The Impact of Kidney Injury in Cystic Fibrosis

The incidence of kidney injury in CF may be significantly higher than in the non-CF population. Bertenshaw and colleagues estimated the incidence of acute renal failure in individuals with CF to be between 4.6 and 10.1 cases per 10,000 per year, more than 100 times the average rate of kidney injury in children and three to eight times that of adults (Bertenshaw et al. 2007). This survey study identified patients by physician report and defined acute renal failure as, "raised plasma creatinine for age." Despite the use of this broad definition, the majority of subjects included in the study (54 %) had severe renal impairment requiring dialysis, making it likely that many patients with modest changes in SCr, which would constitute AKI by recently validated definitions, were not reported. Therefore, this study may actually underestimate the true incidence of AKI in the CF population.

Acute kidney injury has both short- and long-term ramifications, and even small changes in SCr of 0.3–0.5 mg/dL are independently associated with worse outcomes, including mortality, in children and adults (Moffett and Goldstein 2011; Chertow et al. 2005). Meanwhile, children and adults who sustain AKI are also at increased risk for long-term mortality and the development of chronic kidney disease (Askenazi et al. 2006; Wehbe et al. 2011), including AKI resulting from exposure to nephrotoxic medications (Menon et al. 2014). Data on the impact of AKI episodes in CF patients are more limited. Among adults with CF, episodes of AKI correlate with some markers of chronic kidney impairment (Florescu et al. 2012). This cohort study followed 113 adult patients in the United States with CF for up to 8.5 years. While there were no significant changes in BUN ($p = 0.92$) or SCr ($p = 0.2$) among the entire study population, individuals who experienced an episode of AKI had a significant increase in BUN ($P = 0.002$) and a nearly significant increase in SCr ($P = 0.056$) at the end of follow-up. Only the use of inhaled colistin correlated with AKI episodes ($p = 0.03$).

Unfortunately, there are limited additional data about the long-term outcomes of CF patients in relation to episodes of AKI specifically. Yet, there is mounting evidence that patients with CF sustain repeated renal insults over time from a variety of etiologies. As survival for patients with CF increases, the risk of developing CKD rises. Quon et al. estimated that the age-adjusted prevalence of CKD is about twice that of the United States general population (Quon et al. 2011). A strong association has been described between patients CFRD requiring insulin and the development of CKD (Quon et al. 2011). Meanwhile, others have noted an association between repeated intravenous (IV) aminoglycoside (AG) use and long-term renal impairment (Al-Aloul et al. 2005a). Ultimately, the development of CKD is more likely to result from repeated or chronic insults than progression of primary kidney disease.

Children with CF may also develop chronic renal dysfunction. In a retrospective analysis of children with CF who had GFR measured by ^{99m}Tc-DTPA at a single center, Prestidge et al. found that 6 % (4/63) had evidence of stage 2 CKD (GFR <90 mL/min/1.73 m^2, persistent abnormalities in urinary sediment, abnormal renal imaging): one child with reduced GFR and three others with persistent microscopic hematuria (Prestidge et al. 2011). Although the rate of renal impairment in this study was low, the authors observed that 40–56 % of children had evidence of glomerular hyperfiltration depending on the definition used (GFR >2 standard deviations for age or >90th percentile for age). In diabetic adults without CF, glomerular hyperfiltration precedes the development of albuminuria and subsequent GFR decline (Moriya et al. 2012) and it is possible, as the authors suggest, that glomerular hyperfiltration in CF patients similarly portends subsequent kidney function decline, although this has not been established.

The impact of repeated nephrotoxic insults in the CF population becomes most apparent in patients undergoing lung transplantation. In a large cohort study of pediatric lung transplant recipients, patients with CF had more rapid decline in kidney function following lung transplant than other transplant recipients (Hmiel et al. 2005). In this study, Kaplan-Meier analyses determined that older age at transplant and diagnosis of CF were both associated with loss of renal function over time. Broekroelofs similarly found that rate of renal function loss was greatest among lung transplant recipients who also had CF (−10 mL/min/year, range: −14 to −6 mL/min/year) compared to others (Broekroelofs et al. 2000). Meanwhile, using data from the CF Foundation Patient Registry, Quon determined that the 2-year risk of post-lung transplant kidney dysfunction among CF patients was 35 % (95 % CI: 32–39 %) and the risk increased substantially with increasing age (Quon et al. 2012). There are a number of potential reasons why patients with CF are at high risk for development of renal impairment following lung transplantation: receipt of repeated and chronic nephrotoxic medications prior to and following transplantation (calcineurin inhibitors, antibiotics), diabetic nephropathy, and the presence of kidney stones. Renal reserve may be impaired heading into transplant, because of the numerous insults sustained prior to the procedure, and compounded by infection, receipt of additional antibiotic courses, and advanced diabetic disease afterward (Hmiel et al. 2005). With the high risk of development of CKD for patients with CF undergoing lung transplantation, it is paramount to identify means to reduce insults to the kidney prior to transplant.

Causes of Acute Kidney Injury in Cystic Fibrosis

There are a number of potential causes of AKI in patients with CF including toxins, intrinsic renal disease, obstruction, and other insults. In this population, the most likely inciting factor is the use of nephrotoxic medications. Individuals with CF have frequent lung infections which contribute to a decline in lung function over time, and aggressive antimicrobial therapy is used in both the acute and long-term management of CF lung disease. Unfortunately, a number of antibiotics commonly administered in the CF population, most notably aminoglycosides and colistin, have nephrotoxic effects. Nonsteroidal anti-inflammatory drugs (NSAIDs) are also frequently used in CF to mitigate lung inflammation but can have detrimental effects on the kidney. Other nephrotoxic medications such as antihypertensive medications and immunosuppressants, although less commonly given, diabetic nephropathy, renal stones, and other causes may also contribute to kidney injury in this population; each of these potential causes will be discussed in further detail below.

Antibiotics

Aminoglycosides are a commonly used class of antibiotics in CF because of their activity against Gram-negative bacteria, particularly *Pseudomonas aeruginosa*, the most common pathogen infecting the lungs of patients with CF. These are concentration-dependent antibiotics, meaning that bacterial killing is optimized at high concentrations. To take advantage of this property, AGs are typically administered in high doses once daily to maximize killing and allow sufficient time for clearance of the medication prior to re-dosing. However, nephrotoxicity is a known side effect of AGs and the cellular mechanisms leading to toxicity are complex. Aminoglycoside nephrotoxicity results from accumulation of drug within proximal tubule cells leading to cytotoxicity, apoptosis, and cell death (Rougier et al. 2004). After glomerular filtration, a portion of the drug binds to an endocytic receptor, megalin, located on the apical surface of the proximal tubule epithelial cell and is endocytosed (Moestrup et al. 1995). Expression of megalin is directly related to the degree of drug accumulation as it is the principle receptor for AG uptake in the kidney (Schmitz et al. 2002), as well as a number of other important ligands. Following endocytosis, the drug accumulates within lysosomes, causes damage to membrane phospholipids and is released into the cytosol where cellular toxicity occurs (Servais et al. 2005). Tubule cell damage and apoptosis then lead to a reduction in the glomerular filtration rate (GFR) and impaired kidney function (Lopez-Novoa et al. 2011).

Most reports of AKI in patients with CF have implicated AG as the cause (Bertenshaw et al. 2007; Al-Aloul et al. 2005b; Drew et al. 2003; Kennedy et al. 2005; Smyth et al. 2008). In a survey of CF centers in the United Kingdom, an AG was administered prior to onset of AKI in 88 % of cases reported (Bertenshaw et al. 2007). An additional risk factor such as dehydration, underlying renal disease, or long-term receipt of a nephrotoxic drug also often accompanies cases of aminoglycoside-associated AKI (Smyth et al. 2008). Yet, the true incidence of

AG-associated AKI in the CF population had not been known. This is because detection of AKI is reliant upon systematic SCr measurement, which is rarely performed. Among children with CF, AKI rates during AG courses of up to 20 % have been described when SCr is monitored daily (Downes et al. 2014). But, detection of AKI is significantly impacted by the frequency of SCr measurement. Nonsystematic SCr measurement, which is common practice, and dependence upon a suboptimal marker (SCr) lead to an underestimation of the actual incidence of AKI.

Similar to AG, the antibiotic colistin has broad Gram-negative activity and is used often in patients with CF to treat more resistant pathogens. It is a polymyxin antibiotic whose nephrotoxic potential is recognized but not well understood. The mechanism of nephrotoxicity is thought to be similar to the mechanism by which the drug exhibits its antibacterial activity: increasing cell membrane permeability (Lewis and Lewis 2004). Damage to renal tubule cells leads to acute tubular necrosis, mitochondrial dysfunction, and impaired kidney function (Dai et al. 2014). Historically, colistin was associated with nephrotoxicity in up to 50 % of recipients (Falagas and Kasiakou 2006). However, newer formulations of the drug, careful monitoring of patients, and avoidance of coadministration with other known nephrotoxins have led to a reduction in reported toxicity.

The nephrotoxic potential of colistin in patients with CF is conflicting. In a retrospective review of 52 patients receiving 135 courses of colistin at a single center, Ledson and colleagues found that there was no change in renal function following receipt of the drug among the 122 evaluable courses (Ledson et al. 1998); colistin was used in combination with other drugs in 85 % of courses in this study. Meanwhile, in a randomized trial of IV colistin monotherapy versus combination antipseudomonal therapy (Conway et al. 1997), recipients of monotherapy did not demonstrate a change in creatinine clearance after 12 days, while those who received IV colistin in combination with another non-AG antibiotic had a significant decline by day 12 (day 1 = 109 mL/min vs. day 12 = 91 mL/min, $p < 0.01$). Nevertheless, reports of kidney injury with colistin are less prevalent than with IV aminoglycosides in CF, perhaps owing to the different frequency of use of these agents.

There have been no studies directly comparing the development of kidney injury from AG vs. colistin in CF patients. But, receipt of repeated courses of nephrotoxic antibiotics may be associated with long-term renal damage even in the absence of detected SCr-based AKI. In a prospective study of 80 CF outpatients with *Pseudomonas aeruginosa*, Al-Aloul and colleagues found a strong correlation between IV AG use and diminishing kidney function ($r = -0.32$, $P = 0.0055$) as determined by measured creatinine clearance (Al-Aloul et al. 2005a). Figure 2 displays the inverse correlation between kidney function and number of courses of IV nephrotoxic antibiotics found in this study. The association between decreased renal function and repeated antimicrobial exposure was not significant for regimens including IV colistin with a beta-lactam antibiotic ($r = 0.02$, $p = 0.83$). However, there was a significant association between renal function decline and receipt of IV aminoglycosides with a beta-lactam antibiotic ($r = -0.35, p = 0.0018$); the effect was more pronounced when an IV aminoglycoside was coadministered with colistin ($r = -0.51, p < 0.001$).

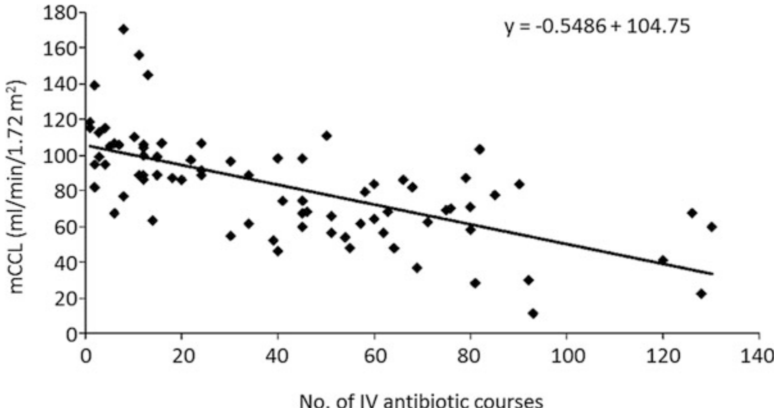

Fig. 2 Correlation between renal function (mCCL) and lifetime use of IV nephrotoxic antibiotics (courses containing aminoglycosides and/or colistin). $R = 0.65$, $P < 0.00001$. *Abbreviations*: *mCCL* measured creatinine clearance (Reproduced with permission from Al-Aloul et al. 2005. ©2004 John Wiley & Sons, Inc)

NSAIDs

Beyond the treatment of pain, nonsteroidal anti-inflammatory drugs (NSAIDs) may have a role in the treatment of chronic lung inflammation in CF patients. However, NSAIDs alter kidney function through their effects on prostaglandins (Weir 2002) and in the setting of altered renal blood flow (dehydration, shock, etc.) may compromise renal perfusion and lead to kidney injury. Despite their potential adverse effects, NSAIDs have rarely been reported to have significant nephrotoxic effects in patients with CF. In a single-center retrospective study of patients on chronic ibuprofen, 50 % patients had to discontinue the therapy due to adverse events (Fennell et al. 2007) but only one (2 %) discontinued the drug due to renal toxicity. Similarly, Lahiri et al. reported that high-dose ibuprofen was not associated with increased non-creatinine biomarkers of kidney injury (Lahiri et al. 2014); these biomarkers will be discussed in further detail below.

Other Nephrotoxins

A number of other medications administered in patients with CF may be toxic to the kidneys, as with other groups of patients. Lung transplantation is an important option for CF patients with severe and end-stage lung disease. Immunosuppressant medications such as the calcineurin inhibitors cyclosporine and tacrolimus have important therapeutic roles in transplant recipients but can lead to rapid decline in kidney function following transplant (Quon et al. 2012; Hmiel et al. 2005; Broekroelofs et al. 2000). These medications contribute to kidney injury and a reduction in GFR by causing vasoconstriction of afferent and efferent glomerular arterioles (Lanese and Conger 1993). Their nephrotoxic effects may be compounded by years of prior

nephrotoxin receipt in CF patients and may unmask decreased renal reserve (Hmiel et al. 2005). Antihypertensives such as loop diuretics and angiotensin-converting enzyme inhibitors may alter renal hemodynamics causing kidney injury (Ferguson et al. 2008). And antimicrobials such as acyclovir and amphotericin have their roles in CF care for the treatment of herpes virus and fungal infection, respectively, but may also be nephrotoxic.

Diabetes

In 2012, nearly 20 % of individuals with CF had cystic fibrosis-related diabetes (CFRD) with more than a third of adults being affected (Cystic fibrosis foundation patient registry 2012 annual data report 2013). Similar to type I and type II diabetes in patients without CF, CFRD causes long-term kidney damage. In a study using registry data from Germany and Austria (Konrad et al. 2013), the rate of diabetic nephropathy (defined as the presence of microalbuminuria) was similar among adults with CFRD (25.2 %) compared to non-CF patients with type I (17.2 %) or type II diabetes (24.7 %, p-values not reported) after adjustment for demographics. Using the US CF Registry data from 2001 to 2008, Quon determined that CFRD requiring insulin therapy was a significant risk factor for the development of stage 3 CKD as defined by an estimated GFR <60 mL/min/1.73 m^2 (Quon et al. 2011). Considering that this study was a retrospective analysis of registry data with reliance on eGFR for detection of CKD, this may actually underestimate the impact of CFRD on CKD development. CFRD requiring insulin therapy is also a significant risk factor for renal dysfunction following lung transplant (HR 1.30; 95 % CI, 1.02–1.67) (Quon et al. 2012). While the microvascular complications of CFRD can lead to chronic renal insufficiency, it is unknown whether CFRD also contributes to episodes of acute kidney injury or compounds the nephrotoxic effects of antibiotics in the CF population.

Stones

Patients with CF are at higher risk for nephrocalcinosis compared to the general population which can contribute to an impairment of kidney function. In a single-center cohort study of pediatric CF patients, the prevalence of risk factors for stone formation was high – hyperoxaluria ($N = 58/83$, 78 %), hypocitraturia (57/76, 75 %), hypercalciuria (16/87, 18 %), and hyperuricuria (15/83, 18 %) (Andrieux et al. 2010). However, no patients had symptomatic kidney stone formation and only 2 % had stones diagnosed by ultrasonography. Some studies have reported a prevalence of nephrolithiasis as high as 21 % (Terribile et al. 2006). Microscopic nephrocalcinosis may also be a frequent occurrence in CF patients, and the increased sodium secretion that occurs in CF may lead to dehydration and low urine volumes, further compounding the risk for stone formation. Repeated exposure to antimicrobials may alter gut flora and decrease the *Oxalobacter formigenes*, which leads to

reduced degradation of oxalate and an increased risk of hyperoxaluria and stone formation (Sidhu et al. 1998).

Markers of Kidney Injury in Cystic Fibrosis

With the myriad of potential causes of kidney injury in patients with CF, the long-term ramifications of repeated and chronic renal insults on kidney function is becoming more apparent as patients with this disease live longer. Traditional markers of kidney function and injury such as serum creatinine are inadequate. And, there is a need for accurate and sensitive markers of kidney injury and incorporation of these markers into routine CF care. Yet, the ideal biomarkers for AKI detection among patients with CF have not been established, and further research is urgently needed.

Novel biomarkers for kidney injury may be clinically useful for early detection of AKI. These biomarkers, of which several have been identified and will be discussed below, are more sensitive than traditional SCr measurements in detecting kidney injury directly and are under investigation for their utility in risk stratification and prognostication in AKI (Parikh et al. 2005). Increased levels have been associated with poor clinical outcomes irrespective of SCr measurements (Haase et al. 2011; Parikh et al. 2005). Serum biomarkers often reflect abnormal kidney function (impaired GFR), while urinary biomarkers may reflect kidney injury and/or function (compromised reabsorption). Depending on the process, urinary biomarkers also have the potential to signify specific sections of the nephron that are affected. This type of specificity could be useful to elucidate the mechanism of underlying injury. However, the determination of the optimal biomarkers for use in specific clinical settings has not been established.

The following sections will discuss the utility of traditional and novel biomarkers for detection of kidney injury in CF. Many of the biomarkers reviewed in this section are described in more detail in other chapters in this book. Therefore, this section will focus primarily on these biomarkers in CF patients specifically. Of note, few studies explicitly examine the role of biomarkers in AKI detection in CF patients. The majority of studies attempt to establish the relationship between biomarkers of interest and GFR. This section summarizes available data and, when applicable, provides guidance as to how kidney injury biomarkers can be used or studied in the future to improve AKI detection in patients with CF.

Serum Creatinine and Estimated GFR

The glomerular filtration rate is widely considered the best measure of kidney function. Direct measurement of GFR, however, is difficult, often costly, and frequently impractical in the hospitalized setting. Serum creatinine is the traditional biomarker most often used for simple assessment of kidney function as well as detection of kidney injury. Creatinine, a product of muscle breakdown, undergoes

glomerular filtration and is excreted in the urine. Because there is minimal extrarenal clearance of creatinine in healthy individuals, renal creatinine clearance is used as a surrogate for kidney function (Perrone et al. 1992). Twenty-four hour urine collection allows for calculation of CCl but is methodologically tedious and prone to error. Therefore, serum creatinine values are used to estimate CCl and GFR through implementation of a number of derived formulae. These formulae tend to be most reliable in the setting of stable kidney function and thus stable SCr. And the validity of these formulas is based on the assumptions that SCr is completely filtered and that the rate of production equals the rate of renal excretion (Perrone et al. 1992). In the setting of unstable renal function or ongoing kidney injury, unfortunately, these criteria are not always met.

Changes in SCr values are nonspecific, often delayed, and do not directly reflect cellular kidney injury. A demonstrable change in SCr is not detected until significant renal mass, roughly 70–80 %, has been affected (Pfaller and Gstraunthaler 1998). Creatinine is formed as a result of muscle breakdown which makes it an additionally problematic marker of kidney function and injury in patients with CF, who frequently have reduced muscle mass compared to healthy patients. Because of this, SCr-based formulae for estimating GFR are often inaccurate and typically underestimate renal impairment in patients with CF (Al-Aloul et al. 2007). Al-Aloul compared measured CCl from timed urine collections with ten SCr-based formulae used to estimate CCl in 74 adult CF patients and 29 healthy, age- and BMI-matched control subjects (Al-Aloul et al. 2007) and concluded that all formulae for estimating CCl were unreliable in CF patients. The correlation between estimated and measured creatinine clearance ranged from 0.55 to 0.7 with a bias of −9.1 to 22.9 depending on the equation used. The formulae were also less accurate in CF patients than in healthy controls. Additionally, the two equations most commonly used clinically for estimating CCl, the Cockcroft-Gault formula (Cockcroft and Gault 1976) and the abbreviated MDRD equation (Rule et al. 2004), grossly overestimated renal function in adult CF patients with reduced CCl (<80 mL/min, Table 1) in their study population.

Although traditional monitoring for kidney injury involves SCr measurement, the sensitivity of this parameter is poor. While more accurate methods of estimation of kidney function are available, such as 24-h urine creatinine collection or nuclear GFR studies, they are typically used to assess GFR at a single time point. These approaches are generally not practical in the hospitalized setting and cannot generally be used for monitoring or early detection of AKI due to the methodological rigor involved in their use.

Aminoglycoside Clearance

Therapeutic drug monitoring (TDM) is used to try to improve efficacy and minimize toxicity from aminoglycosides. The goal of TDM is to maximize effectiveness and safety by determining patient-specific doses and dosing intervals through drug level monitoring. Trough levels are typically monitored to assure adequate clearance of

Table 1 Comparison of mCCl and eCCl (CGF and abbreviated MDRD [four variable]) in renally impaired CF patients (mCCl <80 mL/min)

	N	Mean mCCl (SD)	eCCl formula	Mean eCCl (SD)	% eCCl within 33 % of mCCl	Bias	95 % CI for bias	Limits of agreement
mCCl <80	35	63.5 (12.3)	CGF	81.7 (18.1)	60 %	18.3	13.6–23.0	−9.7–46.3
			aMDRD	79.3 (20.2)	65 %	15.8	10.5–22.0	−17.5–50.1
mCCl ≥80	39	100.7 (13.8)	CGF	102.3 (16.6)	95 %	1.6	−3.5–6.7	−30.4–33.6
			aMDRD	95.1 (16.1)	84 %	−5.6	−11.1–−0.1	−39.6–28.4
Total	74	83.1 (22.9)	CGF	92.6 (20.1)	78 %	9.7	5.5–13.5	−24.9–43.9
			aMDRD	88 (19.7)	77 %	4.9	0.3–9.5	−35.3–45.1

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aMDRD abbreviated Modification of Diet in Renal Disease formula, *CI* confidence interval, *CGF* Cockcroft-Gault formula, *eCCl* estimated creatinine clearance, *mCCl* measured creatinine clearance, *N* number, *SD* standard deviation

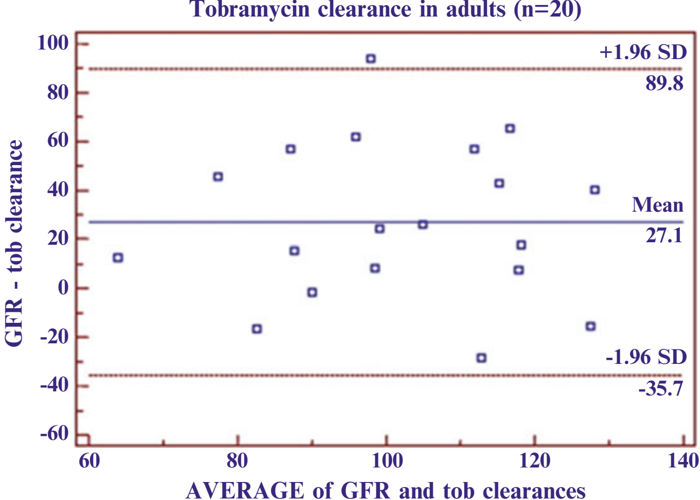
the drug and elevated serum trough concentrations most closely relate to nephrotoxicity (Bertino et al. 1993). Both sophisticated and simple methods have been developed to determine an individual patient's pharmacokinetic parameters (volume of distribution, elimination rate constant, total body clearance) based on serum drug concentrations (Tod et al. 2001). Although there is variability in monitoring practices between CF centers, TDM is almost universally used.

In theory, AG clearance should be directly related to kidney function since these drugs are almost exclusively eliminated via glomerular filtration. Unfortunately, the correlation between GFR and actual drug clearance in CF patients is variable. Some studies (Town et al. 1996) describe a significant correlation between the measured creatinine clearance and tobramycin total body clearance ($r = 0.52, p = 0.02$). Other more recent studies (Soulsby et al. 2010), however, observed that tobramycin clearance correlates poorly with measured GFR in both adults ($r = 0.1$, $p = 0.71$) and children ($r = 0.25$, $p = 0.19$) with CF. Figure 3 displays Bland-Altman plots comparing GFR estimated via tobramycin clearance and measured GFR.

Aminoglycoside clearance is more reflective of kidney function than of kidney injury. Changes in drug clearance over time may suggest that an individual has sustained injury, but the extent of kidney injury needed to result in clinically significant alterations in drug clearance has not been described. Theoretically, longitudinal monitoring of tobramycin clearance could be used to identify chronic renal insufficiency. However, to our knowledge, no studies have demonstrated decreased AG clearance as a marker of CKD in patients with CF.

In a recent population pharmacokinetic study, Alghanem observed that AG clearance appears to be stable over time in patients with CF despite receipt of multiple courses of therapy over several years (Alghanem et al. 2013). The authors evaluated 1,075 aminoglycoside courses in 166 patients aged 14–66 years; subjects received as many as 28 courses over a 15 year period. There were no significant changes in kidney function (based on eCCl) over time, and only a single patient had moderate renal impairment (eCCL 45–58 mL/min). Additionally, there was little change in aminoglycoside clearance from one course to the next (between-occasion variability = 11 %), and no significant trends were detected in AG clearance over time based on the number of prior aminoglycoside courses. The authors concluded that in the population of CF patients they studied, there was no decline in AG clearance over time.

These findings contrast with data from other studies suggesting an increased prevalence of kidney dysfunction over time in patients with CF (Quon et al. 2011; Al-Aloul et al. 2005a). The discrepancy may lie in the variable relationship between AG clearance and eGFR in CF patients. Creatinine clearance had only a weak relationship to AG clearance in the Alghanem study which likely reflects the unreliable nature of SCr-based equations for estimating kidney function in CF patients. Additionally, Alghanem and colleagues relied on drug levels drawn within 72 h of the start of therapy in the majority (83 %) of cases. It is possible that patients with mild/moderate underlying renal dysfunction do not demonstrate impaired drug clearance early in antibiotic courses. The mechanism of AG toxicity is dependent upon accumulation of drug in proximal tubule cells, and decreased AG clearance



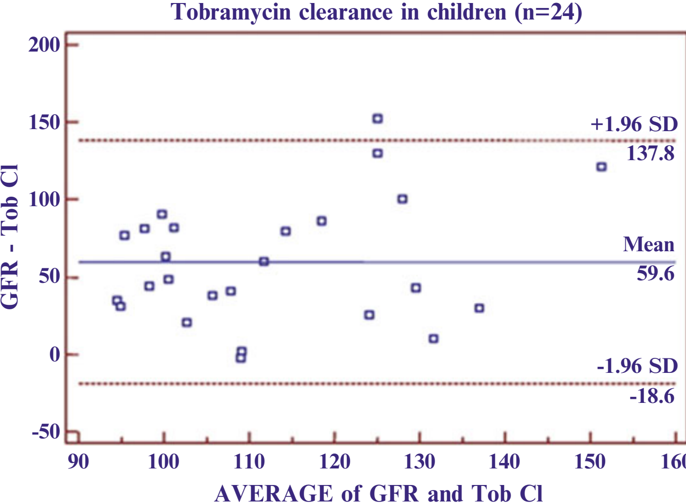


Fig. 3 Bland and Altman analysis for differences between tobramycin clearance and measured GFR in mL/min/1.73 m^2 in adults (**a**) and children (**b**). The *x-axis* represents the average GFR and the *y-axis* represents the difference between the measured GFR and tobramycin clearance (Reproduced with permission from Soulsby et al. 2010. ©2010 Elsevier)

may not manifest until a sufficient amount/duration of drug has been administered. Serial AG level measurements would be needed to determine when individuals pass the cutoff that leads to impaired kidney function, but the frequency of measurement needed to accurately capture this may be impractical. Ultimately, the inconsistent relationships between AG clearance and measured and estimated CCl make it difficult to rely on drug clearance as a marker of kidney injury.

N-Acetyl-β-D-glucosaminidase (NAG)

In the setting of kidney injury, enzymes located within tubular epithelial cells may be released into the urine. *N*-Acetyl-β-D-glucosaminidase (NAG) is one such enzyme. NAG is a proximal tubule lysosomal enzyme and detection in the urine increases in the setting of a number of causes such as nephrotoxic injury and diabetic nephropathy (Skalova 2005). Although not specific to a particular mechanism of injury, it is a sensitive marker of renal tubular injury and has been studied in a variety of patient populations. Typically, NAG is expressed as a ratio with urinary creatinine to account for biologic variability.

In patients with CF, NAG has been primarily studied in the context of nephrotoxin receipt, in particular aminoglycosides, and is a highly sensitive and specific marker of tubular injury in this population (Godson et al. 1988). Urinary levels of NAG increase significantly during courses of IV gentamicin (Godson et al. 1988), amikacin (Halacova et al. 2008), and tobramycin (Steinkamp et al. 1986; Glass et al. 2005; Etherington et al. 2007; Master et al. 2001; Riethmueller et al. 2009; Smyth et al. 2005). Elevated levels of NAG can even be observed during courses of inhaled tobramycin (Guy et al. 2010).

Most studies of NAG have confirmed that urinary concentrations increase despite stable serum creatinine. Glass studied 22 children with normal GFR and stable SCr receiving IV tobramycin three times daily for 14 days (Glass et al. 2005). NAG significantly increased following completion of therapy compared to measurements obtained prior to the start of the drug ($p < 0.0001$). Steinkamp studied 14 subjects before, during, and after receipt of 10 mg/kg/day of IV tobramycin with azlocillin and observed a six- to tenfold increase in urinary NAG during therapy (Steinkamp et al. 1986). Meanwhile, Etherington measured urinary NAG in 88 patients receiving IV tobramycin or colistin on days 1, 14, and at first clinic follow-up (Etherington et al. 2007). Although there were no changes in SCr, a 3.5-fold increase in urinary NAG occurred between day 1 and 14, and NAG excretion was higher in subjects receiving tobramycin compared to colistin (day 14 median NAG ratio, 2.24 vs. 0.98, $p < 0.001$), suggesting an increased risk of tubular toxicity from tobramycin. Additionally, NAG was higher at each time point of the study for subjects with CFRD.

Studies of urinary NAG in CF patients also provide some evidence that tubular injury from nephrotoxic antibiotics may be sustained. In the study by Glass, urinary NAG levels remained higher than pretreatment levels at 4 weeks after the course ($p < 0.001$) (Glass et al. 2005). Meanwhile, Etherington observed that the majority (80 %) of patients who received multiple courses of treatment during the study period had day 1 NAG levels that were significantly higher in subsequent courses ($p < 0.001$) (Etherington et al. 2007). And, almost half (46 %) of patients had an elevated NAG level at their clinic follow-up visit. Although NAG values returned to normal during follow-up assessments in the study by Steinkamp, the study was small ($N = 14$), and follow-up measurements were obtained at 4–120 days after treatment cessation (Steinkamp et al. 1986).

Urinary NAG has also been used to monitor for nephrotoxicity in several studies comparing different dosing regimens of aminoglycosides (Master et al. 2001;

Riethmueller et al. 2009; Smyth et al. 2005). Two of these studies showed lower NAG values with once-daily administration (Master et al. 2001; Smyth et al. 2005), providing evidence for the improved safety of once-daily dosing. Although NAG levels were similar between the once- and thrice-daily tobramycin groups in a randomized trial, significant rises in urinary NAG develop during AG therapy despite stable serum creatinine (Riethmueller et al. 2009).

Urinary NAG is a sensitive marker of proximal tubule kidney injury receiving nephrotoxic medications, and several studies in CF patients have observed increasing and often elevated values over the course of AG therapy without a demonstrable change in serum creatinine. This corroborates that NAG is a more sensitive marker of tubular injury than SCr during aminoglycoside courses and that it may have a role in detection of subclinical kidney injury in patients receiving these medications. However, longitudinal studies are needed which seek to elucidate the relationship between episodes of kidney injury detected by changes in NAG and long-term renal outcomes.

Urinary Albumin

The presence of albuminuria may result from glomerular damage due to increased permeability or may signal proximal tubule dysfunction and decreased reabsorption of the protein (Vaidya et al. 2008). Albumin is freely filtered by the glomerulus, and urinary detection can be increased in the setting of non-pathologic conditions which cause proteinuria, such as dehydration and vigorous exercise. Microalbuminuria, defined as 30–300 mg/L (Vaidya et al. 2008), is often present in patients with diabetes including CF patients with CFRD. In a large cohort study of CF children and adults, CFRD contributed to a sevenfold increased odds (95 % CI: 2.5–20.0, $p =$ 0.0002) of persistent microscopic albuminuria: 10.7 % of patients with CFRD versus 1.6 % of CF patients without CFRD (Lind-Ayres et al. 2011). Transient microalbuminuria was present in a similar percentage of patients with and without CFRD in this study, which was comparable to the rate found in the general population (~6–7 %).

Other factors aside from diabetes may also contribute to the development of albuminuria in CF patients. Lind-Ayres found that a significantly higher percentage (40 %) of patients who had undergone lung transplant, all of whom either had CFRD or glucose intolerance, had persistent microscopic albuminuria. Meanwhile, in a small cohort study assessing proteinuria via 24-h urine collection, CF genotype was the only factor associated with the presence of high (>150 mg/day, $N = 6/22$) vs. low (<150 mg/day, $N = 16/22$) proteinuria (Cemlyn-Jones and Gamboa 2009). The multifactorial nature of albuminuria and its high prevalence in the CF population limits the utility of albuminuria as a marker of acute kidney injury. Persistent detection of microalbuminuria heralds the onset of nephropathy in patients with CFRD. But, the predictive capability of a single measurement is limited. Whether albuminuria can be used as a reliable marker of acute kidney injury is yet to be determined. Additional studies which provide data on serial urine albumin measurements would be needed to determine its potential role for it in AKI detection.

Table 2 Results for correlations between measured GFR and estimated renal function (95 % confidence interval reported in brackets)

Test	*R* value (adults)	*R* value (children)
Cystatin C	0.64 [0.43–0.78] $P = 0.0059$	0.61 [0.39–0.76] $P = 0.0011$
Cockcroft and Gault equation (adults) or Schwartz (children)	0.51 [0.36–0.75] $P = 0.0252$	0.60 [0.34–0.74] $P = 0.0015$
Tobramycin clearance	0.10 [−0.83–0.52] $P = 0.7117$	0.25 [−0.04–0.50] $P = 0.1920$
aMDRD equation (adults) or updated Schwartz (children)	0.29 [−0.17–0.65] $P = 0.2145$	0.60 [0.28–0.80] $P = 0.001$

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Cystatin C (CysC)

Cystatin C is a low molecular weight protein that is freely filtered by the glomerulus and reabsorbed by proximal tubule cells via megalin-assisted endocytosis (Kaseda et al. 2007). It does not undergo tubular secretion, and its function is to serve as an extracellular inhibitor of cysteine proteases. Injury to proximal tubule cells leads to increased excretion in the urine, while elevated plasma levels are reflective of impaired glomerular filtration. Therefore, it may be a useful marker of proximal tubule injury as well as a functional marker of impaired GFR. Numerous studies have examined its role as a serum marker of GFR in a variety of populations, including CF.

Soulsby compared the correlation between measured GFR, using a radioisotope technique, and eGFR using SCr-based equations, serum CysC levels, and tobramycin clearance in adults and children with CF (Soulsby et al. 2010). The results for the correlations are shown in Table 2. CysC had the strongest correlation with measured GFR for both adults and children. However, CysC had no advantage over SCr-based equations for detecting the 4/47 patients with impaired kidney function (measured GFR <90 mL/min/1.73 m^2). The sensitivity and specificity of CysC for detection of decreased GFR was 100 % and 85.7 %, respectively. There was very poor correlation between tobramycin clearance and measured GFR ($R = 0.1$ and 0.25 for adults and children, respectively), and the correlation between tobramycin clearance and CysC was not reported. Given the low prevalence of renal dysfunction in this population, the authors concluded that CysC offers no benefit over SCr-based estimates of GFR.

This study contrasts with an earlier study by Beringer which demonstrated superiority of CysC to SCr-based methods for GFR estimation in both CF patients and healthy, age-matched controls (Beringer et al. 2009). GFR estimates based on a CysC-based equation (GFR = 100/CysC – 14 Tidman et al. 2008) provided greater precision in both the CF and control populations. And, for those with CF, the CysC-based equation demonstrated a significantly higher AUC for the prediction of impaired kidney function (measured GFR <90 mL/min/1.73 m^2) compared with the Cockcroft-Gault equation (AUC 0.928 vs. 0.556, $p = 0.005$) and aMDRD equation (AUC 0.928 vs. 0.539, $p = 0.003$).

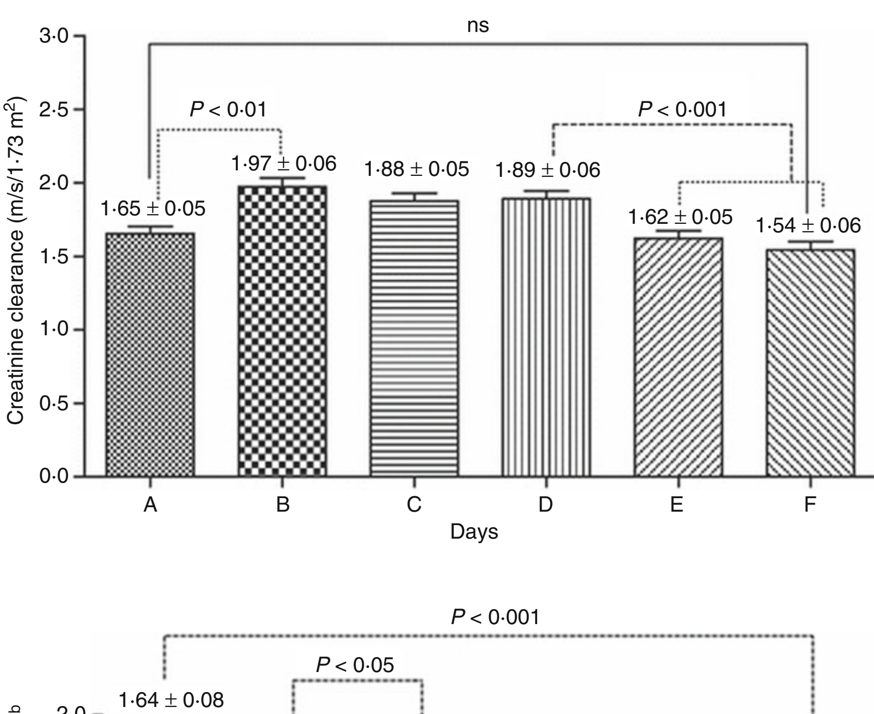
Serum CysC has also shown utility during AG courses in CF patients. Halacova measured serum CysC, CysC clearance, creatinine clearance, urinary NAG, and amikacin clearance in 71 patients receiving intermittent infusion amikacin therapy for 12 days (Halacova et al. 2008). Serum CysC levels increased throughout amikacin treatment ($P < 0.001$, Dunnett's multiple comparisons test) and 80 % of patients demonstrated CysC levels above the normal range. Consequently, the estimated GFR using a CysC-based equation demonstrated a significant decline over the course of amikacin treatment. Conversely, there were no significant changes in creatinine clearance or serum creatinine from day 0 compared with day 12, although serum creatinine was above the normal range in 45 % ($N = 32$) of patients on day 12 of therapy. Figure 4 shows a comparison of the creatinine clearance and CysC clearance values over the course of amikacin in this study. The GFR estimated by SCr was significantly higher than that predicted by CysC: 1.76 ± 0.02 versus 1.18 ± 0.04 mL/s/1.73 m^2 ($\sim 105.6 \pm 1.2$ vs. 70.8 ± 2.4 mL/min/1.73 m^2), $p < 0.0001$. ROC analyses demonstrated that serum CysC and CysC clearance were better predictors of amikacin clearance than creatinine clearance: AUC values of the ratios of amikacin clearance to creatinine clearance, CysC, and CysC clearance on day 12 compared to day 0 were 0.51, 0.92, and 0.84, respectively.

Urinary CysC may also be beneficial for AKI monitoring during aminoglycosides, which are reabsorbed in the proximal tubule via the same endocytic receptor, megalin, as CysC. In a rat model, significant changes in urinary CysC could be detected as early as day 1 of gentamicin therapy (Hoffmann et al. 2010). However, to our knowledge, this urinary biomarker has not been studied in patients with CF. It holds promise for the noninvasive detection of AKI during aminoglycoside courses and warrants investigation.

Beta-2-Microglobulin (β_2M)

Beta-2-microglobulin is another low molecular weight protein which, similar to CysC, is filtered by the glomerulus and reabsorbed in the proximal tubule. It is the light chain of the major histocompatibility class I molecule and, unlike CysC, is not a useful serum biomarker due to its expression on the cell surface of all nucleated cells (Ferguson et al. 2008). Its role as a kidney injury biomarker is limited to detection in the urine which increases in the setting of impaired proximal tubule function. In a rat model of nephrotoxicity (Sasaki et al. 2011), β_2M increased on day 1 of gentamicin, and the rate of increase was higher than other urinary biomarkers (CysC, NGAL, NAG).

Data on the role of β_2M in patients with CF are limited. In a prospective case-control study evaluating the role of serum β_2M as a marker of lung inflammation, Kearns et al. measured β_2M in CF outpatients ($N = 12$), CF patients receiving antibiotics for a pulmonary exacerbation ($N = 6$), and healthy controls ($N = 10$) (Kearns et al. 1989). Serum β_2M values were significantly lower in healthy controls than both CF groups ($p < 0.05$), but there were no differences in urinary values of β_2M between healthy controls and patients with CF. Meanwhile, in a randomized



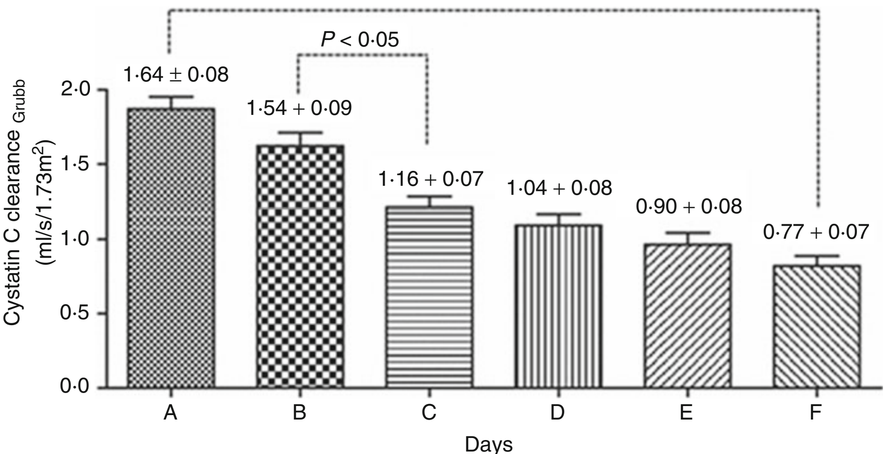


Fig. 4 Comparison of creatinine clearance (**a**) and cystatin C clearance (**b**) during amikacin therapy in CF patients. Data are expressed as column bars (mean ± SEM). *A*: Day before start of amikacin treatment (day 0). *B–F* 3rd, 5th, 7th, 10th, and 12th day of amikacin treatment (Reproduced with permission from Halacova et al. 2008. ©2008 Blackwell Publishing Ltd)

controlled trial of once- versus thrice-daily IV tobramycin, combined with ceftazidime, urinary β_2M was higher in the thrice-daily group (0.87 ± 0.5 mg/L vs. 0.18 ± 0.2 mg/L, P <0.01), although values were in the normal range for both groups (Vic et al. 1998). The study population was small (N = 22) but SCr was unchanged over the course of therapy in both groups. Unfortunately, this data

combined with the fact that β_2M is unstable in acidic urine make it an impractical kidney injury biomarker in the clinical setting.

Retinol-Binding Protein (RBP)

Retinol-binding protein (RBP) is a hepatically synthesized chaperone for vitamin A transport to tissues. It is freely filtered by the glomerulus and reabsorbed in the proximal tubule via megalin (Christensen et al. 1999). RBP is a sensitive marker of tubule dysfunction and can be detected in the urine soon after nephrotoxin exposure (Ferguson et al. 2008). This biomarker has been studied preliminarily in CF patients. Glass measured urinary RBP and NAG in 22 children with CF receiving a 14-day course of IV tobramycin (Glass et al. 2005). Measurements were performed immediately prior to and following therapy, as well as 4 weeks following completion of therapy. Urinary NAG ($P < 0.001$) and RBP ($P = 0.03$) both increased over the course of therapy. Unlike NAG, however, urinary RBP returned to pretreatment levels at 4-week follow-up. Interestingly, pretreatment levels of RBP were elevated in 18 of 21 subjects with prior AG receipt vs. zero for NAG. However, there were no differences in RBP following therapy based on the number of prior aminoglycoside courses.

Given that RBP is a chaperone for vitamin A transport, serum levels may be affected by nutritional status or the presence of liver disease in CF patients. In fact, patients with CF have lower plasma RBP levels than age- and sex-matched controls (Mrugacz et al. 2005) which could be a result of underlying pancreatic insufficiency associated with this disease. In this context, the findings by Glass that urinary RBP both: (a) increased during therapy and (b) was higher at baseline among subjects with prior AG receipt are particularly interesting. These data suggest that RBP may be a highly sensitive marker for both acute and chronic tubular dysfunction in the CF population. Additional studies assessing the relationship between aminoglycoside receipt and RBP levels should be performed, and the time course of elevation of RBP during aminoglycosides needs to be elucidated.

Neutrophil Gelatinase-Associated Lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein involved in iron homeostasis whose expression is upregulated in the setting of ischemia or infection. In humans, increased urinary levels can be detected following a variety of kidney insults and becomes elevated prior to SCr-based AKI develops (Gaspari et al. 2010; Hirsch et al. 2007). Increased levels have been associated with poor clinical outcomes irrespective of SCr changes (Haase et al. 2011; Singer et al. 2011). Urinary NGAL concentrations may reflect dysfunction in glomerular filtration, impaired proximal tubule reabsorption, and increased production by distal nephrons, depending on the mechanism of injury (Kuwabara et al. 2009). Similar to RBP

and cystatin C, urinary excretion is increased in the setting of nephrotoxic proximal tubule injury (Kuwabara et al. 2009).

The role of NGAL as a marker of kidney injury in CF has not been defined. In a study evaluating serum NGAL (Zughaier et al. 2013), values were higher in patients with CF compared to healthy controls ($P < 0.001$) yet similar when compared among CF patients with stable disease vs. those experiencing a pulmonary exacerbation. Meanwhile, when peripheral monocytes of both CF and healthy patients were infected with *Pseudomonas aeruginosa*, NGAL secretion increased. This raises the possibility that serum NGAL varies in response to lung infection and whether this influences its utility as a kidney injury urinary biomarker has been debated (Nazareth and Walshaw 2013). Nevertheless, the relationship between serum and urinary concentrations in the setting of proximal tubule injury, as would result from aminoglycoside exposure, has not been explored in CF patients. Animal studies show a time-dependent increase in urinary NGAL following administration of gentamicin (Zhou et al. 2014) suggesting that it may be a useful marker to determine the optimal duration of aminoglycoside therapy in CF patients. Additionally, the trajectory of NGAL values over the course of therapy may be a good indicator of drug accumulation given the saturability of megalin-facilitated endocytosis.

Kidney Injury Molecule-1 (KIM-1)

Kidney injury molecule-1 (KIM-1) is a transmembrane protein located predominantly (~90 %) in the proximal tubule (Zhou et al. 2008; Chiusolo et al. 2010). Its gene expression, and subsequent protein detection in the urine, is increased in the setting of both ischemia and toxin administration, specifically gentamicin (Zhou et al. 2008; Chen et al. 2010). Changes in KIM-1 precede rises in SCr following ischemic and toxic insults (Tu et al. 2014; Torregrosa et al. 2015), making it a useful biomarker of AKI development and severity.

As with many biomarkers of kidney injury, KIM-1 has not been extensively studied in the CF population. Urinary KIM-1, NAG, and protein were studied in 52 children and young adults to determine the impact of high-dose ibuprofen use on kidney biomarkers (Lahiri et al. 2014). Half of subjects were on high-dose ibuprofen therapy, and there were no differences in mean biomarker concentrations between the groups: KIM-1 $= 0.306 \pm 0.28$ ng/mg creatinine in subjects on ibuprofen vs. 0.381 ± 0.28 ng/mg creatinine in subjects not receiving the medication ($P = 0.34$). The authors did observe a correlation between KIM-1 values and lifetime AG exposure ($r = 0.35$, $P = 0.012$), although other factors which could influence this association (age, recent AG exposure, receipt of oral antibiotics, etc.) were not explored. Of note, this was a small study conducted in relatively healthy CF patients for whom urinary biomarkers were measured at only a single point in time. Therefore, results may not be reflective of the long-term effects of ibuprofen on the kidney. Longitudinal studies are needed in patients with CF to define its role in detection of AKI.

Other Urinary Biomarkers

There are a number of other novel AKI biomarkers that have been studied in non-CF populations. Interleukin-18 (IL-18), liver-type fatty acid-binding protein (L-FABP), and clusterin, for example, may have a role in AKI detection or prognostication. But studies are needed in patients with CF to determine their utility in this population.

Biomarkers and the Future of Kidney Injury in Cystic Fibrosis

Kidney injury is becoming an increasingly recognized issue in patients with CF. The untoward effects of repeated antibiotic courses, CF-related diabetes, and other insults on the kidney lead to an increased risk of development of chronic kidney disease in this population (Al-Aloul et al. 2005a; Wehbe et al. 2012; Quon et al. 2011). As lifetime survival in patients with CF increases, the long-term ramifications of this disease and the associated treatments on renal health are becoming more evident. It becomes paramount, therefore, to identify and employ strategies which seek to characterize those at highest risk for AKI, improve detection of kidney injury, and mitigate its long-term risks.

While diabetes may provide the highest threat to long-term kidney function, the major risk factor for development of AKI in CF patients is the receipt of nephrotoxic antibiotics such as aminoglycosides. There are no well-recognized methods for decreasing the toxic effects of these antibiotics, aside from early discontinuation of therapy. Unfortunately, avoidance of these medications is not a feasible or advisable approach considering the impact of recurrent and chronic lung infections in this population. While there is general agreement that monitoring of aminoglycoside drug levels and renal function is necessary, there is a lack of consistency regarding the optimal monitoring strategy to both identify and prevent AKI. Traditional monitoring for kidney injury via measurement of serum creatinine is suboptimal.

Urinary biomarkers are highly sensitive for kidney injury. In general, however, they have not been extensively studied in patients with CF, and their role in monitoring for kidney injury in these patients has not been defined. There are a number of avenues for future research in this field. In theory, biomarkers could be used to risk stratify CF patients prior to start of nephrotoxic therapy and indicate specific patients in whom alternative antibiotics should be considered. An improved correlation between biomarkers and aminoglycoside pharmacokinetic parameters (AUC, clearance) may allow for noninvasive and rapid monitoring of renal drug handling. Biomarkers may detect AKI earlier than with SCr and promote implementation of nephro-protective strategies in patients receiving nephrotoxins. Alternatively, they could be used to monitor the impact of interventions which seek to reduce kidney injury.

The majority of studies conducted in CF patients have focused on determining which biomarker is most reflective of kidney function in this population. While important, the overall utility of these novel biomarkers lies in their ability to improve the safety of nephrotoxic medications, promote earlier detection of AKI, and identify

patients at higher risk for poor short- and long-term outcomes. Ultimately, biomarker-driven trials will be needed prior to their implementation in the clinical setting.

Potential Applications to Prognosis, Other Diseases, or Conditions

A number of urinary and serum biomarkers have been identified which are highly sensitive for the detection of kidney injury. These biomarkers increase in the serum or urine following a variety of insults such as nephrotoxin receipt, ischemia, and sepsis. Although these biomarkers have not been extensively studied in the CF population, they show promise for improving the earlier detection of AKI compared to traditional biomarkers in a variety of clinical scenarios. Urinary biomarkers also have prognostic implications and have been linked to poor outcomes irrespective of serum creatinine. These biomarkers could be used clinically to identify patients at highest risk for morbidity and mortality. Urinary and serum biomarkers are applicable in a variety of settings where patients are at risk for kidney injury: critical illness, patients receiving nephrotoxins, cardiac bypass, and other. Since the mechanism and severity of kidney injury relates to release/expression/excretion of these biomarkers, they should have utility in multiple patient populations at risk for or sustaining kidney injury.

Summary Points

- Cystic fibrosis (CF) is a genetic disease that predisposes patients to recurrent and chronic respiratory tract infections.
- Patients with CF are at risk for development of acute kidney injury (AKI) due to receipt of nephrotoxic medications, particularly antibiotics, as well as long-term renal damage from long-standing diabetes and repeated nephrotoxin exposures.
- With advances in medical therapies leading to substantial improvement in lifetime survival among CF patients, the long-term ramifications of the disease and its treatments on the kidney may become apparent.
- Chronic kidney disease is prevalent in patients with CF, and means to detect and mitigate kidney insults are important to stem long-term deleterious effects.
- The traditional marker of kidney injury and function, serum creatinine, is unreliable in patients with CF as it often overestimates true kidney function and does not detect kidney injury until a significant number of nephrons have been affected.
- Biomarkers, such as cystatin C (CysC), retinol-binding protein (RBP), kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and others, hold significant promise for early detection of AKI, risk stratification, and prognosis.

- Additional research is urgently needed to explore and define the potential roles of these novel biomarkers in detection of both acute kidney injury and chronic kidney disease in the CF population.

References

Al-Aloul M, Miller H, Alapati S, Stockton PA, Ledson MJ, Walshaw MJ. Renal impairment in cystic fibrosis patients due to repeated intravenous aminoglycoside use. Pediatr Pulmonol. 2005a;39(1):15–20.

Al-Aloul M, Miller H, Stockton P, Ledson MJ, Walshaw MJ. Acute renal failure in CF patients chronically infected by the Liverpool epidemic Pseudomonas aeruginosa strain (LES). J Cyst Fibros. 2005b;4(3):197–201.

Al-Aloul M, Jackson M, Bell G, Ledson M, Walshaw M. Comparison of methods of assessment of renal function in cystic fibrosis (CF) patients. J Cyst Fibros. 2007;6(1):41–7.

Alghanem S, Paterson I, Touw DJ, Thomson AH. Influence of multiple courses of therapy on aminoglycoside clearance in adult patients with cystic fibrosis. J Antimicrob Chemother. 2013;68(6):1338–47.

Andreoli SP. Acute kidney injury in children. Pediatr Nephrol. 2009;24(2):253–63.

Andrieux A, Harambat J, Bui S, Nacka F, Iron A, Llanas B, Fayon M. Renal impairment in children with cystic fibrosis. J Cyst Fibros. 2010;9(4):263–8.

Askenazi DJ, Feig DI, Graham NM, Hui-Stickle S, Goldstein SL. 3–5 year longitudinal follow-up of pediatric patients after acute renal failure. Kidney Int. 2006;69(1):184–9.

Bellomo R, Ronco C, Kellum JA, Mehta RL, Palevsky P. Acute renal failure – definition, outcome measures, animal models, fluid therapy and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. Crit Care. 2004;8(4):R204–12.

Beringer PM, Hidayat L, Heed A, Zheng L, Owens H, Benitez D, Rao AP. GFR estimates using cystatin C are superior to serum creatinine in adult patients with cystic fibrosis. J Cyst Fibros. 2009;8(1):19–25.

Bertenshaw C, Watson AR, Lewis S, Smyth A. Survey of acute renal failure in patients with cystic fibrosis in the UK. Thorax. 2007;62(6):541–5.

Bertino Jr JS, Booker LA, Franck PA, Jenkins PL, Franck KR, Nafziger AN. Incidence of and significant risk factors for aminoglycoside-associated nephrotoxicity in patients dosed by using individualized pharmacokinetic monitoring. J Infect Dis. 1993;167(1):173–9.

Broekroelofs J, Navis GJ, Stegeman CA, van der Bij W, de Boer WJ, de Zeeuw D, de Jong PE. Long-term renal outcome after lung transplantation is predicted by the 1-month postoperative renal function loss. Transplantation. 2000;69(8):1624–8.

Cemlyn-Jones J, Gamboa F. Proteinuria in cystic fibrosis: a possible correlation between genotype and renal phenotype. J Bras Pneumol. 2009;35(7):669–75.

Chen F, Smith R, Gu YZ, Collins ND, Nioi P. Toxicoepigenetic alteration of the kidney injury molecule 1 gene in gentamicin-exposed rat kidney. Toxicol Sci. 2010;117(2):375–80.

Chertow GM, Burdick E, Honour M, Bonventre JV, Bates DW. Acute kidney injury, mortality, length of stay, and costs in hospitalized patients. J Am Soc Nephrol. 2005;16(11):3365–70.

Chiusolo A, Defazio R, Zanetti E, Mongillo M, Mori N, Cristofori P, Trevisan A. Kidney injury molecule-1 expression in rat proximal tubule after treatment with segment-specific nephrotoxicants: a tool for early screening of potential kidney toxicity. Toxicol Pathol. 2010;38(3):338–45.

Christensen EI, Moskaug JO, Vorum H, Jacobsen C, Gundersen TE, Nykjaer A, Blomhoff R, Willnow TE, Moestrup SK. Evidence for an essential role of megalin in transepithelial transport of retinol. J Am Soc Nephrol. 1999;10(4):685–95.

Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron. 1976;16(1):31–41.

Conway SP, Pond MN, Watson A, Etherington C, Robey HL, Goldman MH. Intravenous colistin sulphomethate in acute respiratory exacerbations in adult patients with cystic fibrosis. Thorax. 1997;52(11):987–93.

Crawford I, Maloney PC, Zeitlin PL, Guggino WB, Hyde SC, Turley H, Gatter KC, Harris A, Higgins CF. Immunocytochemical localization of the cystic fibrosis gene product CFTR. Proc Natl Acad Sci U S A. 1991;88(20):9262–6.

Cystic fibrosis foundation patient registry 2012 annual data report. Bethesda; 2013. Maryland

Dai C, Li J, Tang S, Xiao X. Colistin-induced nephrotoxicity in mice involves the mitochondrial, death receptor, and endoplasmic reticulum pathways. Antimicrob Agents Chemother. 2014;58 (7):4075–85.

Downes KJ, Rao MB, Kahill L, Nguyen H, Clancy JP, Goldstein SL. Daily serum creatinine monitoring promotes earlier detection of acute kidney injury in children and adolescents with cystic fibrosis. J Cyst Fibros. 2014;13(4):435–41.

Drew J, Watson AR, Smyth A. Acute renal failure and cystic fibrosis. Arch Dis Child. 2003;88 (7):646.

Etherington C, Bosomworth M, Clifton I, Peckham DG, Conway SP. Measurement of urinary *N*-acetyl-b-D-glucosaminidase in adult patients with cystic fibrosis: before, during and after treatment with intravenous antibiotics. J Cyst Fibros. 2007;6(1):67–73.

Falagas ME, Kasiakou SK. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. Crit Care. 2006;10(1):R27.

Fennell PB, Quante J, Wilson K, Boyle M, Strunk R, Ferkol T. Use of high-dose ibuprofen in a pediatric cystic fibrosis center. J Cyst Fibros. 2007;6(2):153–8.

Ferguson MA, Vaidya VS, Bonventre JV. Biomarkers of nephrotoxic acute kidney injury. Toxicology. 2008;245(3):182–93.

Florescu MC, Lyden E, Murphy PJ, Florescu DF, Fillaus J. Long-term effect of chronic intravenous and inhaled nephrotoxic antibiotic treatment on the renal function of patients with cystic fibrosis. Hemodial Int. 2012;16(3):414–9.

Gaspari F, Cravedi P, Mandala M, Perico N, de Leon FR, Stucchi N, Ferrari S, Labianca R, Remuzzi G, Ruggenenti P. Predicting cisplatin-induced acute kidney injury by urinary neutrophil gelatinase-associated lipocalin excretion: a pilot prospective case-control study. Nephron Clin Pract. 2010;115(2):c154–60.

Glass S, Plant ND, Spencer DA. The effects of intravenous tobramycin on renal tubular function in children with cystic fibrosis. J Cyst Fibros. 2005;4(4):221–5.

Godson C, Ryan MP, O'Halloran D, Bourke S, Brady HR, FitzGerald MX. Investigation of aminoglycoside nephrotoxicity in cystic fibrosis patients. Scand J Gastroenterol Suppl. 1988;143:70–3.

Guy EL, Bosomworth M, Denton M, Conway SP, Brownlee KG, Lee TW. Serum tobramycin levels following delivery of tobramycin (Tobi) via eFlow advanced nebuliser in children with cystic fibrosis. J Cyst Fibros. 2010;9(4):292–5.

Haase M, Devarajan P, Haase-Fielitz A, Bellomo R, Cruz DN, Wagener G, Krawczeski CD, Koyner JL, Murray P, Zappitelli M, Goldstein SL, Makris K, Ronco C, Martensson J, Martling CR, Venge P, Siew E, Ware LB, Ikizler TA, Mertens PR. The outcome of neutrophil gelatinase-associated lipocalin-positive subclinical acute kidney injury: a multicenter pooled analysis of prospective studies. J Am Coll Cardiol. 2011;57(17):1752–61.

Halacova M, Kotaska K, Kukacka J, Vavrova V, Kuzelova M, Ticha J, Prusa R. Serum cystatin C level for better assessment of glomerular filtration rate in cystic fibrosis patients treated by amikacin. J Clin Pharm Ther. 2008;33(4):409–17.

Hirsch R, Dent C, Pfriem H, Allen J, Beekman 3rd RH, Ma Q, Dastrala S, Bennett M, Mitsnefes M, Devarajan P. NGAL is an early predictive biomarker of contrast-induced nephropathy in children. Pediatr Nephrol. 2007;22(12):2089–95.

Hmiel SP, Beck AM, de la Morena MT, Sweet S. Progressive chronic kidney disease after pediatric lung transplantation. Am J Transplant. 2005;5(7):1739–47.

Hoffmann D, Fuchs TC, Henzler T, Matheis KA, Herget T, Dekant W, Hewitt P, Mally A. Evaluation of a urinary kidney biomarker panel in rat models of acute and subchronic nephrotoxicity. Toxicology. 2010;277(1–3):49–58.

Kaseda R, Iino N, Hosojima M, Takeda T, Hosaka K, Kobayashi A, Yamamoto K, Suzuki A, Kasai A, Suzuki Y, Gejyo F, Saito A. Megalin-mediated endocytosis of cystatin C in proximal tubule cells. Biochem Biophys Res Commun. 2007;357(4):1130–4.

Kearns GL, Berry PL, Bocchini Jr JA, Hilman BC, Wilson JT. Renal handling of beta 2-microglobulin in patients with cystic fibrosis. DICP. 1989;23(12):1013–7.

Kennedy SE, Henry RL, Rosenberg AR. Antibiotic-related renal failure and cystic fibrosis. J Paediatr Child Health. 2005;41(7):382–3.

Konrad K, Scheuing N, Badenhoop K, Borkenstein MH, Gohlke B, Schofl C, Seufert J, Thon A, Holl RW. Cystic fibrosis-related diabetes compared with type 1 and type 2 diabetes in adults. Diabetes Metab Res Rev. 2013;29(7):568–75.

Kuwabara T, Mori K, Mukoyama M, Kasahara M, Yokoi H, Saito Y, Yoshioka T, Ogawa Y, Imamaki H, Kusakabe T, Ebihara K, Omata M, Satoh N, Sugawara A, Barasch J, Nakao K. Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. Kidney Int. 2009;75(3):285–94.

Lahiri T, Guillet A, Diehl S, Ferguson M. High-dose ibuprofen is not associated with increased biomarkers of kidney injury in patients with cystic fibrosis. Pediatr Pulmonol. 2014;49 (2):148–53.

Lanese DM, Conger JD. Effects of endothelin receptor antagonist on cyclosporine-induced vasoconstriction in isolated rat renal arterioles. J Clin Invest. 1993;91(5):2144–9.

Ledson MJ, Gallagher MJ, Cowperthwaite C, Convery RP, Walshaw MJ. Four years' experience of intravenous colomycin in an adult cystic fibrosis unit. Eur Respir J. 1998;12(3):592–4.

Lewis JR, Lewis SA. Colistin interactions with the mammalian urothelium. Am J Physiol Cell Physiol. 2004;286(4):C913–22.

Lind-Ayres M, Thomas W, Holme B, Mauer M, Caramori ML, Moran A. Microalbuminuria in patients with cystic fibrosis. Diabetes Care. 2011;34(7):1526–8.

Lopez-Novoa JM, Quiros Y, Vicente L, Morales AI, Lopez-Hernandez FJ. New insights into the mechanism of aminoglycoside nephrotoxicity: an integrative point of view. Kidney Int. 2011;79 (1):33–45.

Master V, Roberts GW, Coulthard KP, Baghurst PA, Martin A, Roberts ME, Onishko CR, Martin AJ, Linke RJ, Holmes M, Jarvinen A, Kennedy D, Colebatch KA, Hansman D, Parsons DW. Efficacy of once-daily tobramycin monotherapy for acute pulmonary exacerbations of cystic fibrosis: a preliminary study. Pediatr Pulmonol. 2001;31(5):367–76.

Menon S, Kirkendall ES, Nguyen H, Goldstein SL. Acute kidney injury associated with high nephrotoxic medication exposure leads to chronic kidney disease after 6 months. J Pediatr. 2014;165(3):522–7.e2.

Moestrup SK, Cui S, Vorum H, Bregengard C, Bjorn SE, Norris K, Gliemann J, Christensen EI. Evidence that epithelial glycoprotein 330/megalin mediates uptake of polybasic drugs. J Clin Invest. 1995;96(3):1404–13.

Moffett BS, Goldstein SL. Acute kidney injury and increasing nephrotoxic-medication exposure in noncritically-ill children. Clin J Am Soc Nephrol. 2011;6(4):856–63.

Moriya T, Tsuchiya A, Okizaki S, Hayashi A, Tanaka K, Shichiri M. Glomerular hyperfiltration and increased glomerular filtration surface are associated with renal function decline in normo- and microalbuminuric type 2 diabetes. Kidney Int. 2012;81(5):486–93.

Mrugacz M, Tobolczyk J, Minarowska A. Retinol binding protein status in relation to ocular surface changes in patients with cystic fibrosis treated with daily vitamin A supplements. Eur J Pediatr. 2005;164(4):202–6.

National Kidney Foundation K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification and stratification. Am J Kidney Dis. 2002;39:S1–S266.

Nazareth D, Walshaw M. A review of renal disease in cystic fibrosis. J Cyst Fibros. 2013;12 (4):309–17.

Parikh CR, Abraham E, Ancukiewicz M, Edelstein CL. Urine IL-18 is an early diagnostic marker for acute kidney injury and predicts mortality in the intensive care unit. J Am Soc Nephrol. 2005;16(10):3046–52.

Perrone RD, Madias NE, Levey AS. Serum creatinine as an index of renal function: new insights into old concepts. Clin Chem. 1992;38(10):1933–53.

Pfaller W, Gstraunthaler G. Nephrotoxicity testing in vitro – what we know and what we need to know. Environ Health Perspect. 1998;106 Suppl 2:559–69.

Prestidge C, Chilvers MA, Davidson AG, Cho E, McMahon V, White CT. Renal function in pediatric cystic fibrosis patients in the first decade of life. Pediatr Nephrol. 2011;26(4):605–12.

Quon BS, Mayer-Hamblett N, Aitken ML, Smyth AR, Goss CH. Risk factors for chronic kidney disease in adults with cystic fibrosis. Am J Respir Crit Care Med. 2011;184(10):1147–52.

Quon BS, Mayer-Hamblett N, Aitken ML, Goss CH. Risk of post-lung transplant renal dysfunction in adults with cystic fibrosis. Chest. 2012;142(1):185–91.

Riethmueller J, Ballmann M, Schroeter TW, Franke P, von Butler R, Claass A, Junge S, Doering G, Stern M. Tobramycin once- vs thrice-daily for elective intravenous antipseudomonal therapy in pediatric cystic fibrosis patients. Infection. 2009;37(5):424–31.

Rougier F, Claude D, Maurin M, Maire P. Aminoglycoside nephrotoxicity. Curr Drug Targets Infect Disord. 2004;4(2):153–62.

Rule AD, Larson TS, Bergstralh EJ, Slezak JM, Jacobsen SJ, Cosio FG. Using serum creatinine to estimate glomerular filtration rate: accuracy in good health and in chronic kidney disease. Ann Intern Med. 2004;141(12):929–37.

Sasaki D, Yamada A, Umeno H, Kurihara H, Nakatsuji S, Fujihira S, Tsubota K, Ono M, Moriguchi A, Watanabe K, Seki J. Comparison of the course of biomarker changes and kidney injury in a rat model of drug-induced acute kidney injury. Biomarkers. 2011;16(7):553–66.

Schmitz C, Hilpert J, Jacobsen C, Boensch C, Christensen EI, Luft FC, Willnow TE. Megalin deficiency offers protection from renal aminoglycoside accumulation. J Biol Chem. 2002;277 (1):618–22.

Servais H, Van Der Smissen P, Thirion G, Van der Essen G, Van Bambeke F, Tulkens PM, Mingeot-Leclercq MP. Gentamicin-induced apoptosis in LLC-PK1 cells: involvement of lysosomes and mitochondria. Toxicol Appl Pharmacol. 2005;206(3):321–33.

Sidhu H, Hoppe B, Hesse A, Tenbrock K, Bromme S, Rietschel E, Peck AB. Absence of *Oxalobacter formigenes* in cystic fibrosis patients: a risk factor for hyperoxaluria. Lancet. 1998;352(9133):1026–9.

Singer E, Elger A, Elitok S, Kettritz R, Nickolas TL, Barasch J, Luft FC, Schmidt-Ott KM. Urinary neutrophil gelatinase-associated lipocalin distinguishes pre-renal from intrinsic renal failure and predicts outcomes. Kidney Int. 2011;80(4):405–14.

Skalova S. The diagnostic role of urinary *N*-acetyl-beta-D-glucosaminidase (NAG) activity in the detection of renal tubular impairment. Acta Medica (Hradec Kralove). 2005;48(2):75–80.

Smyth A, Tan KH, Hyman-Taylor P, Mulheran M, Lewis S, Stableforth D, Prof Knox A. Once versus three-times daily regimens of tobramycin treatment for pulmonary exacerbations of cystic fibrosis – the TOPIC study: a randomised controlled trial. Lancet. 2005;365(9459):573–8.

Smyth A, Lewis S, Bertenshaw C, Choonara I, McGaw J, Watson A. Case-control study of acute renal failure in patients with cystic fibrosis in the UK. Thorax. 2008;63(6):532–5.

Soulsby N, Greville H, Coulthard K, Doecke C. What is the best method for measuring renal function in adults and children with cystic fibrosis? J Cyst Fibros. 2010;9(2):124–9.

Steinkamp G, Lutge M, Wurster U, Schulz-Baldes JG, Grone HJ, Ehrich JH. Renal function in cystic fibrosis: proteinuria and enzymuria before and after tobramycin therapy. Eur J Pediatr. 1986;145(6):526–31.

Terribile M, Capuano M, Cangiano G, Carnovale V, Ferrara P, Petrarulo M, Marangella M. Factors increasing the risk for stone formation in adult patients with cystic fibrosis. Nephrol Dial Transplant. 2006;21(7):1870–5.

Tidman M, Sjostrom P, Jones I. A Comparison of GFR estimating formulae based upon s-cystatin C and s-creatinine and a combination of the two. Nephrol Dial Transplant. 2008;23(1):154–60.

Tod MM, Padoin C, Petitjean O. Individualising aminoglycoside dosage regimens after therapeutic drug monitoring: simple or complex pharmacokinetic methods? Clin Pharmacokinet. 2001;40 (11):803–14.

Torregrosa I, Montoliu C, Urios A, Andres-Costa MJ, Gimenez-Garzo C, Juan I, Puchades MJ, Blasco ML, Carratala A, Sanjuan R, Miguel A. Urinary KIM-1, NGAL and L-FABP for the diagnosis of AKI in patients with acute coronary syndrome or heart failure undergoing coronary angiography. Heart Vessels. 2015 Nov;30(6):703–11.

Town DJ, Vinks AA, Jacobs F, Heijerman HG, Bakker W. Creatinine clearance as predictor of tobramycin elimination in adult patients with cystic fibrosis. Ther Drug Monit. 1996;18 (5):562–9.

Tu Y, Wang H, Sun R, Ni Y, Ma L, Xv F, Hu X, Jiang L, Wu A, Chen X, Chen M, Liu J, Han F. Urinary netrin-1 and KIM-1 as early biomarkers for septic acute kidney injury. Ren Fail. 2014 Nov;36(10):1559–63.

Vaidya VS, Ferguson MA, Bonventre JV. Biomarkers of acute kidney injury. Annu Rev Pharmacol Toxicol. 2008;48:463–93.

Vic P, Ategbo S, Turck D, Husson MO, Launay V, Loeuille GA, Sardet A, Deschildre A, Druon D, Arrouet-Lagande C. Efficacy, tolerance, and pharmacokinetics of once daily tobramycin for pseudomonas exacerbations in cystic fibrosis. Arch Dis Child. 1998;78(6):536–9.

Wehbe E, Brock R, Budev M, Xu M, Demirjian S, Schreiber MJ Jr, Stephany B. Short-term and long-term outcomes of acute kidney injury after lung transplantation, J Heart Lung Transplant. 2011. Epub ahead of print.

Wehbe E, Brock R, Budev M, Xu M, Demirjian S, Schreiber MJ, Stephany B. Short-term and long-term outcomes of acute kidney injury after lung transplantation. J Heart Lung Transplant. 2012;31(3):244–51.

Weir MR. Renal effects of nonselective NSAIDs and coxibs. Cleve Clin J Med. 2002;69 Suppl 1: Si53–8.

Zhou Y, Vaidya VS, Brown RP, Zhang J, Rosenzweig BA, Thompson KL, Miller TJ, Bonventre JV, Goering PL. Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in urine and kidney following acute exposure to gentamicin, mercury, and chromium. Toxicol Sci. 2008;101(1):159–70.

Zhou X, Ma B, Lin Z, Qu Z, Huo Y, Wang J, Li B. Evaluation of the usefulness of novel biomarkers for drug-induced acute kidney injury in beagle dogs. Toxicol Appl Pharmacol. 2014;280 (1):30–5.

Zughaier SM, Tangpricha V, Leong T, Stecenko AA, McCarty NA. Peripheral monocytes derived from patients with cystic fibrosis and healthy donors secrete NGAL in response to Pseudomonas aeruginosa infection. J Investig Med. 2013;61(6):1018–25.

Biomarkers in IgA Nephropathy

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Mohsen Nafar and Shiva Samavat

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Abstract

IgA nephropathy (IgAN) is the most common glomerulonephritis worldwide, diagnosis of which is dependent on kidney biopsy with its invasive nature. Besides diagnosis, histologic lesions are predictors of prognosis and response to therapy. In addition some clinical and laboratory factors predict the risk of progression to end-stage renal disease, such as hypertension, baseline serum creatinine, and time-averaged proteinuria. Biomarkers are measurable substances that are indicators of specific physiologic or pathologic process. Serum and urine are easily accessible and perfect biofluids. This review is focused on recently discovered biomarkers with diagnostic, prognostic, histologic, and response prediction capacities. Yet none of the described biomarkers could be used instead of biopsy. A long road is ahead to reach the optimal biomarker profile with bedside utility.

Keywords

IgA nephropathy • Diagnostic biomarkers • Prognostic biomarkers • Response to treatment • Proteomics • Metabolomics

Abbreviations

2D PAGE	Two-dimensional polyacrylamide gel electrophoresis
2DE	Two-dimensional gel electrophoresis
ACEi	Angiotensin-converting enzyme inhibitor
AOPPs	Advanced oxidation protein products
ARB	Angiotensin receptor blocker
AUC	Area under curve
BAFFs	B-cell-activating factors
BMP-1	Bone morphogenetic protein-1
CKD	Chronic kidney disease
EGF	Epidermal growth factor
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-stage renal disease
FE IgG	Fractional excretion of IgG
FOXP3	Forkhead box P3
Gd-IgA1	Galactose-deficient IgA1
GS	Glomerular sclerosis
IF/TA	Interstitial fibrosis and tubular atrophy

IgAN	IgA nephropathy
IL	Interleukin
IL-2R	IL-2 receptor
INF-γ	Interferon-γ
KDIGO	Kidney Disease: Improving Global Outcomes
KIM-1	Kidney injury molecule-1
LC-MS/MS	Liquid chromatography/tandem mass spectrometry
LG3	Laminin G-like 3
MAC	Membrane attack complex
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MBL	Mannose-binding lectin
MCP-1	Monocyte chemoattractant protein-1
miRNAs	MicroRNAs
mRNA	Messenger RNA
MVBs	Multivesicular bodies
Nano-HPLC-ESI-MS/MS	Nano-high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry
NFκB	Nuclear factor κB
NMR	Nuclear magnetic resonance
PDGF	Platelet-derived growth factor
RANTES	Regulated upon activation normal T cell expressed and secreted
RAS	Renin–angiotensin system
SELDI	Surface-enhanced laser desorption/ionization
sIgA	Secretory IgA
STAT-3	Signal transducer and activator of transcription 3
sTfR	Soluble transferrin receptor
sVCAM-1	Soluble vascular cell adhesion molecule-1
TAL	Thick ascending limb
TBMN	Thin basement membrane nephropathy
TECs	Tubular epithelial cells
TGF-β	Transforming growth factor-β
TLR-9	Toll-like receptor-9
TMAO	Trimethylamine N-oxide
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cell

Key Facts

Key Facts of IgA Nephropathy

- IgA nephropathy (IgAN) is glomerulopathy diagnosed by dominant or codominant deposition of IgA antibodies in mesangial areas.

- Jean Berger first described IgAN in 1968.
- The fundamental step is aberrantly galactosylated IgA1 (Gd-IgA1).
- Antibody formation against Gd-IgA1 leads to immune complex formation and mesangial deposition of these complexes.
- Genetic factors are involved in the pathogenesis of IgAN.
- The mesangial deposition results in macrophage infiltration and tubulointerstitial inflammation, podocyte activation, and further tubular damage.
- The choice of treatment is dictated by the risk of progression. The higher is the risk of progression, the more aggressive is the immunosuppression.

Key Facts of Uromodulin

- Uromodulin precursor contains 640 amino acids.
- The precursor is processed and becomes mature in thick ascending limb (TAL) of Henle's loop and polymerized upon secretion.
- Uromodulin regulates water and electrolyte transport in TAL. It increases the expression of apical potassium channel.
- It has a role in prevention of urinary tract infection.
- It plays a role in innate immunity and acts as a chemoattractant.
- Mutations in uromodulin gene lead to decreased urinary level of uromodulin and result in interstitial inflammation and fibrosis.

Key Facts of Immunosuppression

- Treatment approach in IgAN depends on the risk of progression.
- The risk of progression is estimated based on the amount of proteinuria, renal function, and the presence of hypertension on presentation.
- Those at intermediate or high risk of progression are suitable for immunosuppressive therapy.
- Those with proliferative disease in the kidney biopsy might have better response to immunosuppressive drugs.
- Corticosteroids, mycophenolate mofetil (MMF), cyclophosphamide, and azathioprine are all studied in IgAN.

Definitions

Immunonephelometric assay Immunonephelometric assay is the technique to measure plasma protein level based on the amount of turbidity and scattering of light caused by small particles in the sample.

ELISA The enzyme-linked immunosorbent assay is based on antigen and antibody interaction and enzyme-induced color changes in substrate. Antigens are attached into wells in a plate. Then an antibody that can bind to the antigen and

is linked to an enzyme is added. The next step is the addition of substrate. The reaction causes color change in the substrate, and the intensity of the color signal is indicative of the amount of antigen present.

MicroRNAs MicroRNAs (miRNAs) are 21–23-nucleotide noncoding RNAs that regulate posttranscriptional gene expression by binding to target mRNA leading to either the degradation of mRNA or inhibition of their transcription.

Metabolomics Metabolomics comprehensively identify the metabolites in a biofluid and help to understand the cellular pathways and therefore the pathogenesis of diseases. Metabolome is a collection of metabolites a physiologic process had left behind. Each tissue has its specific metabolome, but analyzing urine or plasma metabolomes results in more general data, which is not tissue specific.

Proteomics Proteomics is the analysis of the whole protein content of a biofluid. The changes in the proteomes are caused by differences in synthesis or modifications during the course of biologic or pathologic processes. These modifications can be used as specific markers of the process.

Oxidative stress Oxidative stress is an imbalance between pro-oxidant processes and antioxidant defense mechanisms.

Introduction

IgA nephropathy (IgAN) is known as the most common glomerulonephritis in the world. It is characterized by predominant or codominant mesangial deposition of IgA. Light microscopic findings vary from near-normal to crescentic glomerulonephritis. The spectrum of clinical findings is as wide as histologic ones: from asymptomatic hematuria to rapidly progressive glomerulonephritis. This made IgAN a diagnosis based on pathology.

The exact pathogenesis of IgAN is not still completely clear, but abnormally glycosylated polymeric IgA_1 (Gd-IgA_1) and autoantibodies against it are present in the mesangial deposits and the circulation. The origin of this aberrantly glycosylated IgA_1 might be the production of mucosal IgA in the bone marrow (mis-homing). Microbial pathogens activate B cells by binding to TLR-9 and induce class switching and antibody production. The immune complexes containing Gd-IgA_1 then bind to transferrin receptor (CD71) on mesangial cells and lead to in situ cytokine production and complement activation and renal injury.

Once known as a benign glomerulonephritis, IgAN is now one of the causes of end-stage renal disease (ESRD). Approximately 20–40 % of IgAN patients will reach ESRD within 20 years after diagnosis. Thus, timely and noninvasive diagnosis, better understanding of pathogenesis and predictors of clinical course, and response to therapy may help to change patient's destiny (Boyd et al. 2012; Wyatt and Julian 2013; Kim et al. 2012).

Diagnostic Biomarkers

The diagnosis of IgAN is based on kidney biopsy, an invasive and inconvenient procedure with minor risk of bleeding, need for transfusion, and even nephrectomy. In order to develop a noninvasive way, various serum and urine biomarkers were assessed.

Urine Biomarkers

Urine is an accessible and noninvasive biofluid, and its composition reflects the pathogenetic changes in the kidney. Besides filtered proteins, urine contains various cytokines and "fingerprints" of immunologic processes and complements system activation. Although there is no single well-documented urinary marker with approved clinical use, several candidate markers have been studied.

Uromodulin

First identified in the early 1950s, uromodulin is a glycoprotein that is exclusively found in the thick ascending limb (TAL) of Henle's loop. It is synthesized in the form of 640 amino acid precursor. Uromodulin maturation occurs in the endoplasmic reticulum by glycosylphosphatidylinositol anchoring and N-glycosylation. After modification in Golgi, it undergoes some proteolytic modifications, which makes it ready for polymerization and urinary excretion. It has different biologic functions, including protection against urinary tract infection, prevention of renal stone formation, and is involved in innate immunity. It binds to IgG, C_1q, and TNF-α and, on the other hand, has the capability to activate monocytes and neutrophils (Rampoldi et al. 2011).

In an effort to identify a diagnostic biomarker for IgAN, proteomic techniques based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were utilized on urine samples of 32 IgAN patients, and a uromodulin fragment, urinary peptide with *m/z* 1913.14, was identified. The decreased intensity of this peptide was reported to be diagnostic. It was also demonstrated that urine peptide pattern can discriminate IgAN and healthy controls with 100 % sensitivity and specificity. The reported sensitivity and specificity for this peptide pattern to distinguish IgAN from other glomerulopathies were 85.7 % and 76.5 %, respectively (Wu et al. 2010). Following this study, Obara et al. (2012) evaluated urinary IgA–uromodulin complex as a diagnostic marker for IgAN. The ELISA for IgA–uromodulin complex was applied on urine samples of IgAN, disease controls, and healthy controls. They calculated the cutoff of 0.705 from the ROC curve, which was able to distinguish healthy controls from those with kidney disease (including IgAN) with 90.5 % sensitivity, 90.0 % specificity, and 90.4 % diagnosis efficiency. With the cutoff of 2.45, IgA–uromodulin complex discriminated IgAN from other kidney diseases (sensitivity of 92.9 % and specificity of 57.4 %). The results indicated that IgA–uromodulin complex could differentiate active but not inactive form of IgAN

from other renal diseases with hematuria or proteinuria except lupus nephritis. These data suggested IgA–uromodulin complex as a marker of active IgAN.

Soluble Transferrin Receptor (sTfR) or CD71

Aberrantly glycosylated IgA_1 immune complexes interact with cell surface IgA_1 receptor, TfR or CD71. An increased mesangial expression of CD71 was reported in IgAN. Binding of IgA immune complex leads to mesangial proliferation, proinflammatory cytokine production (IL-1, IL-6, and TNF-α), and complement activation (Monteiro 2005).

sTfR (molecular mass 84,910 Da) lacks the first 100 amino acids of TfR and is upregulated in IgAN, which is proposed to be eliminated in urine. In order to assess the urinary sTfR as a diagnostic marker in IgAN, fixed-time immunonephelometry assay was utilized on urine samples of 71 patients with biopsy-proven IgAN. Urinary sTfR/creatinine ratio was significantly higher in patients with active IgAN in comparison to those with inactive disease. Although urinary sTfR was also detected in other morphological types of glomerulopathy, the level was much lower (>5 μg/L vs. 2 μg/L) (Delanghe et al. 2013). Thus according to this study, urinary sTfR could be used as a diagnostic marker for active IgAN.

Interleukin 6 (IL-6)

Cytokines play a main role in clinical and pathological manifestations of IgAN. Produced by activated monocytes and mesangial cells, IL-6 leads to mesangial cell proliferation and has a role in fibrogenic process. On the other hand, IL-6 is a plasma cell differentiating factor and by binding to its gp130 receptor and activation of STAT-3 pathway stimulates Gd-IgA1 production (Reily et al. 2014). Thus, urinary IL-6 (uIL-6) can serve as a potential diagnostic biomarker in IgAN. Elevated levels of uIL-6 were shown in IgAN patients compared with healthy controls at the time of biopsy (Stangou et al. 2009). Of course, in an earlier study, this upregulated uIL-6 also has been shown in IgAN patients but it was not disease specific (Hrvacevic et al. 1996).

Furthermore, uIL-6 and its ratio to epidermal growth factor (EGF) were reported to be related to disease progression in IgAN (Harada et al. 2002; Ranieri et al. 1996). Recently, in a study in children with asymptomatic hematuria, authors demonstrated higher uIL-6 activity with IgAN. Despite sample size limitations, they suggested uIL-6 as a screening tool in children with hematuria and as a guide to perform kidney biopsy (Kanemoto et al. 2014).

Complement Components

Histologic evaluations of IgAN reveal the footprint of complement activation. There are evidences of C_3, properdin, and mannose-binding lectin (MBL) within mesangial deposits, which show alternative and lectin pathway activation. Therefore, complement proteins might be markers of diagnosis and prognosis. In a study, urinary level of properdin and membrane attack complex (MAC) was significantly higher in IgAN patients than healthy controls, but due to lack of

disease controls, this could not be supposed as disease-specific marker. Moreover, there was an incremental pattern in urine MAC and factor H with increasing disease activity, which makes them prognostic rather than diagnostic markers (Onda et al. 2011). In a study on urine samples of 162 IgAN patients, urine MBL levels were significantly higher than the healthy controls (Liu et al. 2012). These studies point to the fact that aside from the pathogenetic role of complement system, the markers of complement system activity could guide diagnosis and follow-up of disease activity.

Laminin G-Like 3 (LG3)

Laminin G-like 3 (LG3) is a product of perlecan cleavage. Perlecan is a proteoglycan and major component of extracellular matrix and basement membranes. Proteoglycans play a main role in charge selectivity of filtration barrier. Data suggested that perlecan gene expression is negatively correlated with albuminuria (Ebefors et al. 2011). Cleavage of perlecan by bone morphogenetic protein-1 (BMP-1)/Tolloid-like proteases results in LG3 fragment formation which has anti-angiogenic activity. Urinary excretion of LG3 fragment has also been reported to be significantly reduced in IgAN patients. Evaluation of urine proteome pattern by MALDI-TOF-MS/MS and Western blotting showed lower excretion of LG3 in IgAN patients in comparison to healthy controls and patients with chronic kidney disease (Rocchetti et al. 2013). Moreover, two studies suggested LG3 urinary level as a possible marker of severity both clinical and histological. They reported an inverse correlation between renal function and urine LG3 (Rocchetti et al. 2013; Surin et al. 2013).

Exosomes and Vesicles

Multivesicular bodies (MVBs) contain cellular and cytoplasmic compartments and exosomes. MVBs fuse with the membrane, and exosomes are secreted to the urinary space. Therefore, exosomes are reflection of biological processes in the kidney and are not affected by filtered proteins or uromodulin (Santucci et al. 2013).

Moon et al. (2011) reported 4 exosomal proteins out of 1,877 urinary exosome proteins, analyzed by LC-MS/MS, to be of diagnostic value in early IgAN. These proteins were aminopeptidase N, vasorin precursor, α-1-antitrypsin, and ceruloplasmin and were able to differentiate IgAN from thin basement membrane nephropathy (TBMN) and healthy controls. Urinary excretion of aminopeptidase N and vasorin precursor was higher in TMBN patients and controls than in those with IgAN. On the contrary, the amount of α-1-antitrypsin and ceruloplasmin was higher in the IgAN group than in normal group and TBMN. Increased amount of α-1-antitrypsin might be due to hematuria. Ceruloplasmin level probably increased due to inflammation as an acute phase reactant. Assessing the area under curve of these markers, ceruloplasmin was the best marker to discriminate IgAN from TBMN.

MicroRNAs

MicroRNAs (miRNAs) are 21–23-nucleotide noncoding RNAs that regulate post-transcriptional gene expression by binding to target mRNA leading to either the degradation of mRNA or inhibition of their transcription. Recent data suggested that changes in parenchymal, serum, or urine miRNAs be responsible in pathogenesis of diseases including IgAN. miRNAs in biofluids such as urine and serum could be used for better understanding of pathogenesis and diagnosis and predicting the clinical course of IgAN (Szeto and Li 2014).

In urine miRNAs can be detected by conventional real-time quantitative PCR. Increased urinary expression of miRNA-146a and miRNA-155 has shown in IgAN patients compared with healthy subjects. These miRNAs have immunoregulatory capacity. miR-146a level was inversely related to proinflammatory cytokine (IL-1β, IL-6, and TNF-α) levels and directly related to RANTES. Urinary level of miR-155 was also related with suppression of IL-1β and TNF. miR-155 was positively correlated with FOXP3, and this finding suggested a role for Tregs in development of IgAN (Wang et al. 2011). In another study the same group demonstrated a significantly lower urinary miR-29b and miR-29c, but higher miR-93 levels in IgAN patients than controls. This miRNA profile is mostly related to TGF-β signaling pathway, renal fibrosis, and glomerular scarring (Wang et al. 2012). Comparing IgAN with diabetic glomerulosclerosis and hypertensive nephrosclerosis, upregulated miR-17 in IgAN could be a potential marker for diagnosis (Szeto et al. 2012). Large population studies and better technical approaches are needed for assessment of this miRNA function and introduction of diagnostic as well as prognostic markers of disease.

Urine Metabolomics

Metabolomics comprehensively identify the metabolites in a biofluid and help to understand the cellular pathways and therefore the pathogenesis of diseases. These metabolites could also be used as diagnostic and prognostic biomarkers (Zhao and Lin 2014).

Nuclear magnetic resonance (NMR) spectroscopy is one of the proper techniques to study these metabolic fingerprints. Compared with healthy subjects, urine of IgAN had elevated levels of creatinine, trimethylamine N-oxide (TMAO), acetate, and betaine and lower levels of hippurate, citrate, and lactate. Del Coco et al. showed a connection between betaine and TNF-α and suggested increased clearance of betaine and therefore lower plasma betaine as a pathogenic event that led to increased TNF-α levels (Del Coco et al. 2012).

Urinary Proteome

In 2005, Park et al. reported a urinary protein map of IgAN by the means of 2D gel electrophoresis (2DE) for the first time. They described under-excretion of 35 proteins in IgAN in comparison to healthy controls and suggested that they might be involved in the pathogenesis of the disorder (Park et al. 2006). Furthermore,

Table 1 Differentially expressed urine proteins in IgAN and healthy subjects

Protein ID	Protein name	Biological process	Fold change
GP2	Pancreatic secretory granule membrane major glycoprotein	Antigen transcytosis by M cells in mucosa-associated lymphoid tissue	−3.7
VASN	Vasorin	Inhibitor of TGF-β signaling	−3.7
EGF	Epidermal growth factor	Innate immune response/positive regulation of cell proliferation	−3.1
CLM9	CMRF35-like molecule 9	Immunity	−2.9
PCDH1	Protocadherin-1	Cell–cell signaling	+8.9
UTER	Uteroglobin	Regulation of inflammatory response	+3.5
DPP4	Dipeptidyl peptidase-4	Positive regulation of cell proliferation/T-cell activation	+3.5
SLAF5	SLAM family member 5/CD84	Blood coagulation/leukocyte migration	+2.7
NHLC3	NHL repeat-containing protein 3	It may be involved in a variety of enzymatic processes, including protein modification through ubiquitination	+1.9

Differentially expressed proteins in urine samples of 13 patients with IgAN in comparison to eight healthy subjects. The biologic processes in which these proteins are involved and the pattern of changes are demonstrated

utilizing 2DE, absence of increased alpha-1-microglobulin in urine samples of IgAN patients is reported as a marker of disease (Yokota et al. 2007).

Recently, in an attempt to identify a diagnostic urinary proteome profile, urine samples of IgAN patients were analyzed by the means of liquid chromatography/tandem mass spectrometry (nLC-MS/MS). Differentially expressed proteins were evaluated, and a diagnostic panel was suggested, which was decreased urinary expression of GP2 (pancreatic secretory granule membrane major glycoprotein), vasorin, EGF, and CLM9 (CMRF35-like molecule 9) and increased urinary expression of protocadherin, uteroglobin, DDPIV (dipeptidyl peptidase-4), NHLC3 (NHL repeat-containing protein 3), and SLAF5 (SLAM family member 5/CD84) (Samavat et al. 2014). These proteins and their proposed biologic role are demonstrated in Table 1.

Serum Biomarkers

Galactose-Deficient IgA1 (Gd-IgA1) and Gd-IgA1-Containing Immune Complexes

Aberrantly glycosylated IgA1 is the key point in the pathogenesis of IgAN. The Gd-IgA1 acts as a new antigen and stimulates autoantibody production and immune complex formation, mostly Gd-IgA1-specific IgG and IgA. In a study on large cohort of IgAN, CKD controls, and healthy volunteers, the prevalence of elevated serum levels of IgA, Gd-IgA1, and glycan-specific IgG and IgA was evaluated by

the means of ELISA. Among these markers, Gd-IgA1-specific IgG had the best performance in diagnosis of IgAN with 89 % sensitivity and 92 % specificity. The marker discriminated IgAN from healthy controls and those with nonimmune kidney diseases (Yanagawa et al. 2014).

Although the elevated serum levels of galactose-deficient IgA1 alone are insufficient for diagnosis, it could be used as a predicting factor for progression of IgAN (Zhao et al. 2012).

Serum BAFF

B cells play a central role in the pathogenesis of IgAN. Pathogen-associated molecular patterns bind to TLR-9 and activate B cells and induce class switching and mucosal IgA production (Boyd et al. 2012). B-cell-activating factor (BAFF) is a cytokine that belongs to the tumor necrosis factor (TNF) ligand family and participates in class switching (Mecklenbrauker et al. 2004).

The role of BAFF and TLR-9 was evaluated in IgAN patients, and serum levels of BAFF and TLR-9 mRNA were elevated in IgAN patients compared with healthy controls and patients with mild glomerular abnormalities. Serum IgA1 level, IgA deposition in mesangium, and TLR-9 protein expression were positively correlated with serum BAFF levels (Li et al. 2014). In another study, Xin et al. also reported a twofold elevation in serum BAFF level in IgAN compared with the healthy controls and demonstrated a correlation between serum BAFF and renal function tests (eGFR). Elevated serum BAFF level (>1.47 ng/ml) was mostly correlated with mesangial hypercellularity, segmental glomerulosclerosis, and interstitial fibrosis and tubular atrophy (IF/TA) (Xi et al. 2013).

Metabolomics Studies

Nuclear magnetic resonance (NMR) spectroscopy is an analytic technique to study the metabolome. In a study, serum samples of 35 IgAN patients and 23 healthy controls were evaluated by NMR, and multivariate pattern recognition analysis was performed. Compared with healthy controls, higher levels of lactate, myo-inositol, phenylalanine, and L6, L5, and L3 lipids were detected in serum of IgAN patients. Lower serum levels of β-glucose, α-glucose, valine, tyrosine, phosphocholine, lysine, isoleucine, glycerophosphocholine, glycine, glutamine, glutamate, alanine, acetate, 3-hydroxybutyrate, and 1-methylhistidine were reported in IgAN patients. Further analysis showed high sensitivity and specificity for diagnosis of IgAN (Sui et al. 2012).

Despite the recent developments, achieving a diagnostic profile is a long road to hit.

Prognostic Biomarkers

IgAN is a common glomerular disease with a significant risk to progress toward ESRD. Various clinical and pathologic features have been described as prognostic factors. Male gender, hypertension, elevated serum creatinine at diagnosis, and

Table 2 Clinical and histologic prognostic factors in IgAN

Risk factor	Reference
Male gender	Nachman et al. 2012
Older age	Nachman et al. 2012
Obesity	Floege and Feehally 2013
Smoking	Floege and Feehally 2013
Sustained hypertension (>140/90)[a]	Floege and Feehally 2013
Persistent hematuria	Nachman et al. 2012
Persistent proteinuria (proteinuria of >1 g per day)[a]	Nachman et al. 2012
Impaired renal function at diagnosis[a]	Moriyama et al. 2014
Tubular atrophy/interstitial fibrosis	Lee et al. 2014
Higher uric acid	Moriyama et al. 2014

[a]The KDIGO guideline for glomerulonephritis currently divides patients according to their risk profile based on proteinuria, blood pressure, and eGFR at the time of diagnosis and during follow-up: low risk (normal eGFR, no hypertension, urine protein <0.5 g/day), intermediate risk (proteinuria >0.5–1 g/day ± reduced eGFR ± hypertension), high risk (rapid loss of eGFR)

persistent proteinuria are most studied clinical characteristics, and glomerular sclerosis, tubular atrophy and interstitial fibrosis, endocapillary hypercellularity, and crescent formation are pathologic features predicting progression (Peters et al. 2011).

These prognostic factors do not have high sensitivity and specificity, and there is a need for more accurate, less invasive, and reproducible ones. The clinical and histologic prognostic factors in IgAN are listed in Table 2.

Urine Kidney Injury Molecule-1 (KIM-1)

KIM-1 is a glycoprotein with immunoglobulin and mucin domain that is expressed on injured proximal tubule cells. The metaloproteinases cleave the ectodomain of shed KIM-1. The ectodomain (90 kDa) is proposed to have a proinflammatory role and cause macrophage recruitment and inflammation following tubular injury. Thus, it is involved in both regenerative and fibrosing processes (Peters et al. 2011; Xu et al. 2011). Urinary KIM-1 level has been evaluated as a predictor of renal outcome in several studies.

Urinary KIM-1 has been shown to be an independent predictor of ESRD along with serum creatinine in IgAN patients with an AUC of 0.86 %. It kept its predictive value even in the subgroup of patients with serum creatinine less than 1.5 mg/dl (Peters et al. 2011).

Of note, the relation between pathologic findings and urinary KIM-1 was diverse. Urinary KIM-1 excretion was not correlated with tubulointerstitial score (Peters et al. 2011). In a study by Xu et al., urinary KIM-1 was correlated with pathologic findings of mesangial proliferation, crescent formation, glomerular sclerosis, and

interstitial fibrosis, but after applying multivariate analysis, no significant correlation was found with any of these pathologic parameters. They also demonstrated a worse renal outcome in a group of patients with urinary KIM-1 higher than 4.17 ng/mg urinary creatinine after more than 12 months of follow-up (Xu et al. 2011).

In a more recent study, increased urinary KIM-1 was reported in IgAN patients compared with healthy subjects, which was not correlated with estimated GFR. In the contrary to the previous studies, urinary KIM-1 was not correlated with initial serum creatinine, which might be due to milder disease. They reported urinary KIM-1 level as a marker of tubulointerstitial injury even in patients with mild renal dysfunction and serum creatinine less 2 mg/dl (Lee et al. 2014).

It seems that urinary KIM-1 level could be used as a marker of renal survival if a large study with a long-term follow-up is conducted.

Fractional Excretion of IgG (FE IgG)

Persistent proteinuria is one of the predictors of outcome in IgAN (Glassock 2008). Data have shown that proteins with high molecular weight (>100 kDa) were associated with decline in kidney function (Mackinnon et al. 2003). IgG is one of the high molecular weight proteins, and its fractional excretion has been reported to be related with progression of renal failure. In a study including 34 IgAN patients, FE IgG with AUC of 0.96 predicted the outcome of reaching stage 5 of CKD. FE IgG with a cutoff of 0.029 had 88.9 % sensitivity and 88 % specificity in predicting the primary outcome of decline in GFR. In the whole cohort, patients with higher FE IgG had 37.1-fold higher risk of disease progression (McQuarrie et al. 2011).

Claudio Bazzi et al. analyzed the relationship of FE IgG with histologic findings, renal outcome, and response to therapy in 37 patients with crescentic IgAN. Apart from FE IgG, the ratio of FE IgG to percentage of glomerular sclerosis (FE IgG/GS) was also evaluated as a predicting factor of progression. FE IgG/GS had 91 % sensitivity and 92 % specificity in predicting progression with an AUC of 0.901, and FE IgG/GS was 16-fold higher in progressors. In a group treated with cyclophosphamide and steroids, FE IgG/GS in combination with serum creatinine of more than 1.74 mg/dl had the highest value in prediction of response to therapy. They reported no response to therapy in 89 % of those with FE IgG/GS of more than 0.0034 (Bazzi et al. 2009a).

Soluble CD89 (sCD89)

CD89 (FcαRI) is an IgA-specific Fc-binding receptor, which is expressed on macrophages, neutrophils, eosinophils, dendritic cells, and Kupffer cells, but not mesangial cells. Binding of IgA to membrane-bound receptor leads to phagocytosis

and inflammatory cytokine release. The soluble form of CD89 (sCD89) might have a role in IgA immune complex formation in IgAN. sCD89 30 kDa isoform makes sCD89–polymeric IgA (sCD89–pIgA) complexes both in healthy subjects and those with glomerular disorders (IgAN or non-IgAN causes) (Boyd and Barratt 2010). Vuong et al. reported decreased serum levels of sCD89–pIgA in IgAN patients with progressive disease (doubling of serum creatinine during at least 1-year follow-up or reaching stage 5 of chronic kidney disease). No correlation has been observed between sCD89–pIgA and disease progression in non-IgAN glomerular disorders. Two different explanations for this finding could be suggested, one is the possibility of deposition of complexes in mesangium and the other is the protective role of shed sCD89–pIgA complexes which prevents anti-IgA1 autoantibodies binding to undergalactosylated IgA1 (Vuong et al. 2010).

Urinary Angiotensinogen

The renin–angiotensin system (RAS) has been long recognized to have pathophysiologic role in renal diseases. Apart from the systemic angiotensin II, intrarenal RAS activation has been proposed to be involved in pathogenesis of glomerular injury and progression toward end-stage renal disease (Kobori et al. 2007). Urinary angiotensinogen has been demonstrated as a reflection of intrarenal RAS activation in several studies (Xu et al. 2014; Kobori et al. 2002). In order to assess the intrarenal RAS activity in IgAN patients, urinary angiotensinogen (U_{AGT}) level in 52 IgAN subjects was evaluated and compared with that of healthy controls and patients with minimal glomerular abnormality (MGA). U_{AGT}/U_{cr} was not significantly different between healthy controls and patients with MGA. But U_{AGT}/U_{cr} was significantly higher in the IgAN group. Data showed a significant increase in tissue expression of angiotensinogen mRNA and angiotensin II by immunostaining (Nishiyama et al. 2011). In a study, U_{AGT} level was correlated with urine protein-to-creatinine ratio and serum creatinine. IgAN patients with U_{AGT} >100 ng/mgCr had higher serum creatinine and worse renal function after therapy than those with U_{AGT} <100 ng/mgCr. These data suggested U_{AGT} as a predictor of outcome in IgAN (Kim et al. 2011).

Complement Components

Complement system activation, both alternative and lectin pathway, has been shown in IgAN by demonstrating mesangial deposition of complement components such as mannose-binding lectin (MBL), C3, C4d, and C5b-9 (Wada and Nangaku 2013). Increased serum levels of C3a (footprint of alternative and lectin pathway activation) and C4a (footprint of lectin pathway activation) have been reported in IgAN (Abou-Ragheb et al. 1992).

In a study on 162 IgAN patients, urinary MBL level was reported higher in IgAN than healthy controls. The patients were divided into three groups based on their Lee's pathologic class, with Lee-I or Lee-II in the first group, Lee-III in the second, and Lee-IV or Lee-V in the third group. Patients in the third group had the highest urinary MBL level. Urinary MBL was in positive correlation with serum creatinine and the degree of proteinuria and severity of pathologic changes (mesangial hypercellularity, endocapillary proliferation, tubular atrophy/interstitial fibrosis, glomerular sclerosis) (Liu et al. 2012).

In a study aimed to explore serum biomarkers in IgAN, serum proteome was analyzed by SELDI system. The pathologic glomerular patterns were scored. Compared to the healthy controls, 93 proteins were differently expressed in IgAN. Among these proteins, the one with protein signal at 8,592 m/z was recognized to be C4a desArg by Western blotting. C4a desArg level evaluated by ELISA was positively correlated with mesangial hypercellularity that acts as a prognostic factor. Thus, C4a desArg could be used as potential biomarker associated with severe glomerular injury (Sogabe et al. 2013).

Studies have shown elevated serum IgA and/or C3 to differentiate IgAN with verity of histologic severity. In addition, Zhang et al. found that the higher serum IgA and the lower serum C3 level are (thus, increased IgA/C3 ratio) in IgAN patients, the more is the chance of progression of kidney injury. IgA/C3 ratio at a cutoff of 3.32 had a sensitivity coefficient of 71.43 % and a specificity coefficient of 68.88 %. Those with serum IgA/C3 $\geq$ 3.32 had lower GFR after mean follow-up duration of 36 months and have reached the primary outcome of 50 % decline in GFR or the need for renal replacement therapy (Zhang et al. 2013).

Inflammatory Cytokines

The pathogenetic role of T helper2 (Th2) has been described in IgAN. Th2 cells upon activation produce IL-2 and express IL-2 receptor (IL-2R) and can induce B-cell class switching and IgA production. The aberrantly galactosylated IgA1 complexes deposit in the mesangium and stimulate mesangial cells. Activated mesangial cells secrete proinflammatory cytokines such as TNF-α, IL-1, IL-6, MCP-1, and PDGF. These cytokines activate tubular epithelial cells (TECs). Activated TECs in turn produce IL-18, which modulates macrophage activity through NFκB pathway. The abovementioned mediators could be used as a prognostic marker since interstitial inflammation and fibrosis are key prognostic indicators in IgAN (Lundberg et al. 2012; Stangou et al. 2013; Torres et al. 2008; Shi et al. 2012).

IL-2 and soluble IL-2R (sIL-2R) have been long shown to be elevated in IgAN (Schena et al. 1989; Parera et al. 1992). In a cross-sectional study on 194 patients with IgAN, sIL-2R was analyzed by Luminex system. sIL-2R level of more than 153.1 pg/ml was associated with progression to either CKD stage 5 or a 50 % decline of eGFR during follow-up or a 30 % decline of eGFR in 5 years of follow-

up. This predictive value was independent of baseline serum creatinine or the amount of albuminuria during the follow-up period. Baseline sIL-2R level was also demonstrated to be predictive of the rate of GFR decrement and was correlated with degree of tubulointerstitial fibrosis (>25 % fibrosis) (Lundberg et al. 2012).

Upregulated urinary cytokines might be a marker of disease activity and mesangial cell proliferation. Studied by Stangou et al., urinary levels of IL-1β, MCP-1, IL-17, INF-γ, and IL-6 were correlated with endocapillary proliferation in pathologic specimen. Baseline serum creatinine level was correlated with IL-1β, MCP-1, and IL-2. Among the mentioned cytokines, urinary IL-6 was predictive of renal outcomes (Stangou et al. 2013).

Expression of MCP-1 in the mesangium induces interstitial macrophage infiltration and profibrotic growth factor production. On the other side, tubular epithelial cells produce epidermal growth factor to counteract with tissue fibrosis in renal tubular injury. Thus, decreased level of EGF and increased level of MCP-1 might cause progressive interstitial damage and fibrosis. In patients with IgAN, decreased urinary EGF/MCP-1 has been reported, in patients with the lowest ratio (less than 8.9) having the most severe pathologic lesions. Of note is detrimental effect of low EGF/MCP-1 ratio on renal survival. Those with EGF/MCP-1 ratio <8.9 had 36 % renal survival after 84 months of follow-up. At the cutoff of 23.2, EGF/MCP-1 ratio had 88.9 % sensitivity and 86.4 % specificity for prediction of serum creatinine doubling or development of ESRD (Torres et al. 2008).

Elevated serum IL-18, produced by activated TECs, is positively correlated with the amount of proteinuria and serum creatinine level and negatively correlated with eGFR and serum albumin. Serum IL-18 with a cutoff value of 323.69 pg/ml has 94.7 % and 78.6 % sensitivity and specificity, respectively, in predicting tubulointerstitial injury. Serum IL-18, tubulointerstitial injury, and serum creatinine at diagnosis were independent predictors of composite end point of creatinine doubling or renal replacement therapy or death of any cause (Shi et al. 2012).

Markers of Oxidative Stress

Oxidative stress is a state of imbalance between oxidative activity and antioxidant defense. The state of oxidative stress as reduction in superoxide dismutase, catalase, and glutathione peroxidase activity and increased advanced oxidation protein products (AOPPs) has been demonstrated in different studies. IgAN patients had higher levels of AOPPs than healthy controls, and this high level of oxidative marker was directly correlated with the amount of proteinuria at diagnosis and time-averaged proteinuria during the course of follow-up. AOPP level above 100.7 μmol/L also had sensitivity and specificity of about 75 % in predicting progressive eGFR loss. The increased proteinuria and the decline in eGFR might be related to the toxic effects of AOPPs on podocytes and increased production of TGF-β (Camilla et al. 2011). Therefore, diminution of oxidative stress could lead to attenuation of rate of decline in eGFR and slowing the progression of disease.

Table 3 Different histologic classifications of IgAN

Histologic classifications	Advantages	Disadvantages
The Lee classification	Simple for routine use	Qualitative description, less reproducibility
	Take into account the severity of crescent formation	Not scoring chronic injury (IF/TA)
		Not scoring endocapillary proliferation as an individual marker
The Haas classification	Simple for routine use	Not scoring endocapillary proliferation as an individual marker
		Not scoring crescent formation as an individual marker
		Combining markers of disease activity (hypercellularity) and chronic injury in same categories
The Oxford classification (MEST)	Individually scoring the pathologic changes (active or chronic lesions)	More complex than the others
	Quantitative and reproducible	Not taking crescents into account

METS: mesangial hypercellularity (M), endocapillary proliferation (E), tubular atrophy/interstitial fibrosis (T), segmental glomerulosclerosis (S)

Even with all these well-conducted studies, drawing a solid conclusion from the information on the prognostic marker in IgAN is too soon. Large cohort studies may lead to more precise results.

Biomarker Correlation with Histologic Findings

Histologic lesions in IgAN have a wide spectrum, from minimal glomerular abnormality, mesangial hypercellularity, endocapillary and extracapillary hypercellularity, segmental glomerulosclerosis, tubular atrophy, and interstitial fibrosis. Each of these findings may indicate a distinct prognostic or therapeutic implication. The last three histologic features are indicators of chronic lesions and are reported to be related to renal outcome. The proliferative lesions may predict response to immunosuppressive therapies. In order to minimize interobserver variations in pathology reports, various classification systems have been developed, including the Lee classification, the Haas classification, and the Oxford classification (Roberts 2014). All these classifications have limitation in predicting prognosis, when compared with clinical prognostic factors. The advantages and disadvantages of each are demonstrated in Table 3.

All of these pathologic classifications are invasive and need kidney biopsy. Several groups had studied correlation of different biomarkers with the pathologic findings. Some of the biomarkers will be reviewed here; the others have been summarized in Table 4.

Table 4 Biomarkers correlated with histologic lesions

Histologic lesion	Biomarker	Reference
Mesangial hypercellularity	Urine MBL	Liu et al. 2012
	C_4a desArg	Sogabe et al. 2013
Endocapillary proliferation	Urine MBL	Liu et al. 2012
Crescent formation	FE IgG	Bazzi et al. 2009
Tubulointerstitial damage	Serum uric acid	Zhou et al. 2014
	sIL-2R	Lundberg et al. 2012
	Serum IL-18	Shi et al. 2012
	FE IgG, FE α_1m	Bazzi et al. 2009
	Urine α_1m and β_2m	Peters et al. 2011
	Urine EGF, IL-6, MCP-1	Stangou et al. 2009
	Urine MBL	Liu et al. 2012
Glomerular sclerosis	miRNA-429	Wang et al. 2010
	FE IgG, FE α_1m	Bazzi et al. 2009
	Urine IL-6	Stangou et al. 2009
	Urine MBL	Liu et al. 2012
	FE IgG, FE α_1m	Bazzi et al. 2009

α_1m α_1-microglobulin, *β_2m* β_2-microglobulin, *EGF* epidermal growth factor, *FE α_1m* fractional excretion of α_1-microglobulin, *FE IgG* fractional excretion of IgG, *IL-6* interleukin 6, *IL-18* interleukin 18, *MBL* mannose-binding lectin, *miRNA-429* microRNA-429, *MCP-1* monocyte chemoattractant protein-1, *sIL-2R* soluble interleukin 2 receptor

Podocalyxin

Podocalyxin is an apical membrane protein of podocytes. Its extracellular portion consists of a juxtamembrane stalk and four different domains: O-glycosylated, sialylated, N-glycosylated, and a globular domain. The intracellular domain of podocalyxin interacts with Na^+/H^+ exchanger regulatory factors 1 and 2, ezrin, and actin cytoskeleton of podocyte. It has a critical role in the development of glomeruli and slit diaphragm. Urine level of podocalyxin is related to the degree of podocyte injury in IgAN. In a study in adult IgAN patients, a significant correlation has been reported between the urinary level of this protein and the severity of acute extracapillary abnormalities (Sekulic and Sekulic 2013). This marker can be used to predict the renal lesions and possibly the prognosis.

TGF-β and IL-6

In a study aimed to evaluate response to treatment with steroids, urinary TGF-β and IL-6 levels were evaluated before and after treatment in IgAN patients. The baseline urinary TGF-β level was significantly higher in patients with mesangial hypercellularity, mesangial expansion, and crescent formation, and urinary IL-6 level was directly related to glomerulosclerosis (Kalliakmani et al. 2011). There is a study that showed a significant correlation between chronicity of IgAN and

glomerulosclerosis (Stangou et al. 2009). Conversely, urine IL-6 has been correlated with endocapillary proliferation along with other proinflammatory cytokines such as IL-1β, MCP-1, IL-17, and INF-γ (Stangou et al. 2013), and the pediatric patients' urine IL-6 level was positively correlated with mesangial hypercellularity, crescent formation, and endocapillary proliferation (Kanemoto et al. 2014). TGF-β and IL-6 as markers of disease severity and pathologic findings should be evaluated in large-scale study.

Thioredoxin

Thioredoxin is a redox-active protein and a marker of oxidative stress that has been evaluated in IgAN patients (Nosaki et al. 2012). In this study serum level of thioredoxin above 40 ng/ml was significantly associated with mesangial cell proliferation. Although it was not specific for IgAN and it was also seen in other causes of mesangial proliferation such as lupus nephritis, it still is a valuable marker that needs to be proven in larger studies.

Soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1)

sVCAM-1 is known as a marker of endothelial injury. Following endothelial damage and activation, sVCAM-1 is cleaved from the membrane-binding domain and released into the circulation. Severe pathologic finding of crescent formation is the consequence of endothelial injury. sVCAM-1 was related to active crescentic lesion rather than chronic glomerular lesions. Those with tubular atrophy and interstitial fibrosis score (T in Oxford classification) had significantly higher sVCAM-1 (Zhu et al. 2013).

Urine Secretory IgA (sIgA)

The sIgA has been shown to have a role in pathogenesis of IgAN, and increased urinary levels of it were evident in patients when compared with healthy controls. Data suggested that this increased level is associated with increased scores of mesangial proliferation, crescent formation, and higher score of tubular atrophy and interstitial fibrosis. The higher the urine sIgA was, the more severe the pathologic findings were (Tan et al. 2009). Thus, this marker might be used as a predictor of pathologic changes in IgAN.

Proteomic Analysis of Urine

In a proteomic analysis of urine samples of 13 IgAN patients using liquid chromatography/tandem mass spectrometry (nLC-MS/MS), Kalantari et al. reported a

Table 5 Biomarker expression and their correlation with eGFR

Protein ID	Protein name	Up-/ downregulation	Correlation with eGFR
AFAM	Afamin (alpha-albumin)	↓	Positive
A2GL1	Leucine-rich alpha-2 glycoprotein	↓	Positive
CERU	Ceruloplasmin	↓	Positive
AMBP	Alpha-1-microgolbulin	↑	Negative
HEMO	Hemopexin	↑	Negative
APOA1	Apolipoprotein A-I	↑	Negative
CO3	Complement C3	↑	Negative
VTDB	Vitamin D-binding protein	↑	Negative
APOA4	Apolipoprotein A-IV	↑	Negative
B2MG	Beta-2-microglobulin	↑	Negative
RET4	Retinol-binding protein 4	↑	Negative

All the markers significantly correlate with eGFR with a p value of <0.006

panel of biomarkers that can differentiate various pathological subclasses based on Oxford parameters specially endocapillary proliferation. These candidate biomarkers were afamin, leucine-rich alpha-2-glycoprotein, ceruloplasmin, alpha-1-microgolbulin, hemopexin, apolipoprotein A-I, complement C3, vitamin D-binding protein, beta-2-microglobulin, and retinol-binding protein 4. Changes in urinary expression of these markers were correlated with eGFR, as shown in Table 5 (Kalantari et al. 2013).

It seems that a single marker might be insufficient to differentiate pathologic changes and predict disease course. A panel of biomarkers, serum, or urine might be helpful.

Biomarkers of Response to Therapy

Better understanding of pathogenesis of IgAN helps targeted therapy. Floege has proposed five pathogenetic steps. The first step is overproduction of Gd-IgA1, which is thought to be from tonsillar origin. Therefore tonsillectomy is considered as a therapeutic option, although the results on the course of IgAN are inconsistent. The next step is antibody formation against Gd-IgA1 (autoimmunity). To address this step, steroids and immunosuppressive therapy come to action. Deposition of the immune complexes in mesangium and binding to mesangial IgA receptors (CD71) induce inflammation and complement activation. Removal of glomerular IgA, blocking the receptor, and prevention of complement activation are the proposed approaches to target third and fourth steps of the pathogenesis. The last two steps are cellular damage and growth factor (such as platelet-derived growth factor, TGF-β) and profibrotic mechanism activation (Floege 2011).

Table 6 Biomarkers as predictors of response to therapy

Treatment	Biomarkers	Reference
RAS blockade	Urine kininogen, ITIH4, transthyretin	Rocchetti et al. 2008
	Urinary KIM-1	Seo et al. 2013
	FE IgG	Bazzi et al. 2009
Fish oil	Serum oxylipins	Zivkovic et al. 2012
Tonsillectomy ± steroids	Serum Gd-IgA1	Nakata et al. 2014
	Changes in GalNAc content of IgA1	Iwantani et al. 2012
	IgA–uromodulin complex	Obara et al. 2012
	Serum thioredoxin	Nosaki et al. 2012
Immunosuppressive therapy	Serum IL-18	Shi et al. 2012
	Urinary MBL	Liu et al. 2012
	FE IgG/GS	Bazzi et al. 2009

FE IgG fractional excretion of IgG, *FE IgG/GS* FE IgG to glomerulosclerosis ratio, *GalNAc* N-acetylgalactosamine, *Gd-IgA1* galactose-deficient IgA1, *ITIH4* inter-α-trypsin-inhibitor heavy chain H4 precursor, *KIM-1* kidney injury molecule-1, *MBL* mannose-binding lectin, *RAS* renin–angiotensin system

Apart from novel anti-growth factor therapy, angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs) are the mainstay of therapy.

According to the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines, in those with persistent proteinuria after 3–6 months of treatment with ACEi or ARBs and estimated GFR of more than 50 cm^3/min per 1.73 m^2, treatment with fish oil or steroids is recommended. Immunosuppressive drugs such as cyclophosphamide, azathioprine, or mycophenolate mofetil are reserved for those with rapidly progressive disease (KDIGO 2012).

Biomarkers can be used as a guide to select patients for specific treatment, follow the response, and predict recurrences (Table 6).

Biomarker Response to Blockade of the Renin–Angiotensin System

In 2008, Rocchetti et al. evaluated response to treatment with ACEi in IgAN patients. Responder patients were defined as more than 50 % reduction in proteinuria and stable GFR after 6 months of treatment with ACEi. They utilized 2D PAGE and nano-HPLC-ESI-MS/MS to analyze the urinary proteome in patients. Patients who responded to treatment had a significantly higher urine kininogen level at the baseline compared to the nonresponders. Additionally, there was a significant increase in urine level of kininogen after treatment in the responder group, but did not reach the normal values. They also reported decreased level of inter-α-trypsin-inhibitor heavy chain H4 precursor (ITIH4, 35 kDa fragment) and increased level of transthyretin in nonresponders when compared with responders (Rocchetti et al. 2008).

Urinary KIM-1 has been introduced as a marker of renal survival. The changes in its level have also been evaluated as a marker of response. Urinary KIM-1 was measured at diagnosis and after about 2 years of treatment with different regimens including a low-salt diet, blood pressure control, pharmacotherapy with angiotensin receptor blockers or angiotensin-converting enzyme inhibitors, and if necessary immunosuppressives (in 13.5 % of patients). The urinary KIM-1/creatinine ratio was decreased to 0.26 [0.12–0.65] ng/mg from the baseline of 1.16 [0.51–1.83] ng/mg after mean follow-up period of 23.56 ± 5.08 months. In about half of the patients, KIM-1 levels became normal. This study suggested KIM-1 as a valuable predictor of response to therapy, but larger study on patients with more severe renal dysfunction is needed to verify it (Seo et al. 2013).

Fractional excretion of IgG (FE IgG) is known as a marker of changes in size selectivity and, as it was mentioned previously, a marker of disease progression in IgAN. Bazzi et al. evaluated the clinical, laboratory, and histologic risk factors of disease progression in two groups of IgAN patients (baseline serum creatinine less than 3 mg/dl and no immunosuppressive therapy) treated with or without ACEi. The risk factors were blood pressure, 24-hour proteinuria, FE IgG, fractional excretion of α1-microglobulin, glomerular sclerosis, and tubulointerstitial damage. Progression was defined as primary end points of end-stage renal disease or serum creatinine doubling or secondary end point of 25 % increase in serum creatinine during follow-up. The cutoff point for progression was calculated for each risk factor with highest sensitivity and specificity. ACE inhibitors were renoprotective in cases with baseline risk factors above the defined cutoff. In these patients, treatment with ACEi reduced the progression rate significantly. Although different players are involved in ACEi renoprotection, FE IgG with the cutoff value of ≥0.006 was the most powerful predictor of renoprotection in this study (Bazzi et al. 2009).

Biomarker Response to Fish Oil

Fish oil (3.3 g/day) is one of the recommended treatments in KDIGO guideline for patients with more than 1 g/day proteinuria and no response to ACEi after 3–6 months (KDIGO 2012). Studies reported different outcomes of using fish oil.

Apart from differences in patient population, drug dosage, and composition, fatty acid metabolism variances might be the cause of inconsistency in response to therapy. Fish oil is rich in ω-3 fatty acids (FAs). FA metabolism leads to production of oxylipins. Oxylipins are produced by enzymatic and nonenzymatic oxidation of FAs. Enzymatic oxidation involves the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways. Oxylipins play an important role in different signaling pathways, such as coagulation cascade, vasodilation, and cellular immunity and autoimmunity. Analyzing oxylipin profile in IgAN patients with diverse response to therapy may help predicting fish oil responsiveness. LC-MS/MS was used to identify oxylipin profile in serum of IgAN patients under treatment with fish oil. When responders (those with at least 25 % decrease in

proteinuria) were compared with nonresponders, the major difference was among arachidonic acid and linoleic acid metabolites in the LOX pathways. Lower levels of hydroxyeicosatetraenoic acids (HETEs) and the leukotriene B_4 (LTB_4) were associated with kidney function improvement. Fish oil can result in reduction of these metabolites (Zivkovic et al. 2012).

Biomarker Response to Tonsillectomy ± Steroids

Serum galactose-deficient IgA1 (Gd-IgA1) has the leading role in the pathogenesis of IgAN, thus following its serum level during the course of treatment might be helpful. In a study, changes in serum level of Gd-IgA1 with treatment were evaluated. Patients were treated with tonsillectomy followed by steroids if not responding to tonsillectomy alone. Clinical response was defined as remission of hematuria and protein/creatinine ratio less than 0.15 g/g. Twenty-two out of 37 patients responded to tonsillectomy alone, 13 patients responded to tonsillectomy and steroids, and 2 responded to neither. The changes in serum Gd-IgA1 was parallel to the clinical response, declined in those with remission in proteinuria and hematuria. Thus, apart from its pathogenetic role, serum Gd-Iga1 could be used as a marker of clinical response (Nakata et al. 2014).

Aberrant O-glycosylation of IgA1 including hyposialylation, reduced galactose (Gal) number, and reduced N-acetylgalactosamine (GalNAc) number in the hinge region is the cornerstone of IgAN. The variations in glycosylation pattern after tonsillectomy and steroid therapy and the molar content of GalNAc and galactose in the hinge region were evaluated as a marker of response. There was a significant increase in GalNAc in patients in remission but the level was still lower than healthy controls. Therefore, quantitative changes in GalNAc content of IgA1, analyzed by MALDI-TOF-MS, might be a new marker of therapeutic response (Iwantani et al. 2012).

As previously mentioned, elevated IgA–uromodulin complex level could be a diagnostic marker of active IgAN. Thus, it could be used as a criterion for selection of patients in early active phase to be treated with tonsillectomy and steroid (Obara et al. 2012).

Data have suggested that oxidative stress enhances the nephrotoxicity of aberrantly galactosylated IgA1. It is proposed that improvement in oxidative stress in response to either steroids or tonsillectomy might be related to the clinical response. Thioredoxin is a redox-active protein, which was used as a marker of oxidative stress in patients with IgAN. Serum and urine levels of thioredoxin were measured with ELISA before and after tonsillectomy. Serum thioredoxin level in IgAN patients was higher at baseline compared with healthy controls and was significantly decreased after tonsillectomy but did not reach the levels in controls (Nosaki et al. 2012).

Most of the abovementioned studies have included small number of patients, and a large clinical trial is needed to draw a net conclusion.

Biomarker Response to Immunosuppressive Therapy

Most of the studies, which reported a biomarker as predictor of response to immunosuppressive therapy, were single centered with small number of cases short follow-up. Beyond all, they were mostly designed for outcomes of progression and prognosis.

KDIGO recommends steroids in IgAN patients with nephrotic-range proteinuria and in patients with GFR of more than 50 ml/min/1.73 m^2 and proteinuria of more than 1 g/day after 3–6 months of treatment with RAS blockers (KDIGO 2012).

In order to study disease activity after remission of proteinuria with steroids, urine IL-6 and TGF-β were measured in 21 patients with IgAN at diagnosis and at the end of treatment. Following treatment despite a significant reduction in proteinuria, the urinary level of these factors remains high, which might represent ongoing inflammation (Kalliakmani et al. 2011). Serial measurements of factors and longer follow-up might help to define a tool for clinical course monitoring and patient selection for steroid therapy. In a different study, baseline serum level of IL-18 predicted the response to steroid therapy. The higher the serum IL-18 was, the more was the probability of the nonresponder to therapy. This correlation might be due to association of this marker with tubulointerstitial damage (Shi et al. 2012).

When steroids were used in combination with azathioprine in subjects nonresponsive to RAS blocker and fish oil, affected urinary markers of inflammation including IL-6 were suppressed (Stangou et al. 2013).

Liu et al. in a study aimed to evaluate the value of urinary MBL in prognosis and progression of IgAN included patients at diagnosis who have been followed for $28{\cdot}6 \pm 5{\cdot}9$ months, and they were treated either with non-immunosuppressive therapy (ACEi, ARB, fish oil, antiplatelet) or steroids alone or in combination with cytostatics, cyclosporin A, and mycophenolate mofetil. In both groups (non-immunosuppressive vs. immunosuppressive), patients who had reached remission had lower baseline urinary MBL level (Liu et al. 2012). Thus baseline urinary MBL could serve as a predictor of response rather than a surrogate of response to therapy.

The pathologic finding of crescentic glomerulonephritis with rapidly progressive renal failure is one of the grave clinical features of IgAN. Different therapeutic approaches have been employed to treat this condition including combined treatment with steroids and cyclophosphamide. Thirty-seven crescentic IgAN patients were investigated. The relationship between the fractional excretion of IgG (FE IgG) and histologic lesions and response to therapy was analyzed. FE IgG to glomerulosclerosis ratio (FE IgG/GS) and serum creatinine in combination were the most accurate predictors of responsiveness to therapy. Patients with FE IgG/SG ≥ 0.00034 and sCr ≥ 1.74 mg/dl were at 100 % risk of progression even with treatment. So it could be used in patient selection for immunosuppression therapy (Bazzi et al. 2009).

According to the evidences, FE IgG/SG is the best predictor of response to treatment with RAS blocker and steroid and cyclophosphamide. Further multicenter large-scale trails are needed to evaluate predicting markers of response.

Although great evolutions have occurred in protein isolation techniques, validation of these biomarkers in large population studies and bringing them from bench to beside are at their infancy.

Potential Applications to Prognosis, Other Diseases, or Conditions

Urine is a biofluid, which can be easily collected in a noninvasive procedure. It is representative of local processes in the kidney and least affected by systemic processes. It could be examined repeatedly during the course of disease. Thus, it seems to be a perfect sample for biomarker evaluation in glomerular disease. Moreover, according to available studies, a panel of biomarkers rather than a single biomarker seems to be more accurate and practical. Accordingly, proteomics and metabolomics studies might be of more help in the path to design diagnostic, prognostic, and predictive biomarkers, as they are representative of active ongoing processes in the kidney. In that way, light would be shed on pathogenetic pathways too, and this would help for targeted therapy.

In order to extract a panel of biomarkers in different fields of glomerular diseases, it is practical to find a proteomics or metabolomics bank with various samples from patients with different glomerular diseases (minimal change disease, focal segmental glomerulosclerosis, membranous nephropathy, etc.). This will help to define disease-specific biomarker profile and little by little will fade the need to perform kidney biopsy in order to make a diagnosis or predict the outcome of the glomerulopathy.

Summary Points

- This chapter focuses on novel biomarkers in IgA nephropathy.
- IgA nephropathy is one of the most common glomerulonephritis worldwide, diagnosis of which is dependent on renal biopsy.
- Different clinical and pathological factors influence the outcome and response to therapy.
- As an easily accessible biofluid sample, urine protein and metabolite profile is the most studied markers in IgAN.
- In a search for noninvasive biomarkers for diagnosis of IgA nephropathy, various proteomics methods (2DE, MALDI-TOF-MS, LC-MS/MS, SELDI-MS/MS) or metabolomics (NMR) were used.
- Uromodulin, sTfR, IL-6/EGF, and complement components have been repeatedly reported as diagnostic urinary markers.
- Analysis of urine exosomes using LC-MS/MS, evaluation of urine metabolomics by the means of NMR spectroscopy, or urine proteome study by nLC-MS/MS are new approaches for noninvasive diagnosis of IgAN. These data also could help clarifying the pathogenesis of disease.

- Numerous studies have reported urinary KIM-1, FE IgG, and inflammatory cytokines as predictor of renal outcome in IgAN.
- Various markers are proposed as predictor of response to immunosuppressive therapy. The most widely studied is FE IgG.
- A wide range of studies have conducted and various markers have been introduced, but large cohort studies are still needed to reach the goal of noninvasive diagnostic, pathogenetic, and prognostic markers in IgAN.

References

Abou-Ragheb HH, Williams AJ, Brown CB, et al. Plasma levels of the anaphylatoxins C3a and C4a in patients with IgA nephropathy/Henoch-Schönlein nephritis. Nephron. 1992;62:22–6.

Bazzi C, Rizza V, Raimondi S, et al. In crescentic IgA nephropathy, fractional excretion of IgG in combination with nephron loss is the best predictor of progression and responsiveness to immunosuppression. Clin J Am Soc Nephrol. 2009a;4:929–35.

Bazzi C, Rizza V, Paparella M, et al. Fractional urinary excretion of IgG is the most powerful predictor of renoprotection by ACE inhibitors in IgA nephropathy. J Nephrol. 2009b;22:387–96.

Boyd JK, Barratt J. Immune complex formation in IgA nephropathy: CD89 a 'saint' or a 'sinner'? Kidney Int. 2010;78:1211–3.

Boyd JK, Cheung CK, Molyneux K, et al. An update on the pathogenesis and treatment of IgA nephropathy. Kidney Int. 2012;81:833–43.

Camilla R, Suzuki H, Dapra V, et al. Oxidative stress and galactose-deficient IgA1 as markers of progression in IgA nephropathy. Clin J Am Soc Nephrol. 2011;6:1903–11.

Del Coco L, Assfalg M, D'Onofrio M, et al. A proton nuclear magnetic resonance-based metabolomics approach in IgA nephropathy urinary profiles. Metabolomics. 2012. doi:10.1007/s11306-012-0489-2.

Delanghe SE, Speeckaert MM, Segers H, et al. Soluble transferrin receptor in urine, a new biomarker for IgA nephropathy and Henoch–Schönlein purpura nephritis. Clin Biochem. 2013;46:591–7.

Ebefors K, Granqvist A, Ingelsten M, et al. Role of glomerular proteoglycans in IgA nephropathy. PLoS One. 2011;6(4):e18575.

Floege J. The pathogenesis of IgA nephropathy: what is new and how does it change therapeutic approaches? Am J Kidney Dis. 2011;58(6):992–1004.

Floege J, Feehally J. Treatment of IgA nephropathy and Henoch–Schonlein nephritis. Nat Rev Nephrol. 2013;9:320–7.

Glassock RJ. IgA nephropathy: challenges and opportunities. Cleve Clin J Med. 2008;75:569–76.

Harada K, Akai Y, Kurumatani N, et al. Prognostic value of urinary interleukin 6 in patients with IgA nephropathy: an 8-year follow-up study. Nephron. 2002;92:824–6.

Hrvacevic R, Topalov D, Stojanovic R, et al. Serum and urinary interleukin-6 levels in patients with primary glomerulonephritis. Srp Arh Celok Lek. 1996;124:40–2.

Iwantani H, Inoue T, Wada Y, et al. Quantitative change of IgA hinge O-glycan composition is a novel marker of therapeutic responses of IgA nephropathy. Biochem Biophys Res Commun. 2012;428:339–42.

Kalantari S, Rutishauser D, Samavat S, et al. Urinary prognostic biomarkers and classification of IgA nephropathy by high resolution mass spectrometry coupled with liquid chromatography. PLoS One. 2013;8(12):e80830. doi:10.1371/journal.pone.0080830.

Kalliakmani P, Nakopoulou L, Tsakas S, et al. Urinary interleukin-6 (IL-6) and transforming growth factor (TGF-β) levels in corticosteroid-treated patients with IgA nephropathy. Clin Nephrol. 2011;76(2):144–50.

Kanemoto K, Matsumura R, Anzai M, et al. Urinary excretion of interleukin-6 in pediatric IgA nephropathy patients. J Nephrol Therapeutic. 2014; S11: doi:10.4172/2161-0959.S11-004.

Kidney Disease: Improving Global Outcomes (KDIGO) Glomerulonephritis Work Group. Clinical practice guidelines for glomerulonephritis – chapter 10: immunoglobulin A nephropathy. Kidney Int Suppl. 2012;2:209–17.

Kim YG, Song SB, Lee SH, et al. Urinary angiotensinogen as a predictive marker in patients with immunoglobulin A nephropathy. Clin Exp Nephrol. 2011;15(5):720–6.

Kim JK, Kim JH, Lee SC, et al. Clinical features and outcomes of IgA nephropathy with nephrotic syndrome. Clin J Am Soc Nephrol. 2012;7:427–36.

Kobori H, Harrison-Bernard LM, Navar LG. Urinary excretion of angiotensinogen reflects intrarenal angiotensinogen production. Kidney Int. 2002;61(2):579–85.

Kobori H, Nangaku M, Navar LG, et al. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. Pharmacol Rev. 2007;59:251–87.

Lee H, Hwang JH, Paik JH, et al. Long-term prognosis of clinically early IgA nephropathy is not always favorable. BMC Nephrol. 2014a;15:94.

Lee YH, Kim YG, Lee SH, et al. Clinicopathological role of kidney injury molecule-1in immunoglobulin A nephropathy. Kidney Res Clin Pract. 2014b;33:139–43.

Li W, Peng X, Liu Y, Liu H, et al. TLR9 and BAFF: their expression in patients with IgA nephropathy. Mol Med Rep. 2014;10(3):1469–74.

Liu LL, Jiang Y, Wang LN, Liu N. Urinary mannose-binding lectin is a biomarker for predicting the progression of immunoglobulin (Ig) A nephropathy. Clin Exp Immunol. 2012;169(2):148–55.

Lundberg S, Lundahl J, Gunnarsson I, et al. Soluble interleukin-2 receptor alfa predicts renal outcome in IgA nephropathy. Nephrol Dial Transplant. 2012;27:1916–23.

Mackinnon B, Shakerdi L, Deighan CJ, et al. Urinary transferrin, high molecular weight proteinuria and the progression of renal disease. Clin Nephrol. 2003;59:252–8.

McQuarrie EP, Shakerdi L, Jardine AG, et al. Fractional excretions of albumin and IgG are the best predictors of progression in primary glomerulonephritis. Nephrol Dial Transplant. 2011;26:1563–9.

Mecklenbrauker I, Kalled SL, Leitges M, et al. Regulation of B-cell survival by BAFF-dependent PKC delta mediated nuclear signalling. Nature. 2004;431(7007):456–61.

Monteiro RC. New insights in the pathogenesis of IgA nephropathy. Nefrologia. 2005;25 Suppl 2:82–6.

Moon P, Lee J, You S, et al. Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy. Proteomics. 2011;11:2459–75.

Moriyama T, Tanaka K, Iwasaki C, et al. Prognosis in IgA nephropathy: 30-year analysis of 1,012 patients at a single center in Japan. PLoS One. 2014;9(3):e91756. doi:10.1371/journal.pone.0091756.

Nachman PH, Jennette JC, Falk RJ. Primary glomerular disease. In: Taal MW et al., editors. Brenner %26 Rector's the kidney. 9th ed. Philadelphia: Saunders; 2012. p. 1146–8.

Nakata J, Suzuki Y, Suzuki H, et al. Changes in nephritogenic serum galactose-deficient IgA1 in IgA nephropathy following tonsillectomy and steroid therapy. PLoS One. 2014;9(2):e89707. doi:10.1371/journal.pone.0089707.

Nishiyama A, Konishi Y, Ohashi N, et al. Urinary angiotensinogen reflects the activity of intrarenal renin–angiotensin system in patients with IgA nephropathy. Nephrol Dial Transplant. 2011;26:170–7.

Nosaki T, Uto H, Takami Y, et al. High serum thioredoxin levels are reduced after tonsillectomy in patients with IgA nephropathy. Intern Med. 2012;51:559–65.

Obara T, Mizoguchi S, Shimozuru Y, et al. The complex of immunoglobulin A and uromodulin as a diagnostic marker for immunoglobulin A nephropathy. Clin Exp Nephrol. 2012;16: 713–21.

Onda K, Ohsawa I, Ohi H, et al. Excretion of complement proteins and its activation marker C5b-9 in IgA nephropathy in relation to renal function. BMC Nephrol. 2011;12:64.

Parera M, Rivera F, Egido J, et al. The role of interleukin 2 (IL-2) and serum-soluble IL-2 receptor cells in idiopathic IgA nephropathy. Clin Immunol Immunopathol. 1992;63:196–9.

Park MR, Wang EH, Jin DC, et al. Establishment of a 2-D human urinary proteomic map in IgA nephropathy. Proteomics. 2006;6:1066–76.

Peters HPE, Waanders F, Meijer E, et al. High urinary excretion of kidney injury molecule-1 is an independent predictor of end-stage renal disease in patients with IgA nephropathy. Nephrol Dial Transplant. 2011;26:3581–8.

Rampoldi L, Scolari F, Amoroso A, et al. The rediscovery of uromodulin (Tamm–Horsfall protein): from tubulointerstitial nephropathy to chronic kidney disease. Kidney Int. 2011;80:338–47.

Ranieri E, Gesualdo L, Petrarulo F, Schena FP. Urinary IL- 6/EGF ratio: a useful prognostic marker for the progression of renal damage in IgA nephropathy. Kidney Int. 1996;50:1990–2001.

Reily C, Ueda H, Huang Z, et al. Cellular signaling and production of galactose-deficient IgA1 in IgA nephropathy, an autoimmune disease. J Immunol Res. 2014: 10 pp. doi:10.1155/2014/197548.

Roberts ISD. Pathology of IgA nephropathy. Nat Rev Nephrol. 2014;10:445–54.

Rocchetti MT, Centra M, Papale M, et al. Urine protein profile of IgA nephropathy patients may predict the response to ACE-inhibitor therapy. Proteomics. 2008;8:206–16.

Rocchetti MT, Papale M, d'Apollo AM, et al. Association of urinary laminin G-like 3 and free K light chains with disease activity and histological injury in IgA nephropathy. Clin J Am Soc Nephrol. 2013;8:1115–25.

Samavat Sh, Kalantari Sh, Nafar M, et al. Diagnostic urinary proteome profile for IgA nephropathy. IJKD. 2015;9:239–48.

Santucci L, Candiano G, Bruschi M, et al. Urinary proteome in a snapshot: normal urine and glomerulonephritis. J Nephrol. 2013;26(4):610–6.

Schena FP, Mastrolitti G, Jirillo E, et al. Increased production of interleukin-2 and IL-2 receptor in primary IgA nephropathy. Kidney Int. 1989;35:875–9.

Sekulic M, Sekulic SP. A compendium of urinary biomarkers indicative of glomerular podocytopathy. Pathol Res Int. 2013; 2013: 18 pp. doi:10.1155/2013/782395, Article ID 782395.

Seo MS, Park MY, Choi SJ, et al. Effect of treatment on urinary kidney injury molecule-1 in IgA nephropathy. BMC Nephrol. 2013;14:139.

Shi B, Ni Z, Cao L, et al. SerumIL-18 is closely associated with renal tubulointerstitial injury and predicts renal prognosis in IgA nephropathy. Mediat Inflamm. 2012: 9 pp. doi:10.1155/2012/728417, Article ID 728417.

Sogabe A, Uto H, Kanmuri S, et al. Correlation of serum levels of complement C4a desArg with pathologically estimated severity of glomerular lesions and mesangial hypercellularity scores in patients with IgA nephropathy. Int J Mol Med. 2013;32:307–14.

Stangou M, Alexopoulos E, Papagianni A, et al. Urinary levels of epidermal growth factor, interleukin-6 and monocyte chemoattractant protein-1 may act as predictor markers of renal function outcome in immunoglobulin A nephropathy. Nephrol (Carlton). 2009;14(6):613–20.

Stangou M, Papagianni A, Bantis C, et al. Up-regulation of urinary markers predict outcome in IgA nephropathy but their predictive value is influenced by treatment with steroids and azathioprine. Clin Nephrol. 2013;80:203–10.

Sui W, Li L, Che W, et al. A proton nuclear magnetic resonance-based metabonomics study of metabolic profiling in immunoglobulin a nephropathy. Clinics. 2012;67(4):363–73.

Surin B, Sachon E, Rougier J, et al. LG3 fragment of endorepellin is a possible biomarker of severity in IgA nephropathy. Proteomics. 2013;13:142–52.

Szeto CC, Li PKT. MicroRNAs in IgA nephropathy. Nat Rev Nephrol. 2014;10:249–56.

Szeto CC, Ching-Ha KB, Ka-Bik L, et al. Micro-RNA expression in the urinary sediment of patients with chronic kidney diseases. Dis Markers. 2012;33:137–44.
Tan Y, Zhang JJ, Liu G, et al. The level of urinary secretory immunoglobulin A (sIgA) of patients with IgA nephropathy is elevated and associated with pathological phenotypes. Clin Exp Immunol. 2009;156:111–6.
Torres DD, Rossini M, Manno C, et al. The ratio of epidermal growth factor to monocyte chemotactic peptide-1 in the urine predicts renal prognosis in IgA nephropathy. Kidney Int. 2008;73:327–33.
Vuong MT, Hahn-Zoric M, Lundberg S, et al. Association of soluble CD89 levels with disease progression but not susceptibility in IgA nephropathy. Kidney Int. 2010;78:1281–7.
Wada T, Nangaku M. Novel roles of complement in renal diseases and their therapeutic consequences. Kidney Int. 2013;84:441–50.
Wang G, Kwan BC, Lai FM, et al. Expression of microRNAs in the urinary sediment of patients with IgA nephropathy. Dis Markers. 2010;28:79–86.
Wang G, Kwan BC, Lai FM, et al. Elevated levels of miR-146a and miR-155 in kidney biopsy and urine from patients with IgA nephropathy. Dis Markers. 2011;30:171–9.
Wang G, Kwan BC, Lai FM, et al. Urinary miR-21, miR-29, and miR-93: novel biomarkers of fibrosis. Am J Nephrol. 2012;36(5):412–8.
Wu J, Wang N, Wang J, et al. Identification of a uromodulin fragment for diagnosis of IgA nephropathy. Rapid Commun Mass Spectrom. 2010;24:1971–8.
Wyatt RJ, Julian BA. IgA nephropathy. N Engl J Med. 2013;368:2402–14.
Xin G, Shi W, Xu LX, et al. Serum BAFF is elevated in patients with IgA nephropathy and associated with clinical and histopathological features. J Nephrol. 2013;26(4):683–90.
Xu PC, Zhang JJ, Chen M, et al. Urinary kidney injury molecule-1 in patients with IgA nephropathy is closely associated with disease severity. Nephrol Dial Transplant. 2011;0:1–8.
Xu Z, Xu B, Xu C. Urinary angiotensinogen as a potential biomarker of intrarenal renin-angiotensin system activity in Chinese chronic kidney disease patients. Ir J Med Sci. 2015;184(2):297–304.
Yanagawa H, Suzuki H, Suzuki Y, et al. A panel of serum biomarkers differentiates IgA nephropathy from other renal diseases. PLoS One. 2014;9(5):e98081. doi:10.1371/journal.pone.0098081.
Yokota H, Hiramoto M, Okada H, et al. Absence of increased alpha-1-microglobulin in IgA nephropathy proteinuria. Mol Cell Proteomics. 2007;6:738–44.
Zhang J, Wang C, Tang Y, et al. Serum immunoglobulin A/C3 ratio predicts progression of immunoglobulin A nephropathy. Nephrology. 2013;18:125–31.
Zhao YY, Lin RC. UPLC–MS^E application in disease biomarker discovery: the discoveries in proteomics to metabolomics. Chem Biol Interact. 2014;215:7–16.
Zhao N, Hou P, Lv J, et al. The level of galactose-deficient IgA1 in the sera of patients with IgA nephropathy is associated with disease progression. Kidney Int. 2012;82:790–6.
Zhou J, Chen Y, Liu Y, et al. Plasma uric acid level indicates tubular interstitial lesions at early stage of IgA nephropathy. BMC Nephrol. 2014;15:11.
Zhu L, Shi S, Liu L, et al. Increased plasma sVCAM-1 is associated with severity in IgA nephropathy. BMC Nephrol. 2013;14:21.
Zivkovic AM, Yang J, Georgi K, et al. Serum oxylipin profiles in IgA nephropathy patients reflect kidney functional alterations. Metabolomics. 2012;8(6):1102–13.

MicroRNAs as Biomarkers of Diabetic Nephropathy

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Abstract

Over the course of the past decade, miRNAs (microRNA) have established themselves as important players in many aspects of biology, not least of all in disease pathology. Indeed, microRNA (miRNA) dysregulation has been demonstrated in numerous diseases and in almost all tissues with a number of them displaying promise as therapeutic targets. In recent years, the presence of miRNA in various biofluids, including blood and urine, has been well documented. Importantly, there have been a number of studies demonstrating that miRNA profiles in these biofluids undergo distinct shifts in both the levels of particular

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miRNA species and also which specific miRNA is present. This has sparked interest in their potential as noninvasive biomarkers for disease. Interestingly, the vast majority of these miRNAs have no current known role in their respective diseases. Furthermore, recent discoveries of exosome-bound miRNA being excreted from cells into both the urine and blood have sparked further interest in the field. Here, we review the current literature regarding clinical investigation of miRNAs as diagnostic and prognostic markers for diabetic nephropathy. Specifically, we discuss those studies utilizing miRNA profiles in blood, urine, and also exosomes, their importance to the field miRNA biomarker research, and any potential issues arising from these studies.

Keywords
Diabetic nephropathy • Diabetes • MicroRNA • Biomarkers • Urine • Plasma • Serum • Exosome

Abbreviations

BMI	Body mass index
CKD	Chronic kidney disease
DGS	Diabetic glomerulosclerosis
DM	Diabetes mellitus
DN	Diabetic nephropathy
ECM	Extracellular matrix
FPG	Fasting plasma glucose
FSGS	Focal segmental glomerulosclerosis
HTN	Hypertensive nephropathy
IgAN	IgA nephropathy
MCN	Minimal change nephropathy
MGN	Membranous nephropathy
miRNA/miR	Microribonucleic acid
mRNA	Messenger ribonucleic acid
OGGT	Oral glucose tolerance test
PKD	Polycystic kidney disease
qRT-PCR	Quantitative real-time polymerase chain reaction
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
T2D	Type 2 diabetes
UAER	Urinary albumin excretion rate

Key Facts of Diabetic Nephropathy

- A significant proportion of both type 1 and type 2 diabetics develop diabetic nephropathy.

- The condition is progressive and involves renal fibrosis and destruction of tissue architecture, microvascular damage, and loss of function of the glomerulus, the filtration unit of the kidney.
- Diabetic nephropathy is the leading cause of end-stage renal disease which results in eventual renal failure and therefore requirement of either renal transplantation or hemodialysis.
- Prognosis for those with end-stage renal failure is poor and is further worsened by concomitant cardiovascular complications.
- There is a reciprocal relationship between cardiovascular complications and diabetic nephropathy meaning those with one complication are at higher risk of developing the other.
- Diabetic nephropathy can progress for 5–10 years without any adverse physiological manifestation and therefore is often somewhat advanced at the time of diagnosis.
- There are no therapeutic compounds available that effectively prevent the development or progression of diabetic nephropathy.
- With the exception of cardiovascular complications, there are no means to accurately predict those that will develop diabetic nephropathy.

Definitions

Biomarker Any biological substance (protein, DNA/RNA sequence, metabolite) which is measurable and enables diagnosis or prognosis of a disease.

Diabetic nephropathy A degenerative complication of T1D and T2D ultimately leading to end-stage renal disease.

Exosome 50–100-nm lipid vesicles excreted directly from the cell plasma membrane and are transported in the blood or urine.

Gene array Silica chips containing oligonucleotide tags designed to capture specific mRNA sequences.

Glomerulosclerosis An aspect of diabetic nephropathy which entails glomerular hypertrophy, microvascular endothelial dysfunction, and loss of podocytes.

High-throughput sequencing A technology which sequences every tagged RNA molecule in a given sample.

MicroRNA 22–25 nucleotide noncoding RNA sequences posttranscriptionally regulate protein production.

MicroRNA family A group of miRNA which contain identical seed regions which allow them to target mRNA for posttranscriptional regulation.

mRNA A sequence that is transcribed from the genome and translated into protein by ribosomes.

Plasma Blood preparation with blood cells removed by centrifugation.

qRT-PCR panel Fixed arrays of specific mRNA or miRNA preloaded on commercial qRT-PCR plates.

Serum Blood preparation with cells removed by clotting followed by centrifugation.

Tubulointerstitial fibrosis Deposition and accumulation of extracellular matrix proteins in interstitial space of the kidney.

Introduction

MicroRNA research has flourished since the initial discovery of their presence in humans. Recent years have seen numerous volumes published on miRNA and their relevance to specific areas of transcriptional and medical biology. This chapter aims to highlight the developments in a forefront region of miRNA research, miRNA biomarkers. Specifically, the detection of miRNA in biofluids including urine and plasma will be discussed. These topics will be focused on their relevance to the early diagnosis of diabetic nephropathy and also the identification of those at risk of progressive diabetic renal disease. Consideration will also be given to potential benefit of this field to nondiabetic nephropathies.

Need for Better Biomarkers

Clinicians are recommended to test T2D patients at the time of diagnosis and every year after this for urinary protein exceeding 30–300 mg/24 h thus indicating impaired renal function as defined by microalbuminuria. While this test is well established and a rather accurate measure of renal and, in particular, glomerular health, the test fails to identify those at risk of DN, and therefore no action can be taken to prevent its onset. Moreover, tissue and cellular damage incurred during DN is progressive and currently irreversible (Dronavalli et al. 2008). There is a pertinent need to improve evaluation of both renal damage and also identification of diabetic subjects at risk of developing DN.

A number of experimental markers of renal damage are reported in the literature or are available to clinicians but are generally either cumbersome or have known inaccuracies. Creatinine clearance has a tendency to over- or underestimate renal function in both healthy and diseased subjects (Wuyts et al. 2003). Furthermore, there are several formulae for calculating creatinine clearance with each method accounting for different physiological parameters and possessing differing accuracies. Direct GFR measurement by way of monitoring clearance of infused inulin is

both cumbersome and prone to inaccuracies (Hsu and Bansal 2011). However, inulin clearance is considered a more accurate, earlier marker for renal damage than albuminuria or creatinine clearance alone. However, there are issues with inter-lab consistency of results due largely to management of this rigorous test.

There are numerous proteins or metabolites that indicate potential to be used as predictive markers to identify those at risk of going on to develop DN. The presence of a number of podocyte proteins can indicate podocyte damage and stress, the presence and concentration of a number of immunoglobulin G isoforms can both predict and diagnose progression of glomerular damage, and KIM-1, a marker of tubular epithelial cell proliferation, can indicate tubular damage (Moresco et al. 2013; Wang et al. 2013a). There are many other markers reported in the literature that demonstrate effectiveness in diagnosing DN or identifying those at risk of development. However, a number of these markers may not be truly specific to DN and may indicate other forms of renal disease as is the case with transferring and primary glomerulonephritis (Wang et al. 2013a). Additionally, many of these markers are expensive to analyze and possess inter-assay variability due to testing methods and protein stability.

Thus, the need for highly stable, cost-effective, and reproducible markers of DN and pre-DN subjects is present. MicroRNA is well poised to fill this need. MicroRNA is highly stable at room temperature (Mall et al. 2013), their dysregulation is often cell/tissue specific (Babak et al. 2004), and their expression profiles can indicate DN or the potential for developing DN (Zampetaki et al. 2010). Furthermore, targeted miRNA analysis is comparatively cheap, reproducible, and well suited to high-throughput qRT-PCR analysis such as that found in large diagnostic labs. Finally, isolation of these novel biomarkers from either urine or plasma is relatively straightforward and quite forgiving compared to numerous other protein- or metabolite-type biomarkers, both established and experimental (Fig. 1).

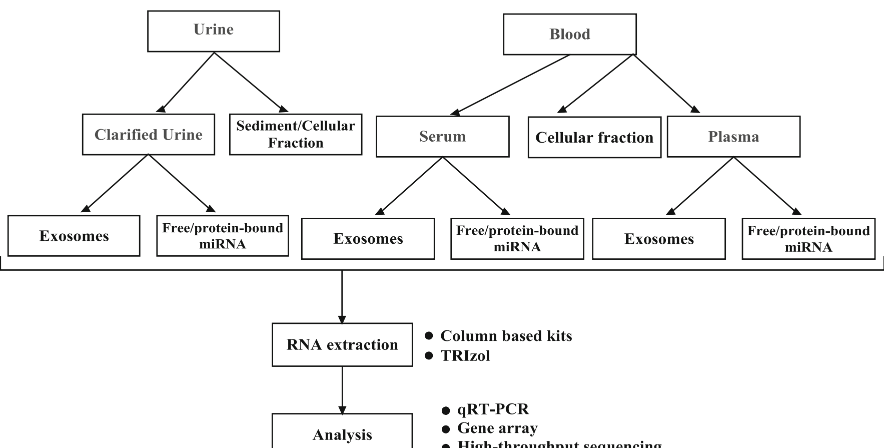

Fig. 1 Depiction of the various samples obtainable for miRNA analysis from both blood and urine and the methods by which they may be analyzed

miRNA in Biofluids

Biofluids, in particular plasma, are attractive for the diagnosis of a number of pathologies as it may carry indications of disease in any region of the body given that the appropriate markers are observed. While this concept holds true for well-defined conditions such as cancer, where miRNA research has flourished, the same cannot be said for more complex diseases such as type 2 diabetes (Chen et al. 2008). The multifactorial nature of diabetes results in damage to an array of tissues, and as such a single biomarker is unlikely to provide sufficient information on the state of disease. Accordingly, miRNA, which often possesses tissue- or cell-type-specific profiles, may provide an excellent alternative to traditional clinical biomarkers (Babak et al. 2004).

miRNA is remarkably stable in plasma as their ~22-nucleotide structure is both mechanically and thermodynamically sound. This inherent stability is further enhanced by being paired with protective chaperones such as Ago2 or being encased in small lipid vesicles such as exosomes (Creemers et al. 2012). While the regulatory mechanisms of exosomal and extracellular miRNA are currently unclear, current research indicates that the profile of the miRNA populations is both cell specific and disease specific. These plasma-borne miRNAs have also been demonstrated to be taken up by nonhost cells and exert regulatory effects on the target cell translational processes in vitro (Momen-Heravi et al. 2014). miRNA may therefore play a role in communication between tissues in both homeostatic and pathological processes and thus further enhance the prospect of miRNA biomarker therapeutics.

While it is true that plasma represents the whole body miRNA secretosome, urine more specifically represents that of the kidney and its components. The cells of the kidney secrete exosomal and protein-bound miRNA into the urinary stream and are equally as stable as those in plasma (Mall et al. 2013). Analysis of urinary miRNA has the potential to specifically identify the health of the diabetic kidney and may also provide a measure of the likelihood that a patient will progress to diabetic nephropathy.

miRNA in Renal Disease

miRNAs have rapidly proven themselves as important regulators in a vast array of tissues in addition to playing roles in development and homeostasis (Sayed and Abdellatif 2011; Guo et al. 2014). The kidney is no exception, with much work published in regard to a great number of nephropathies. In particular, renal carcinomas (Nakada et al. 2008), PKD (Wessely and Tran 2012), IgAN (Szeto and Li 2014), and, importantly, DN (McClelland and Kantharidis 2014) have all been found to be mediated in part by a growing number of miRNAs.

A small group of miRNAs have been repeatedly shown to be involved in glomerulosclerotic and tubulointerstitial injury. These major renal pathologies are predominately mediated by the resident cells of the glomerular and tubular structures of the kidney, respectively (Schena and Gesualdo 2005). Surprisingly, in the diabetic milieu, these cells exhibit similar changes in cellular physiology such as increased extracellular matrix production and concomitant reduction on ECM turnover

proteins, increased growth factor production and secretion, cellular hypertrophy, loss of cell-cell contacts, and disturbances to basement membranes. The processes have been shown to be dependent upon dysregulation of a central set of miRNAs. This list includes the miR-29 and miR-200 families, miR-192/215 and miR-21 (McClelland et al. 2014). There have been a considerable number of miRNAs added to this list in recent years and this list continues to grow. Some of these, such as the miR-30 and let-7 families, appear in biofluids alongside miRNA of no known relevance to DN (Zhou et al. 2013). The presence of these miRNAs suggests potential to be effective biomarkers for the detection and diagnosis of DN.

Despite the state of knowledge regarding miRNA and DN, there have been surprisingly few studies specifically investigating their use as biomarkers for diabetic kidney disease, though recent years have seen increasing interest in the field. A number of studies have demonstrated the validity of biofluid miRNA profiles in not only diagnosing DN but also identifying those at risk of developing DN. These studies clearly authenticate the validity of miRNA as potential biomarkers in the diagnosis of DN and those at risk of developing DN. Validation of these miRNA biomarker profiles has obvious implications for early detection and subsequently the timely interventions required to prevent or attenuate development and progression of an otherwise degenerative condition.

Circulating miRNA

Circulating miRNAs have been the subject of a number of recent studies. These studies have focused on both diagnosis of diabetes in general and also of DN. A number of studies have also sought to identify miRNA in plasma or serum which may differentially diagnose various stages of diabetic nephropathy.

The seminal paper on serum miRNA signatures in disease was conducted by Chen et al. (2008). The authors performed Solexa sequencing on serum and blood cell fractions from subjects with lung cancer, colorectal cancer, and diabetes and healthy controls. Remarkable similarity in the miRNA profiles from both plasma and cellular fractions between each of the diseased groups was reported. Specifically, 23 miRNAs were detected in the serum of those with lung cancer or diabetes that were not detected in the sera of healthy controls. Conversely, there were 16 miRNAs detected in healthy sera that were not detected in that from diseased subjects. Of particular interest, there were 39 miRNAs uniquely detected in sera from lung cancer patient serum compared to diabetic samples and with three unique to diabetic sera.

The study also provided compelling evidence that miRNAs are differentially expressed in serum and are therefore suitable biomarkers for the noninvasive determination of disease states. Although there were 69 miRNAs detected in both serum and cellular fractions of lung cancer patients, there were further 28 and 63 unique miRNAs detected in normal and diseased serum, respectively. Furthermore, when miRNA profiles were compared within the lung cancer group, there were 57 common miRNAs yet 76 and 15 unique miRNAs in plasma and cellular fractions, respectively. Likewise, there were 84 common miRNAs between plasma and cellular fraction from

diabetic subjects and 17 and 27 unique miRNAs in these fractions, respectively. This study provided the catalyst for research into biofluid-borne miRNAs and their role as both signaling molecules and as clinical biomarkers themselves.

A large prospective study by Zampetaki et al. followed over 800 subjects for 15 years and revealed significant changes in five miRNAs in subjects who developed DM over the course of the study (Zampetaki et al. 2010). Additionally, 8 of 13 miRNAs analyzed were dysregulated in those with DM at the onset of the study. However, a number of these, namely, miR-197, miR-28-3p, and miR-150, were not reported in the Chen study though this may be an artifact of analysis platforms and their relative sensitivities or, more importantly, disease-specific expression (Chen et al. 2008). Alternatively, as Zampetaki utilized plasma miRNA as opposed to serum miRNA, these discrepancies may lie in sample preparation. The authors also reported that miR-126 secretion was decreased in high glucose-treated endothelial cells. These changes also correlate with onset and diagnosis of peripheral vascular disease in the study cohort.

Due to the role of endothelial dysfunction in the glomerular aspects of DN, decreases in miR-126 in urine may prove to be an effective marker for glomerular injury (Karalliedde and Gnudi 2011). However, miR-126 is elevated in the urine of subjects with DN compared to non-nephropathic diabetics or healthy controls (Liu et al. 2014). In further contrast to the study by Zampetaki, these investigators found no difference in urinary miR-126 between healthy controls and DM subjects without DN. Considering the in vitro data from the Zampetaki study, it is likely that decreased circulating miR-126 is likely more indicative of cardiovascular risk/ disease rather than DN specifically. However, given clear links between cardiovascular disease and DN, the expression of this miRNA remains important (Van Buren and Toto 2011). Regardless, circulating miR-126 has been recently supported as a suitable biomarker for detecting those that are susceptible to DM (Zhang et al. 2013).

A study by Kong et al. also sought to identify a miRNA signature in serum from patients with and without diabetes using seven T2D-related miRNAs (Kong et al. 2011). All seven miRNAs were found to be elevated in diabetic patients compared to those who were deemed to be at risk of diabetes due to excessive BMI or family history. Interestingly, these miRNAs, with the exception of miR-375, were detected at similar levels in at-risk individual as those with clinically defined prediabetes. The panel of miRNAs was also used to correctly classify diabetic and nondiabetic patients in a blind test. Although the study excluded patients with known nephropathy, it is interesting to note that these miRNAs, with the exception of miR-9 and miR-375, have been implicated in renal pathology or have been identified in urine (Wang et al. 2012a Li et al. 2013; Shi et al. 2013; He et al. 2014; Huang et al. 2014). A number of these miRNAs were also not detected in the Chen study; however, this may result from differences in microarrays compared to Solexa platforms and more specifically the libraries used to generate the arrays.

miR-135a has been implicated in the development of diabetic nephropathy through targeting of TRPC1 (He et al. 2014). miR-135a was elevated in both plasma and renal biopsy material from subjects with diabetic nephropathy compared to healthy controls and those with DM thus providing a potential plasma biomarker for DN. The authors also reported changes in a number of miRNAs in those with DM

compared to controls with these changes being magnified in those with DN. Of particular interest in this study, a number of miRNAs were reported as upregulated, namely, miR-15a, miR-21, and miR-126. These miRNAs were reported as being downregulated in subjects in the Zampetaki study (Zampetaki et al. 2010). These discrepancies may result from differences in the ethnicity of the patient cohorts employed. Furthermore, as He et al. included subjects with DN, these differences may represent a shift in circulating miRNA during development of DN thus providing an indication of disease progression in any given individual. Supporting this notion, miR-34a was increased in diabetic serum in the Kong cohort which excluded diabetics with no apparent renal disease while being increased in DN subjects in the He cohort (Kong et al. 2011; He et al. 2014).

Single nucleotide polymorphisms are a popular target for studies attempting to identify genetic elements responsible for disease, particularly in those employing genome-wide association analyses. Interestingly, Zhou et al. reported potentially important genetic variation in the promoter region of let-7a-2, a member of the highly conserved let-7 family (Roush and Slack 2008; Zhou et al. 2013). The authors analyzed both miRNA and genomic material from plasma of over 260 Han Chinese, a cohort constituting subjects with DN, diabetics without DN, and also control subjects. A number of let-7 family members were more than twofold downregulated in those with DN compared to diabetics without DN. The authors also reported 22 miRNAs with dysregulated expression between DN subjects and diabetics with no DN. No data was presented for either group compared to controls with the exception of let-7a which was reported as being ~80 % upregulated in DM subjects compared to control. Interestingly, the expression of this miRNA was reduced below control levels in those with DN. This again provides a clear example of a specific miRNA whose expression levels may noninvasively indicate the state of disease in diabetic subjects.

The let-7 family are reported to have roles in diabetic renal fibrosis with their downregulation leading to derepression of TGFβRI and subsequently fibrotic signaling (Brennan et al. 2013; Wang et al. 2014). This downregulation may result from defective genomic regulatory units. Zhou et al. analyzed three SNPs in the promoter region of let-7a and reported that 50 % of DM subjects' genomes contained the CT SNP compared to only 40 % of healthy controls (Zhou et al. 2013). This association increased to 66 % in those that had progressed to DN. Both the TT and the CC phenotype alone presented no correlation with disease state. Of great importance was that 82 % of DM subjects possessed the CC/TT phenotype compared to only 68 % in controls. DN subjects were only slightly higher at 85 %. These findings, both miRNA expression profiles and SNP incidence, provide excellent measures for the diagnosis of DN and also the detection of those at risk of developing DM and identification of those that will progress to DN.

Finally, miR-199a has been detected at elevated levels in serum of those with T2D compared to healthy subjects (Yan et al. 2014). The authors confirmed GLUT4 as a target of miR-199a through in vitro gene knockdown and replacement experiments and demonstrated that miR-199a targeting of GLUT4 resulted in modulation of glucose uptake in L6 skeletal muscle cells. These findings have obvious implications to the development and progression of DN. Increased circulating miR-199a may

potentially be taken up by metabolic tissues thus decreasing GLUT4 expression and concordantly decreasing insulin sensitivity (Michael et al. 2001). The resultant increase in serum glucose has ramifications for the kidney, a tissue whose functional components are composed of cells well documented to be sensitive to chronic hyperglycemic conditions (Brownlee 2001). Clinical investigation of this miRNA in reference to diabetic nephropathy would be of value to the progression of miRNA biomarker research.

Urinary miRNA

Urine is particularly suited for determination of renal health. The cells that comprise the nephron are highly specialized, and each serves a specific function in both the filtration of plasma and the recovery and exclusion of particular metabolites (Smith 1951). As such, the resulting urinary product contains a number of biomolecules which indicate function or dysfunction of various parts of the nephron. miRNA is no exception and the quantity and type of miRNA present may indicate adverse physiology in any of these cells. This urinary miRNA may be detected both in urinary sediment and urinary exosomes which represent shed apoptotic or damaged cells and actively secreted miRNA, respectively (Szeto et al. 2012; Barutta et al. 2013). These features, combined with the relative ease of urinary miRNA purification and stability in conjunction with noninvasive availability of clinical samples, have led to a considerable number of studies in recent years analyzing miRNA from diabetic urine.

A comparative study by Szeto et al. analyzed miRNA in urinary sediments from subjects with IgAN, HTN, and DGS (Szeto et al. 2012). The authors reported significantly less miR-15 in urinary sediments from those with DGS compared to those with IgAN and HTN. There were also decreased levels of miR-21, miR-17, and miR-216a though these differences were not significant. Importantly, expression levels of a number of miRNAs correlated with indicators of renal function and damage including proteinuria, eGFR, glomerulosclerosis, and tubulointerstitial fibrosis. The rate of eGFR decline was inversely correlated with both miR-21 and miR-216a levels. miR-21 has been implicated in fibrotic signaling in proximal tubule epithelial cells and mesangial cells, while miR-216a has a role in glomerulosclerosis (Kato et al. 2010; Dey et al. 2011). Their level in urinary sediments therefore provides important information about the health of the nephron.

The miR-29 family is also implicated in renal fibrosis through regulation of ECM proteins such as collagen (Peng et al. 2013). All three members of this fibrotic family of miRNA were analyzed in urinary supernatant from DM patients with and without abnormal renal function as defined by urinary albumin concentration. Although all three miRNAs were detected in urinary supernatant, only miR-29a was found to be increased those with albuminuria compared to those without. Although miR-29a levels were positively correlated with UAER, no correlation was found with other

indicators of renal function including urea, cystatin, β2-microglobulin levels, creatinine clearance, or eGFR. It is interesting to note that miR-29a, miR-29b, and miR-29c are downregulated in renal tissues from experimental diabetes models which in part leads to increased ECM expression (Wang et al. 2012a). Increased expression in the urine is therefore unexpected. Regardless, these findings by Peng et al. are in line with those of Kong et al. who reported increased circulating miR-29a (Kong et al. 2011).

DN progresses through a number of stages typically marked by the presence and level of albuminuria (Brownlee 2001). This clinical presentation has its origins in the underlying physiology of the nephron and, in particular, the glomerulus. As such, urinary miRNA profiles may differ between patients exhibiting varying degrees of disease. Argyropoulos et al. attempted to ascertain these potential differences by analyzing miRNA expression levels in total urine from subjects with T1D who failed to develop DN over a 20-year period, those who developed DN, and those that presented intermittent or persistent microalbuminuria (Argyropoulos et al. 2013). The authors reported high-level dysregulation of a number of miRNAs between albuminuric and non-albuminuric subjects. Unique signatures were also reported for those with intermittent vs. persistent microalbuminuria and also for those that went on to develop DN over the course of the study compared to those T1D subjects that did not develop DN. Although the panels of miRNA presented in each of the comparisons are largely unique, it should be noted that these very differences highlight the possibility of highly specific miRNA signatures which exist at various stages of DN.

In addition to the unique profiles reported in each group, there are two miRNAs which were reported in multiple comparisons, namely, miR-221-3p and miR-323b-5p (Argyropoulos et al. 2013). Specifically, miR-221-3p was downregulated in those with microalbuminuria compared to those without microalbuminuria and was also decreased in those that developed DN. Conversely, miR-323b-5p was decreased in those with manifest microalbuminuria when compared to those without albuminuria. Interestingly, this miRNA was increased in those with persistent microalbuminuria compared to those with intermittent microalbuminuria indicating that the level of this miRNA may indicate the duration of disease. Although neither of these miRNAs have reported functions in diabetic nephropathy, pathway analysis reveals that miR-221-3p targets a number of genes involved in cell cycle regulation, protein synthesis, and PI3K signaling pathway and is therefore an attractive target for future research (http://diana.cslab.ece.ntua.gr/). Likewise, miR-323b-5p targets multiple genes in proximal tubule-specific pathways and pathways important for tubular function and will also be of interest to basic research (http://diana.cslab.ece.ntua.gr/).

Upon summation of the literature at the time, Yang et al. proposed a miRNA signature for urine analysis in those with DN (Yang et al. 2013). The authors highlight that increased levels of miR-377, miR-192, miR-216/217, and miR-144 in addition to decreased miR-21 and miR-375 may be most indicative of renal health in diabetic patients. This hypothesis was based on previously reported urinary analysis of these miRNAs in addition to their reported roles in cellular physiology. Although there was no clinical study to support this notion, the paper provides a good platform for future urinary miRNA biomarker work.

Exosomal miRNA

Exosomes are 50–100-nm lipid microvesicles that are extruded from the cellular plasma membrane (Raposo and Stoorvogel 2013). Their role in cellular biology and the modes by which they are regulated are relatively unknown. In recent years, evidence has emerged indicating that exosomes may play a fundamental role in cellular and physiology (Camussi et al. 2010). These inferences come from the observation that proteomic and RNA profiles of microvesicles differ significantly to that of their host cells (Koppers-Lalic et al. 2014). Importantly, miRNA profiles also differ significantly suggesting that miRNA may be trafficked between cells and tissues as a means of communication between cells and tissue (Xiao et al. 2012). Indeed, in vitro and in vivo experiments have demonstrated that exosome-bound miRNA may be taken up by non-donor cells and exert posttranscriptional regulatory control in a manner identical to miRNA transcribed from the target cells' genome (Rana et al. 2013).

Although their presence in, and secretion to, biofluids is likely required for proper physiological function, the isotype and quantity of these molecules vary with differing stages of disease and are therefore important for diagnosis (Barutta et al. 2013; Lv et al. 2013a). Importantly, over-/underexpression of specific miRNA species in biofluids may prime individuals for development of disease by modulating key signaling pathways in target cell types.

Exosomes are secreted from cells in both homeostatic and pathological conditions and are relatively stable (Raposo and Stoorvogel 2013; Ge et al. 2014). As such, exosomal miRNA provides an attractive target for clinical biomarker evaluation. Their purification is relatively simple when compared to blood-borne miRNA, and sample collection is completely noninvasive and may be collected at any time (Cheng et al. 2014). Despite the great advantages to be had in the use of exosomal miRNA for the diagnosis of diabetes and more specifically DN, their potential has gone largely unappreciated. There are a great number of reviews discussing the advantages of this approach yet surprisingly few research publications investigating their potential; however, this field is quickly gaining traction.

Progress has recently been bolstered with a technical study by Cheng et al. which determined the efficiency of a number of exosomal isolation methods along with analysis of RNA profiles of exosomes from each method (Cheng et al. 2014). The authors demonstrated that a simple, on-column protocol provided small RNA of sufficient quantity and quality to allow high-throughput massively parallel sequencing. Additionally, the on-column system by Norgen required much shorter processing times (1.5 h vs. 4–4.5 h) and also much less sample volumes (5–10 mL vs. >20 mL), characteristics well suited to diagnostic labs where time, equipment, and samples may be limited. Although high-throughput sequencing is currently not employed in most diagnostic labs, that the authors were able to employ this analysis clearly demonstrates that the RNA obtained is more than suitable for more conventional qRT-PCR applications. It should also be noted that high-throughput sequencing is largely an exploratory technique and as such will not be required once a diagnostic profile has been established.

The study has provided an excellent platform from which future studies may be launched. Following the removal of low-read signals, a total of 66 abundant miRNAs were detected in exosomes isolated with the Norgen kit and 166 miRNAs from ultracentrifugation-based protocols (Cheng et al. 2014). The vast majority of these miRNAs were detected across all subjects in the study thus validating not only consistency in the isolation protocols but also consistency in miRNA expression in healthy individuals. This study therefore provides an important reference for which future studies may compare samples from diseased individuals.

In a study utilizing a cohort containing subjects with DN, FSGS, and IgAN, Lv et al. isolated exosomes by ultracentrifugation from cleared urine (Lv et al. 2013). They reported increasing levels of miR-29c and miR-200c between each of the groups with DN subjects having the lowest expression and IgAN subjects presenting the highest. None of these changes reached statistical significance nor were there any control subjects in the study cohort. Nonetheless, this study demonstrated that exosomal miRNA expression profiles may shift with differing renal pathologies highlighting that microvesicle-bound miRNA may prove to be effective for determining disease state and possibly progression. The authors also reported remarkable stability of exosomal RNA indicating that exosomes provide a highly stable source of miRNA for biomarker analysis (Lv et al. 2013).

In a follow-up report, Lv et al. again reported changes in miR-29c and miR-200c levels in urinary exosomes (Lv et al. 2013). Here, the study cohort comprised those with DN, FSG, IgAN, membranous nephropathy, and mesangial proliferative glomerulosclerosis and healthy controls as defined by UAER. Unfortunately, all pathological groups were pooled in the reported data with no delineation or comparisons drawn between the different groups. Regardless, all three members of the miR-29 family, in addition to miR-200a/b/c, were significantly downregulated in the urinary exosome fractions from the CKD group compared to controls (Lv et al. 2013). Interestingly, when comparing CKD samples based on fibrotic scoring, they found that miR-29a/c and miR-200b/c were significantly higher in those with mild fibrosis compared to those with moderate to severe fibrosis. Although no comparisons were drawn directly between fibrotic and control subjects, it is interesting to speculate, due to the established roles of these miRNAs in fibrotic signaling, that these miRNAs continue to decrease as fibrosis progresses (Wang et al. 2012a; Xiong et al. 2012). Indeed, there was a significant correlation between tubulointerstitial fibrosis and miR-29c levels with the remaining miRNAs showing nonsignificant correlations to fibrotic score.

Barutta et al. analyzed miRNA content of urinary exosomes from those with incipient DN as marked by microalbuminuria and T1D without DN and healthy controls (Barutta et al. 2013). qRT-PCR panel analysis revealed 22 dysregulated miRNAs in microalbuminuric and normoalbuminuric subjects compared to healthy controls. Regrettably, this list was not published. However, the authors did report upregulation of miR-145 and miR-130a in microalbuminuric exosomes compared to both T1D and control samples. The expression of these miRNAs was also increased in T1D subjects compared to controls indicating that these miRNAs may be suitable

Table 1 miRNA which may be prognostic markers for development of DM

miRNA	Change	Source	Prognosis	References
miR-126	Down	Plasma	Marker for risk of developing DM	(Guo et al. 2014)
miR-15a	Down	Plasma	Marker for risk of developing DM	(Guo et al. 2014)
miR-223	Down	Plasma	Marker for risk of developing DM	(Guo et al. 2014)
miR-28-3p	Up	Plasma	Marker for risk of developing DM	(Guo et al. 2014)
miR-29b	Down	Plasma	Marker for risk of developing DM	(Guo et al. 2014)

to track progression of renal damage in those with T1D, at least to the stage of microalbuminuria. Conversely, miR-155 and miR-424 were decreased between groups with microalbuminuria subjects expressing the lowest levels of these miRNA species and healthy controls the highest. Again these expression profiles may be utilized for tracking renal health in those with T1D.

Diagnostic and Prognostic Profiles

A number of the above studies have identified miRNAs which have potential to be used as either prognostic or diagnostic biomarkers (Tables 1 and 2). Of the studies reviewed here, that by Zampetaki holds special significance. This prospective study followed over 800 individuals to the completion of the 15-year study (Zampetaki et al. 2010). Importantly, 19 subjects developed T2D over the final 10 years of the study. These individuals were found to have altered expression of a number of miRNAs at the outset of the study therefore providing a potential miRNA signature for identification of individuals that may develop diabetes in the near future (Table 1). However, this profile finds little support in subsequent studies (Table 3). Indeed, miR-15a, miR-126, and miR-29b are reported as being upregulated (Kong et al. 2011; Argyropoulos et al. 2013; Peng et al. 2013; He et al. 2014). Regardless, the size, design, and length of the study, especially in comparison to other studies reviewed here, add credit to these findings, and as such, subsequent follow-up studies of a similar nature which will either support or refute the findings of Zampetaki are of great importance.

Urinary miRNA profiles were also identified by Argyropoulos who utilized samples from 40 subjects from the Pittsburgh Epidemiology of Diabetes Complications study which entailed a >20-year follow-up period (Argyropoulos et al. 2013). The selected cohort contained four groups of ten subjects each comprising those with T1D that did not develop DN, those who developed DN, those who displayed intermittent microalbuminuria, and those with persistent microalbuminuria. This study resulted in three panels of miRNA which potentially identify microalbuminuric T1D subjects when compared to non-albuminuric T1D subjects and differentiate between intermittent and persistent microalbuminuria and T1D subjects with overt DN compared to those without (Table 2). Surprisingly, the vast majority of miRNAs identified in these panels were not reported at significant levels

Table 2 miRNAs which are differentially expressed in various biofluids and display promise as diagnostic markers for various stages of DM and, more specifically, DN

miRNA	Change	Source	Disease/stage	References
let-628-5p	Up	Urine	Differentiates intermittent from persistent microalbuminuria	(Rana et al. 2013)
miR-1224-3p	Up	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-124a	Up	Serum	Marks progression from pre-DM to T2D	(Karalliedde and Gnudi 2011)
miR-126	Up	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-135a	Up	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-141-3p	Up	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-146a	Up	Serum	Marks progression from pre-DM to T2D	(Karalliedde and Gnudi 2011)
miR-15a	Up	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-17	Up	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-17-5p	Up	Urine	Differentiates intermittent from persistent microalbuminuria	(Rana et al. 2013)
miR-188-3p	Down	Urine	Marks microalbuminuria	(Rana et al. 2013)
miR-1912	Up	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-1913	Up	Urine	Marks microalbuminuria	(Rana et al. 2013)
miR-192	Up	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-194-1	Down	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-205	Down	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-21	Up	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-214-3p	Up	Urine	Marks microalbuminuria	(Rana et al. 2013)
miR-215	Down	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-221-3p	Down	Urine	Marks microalbuminuria	(Rana et al. 2013)
miR-221-3p	Down	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-222-3p	Up	Urine	Differentiates intermittent from persistent microalbuminuria	(Rana et al. 2013)

(*continued*)

Table 2 (continued)

miRNA	Change	Source	Disease/stage	References
miR-29a	Up	Serum	Marks progression from pre-DM to T2D	(Karalliedde and Gnudi 2011)
miR-29a	Up	Urine	Differentiates intermittent from persistent microalbuminuria	(Nakamura et al. 2000)
miR-29b-1-5p	Up	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-29c	Up	Urine	Differentiates intermittent from persistent microalbuminuria	(Nakamura et al. 2000)
miR-30b	Up	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-30d	Up	Serum	Marks progression from pre-DM to T2D	(Karalliedde and Gnudi 2011)
miR-323b-5p	Down	Urine	Marks microalbuminuria	(Rana et al. 2013)
miR-323b-5p	Up	Urine	Differentiates intermittent from persistent microalbuminuria	(Rana et al. 2013)
miR-34a	Up	Serum	Marks progression from pre-DM to T2D	(Karalliedde and Gnudi 2011)
miR-34a	Down	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-355-5p	Up	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-373-5p	Up	Urine	Marks microalbuminuria	(Rana et al. 2013)
miR-373-5p	Down	Urine	Differentiates intermittent from persistent microalbuminuria	(Rana et al. 2013)
miR-375	Up	Serum	Marks progression from pre-DM to T2D	(Karalliedde and Gnudi 2011)
miR-377	Up	Serum	Changes mark the presence of DM and increase with progression of DN	(Koppers-Lalic et al. 2014)
miR-424-5p	Up	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-429	Up	Urine	Marks microalbuminuria	(Rana et al. 2013)
miR-433	Up	Urine	Differentiates intermittent from persistent microalbuminuria	(Rana et al. 2013)
miR-486-3p	Up	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-520h	Down	Urine	Differentiates intermittent from persistent microalbuminuria	(Rana et al. 2013)
miR-524-5p	Down	Urine	Marks microalbuminuria	(Rana et al. 2013)

(*continued*)

Table 2 (continued)

miRNA	Change	Source	Disease/stage	References
miR-552	Up	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-589-5p	Down	Urine	Differentiates intermittent from persistent microalbuminuria	(Rana et al. 2013)
miR-619	Up	Urine	Marks overt diabetic nephropathy	(Rana et al. 2013)
miR-638	Up	Urine	Marks microalbuminuria	(Rana et al. 2013)
miR-765	Up	Urine	Marks microalbuminuria	(Rana et al. 2013)
miR-9	Up	Serum	Marks progression from pre-DM to T2D	(Karalliedde and Gnudi 2011)
miR-92a-3p	Down	Urine	Differentiates intermittent from persistent microalbuminuria	(Rana et al. 2013)
miR-92b	Up	Urine	Marks microalbuminuria	(Rana et al. 2013)

in any of the studies reviewed here. This may merely be the result of T1D pathology compared to T2D (Table 3).

Conversely, these discrepancies may originate from differences in the miRNA libraries used in either high-throughput sequencing, qRT-PCR arrays, or gene arrays which are based on the miRBase database. This is especially likely when one considers that from miRBase 10, which the Chen and Zhou studies utilized, to miRBase 18, which Argyropoulos aligned their data sets to, there have been 379 miRNA records modified, 1217 miRNAs added, and 18 miRNAs deleted from the miRBase libraries (http://www.mirbasetracker.org/). It is also important to note that studies using commercial qRT-PCR arrays and gene arrays may be missing a considerable proportion of miRNA as commercial products are rarely updated as often as miRBase.

Notable Differences in Profiles

As previously discussed, urine and urine-derived exosomes are particularly well suited to diagnosis of renal health as they are rich in nephron miRNA as compared to plasma which may contain miRNA from any number of tissues (Lorenzen and Thum 2012). It is therefore pertinent that the miRNA from these distinct sources be compared in order to arrive at a consensus for what may be viable markers for diabetic nephropathy and its various stages. However, despite the large number of miRNAs reported among the above studies, few miRNAs have been reported to exist at significant levels in more than one biofluid, an observation which may find clarity

Table 3 Comparison of miRNA reported to be differentially expressed in various biofluids in the reviewed DN- and DM-related studies

miRNA	Serum/plasma (references)	Urine (references)	Exosomes (references)
let-628-5p	–	Up (Rana et al. 2013)	–
let-7	Down (Glowacki et al. 2013)	—	–
miR-1224-3p	–	Up (Rana et al. 2013)	–
miR-124a	Up (Karalliedde and Gnudi 2011)	–	–
miR-126	Down (Guo et al. 2014), up (Koppers-Lalic et al. 2014)	–	–
miR-130a	–	–	Up (Momen-Heravi et al. 2014)
miR-135a	Up (Koppers-Lalic et al. 2014)	–	–
miR-141-3p	–	Up (Rana et al. 2013)	–
miR-145	–	–	Up (Momen-Heravi et al. 2014)
miR-146a	Up (Karalliedde and Gnudi 2011)	–	–
miR-150	Down[a] (Guo et al. 2014)	–	–
miR-155	–	–	Down (Momen-Heravi et al. 2014)
miR-15a	Down (Guo et al. 2014), up (Koppers-Lalic et al. 2014)	Down (Michael et al. 2001)	–
miR-17	Up (Koppers-Lalic et al. 2014)	Up (Rana et al. 2013)	–
miR-188-3p	–	Down (Rana et al. 2013)	–
miR-191	Down (Guo et al. 2014), up (Glowacki et al. 2013)	–	–
miR-1912	–	Up (Rana et al. 2013)	–
miR-1913	–	Up (Rana et al. 2013)	–
miR-1915	Up (Glowacki et al. 2013)	–	–
miR-192	Up (Koppers-Lalic et al. 2014)	–	–
miR-194-1	Down (Koppers-Lalic et al. 2014)	–	–
miR-197	Down (Guo et al. 2014)	–	–
miR-200	–	–	Down (Szeto and Li 2014; Wang et al. 2011)
miR-205	Down (Koppers-Lalic et al. 2014)	–	–
miR-20b	Down (Guo et al. 2014)	–	–

(*continued*)

Table 3 (continued)

miRNA	Serum/plasma (references)	Urine (references)	Exosomes (references)
miR-21	Down (Guo et al. 2014), up (Koppers-Lalic et al. 2014)	–	–
miR-214	–	Up (Rana et al. 2013)	–
miR-215	Down (Koppers-Lalic et al. 2014)	–	–
miR-221-3p	–	Down (Rana et al. 2013)	–
miR-222-3p	–	Up (Rana et al. 2013)	–
miR-223	Down (Guo et al. 2014)	–	–
miR-24	Down (Guo et al. 2014)	–	–
miR-26a	Down (Glowacki et al. 2013)	–	–
miR-28-3p	Up (Guo et al. 2014)	–	–
miR-29[b]	Down (Guo et al. 2014), up (Karalliedde and Gnudi 2011)	Up (Nakamura et al. 2000), up (Rana et al. 2013)	Down (Szeto and Li 2014; Wang et al. 2011)
miR-30[b]	Up (Karalliedde and Gnudi 2011, Koppers-Lalic et al. 2014, Glowacki et al. 2013)	–	–
miR-320[b]	Down[a] (Guo et al. 2014), up (Glowacki et al. 2013)	–	–
miR-323b-5p	–	Down (Rana et al. 2013)	–
miR-323b-5p	–	Up (Rana et al. 2013)	–
miR-34a	Up (Karalliedde and Gnudi 2011), down (Koppers-Lalic et al. 2014)	–	–
miR-355-5p	–	Up (Rana et al. 2013)	–
miR-363	Down (Glowacki et al. 2013)	–	–
miR-3665	Up (Glowacki et al. 2013)	–	–
miR-373-5p	–	Up/down (Rana et al. 2013)	–
miR-375	Up (Karalliedde and Gnudi 2011)	–	–
miR-377	Up (Koppers-Lalic et al. 2014)	–	–
miR-3940-5p	Up (Glowacki et al. 2013)	–	–
miR-3960	Up (Glowacki et al. 2013)	–	–
miR-424	–	–	Down (Momen-Heravi et al. 2014)
miR-424-5p	–	Up (Rana et al. 2013)	–
miR-429	–	Up (Rana et al. 2013)	–

(*continued*)

Table 3 (continued)

miRNA	Serum/plasma (references)	Urine (references)	Exosomes (references)
miR-433	–	Up (Rana et al. 2013)	–
miR-4429	Up (Glowacki et al. 2013)	–	–
miR-4454	Up (Glowacki et al. 2013)	–	–
miR-4466	Up (Glowacki et al. 2013)	–	–
miR-4488	Up (Glowacki et al. 2013)	–	–
miR-4707-5p	Up (Glowacki et al. 2013)	–	–
miR-486-3p	–	Up (Rana et al. 2013)	–
miR-486-5p	Down[a] (Guo et al. 2014), up (Glowacki et al. 2013)	–	–
miR-520h	–	Down (Rana et al. 2013)	–
miR-524-5p	–	Down (Rana et al. 2013)	–
mir-552	–	Up (Rana et al. 2013)	–
miR-589-5p	–	Down (Rana et al. 2013)	–
miR-619	–	Up (Rana et al. 2013)	–
miR-638	–	Up (Rana et al. 2013)	–
miR-765	–	Up (Rana et al. 2013)	–
miR-9	Up (Karalliedde and Gnudi 2011)	–	–
miR-92a-3p	–	Down (Rana et al. 2013)	–
miR-92b	–	Up (Rana et al. 2013)	–

[a]Reported as a nonsignificant trend
[b]miRNAs of the same family are listed as a single miRNA species

when considering the potential source of miRNAs in these biofluids (Table 3). Exceptions to this observation are miR-15a and the miR-29 family.

miR-15a was reported by Zampetaki to be downregulated in serum, while He reported this miRNA to be upregulated (Table 3; Zampetaki et al. 2010; He et al. 2014). This difference may lie in the analysis of serum versus plasma as serum preparation requires the clotting of blood before generation of a cleared supernatant (Luque-Garcia and Neubert 2007). The clotting process required to produce serum may induce release of miRNA either in miRNA-protein complexes or in exosomes which are not cleared by the low-speed centrifugation required to remove the cellular fraction (Hunter et al. 2008; Duttagupta et al. 2011; Wang et al. 2012c). This highlights the need for standardization of sample preparation

for biofluid miRNA studies. Although the Szeto data supports that of Zampetaki, it must be noted that the Szeto study did not draw comparisons to control samples but to nondiabetic forms of nephropathy, namely, IgAN and HTN (Szeto et al. 2012). While this is important, control samples are required in future studies to draw conclusion about the validity of changes in miRNA as being indicative of disease, particularly DN.

There is little congruency in the changes of miR-29 family members across the various sample sources (Table 3). For example, miR-29b was reported to be downregulated in serum by Zampetaki and in urinary exosomes by LV, while Argyropoulos reported upregulation in urine (Zampetaki et al. 2010; Argyropoulos et al. 2013; Lv et al. 2013). However, it should be noted that Argyropoulos utilized uncleared urine for miRNA analysis and samples were also obtained from type 1 diabetics, while all other studies reviewed here have utilized samples from type 2 diabetics. Furthermore, uncleared urine may contain considerable cellular material especially in the case of those with advanced nephropathy (Detrisac et al. 1983; Nakamura et al. 2000). It is therefore important to remove this cellular sediment, however small, to avoid occlusion of any obtained data. Furthermore, as highlighted by Szeto, various nephropathies produce differing miRNA profiles (Szeto et al. 2012). This gains particular importance with the Lv study which grouped subjects with DN, FSGS, IgAN, and membranous nephropathy into a single "CKD" group (Lv et al. 2013). Although the data reported by Lv is supported by a number of experimental studies, it is important to report data from individual phenotypes to enable proper identification of disease-specific biomarkers.

There are further differences in changes of particular miRNA reported in serum studies including miR-191, miR-21, the miR-320 family, miR-34a, and miR-486-5p (Table 3). The prospective study by Zampetaki reported decreased levels of miR-191 in those that went on to develop T2D during the course of the 20-year study (Zampetaki et al. 2010). Conversely, Zhou reported that miR-191 was increased in T2D subjects that had progressed to DN compared to those that had not (Zhou et al. 2013). However, the authors did not provide comparisons to control samples, and RNA extraction was performed on whole blood which has obvious implications for the observed miRNA profile. Given the differences in design study and sample preparation, it is difficult to speculate which directional change in miR-191 is most representative of disease state, and therefore further studies are required to clarify this. miR-21 was reported as downregulated in those that developed DM in the Zampetaki study which is contrary to experimental data for this miRNA in both in vitro and in vivo models of diabetic nephropathy (Zampetaki et al. 2010; Denby et al. 2011; Chau et al. 2012; Dey et al. 2012). On the other hand, He et al. found that miR-21 is increased in those with DM compared to controls with further increases seen in those with DN compared to those without DN, a finding supported by Glowacki et al. who observed increased circulating miR-21 in renal allograft subjects (Glowacki et al. 2013; He et al. 2014).

There is a marked difference in the level of miR-34a between the Kong and He studies (Table 3). Kong reported that miR-34a is decreased in serum of those with DM and is further decreased in DN subjects (Kong et al. 2011). Conversely, He

reported that miR-34a was increased in T2D subjects compared to those with prediabetes as defined by OGGT/FPG and those susceptible to T2D (He et al. 2014). This difference is unlikely to be a result of cohort ethnicity as both studies were conducted with Chinese populations. Furthermore, both studies utilized patient serum for their analysis. As with many miRNA biomarker studies, the cohorts enlisted were much smaller than what is typically seen in most other cohort-based studies. The cause of this is generally twofold. Healthy control biopsy material is generally difficult to obtain due to the invasive nature of renal biopsy collection and therefore limits the size and number of studies. Another major consideration is the cost involved in performing analysis of large numbers of samples. As this field is still largely exploratory, it requires utilization of gene arrays or high-throughput sequencing platforms which are costly to run and also require specialist knowledge to assemble and analyze the large amounts of data obtained.

Potential Applications for Other Nephropathies

There have been a number of studies concerning miRNA biomarkers in a wide array of nondiabetic nephropathies (Table 4). Of the studies previously reviewed here, that by Szeto demonstrated differential levels of urinary miRNA species in HTN, DGS, and IgAN (Szeto et al. 2012). Specifically, miR-17 was uniquely upregulated in urine from those with IgAN while there was a nonsignificant increase in the levels of both miR-21 and miR-216a in HTN urine samples. There have been a considerable number of studies into miRNA biomarkers for various nephropathies in urine and plasma, a selection of which will be discussed here.

A study by Neal et al. sought to measure miRNA levels in plasma and urine from subjects in various stages of nondiabetic CKD (Neal et al. 2011). The authors found that not only did the total concentration of miRNA decrease as disease worsened but that the levels of a number of circulating miRNAs were inversely correlated with renal function. Furthermore, a number of these miRNAs were found to be downregulated in various groups compared to controls. Of the miRNAs analyzed, only miR-638 displayed changes in detectable levels contrary to those reported previously in diabetic nephropathy albeit from a different source (Argyropoulos et al. 2013). Interestingly, the levels of these miRNAs were largely unchanged in urine from the various study groups indicating that the changes in plasma miRNA may be originating distal to the kidney and are likely the result of increased blood toxicity due to impaired renal function.

In another study comparing various nephropathies, Wang et al. compared miRNA levels in urinary sediment from subjects with DGS, MCN, FSGS, and MGN (Wang et al. 2013b). Considering the studies' use of urinary sediment rather than clarified urine, not surprisingly, the miRNAs reported to be most dysregulated are those that have been well studied in regard to renal cell dysfunction and fibrotic signaling, namely, miR-29a, miR-192, and miR-200a/c (Chung et al. 2010; Wang et al. 2011, 2012c). With the exception of miR-29a and miR-200a, these miRNAs were found to be downregulated across all groups compared to controls.

Table 4 Summary of miRNA reported to be dysregulated in various nephropathies outside of DN

miRNA	Change	Nephropathy	Reported comparison	References
miR-223	Up	PKD	Differential diagnosis of PKD to non-PKD CKD	(Ben-Dov et al. 2014)
miR-199a	Up	PKD	Differential diagnosis of PKD to non-PKD CKD	(Ben-Dov et al. 2014)
miR-199b	Up	PKD	Differential diagnosis of PKD to non-PKD CKD	(Ben-Dov et al. 2014)
miR-146a	Up	Lupus nephritis	Identify lupus nephritis over control	(Wang et al. 2012b)
miR-155	Up	Lupus nephritis	Identify lupus nephritis over control	(Wang et al. 2012b)
miR-15	Down	DGS	Differential expression in DGS to HTN and IgAN	(Michael et al. 2001)
miR-17	Up	IgAN	Differential expression in IgAN to DGS and HTN	(Michael et al. 2001)
miR-216a	Up	HTN	Differential expression in HTN to DGS and IgAN	(Michael et al. 2001)
miR-210	Down	Nondiabetic CKD	Differential expression in stage 3/4 CKD, ESRD, and controls	(Zampetaki et al. 2010)
miR-16	Down	Nondiabetic CKD	Differential expression in stage 3/4 CKD, ESRD, and controls	(Zampetaki et al. 2010)
miR-155	Down	Nondiabetic CKD	Differential expression in stage 3/4 CKD, ESRD, and controls	(Zampetaki et al. 2010)
miR-638	Down	Nondiabetic CKD	Differential expression in stage 4 CKD and ESRD to controls	(Zampetaki et al. 2010)
miR-210	Down	Nondiabetic CKD	Differential expression in stage 4 CKD and ESRD to controls	(Zampetaki et al. 2010)
miR-16	Down	Nondiabetic CKD	Differential expression in stage 4 CKD and ESRD to controls	(Zampetaki et al. 2010)
miR-192	Down	DGS	Differential expression compared to MCN/FSGS, MGN, and controls	(Zampetaki et al. 2010)
miR-192	Up	MCN/FSGS, MGN	Differential expression compared to DGS and controls	(Zampetaki et al. 2010)
miR-200c	Down	DGS, MGN	Differential expression compared to MCN/FSGS and controls	(Zampetaki et al. 2010)
miR-200c	Up	MCN/FSGS	Differential expression compared to DGS, MGN, and controls	(Zampetaki et al. 2010)
miR-638	Down	DGS, MCN/FGSG, MGN	Indicates abnormal renal pathology	(Zampetaki et al. 2010)

Additionally, in support of the Neal study, miR-638 was decreased in all groups compared to controls (Neal et al. 2011). In support of miRNA biomarkers acting as a means for differential diagnosis, levels of various miRNAs were correlated with different clinical parameters between the studied groups (Table 5). Furthermore, differential correlations were also seen between miRNA levels and histological

Table 5 Summary of correlations between miRNA levels in various nephropathies and clinical/histological markers

miRNA	Proteinuria	eGFR	GS	TIF
miR-21	–	DN	–	DN
miR-29a	–	–	–	DN, MGN
miR-29b	–	DN[a]	–	DN
miR-29c	–	–	–	–
miR-122	–	–	DN	DN
miR-141	–	MCN/FSGS	–	–
miR-150	–	DN	MGN	–
miR-184	–	MCN/FSGS	–	MGN
miR-192	DN	MCN/FSGS	–	MGN
miR-198	DN[a]	–	–	–
miR-200a	–	–	–	–
miR-200b	–	MCN/FSGS	–	–
miR-200c	–	MCN/FSGS	DN[a]	–
miR-205	–	MCN/FSGS	MGN[a]	–
miR-375	–	MCN/FSGS	–	MGN
miR-429	–	–	DN	–
miR-638	DN[a]	DN	–	–

GS glomerulosclerosis, *TIF* tubulointerstitial fibrosis
[a]Nonsignificant correlations (p <0.06), adapted from Wang et al. (2013)

parameters between the various groups. These correlations clearly illustrate both the complexity and redundancy of miRNA signaling systems. By their very nature, dysregulation of any number of miRNA can ultimately result in similar outcomes. However, given that miRNA is regulated as any other gene, their profiles can hint at the source of the insult therefore aiding in differential diagnosis.

Changes in urinary miRNA in PKD have also been reported in comparison to various stages of non-PKD CKD providing a further measure of differential diagnostic capability (Ben-Dov et al. 2014). This study compared urinary sediment, urinary exosomes, and various cultured cell lines including primary proximal tubule cells. Urine samples were obtained from males and females within each group, and all samples were analyzed by high-throughput sequencing. This study produced a massive amount of data, all of which is available online. The authors highlight that there were increased levels of miR-223 and miR-199a/b in urinary sediments of those with PKD compared to non-PKD CKD. Interestingly, miR-223 was increased in DM plasma though, as already demonstrated, there can be vast differences in miRNA profiles pending the source of the sample, both in regard to biofluids and disease state of the donor (Zampetaki et al. 2010).

Lupus nephritis is a transient complication of systemic lupus erythematosus which may be caused by a number of distinct glomerular phenotypes (Weening et al. 2004). Furthermore, not all lupus patients will develop some form of nephritis, and a single patient may display differing phenotypes throughout their lifetime.

Although lupus nephritis is generally easily treated, each phenotype requires specialized regimens, and therefore effective diagnosis without repeated biopsies is required (Szeto 2014). Although the various phenotypes have not been studied in regard to miRNA, Wang et al. reported that miR-145 and miR-155 were both increased in urinary sediment of lupus nephritis subjects compared to controls (Wang et al. 2012b). Furthermore, miR-145 levels correlated with GFR and miR-155 with the level of proteinuria and also with SLEDAI score, an index used in clinical studies which demonstrate lupus activity.

These examples are but a few of the many studies demonstrating the effectiveness of utilizing miRNA as biomarkers for disease. However, as with many of those published in the field of diabetic nephropathy, many of these have utilized small cohorts of patients. The reasons for this are the same as those for reviewed studies concerning DN, cost and sample availability. Regardless, it is obvious that miRNAs have a promising future as diagnostic and prognostic tools given enough time and research interest.

Concluding Remarks

This chapter has sought to highlight the current knowledge regarding the use of miRNA in the diagnosis and detection of those with DN and also those at risk of developing DN. It should be apparent that, although there has been a considerable amount of work conducted in the field, there is still much to be done. Although the above studies have clearly demonstrated that miRNAs show great potential as clinical biomarkers, there remain a number of diverse challenges to be overcome before miRNA can be effectively utilized in the clinic.

The most basic of these is technical congruence. The need for experimental standardization is particularly highlighted by the Cheng study which demonstrated differences in miRNA profiles from exosomes isolated through utilization of different commercially available kits (Cheng et al. 2014). Furthermore, purification of miRNA from blood and urine needs to be standardized due to the presence of whole cells and cellular debris which unequivocally contain miRNA profiles which differ to that of free or exosome-bound miRNA. Additionally, cellular fractions of plasma and urine undoubtedly contain RNases which are free to degrade miRNA upon cell lysis during miRNA isolation. These factors likely contribute to intra-study differences and clearly necessitate standardization of an inherently sensitive assay.

Another major concern in current miRNA biomarker studies is the cohort size in the bulk of studies. While most clinical studies regarding pharmaceutical intervention or protein-based biomarkers typically involve hundreds of subjects, miRNA studies are typically restricted to no more than 20 subjects with many being limited to fewer than 10 subjects per group. While it is easy to account this to sample availability, one needs to keep in mind that miRNA biomarkers are typically sought in noninvasive, freely available biofluids. For effective analysis of miRNA profiles to be enabled, high-throughput sequencing methods must be employed which are costly and require specialist knowledge. This cost can be offset by the use of

conventional qRT-PCR analysis at the cost of analytical depth. It is therefore important that governments and other funding bodies recognize the costs involved in establishing a putative miRNA profile which may be used in a clinical setting. While this cost will initially undoubtedly be high, it will be greatly offset by greater clinical management of those at risk of, and those with, DN.

In addition to the cost of sample analysis, there are added costs of prospective studies such as those conducted by Zampetaki. Prospective studies are required to enable development of miRNA profiles which may identify individuals at risk of diabetes and, more specifically, DN. These types of studies provide the opportunity to establish profiles which indicate the various stages of DN progression. However, the cost of following large cohorts of patients is largely prohibitive to a developing field of research. The desired outcomes of prospective studies also necessitate multiple high-throughput sequencing of plasma/urine/exosomes from numerous individuals over time which dramatically add to an already large project cost. Again, if these costs can be met, we will see a shift in clinical diagnostic paradigms which would be rapidly seen as reduction in costs incurred to the public health sector.

There is a great need for improved diagnosis of DN and also those diabetics at risk of developing DN. This need is generated both by a desire to improve patient quality of life and also to reduce financial burden on public health systems associated with ongoing cost of care. Furthermore, given the poor prognosis for those that progress to end-stage renal disease coupled with a lack of organ donation, the need for establishment of improved diagnostic protocols becomes much more evident. Establishment of these profiles may also lead to discovery of suitable miRNA targets for therapeutic intervention in both extant and developing DN.

miRNA research is an exceedingly young field considering their discovery in humans almost 15 years ago. It is exceptional that we are already beginning to see the potential for miRNAs as noninvasive biomarkers for DN and other nephropathies. However, we must learn to crawl before we can walk and walk before we can run, although it seems that we have taken our first steps toward our goals of improved prognostic and diagnostic tools. With continued funding, protocol standardization, and sample availability, there will be great progress in filling a pertinent need in a burgeoning public health sector. For those involved in miRNA research, these are exciting times with each day bringing us one step closer to establishing biomarker profiles.

Summary Points

- Blood contains miRNA as either free, protein-bound complexes or packaged into exosomes and can indicate dysfunction of a number of tissues.
- Various components of the nephron secrete miRNA into the urinary stream, and therefore urinary miRNA provides an excellent marker for renal health.
- miRNA is dysregulated in diabetic nephropathy, and the profile of secreted miRNA, both in serum/plasma and urine, is also dysregulated.

- Circulating miRNA and those excreted in urine are highly stable and easily extracted therefore providing a source of noninvasive biomarkers for diabetic nephropathy.
- Potential miRNA profiles, from both urine and plasma/serum, have been identified which can differentially diagnose specific stages of diabetic nephropathy including the onset of microalbuminuria.
- Prediction of those that will develop type 2 diabetes or type 2 diabetic subjects that will progress to diabetic nephropathy is also possible.

References

Argyropoulos C, Wang K, et al. Urinary microRNA profiling in the nephropathy of type 1 diabetes. PLoS One. 2013;8(1):e54662.
Babak T, Zhang WEN, et al. Probing microRNAs with microarrays: tissue specificity and functional inference. RNA. 2004;10(11):1813–9.
Barutta F, Tricarico M, et al. Urinary exosomal microRNAs in incipient diabetic nephropathy. PLoS One. 2013;8(11):e73798.
Ben-Dov IZ, Tan Y-C, et al. Urine microRNA as potential biomarkers of autosomal dominant polycystic kidney disease progression: description of miRNA profiles at baseline. PLoS One. 2014;9(1):e86856.
Brennan EP, Nolan KA, et al. Lipoxins attenuate renal fibrosis by inducing let-7c and suppressing TGFβR1. J Am Soc Nephrol. 2013;24(4):627–37.
Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature. 2001;414 (6865):813–20.
Camussi G, Deregibus MC, et al. Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int. 2010;78(9):838–48.
Chau BN, Xin C, et al. MicroRNA-21 promotes fibrosis of the kidney by silencing metabolic pathways. Sci Transl Med. 2012;4(121):121ra118.
Chen X, Ba Y, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18(10):997–1006.
Cheng L, Sharples RA, et al. Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. J Extracell Vesicles. 2014;3. doi:10.3402/jev.v3403.23743.
Chung ACK, Huang XR, et al. miR-192 mediates TGF-β/Smad3-driven renal fibrosis. J Am Soc Nephrol. 2010;21(8):1317–25.
Creemers EE, Tijsen AJ, et al. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ Res. 2012;110(3):483–95.
Denby L, Ramdas V, et al. miR-21 and miR-214 are consistently modulated during renal injury in rodent models. Am J Pathol. 2011;179(2):661–72.
Detrisac CJ, Mayfield RK, et al. In vitro culture of cells exfoliated in the urine by patients with diabetes mellitus. J Clin Invest. 1983;71(1):170–3.
Dey N, Das F, et al. MicroRNA-21 orchestrates high glucose-induced signals to TOR complex 1, resulting in renal cell pathology in diabetes. J Biol Chem. 2011;286(29):25586–603.
Dey N, Ghosh-Choudhury N, et al. TGFbeta-stimulated microRNA-21 utilizes PTEN to orchestrate AKT/mTORC1 signaling for mesangial cell hypertrophy and matrix expansion. PLoS One. 2012;7(8):e42316.
Dronavalli S, Duka I, et al. The pathogenesis of diabetic nephropathy. Nat Clin Pract End Met. 2008;4(8):444–52.
Duttagupta R, Jiang R, et al. Impact of cellular miRNAs on circulating miRNA biomarker signatures. PLoS One. 2011;6(6):e20769.

Ge Q, Zhou Y, et al. miRNA in plasma exosome is stable under different storage conditions. Molecules. 2014;19(2):1568–75.

Glowacki F, Savary G, et al. Increased circulating miR-21 levels are associated with kidney fibrosis. PLoS One. 2013;8(2):e58014.

Guo Z, Maki M, et al. Genome-wide survey of tissue-specific microRNA and transcription factor regulatory networks in 12 tissues. Sci Rep. 2014;4:5150.

He F, Peng F, et al. MiR-135a promotes renal fibrosis in diabetic nephropathy by regulating TRPC1. Diabetologia. 2014;57(8):1726–36.

Hsu C-Y, Bansal N. Measured GFR as "Gold Standard" – all that glitters is not gold? Clin J Am Soc Nephrol. 2011;6(8):1813–4.

Huang Y, Liu Y, et al. Involvement of inflammation-related miR-155 and miR-146a in diabetic nephropathy: implications for glomerular endothelial injury. BMC Nephrol. 2014;15 (142):1471–2369.

Hunter MP, Ismail N, et al. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One. 2008;3(11):e3694.

Karalliedde J, Gnudi L. Endothelial factors and diabetic nephropathy. Diabetes Care. 2011;34 Suppl 2:S291–6.

Kato M, Wang L, et al. Post-transcriptional up-regulation of Tsc-22 by Ybx1, a target of miR-216a, mediates TGF-β-induced collagen expression in kidney cells. J Biol Chem. 2010;285 (44):34004–15.

Kong L, Zhu J, et al. Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. Acta Diabetol. 2011;48(1):61–9.

Koppers-Lalic D, Hackenberg M, et al. Nontemplated nucleotide additions distinguish the small RNA composition in cells from exosomes. Cell Rep. 2014;8(6):1649–58.

Li D, Lu Z, et al. MiR-124 is related to podocytic adhesive capacity damage in STZ-induced uninephrectomized diabetic rats. Kidney Blood Press Res. 2013;37(4–5):422–31.

Liu Y, Gao G, et al. Stability of miR-126 in urine and its potential as a biomarker for renal endothelial injury with diabetic nephropathy. Int J Endocrinol. 2014;2014:393109.

Lorenzen JM, Thum T. Circulating and urinary microRNAs in kidney disease. Clin J Am Soc Nephrol. 2012;7:1528–33.

Luque-Garcia JL, Neubert TA. Sample preparation for serum/plasma profiling and biomarker identification by mass spectrometry. J Chromatogr A. 2007;1153(1–2):259–76.

Lv L-L, Cao Y, et al. Isolation and quantification of microRNAs from urinary exosomes/ microvesicles for biomarker discovery. Int J Biol Sci. 2013a;9(10):1021.

Lv LL, Cao YH, et al. MicroRNA-29c in urinary exosome/microvesicle as a biomarker of renal fibrosis. Am J Physiol Renal Physiol. 2013b;305(8):14.

Mall C, Rocke DM, et al. Stability of miRNA in human urine supports its biomarker potential. Biomark Med. 2013;7(4):623–31.

McClelland AD, Kantharidis P. microRNA in the development of diabetic complications. Clin Sci. 2014;126(2):95–110.

McClelland A, Hagiwara S, et al. Where are we in diabetic nephropathy: microRNAs and biomarkers? Curr Opin Nephrol Hypertens. 2014;23(1):80–6.

Michael LF, Wu Z, et al. Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. Proc Natl Acad Sci. 2001;98(7):3820–5.

Momen-Heravi F, Bala S, et al. Exosome-mediated delivery of functionally active miRNA-155 inhibitor to macrophages. Nanomedicine. 2014;10(7):1517–27.

Moresco RN, Sangoi MB, et al. Diabetic nephropathy: traditional to proteomic markers. Clin Chim Acta. 2013;421:17–30.

Nakada C, Matsuura K, et al. Genome-wide microRNA expression profiling in renal cell carcinoma: significant down-regulation of miR-141 and miR-200c. J Pathol. 2008;216(4):418–27.

Nakamura T, Ushiyama C, et al. Urinary excretion of podocytes in patients with diabetic nephropathy. Nephrol Dial Transplant. 2000;15(9):1379–83.

Neal CS, Michael MZ, et al. Circulating microRNA expression is reduced in chronic kidney disease. Nephrol Dial Transplant. 2011;26(11):3794–802.
Peng H, Zhong M, et al. Urinary miR-29 correlates with albuminuria and carotid intima-media thickness in type 2 diabetes patients. PLoS One. 2013;8(12):e82607.
Rana S, Malinowska K, et al. Exosomal tumor microRNA modulates premetastatic organ cells. Neoplasia (New York, NY). 2013;15(3):281–95.
Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. 2013;200(4):373–83.
Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol. 2008;18(10):505–16.
Sayed D, Abdellatif M. MicroRNAs in development and disease. Physiol Rev. 2011;91(3):827–87.
Schena FP, Gesualdo L. Pathogenetic mechanisms of diabetic nephropathy. J Am Soc Nephrol. 2005;16(3 Suppl 1):S30–3.
Shi S, Yu L, et al. Smad2-dependent downregulation of miR-30 is required for TGF-β-induced apoptosis in podocytes. PLoS One. 2013;8(9):e75572.
Smith HW. The kidney: structure and function in health and disease. Oxford: Oxford University Press; 1951.
Szeto C-C. Urine miRNA in nephrotic syndrome. Clin Chim Acta. 2014;436:308–13.
Szeto CC, Li PK. MicroRNAs in IgA nephropathy. Nat Rev Nephrol. 2014;10(5):249–56.
Szeto C-C, Ching-Ha KB, et al. Micro-RNA expression in the urinary sediment of patients with chronic kidney diseases. Dis Markers. 2012;33(3):137–44.
Van Buren PN, Toto R. Hypertension in diabetic nephropathy: epidemiology, mechanisms, and management. Adv Chronic Kidney Dis. 2011;18(1):28–41.
Wang B, Koh P, et al. miR-200a prevents renal fibrogenesis through repression of TGF-β2 expression. Diabetes. 2011;60(1):280–7.
Wang B, Komers R, et al. Suppression of microRNA-29 expression by TGF-beta1 promotes collagen expression and renal fibrosis. J Am Soc Nephrol. 2012a;23(2):252–65.
Wang G, Tam L-S, et al. Expression of miR-146a and miR-155 in the urinary sediment of systemic lupus erythematosus. Clin Rheumatol. 2012b;31(3):435–40.
Wang K, Yuan Y, et al. Comparing the microRNA spectrum between serum and plasma. PLoS One. 2012c;7(7):e41561.
Wang C, Li C, et al. New urinary biomarkers for diabetic kidney disease. Biomark Res. 2013a;1(1):1–4.
Wang G, Kwan BC-H, et al. Urinary sediment miRNA levels in adult nephrotic syndrome. Clin Chim Acta. 2013b;418:5–11.
Wang B, Jha JC, et al. Transforming growth factor-beta1-mediated renal fibrosis is dependent on the regulation of transforming growth factor receptor 1 expression by let-7b. Kidney Int. 2014;85(2):352–61.
Weening JJ, D'Agati VD, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. J Am Soc Nephrol. 2004;15(2):241–50.
Wessely O, Tran U. Small RNAs have a Big effect on polycystic kidney disease. J Am Soc Nephrol. 2012;23(12):1909–10.
Wuyts B, Bernard D, et al. Reevaluation of formulas for predicting creatinine clearance in adults and children, using compensated creatinine methods. Clin Chem. 2003;49(6):1011–4.
Xiao D, Ohlendorf J, et al. Identifying mRNA, MicroRNA and protein profiles of melanoma exosomes. PLoS One. 2012;7(10):e46874.
Xiong M, Jiang L, et al. The miR-200 family regulates TGF-beta1-induced renal tubular epithelial to mesenchymal transition through smad pathway by targeting ZEB1 and ZEB2 expression. Am J Physiol Renal Physiol. 2012;302(3):19.
Yan S-T, Li C-L, et al. MiR-199a is overexpressed in plasma of type 2 diabetes patients which contributes to type 2 diabetes by targeting GLUT4. Mol Cell Biochem. 2014;397(1–2):45–51.
Yang Y, Xiao L, et al. Urine miRNAs: potential biomarkers for monitoring progression of early stages of diabetic nephropathy. Med Hypotheses. 2013;81(2):274–8.

Zampetaki A, Kiechl S, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res. 2010;107(6):810–7.
Zhang T, Lv C, et al. Plasma miR-126 Is a potential biomarker for early prediction of type 2 diabetes mellitus in susceptible individuals. BioMed Res Int. 2013;2013:761617.
Zhou J, Peng R, et al. A potentially functional polymorphism in the regulatory region of let-7a-2 is associated with an increased risk for diabetic nephropathy. Gene. 2013;527(2):456–61.

Biomarkers in Focal Segmental Glomerulosclerosis

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Mohsen Nafar and Shiva Kalantari

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Abstract

Focal segmental glomerulosclerosis (FSGS) is a podocyte-related disease and one of the common causes of idiopathic glomerulonephritis. The pathogenesis of FSGS is not well understood and still under study; however, some clues have been found regarding implications of critical signaling pathways. Diagnosis of FSGS like other glomerulopathies is based on renal biopsy. Although biopsy considered a gold standard in nephrology world for diagnosis, it is invasive and not always possible to be performed. Discovery of the biomolecules which are easily measurable, noninvasive, specific, and sensitive and their changes reflect the type and stage of the disease can simplify the diagnosis. These biomolecules are referred to as "biomarkers" and can be complementary to biopsy for faster and more accurate diagnosis. The advances in biomedicine fields and nascency of the high-throughput platforms such as proteomics, transcriptomics, metabolomics, and other related "omics" have brought the novel way of detection biomarkers in various diseases. In this review, we focus on the recently presented biomarkers for diagnosis, prognosis, and prediction of the responsiveness to drugs for FSGS detected by different methods. However, most of the current biomarkers are still under more examination; they will be dependable complementary of traditional diagnostic methods in the future.

Keywords

Focal segmental glomerulosclerosis • Diagnostic biomarker • Prognostic biomarker • Predictive biomarker • Proteomics • Metabolite biomarker • Ribonucleic acid biomarker • Podocyte

Abbreviations

2DE	Two-dimensional electrophoresis
ACE	Angiotensin-converting enzyme
ADR	Adriamycin
ARB	Angiotensin receptor blocker
CLCF1	Cardiotrophin-like cytokine factor 1
ConA	Concanavalin A
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial–mesenchymal transition
ESRD	End-stage renal disease
FDR	False discovery rate
FN	Fibronectin
FSGS	Focal segmental glomerulosclerosis

GBM	Glomerular basement membrane
GC-MS	Gas chromatography mass spectrometry
GFR	Glomerular filtration rate
GPI	Glycosylphosphatidylinositol
HLA	Human leukocyte antigen
IEF	Isoelectrofocusing
IGFBP	Insulin-like growth factor-binding protein
INS	Idiopathic nephrotic syndrome
LC	Liquid chromatography
LN	Lupus nephritis
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MCD	Minimal change disease
MCNS	Minimal change nephrotic syndrome
MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
MMF	Mycophenolate mofetil
MMP9	Matrix metallopeptidase 9
MN	Membranous nephropathy
MS	Mass spectrometry
NGAL	Neutrophil gelatinase-associated lipocalin
NMR	Nuclear magnetic resonance
NOS	Not otherwise specified
RT-qPCR	Real-time quantitative polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI-TOF	Surface-enhanced laser desorption/ionization time of flight
SRM	Single reaction monitoring
SRNS	Steroid-resistant nephrotic syndrome
SSNS	Steroid-sensitive nephrotic syndrome
suPAR	Soluble urokinase plasminogen activator receptor
TGF-ß	Transforming growth factor-ß
TWEAK	TNF-like weak inducer of apoptosis
UPAR	Urokinase plasminogen activator receptor

Key Facts

Key Facts of TGF-ß Pathway

- TGF-ß signaling regulates a variety of cellular processes in embryonic and mature cells such as: apoptosis, cell proliferation, and differentiation.
- This pathway is a conserved signaling pathway from invertebrates to higher vertebrates.
- The signaling is begun by binding TGF-ß ligands to receptors on the cell surface, activation of phosphorylation cascade of Smad proteins, translocation of the Smad complexes into the nucleus, and regulation of the target genes accompanied with other nuclear factors.

- TGF-ß is able to trigger extracellular component production in glomeruli and hence is involved in pathogenesis of glomerular diseases including FSGS.
- Collagens (such as collagen type III), fibronectin, and proteoglycans are some of the synthesized ECM proteins in damaged glomeruli.
- Induction of ECM component production leads to the increase of permeability of glomeruli which results in proteinuria.

Key Facts of Notch Signaling

- Notch signaling is initiated by binding the ligands to the notch receptors which switch on a series of proteolytic cleavage steps of notch. The final product of these proteolytic steps is a transcription factor that can induce the target genes.
- The target genes of activated notch protein during the signaling pathway are Hes and Hey transcription factors that are responsible for negative regulation of tissue-specific differentiation.
- Notch signaling is important for developing the glomeruli and differentiation of podocytes.
- Continued activity of notch signaling in mature podocytes can be harmful and damage these cells.

Key Facts of Wnt Signaling

- Wnt signaling is reported for the first time in 1987 during the identification of an oncogene named int1. This gene is homolog to wingless gene in *Drosophila*. Therefore, the name “Wnt” comes from “int” and “wingless.”
- It is divided into two signaling pathways: canonical and noncanonical. The noncanonical is further divided into two pathways: planar cell polarity pathway and Wnt/calcium pathway.
- Canonical is considered as a pathway which is dependent to ß-catenin, and noncanonical is considered as a pathway which is independent to ß-catenin.
- Each of the signaling pathways is triggered by different kinds of receptors. For example, canonical pathway is triggered by Wnt1, Wnt3a, and Wnt8, while noncanonical pathway is triggered by Wnt5a and Wnt11.
- Wnt proteins are secreted glycoproteins that act through frizzled family receptors.
- Some of the Wnt protein functions include: cell migration, cell differentiation, and cell proliferation.
- Canonical pathway can be inhibited by negative feedback of one of its target genes named DKK1.
- Activation of Wnt-ß-catenin pathway through integrin-linked kinase in the podocytes has been reported recently as one of the mechanisms involved in FSGS.

Key Facts of NF-κB Signaling

- NF-κB is the abbreviation of nuclear factor kappa-light-chain enhancer of activated B cells.
- This protein is a transcription factor that has a critical role in regulation of both innate and adaptive immune systems.
- This family of DNA-binding protein has five members including: RelA, NF-κB1, NF-κB2, cRel, and RelB.
- Activation of NF-κB signaling may affect podocyte cytoskeleton which ends up to impaired glomerular permeability and leaking proteins into urine.
- Repression of NF-κB signaling is one of the mechanisms of glucocorticoid therapy.

Key Facts of TWEAK

- TWEAK is the abbreviation of tumor necrosis factor (TNF)-like weak inducer of apoptosis.
- TWEAK is a type of cytokine that binds to its receptor Fnt14 (FGF-inducible molecule 14) and stimulates proinflammatory responses, angiogenesis, and cell growth/death.
- It is involved in some inflammatory and autoimmune diseases including lupus nephritis (LN).
- Fnt14 in the kidney is located in podocytes, tubular, and mesangial cells.

Definitions

Podocyte A type of highly differentiated cells in the glomerulus which composed glomerular filtration barrier besides glomerular basement membrane and endothelial cells.

End-stage renal disease The last stage of progression of kidney disease that renal function is lost and dialysis or renal transplantation is recommended.

Circulating permeability factors Circulating proteins that cause abnormal permeability of glomeruli to the plasma proteins. These factors might be related to dysfunction of T cells.

Extracellular matrix A collection of secreted molecules from the cells is mostly composed of fibrous proteins and glycosaminoglycans which structurally and biochemically support the cells. Cell-to-cell communication, cell adhesion, and regulating the cell dynamics are some of the functions of extracellular matrix.

Glomerular filtration rate A calculated factor which can serve as a test for evaluation of the kidney function or judgment about the progression of kidney disease. This factor describes the rate of filtered plasma which passes through the kidney.

MALDI-TOF technique One of the proteomic techniques for identification of proteins extracted from different sources of samples using mass spectrometer. The extracted proteome is mixed with a kind of matrix and ionized by laser and delivered to an analyzer named "time of flight" which is connected to a detector. A user can identify the molecules (e.g., proteins) using these experimental spectra that are matched with theoretical spectra available in the database.

Single reaction monitoring technique This is a mass spectrometric-based technique for determining the absolute abundance of a molecule (e.g., protein) in the sample. The abundance of the molecule is determined by a calibration curve of a known molecule.

SELDI-TOF technique One of the proteomic techniques for profiling the proteome of different types of samples using mass spectrometer. This technique does not need sample preparation procedure and may serve as a diagnostic tool. Low resolution and lack of reproducibility are some of the limitations of this technique.

Introduction

Focal segmental glomerulosclerosis (FSGS) is a podocyte-derived glomerular disease described for the first time by Arnold Rich in 1959 (Rich 1959). This clinical-pathological syndrome has multiple etiologies and pathogenic mechanisms, and therefore it might be a combination of several pathological conditions and not a single disease (Falk et al. 2000). The incidence of FSGS has been increased in the past two decades and expected to be increasing since the proportion of patients with primary FSGS has been raised recently and reached to approximately 25 % of adult nephropathies (Falk et al. 2000).

In fact the clinical features of FSGS are the features of a nephrotic syndrome and include: peripheral edema, marked proteinuria (with or without nephrotic range), hypertension, hypoalbuminemia, hyperlipidemia, and progressive loss of renal function. It may be found either as primary or secondary forms. Primary FSGS sometimes has no identifiable cause or known etiology (idiopathic) and linked to genetic mutations in podocyte-specific proteins (e.g., membrane and podocyte slit diaphragm proteins), while secondary FSGS may occur in response to previous glomerular injury, glomerular hypertension, hypertrophy, drug toxicity (e.g., pamidronate, adriamycin), some malignancies or viral infections (e.g., HIV infection), obesity, and reduced renal mass. FSGS as one of the most common causes of primary glomerular disease in children and adults may progress to end-stage renal disease (ESRD) with a relatively high risk.

Pathological features of FSGS on light microscopy are recognized by sclerotic lesions that affect some of the glomeruli (focal) and a portion of the glomerular tuft (segmental). Accumulation of foam cells in glomerular capillary, swelling of epithelial cells, collapse of capillaries, and expansion of extracellular matrix lead to hyalinosis that also may be observed in microscopic section (Falk et al. 2000). Diffuse effacement of foot process in podocytes is a significant finding on electron microscopy.

Based on these pathological features, five histological variants were described: perihilar variant, cellular variant, tip variant, collapsing variant, and FSGS not otherwise specified (NOS) (Deegens et al. 2008).

It is suggested that there is a relationship between the frequency of these variants, race, and ethnicity. Accordingly, collapsing and cellular variants are dominant in African American population, while they are relatively rare in Indian and Dutch population (Deegens et al. 2008). In contrast tip variant is more frequent in whites (Chun et al. 2004). In general, the frequency of FSGS variants according to published literature is different. Accordingly NOS is the most frequent (up to 72.5 %) and collapsing is the least frequent (up to 24 %) variant.

These variants are also different in prognosis. In general, collapsing variant is known to have the worst renal survival rate and hence has worse prognosis compared to other variants, while the tip variant has clinical features similar to that of minimal change disease (MCD) and responsive to steroid therapy, hence considered to have good prognosis. Cellular variant is also known to have an intermediate prognosis between the two variants.

The pathogenesis of FSGS is poorly understood. However, FSGS is known to be caused by an abnormality in podocytes (highly differential epithelial cells of the glomerulus).

Transforming growth factor-ß (TGF-ß) is the possible mediator of lesions in different renal injuries including FSGS via induction of podocyte injury through apoptosis, proliferation, and epithelial–mesenchymal transition (EMT) and increased production of extracellular matrix proteins including collagen (Lee 2013).

The role of mutation in specific genes is also known to be involved in pathogenesis of FSGS. Some of these genes are podocin (NPHS2), nephrin (NPHS1), actinin-4 (ACTN-4), CD2-associated protein (CD2AP), WT1 transient receptor potential cation 6 (TRPC6), and phospholipase C (PLCE1/NPHS3) (Woroniecki and Kopp 2007).

Injury of epithelial and endothelial cells as a consequence of compensatory capillary hypertension after loss of nephrons as well as mesangial transformation is another hypothesis for FSGS pathogenesis (Falk et al. 2000). Lipid metabolic disorders, abnormalities of the coagulation pathway, and alterations in T cell function also may play important role in FSGS. Association of some specific HLA types is known to be involved in familial and even nonfamilial forms of FSGS. Accordingly, people who have these alleles of HLA are more prone to FSGS: HLA-DR4, HLA-B12, HLA-DRw8, HLA-DRw5, HLA-B8, HLA-DR3 and HLA-DR7, and HLA-DR4303 and HLA-Bw53 (Falk et al. 2000).

There are also some evidence regarding involvement of Wnt/Ctnnb1 pathway and notch signaling in podocytes of patients and animal models with FSGS. TGF-ß is believed to act upstream of both pathways (Kato and Susztak 2012).

There are also several reports regarding the possible role of permeability factors in FSGS pathogenesis via an effect on podocyte-specific proteins (nephrin and podocin), phosphorylation of cellular proteins in the podocyte, activity of serine proteases, and activity of integrin-like kinase that may lead to detachment of podocyte from GBM (Sharma et al. 2004; Hattori et al. 2008; Carraro et al. 2004).

Treatment of FSGS follows two major goals: complete remission of proteinuria and preservation of renal function. However, complete remission might not occur in all cases because of heterogeneity of the disease and different responsiveness to therapeutic strategies. Initial immunosuppressive therapy with corticosteroids including prednisone, prednisolone, and methylprednisolone is the first line of treatment for idiopathic nephrotic syndrome; however, a proportion of patients might not respond to this therapy. The mechanism of action of corticosteroids in FSGS still has some ambiguities and has not been completely elucidated. Calcineurin inhibitors such as cyclosporine A and tacrolimus may be used as initial therapy, especially in patients at increased risk for corticosteroids toxicity (Kato and Susztak 2012). Angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs), rosiglitazone (a peroxisome proliferator-activated receptor-γ), antiproliferative agents such as mycophenolate mofetil (MMF), adalimumab (a human monoclonal antibody), abatacept, and rituximab (a chimeric monoclonal antibody) are the other possible treatments which could be used for FSGS.

It is worthy to note that FSGS patients may respond to these therapies (especially to steroid drugs) differently. Since prediction of responsiveness of FSGS patients to steroid drugs is almost impossible at presentation, the steroid-resistant patients might be imposed to side effects of an ineffective therapy and lose the time for appropriate therapy. In spite of some attempts for discovery of the biomarkers responsible for different responses, prediction of responsiveness before medication is still impossible.

In addition, diagnosis of FSGS underlies on kidney biopsy. As FSGS variants show different responsiveness to therapies, recognition of variant could be helpful to choose the therapeutic strategy. Furthermore, some investigators have argued that achievement of complete or partial remission of nephrotic syndrome (Hattori et al. 2008) and quality of the responsiveness to therapies could be a better predictor of outcome in FSGS than the histological variant. Biomarker discovery researches are promising in this regard to extricate nephrologists from invasive biopsy and bewilderment in choosing the best responsive and least risky therapy. Proteomics and metabolomics as well as transcriptomics, the major platforms in biomarker discovery, are valuable for differentiating between variants and also for diagnosis and therefore reducing the rate of inefficient therapies; however, such these studies are few.

In this review we focus on different types of biomarkers reported in the literature for diagnosis and prognosis purposes as well as prediction of the responsiveness to therapy for FSGS.

Potential Applications to Prognosis, Other Diseases, or Conditions

However, FSGS is a well-characterized glomerular disease with defined features (e.g., podocyte injury and histological patterns); its hallmarks are common with other glomerular diseases which end up to nephrotic syndrome. A few of candidate biomarkers that were reported in the literature and were named in the present review such as alpha-1-antitrypsin, beta-2-microglobulin, and transferrin were detected in other glomerular diseases as well (including IgA nephropathy, membranous glomerulonephritis, and diabetic nephropathy). Detection of these nonspecific candidates may let to provide a rational for this hypothesis: "however, all the glomerular diseases have distinct specific mechanism; they are interconnected with each other in some parts which lead to represent the same symptoms including proteinuria and swelling." Therefore, if we could recognize this shared pattern of pathogenesis and sketching a therapy for that, a broad spectrum of renal disease may be treated. This is not an unattainable dream. Mapping of disease network for each glomerular disease using available biomarkers and available information in the databases and comparing them via advanced bioinformatic methods is one of the possible ways to make this dream come true. Several attempts have been started to map a network for some renal diseases recently that are still in progress.

Owing to this viewpoint and application, the nonspecific candidate biomarkers should be considered as valuable as specific candidates. On the other hand, the relative or absolute abundance of these nonspecific biomarkers might be quite different in glomerular diseases which could be applied in the quantitative diagnostic protein arrays as a "panel of diagnostic/prognostic biomarkers" in the future.

Diagnostic Biomarkers

Protein Biomarkers

This section focuses on protein candidates for discrimination of FSGS from other types of glomerular diseases or normal subjects reported in the literature (see Table 1). The other kinds of biomarkers including mRNAs and small metabolites have been discussed in the sections "Ribonucleic Acid Biomarkers" and "Metabolite Biomarkers."

suPAR

suPAR stands for soluble urokinase plasminogen activator receptor, a circulating form of uPAR, which is a membrane glycosylphosphatidylinositol (GPI)-anchored protein in numerous types of cells including podocytes (Jefferson and Shankland 2013). suPAR is produced through proteolytic cleavage of uPAR in the linker

Table 1 Diagnostic protein biomarker candidates for FSGS

Protein ID	Protein name	Source	Analytical technique	Reference
suPAR	Soluble urokinase plasminogen activator receptor	Serum/ urine/ cultured podocyte	ELISA kit/1DE-LC-MS/ MS	Wei et al. 2011, 2012; Huang et al. 2014; Matsumoto et al. 2010
CD80	T lymphocyte activation antigen CD80	Serum/ urine	ELISA kit	Cara-Fuentes et al. 2013
NGAL	Neutrophil gelatinase-associated lipocalin	Serum/ urine	Enzyme immunoassay	Chehade et al. 2013; Proletov et al. 2013
Cys C	Cystatin C	Serum/ urine	Enzyme immunoassay	Proletov et al. 2013
IGFBP-1	Insulin-like growth factor-binding protein 1	Urine	–	Worthmann et al. 2010
IGFBP-3	Insulin-like growth factor-binding protein 3	Urine	–	Worthmann et al. 2010
IGFBP-rP1	Insulin-like growth factor-binding protein-related protein-1	Cultured podocytes	1DE-LC-MS/MS	Matsumoto et al. 2010
IBP7	Insulin-like growth factor-binding protein 7	Urine	Nano-LC-MS/MS	Nafar et al. 2014
A1AT1	Alpha-1-antitrypsin	Urine	Nano-LC-MS/MS, 2DE-MALDI-TOF, magnetic bead peptide enrichment-MALDI-TOF	Nafar et al. 2014; Candiano et al. 2006; Navarro-Muñoz et al. 2012; Pérez et al. 2014

Different protein biomarkers detected in urine, serum, or cultured cells which were discriminated FSGS subjects from normal subjects or other glomerular diseases and hence have diagnostic value

region between domains D_I and D_{II} and released in the circulation in the form of different fragments (Smith and Marshall 2010) and hence categorized as a circulating permeability factor.

It is believed that suPAR acts through binding to and activating beta 3 integrin signaling in podocytes. Two members of the integrin family (α3β1 and αvβ3) have a major role in podocyte structure and function in which their aberrant activity via binding to suPAR may lead to foot process effacement followed by proteinuria (Wei et al. 2011). Contribution of suPAR-mediated signaling in FSGS is suggested by Wei and colleagues in 2011 (Wei et al. 2011).

The role of circulating permeability factors in pathogenesis of FSGS has been discussed in the literature and postulated to the following reasons: (a) rapid recur of

primary FSGS after kidney transplantation in adults and children (30 % and 50 %, respectively) and delayed relapse using plasmapheresis before transplantation, (b) occurrence of proteinuria in rats after injection of plasma fractions of patients with FSGS, (c) increment of albumin permeability in an ex vivo model of isolated glomerulus after addition of sera of FSGS patients, and (d) transmission of FSGS from mother to fetus (McCarthy et al. 2010). Therefore, one can hypothesize that suPAR as a circulating permeability factor which probably stems from injured podocytes might be detected in serum or urine of FSGS patients and has diagnostic value. Several research groups have tested this hypothesis from 2011 up to now (Wei et al. 2011; McCarthy et al. 2010; Meijers et al. 2014). Wei and colleagues for the first time reported elevated levels of serum suPAR in FSGS subjects compare with other glomerular diseases (Wei et al. 2011). They reported elevated serum suPAR level in native and recurrent FSGS cases and then verified this report in two different cohorts composed of the FSGS Clinical Trial and the PodoNet European FSGS consortium (Wei et al. 2011, 2012). Versus the fans of serum suPAR diagnostic value in FSGS, Bock and Meijers believe that serum suPAR cannot discriminate FSGS patients from non-FSGS patients or healthy controls (Bock et al. 2013; Meijers et al. 2014).

Jefferson has reviewed the limitations of the studies which demonstrated elevated levels of serum suPAR as follows: (1) Lack of enough sensitivity (only a part of FSGS subjects in each group had elevated suPAR level). This might be due to heterogeneity of the studied population or confounding factors that were not considered. (2) Sampling in different courses of disease in which levels of suPAR might be variable. (3) Association of FSGS with other conditions which might result in increased suPAR level such as inflammatory disorders, atherosclerotic disease, myocardial infarction, systemic lupus erythematosus, and some of the cancers (Jefferson and Shankland 2013). Although suPAR was detected in some studies as a differential molecule in FSGS compared with healthy condition or other glomerular diseases, being not specific to FSGS (it showed elevated level also in membranous nephropathy (Wei et al. 2011)), inconsistent and irreproducible results in other cohorts (Bock et al. 2013; Meijers et al. 2014) bring the value of suPAR as a valid biomarker under the question. While suPAR level in serum is controversial, there are no conflicting results in measuring urinary levels of suPAR in FSGS up to now.

Urinary suPAR was measured in a robust and well-designed study using ELISA kit in 62 patients with primary FSGS (Huang et al. 2014). suPAR level is, then, compared with minimal change disease (MCD), membranous nephropathy (MN), secondary FSGS, and normal subjects. Subsequently, the effect of urinary suPAR on activating β3 integrin was investigated in cultured podocytes. The conclusion was interesting: urinary suPAR level is specifically increased in primary FSGS (especially in cellular variant) and β3 integrin signaling is induced by urinary suPAR level in human podocytes (Huang et al. 2014).

Taken together, suPAR is implied in pathogenesis of podocyte disorders such as FSGS, and future investigations on its molecular mechanism will definitely help to better insight of the disease. Although suPAR has pathogenic value, its diagnostic value is still unclear. We suggest that measuring urinary suPAR might be more

promising in biomarker discovery of FSGS compared with serum suPAR which has a heterogeneous population of fragments.

CD80

CD80 also known as B7-1 is a transmembrane protein which is mostly expressed on the surface of B lymphocytes and antigen-presenting cells (Henry et al. 1999). Expression of CD80 on podocytes in some patients with primary FSGS suggests the contribution and relationship between this protein and FSGS (Reiser and Mundel 2004). Remodeling of actin cytoskeleton and modulation of components of the slit diaphragm due to expression of CD80 on podocytes under stress conditions might justify its contribution in the pathogenesis of FSGS (Reiser and Mundel 2004; Reiser et al. 2004).

Recently, Cara-Fuentes demonstrated CD80 as a differential biomarker between genetic form and primary FSGS and thus considered it as a diagnostic urinary biomarker accompanied with serum suPAR (Cara-Fuentes et al. 2013). In another study by the same group, CD80 is represented as a differential biomarker between MCD and FSGS (Cara-Fuentes et al. 2014). The latter has been reviewed by Davin in a letter in *Pediatric Nephrology* journal recently (Davin 2014). Devin believes in bias in their conclusion regarding pathophysiological role of CD80 in MCD patients and not in FSGS and linked this bias to higher number of values considered in MCD compared with in FSGS. On the other hand, their claim was discordant with the report of Yu et al., based on positive staining for B7-1 (CD80) along peripheral capillary walls in biopsy slides of patients with primary FSGS (two of three) and MCD (three of five). In general, Yu et al. showed clear involvement of CD80 at least in some cases in the pathogenesis of FSGS and suggested CD80 as a treatment biomarker for FSGS and a target for abatacept (Yu et al. 2013).

Albeit there is conflict about the pathophysiological role of CD80 in FSGS in the mentioned studies, diagnostic value of CD80 cannot be excluded. Evaluating CD80 is suggested with other high-resolution techniques and not only by commercially available kits and in larger cohorts.

uNGAL

Neutrophil gelatinase-associated lipocalin (NGAL), also called lipocalin 2 or siderocalin or 24p3, is a member of lipocalin superfamily (Flower 1996). NGAL has several functions including participation in innate immune system (Goetz et al. 2002), activation or repression of iron-responsive genes (Yang et al. 2002), and induction of apoptosis through deprivation of trophic factors (Devireddy et al. 2005). This protein was detected originally in form of bound with MMP9 in human activated neutrophils (Kjeldsen et al. 1993). High expression of NGAL in human kidney in response to injury and correlation of its serum and urinary level with the severity of renal injury have been reported in various studies, and, hence, it is considered as a promising biomarker in clinical nephrology. NGAL, specially its urinary isoform (uNGAL), is mostly referred to as a sensitive biomarker for

progression of acute kidney injury (Makris et al. 2009). Diagnostic value of this protein has been demonstrated for FSGS recently. Chehade et al. showed uNGAL could significantly discriminate between healthy children and those with FSGS ($p = 0.007$) and between children with MCD and those with FSGS ($p = 0.01$) (Chehade et al. 2013). Proletov et al. also measured uNGAL and Cyc C in 104 patients with primary glomerulopathies including FSGS. Urinary Cys C and NGAL excretion in their study correlated with the degree of glomerulosclerosis and proteinuria and the reduced glomerular filtration rate (GFR) regardless of the method of its determination (Proletov et al. 2013). According to Korzeniecka-Kozerska's findings, MMP-9/NGAL ratio may serve as differentiation marker between minimal change nephrotic syndrome (MCNS) and FSGS in nephrotic children (Korzeniecka-Kozerska et al. 2013). Although Kozerska's study had some limitations, it was well designed. The limitations were small group of patients who were under treatment at enrollment, lack of biopsy for MCNS group, and use nonparametric statistics to analyze the data. The positive point of their study was subdividing the cohort into two groups and subjected them to examination twice (before treatment and after regression of proteinuria) which shows a wise study design.

Although there are not enough definitive studies regarding the role played by this molecule in the pathogenesis of kidney diseases and specially FSGS, this seems to be a promising diagnostic marker of FSGS besides its well-known potential value for monitoring the progression of chronic kidney diseases.

IGFBPs

Circulating growth factors that are filtered from the bloodstream and pass the glomerular filtration barrier influenced glomeruli and seem to be important candidates for renal disease pathogenesis as well as diagnosis. Several members of insulin-like growth factor-binding protein family with the gene name of IGFPB have been demonstrated as a diagnostic urinary marker for FSGS. Urinary excretion of IGFBP-1 and IGFBP-3 was reported by Worthmann and Peters as discriminating proteins between FSGS and MCD patients (Worthmann et al. 2010). They could find a relationship between the local expression of IGFBPs in podocytes as well as endothelial cells and the pathogenesis of glomerular disease. The induction of podocyte expression of these proteins by TGF-beta and bradykinin in this study suggested the involvement of TGF-beta signaling in the pathogenesis of FSGS. A recent proteomic study on podocyte cultured cells and mouse model of FSGS validated the role of IGFBP-rP1 in pathogenesis of podocyte disorders like FSGS (Matsumoto et al. 2010) and strengthened the probability of nomination of IGFBP family as biomarker. A recent data regarding noninvasive urinary diagnostic biomarkers for FSGS also revealed another member of insulin-like growth factor-binding proteins, namely, IBP7 (Nafar et al. 2014). Despite the small cohort of this study, detection of IBP7 and other suggested biomarkers in this study using a high-resolution mass spectrometry technique and high quality of multivariate statistical analysis were valuable.

Alpha-1-antitrypsin

Alpha-1-antitrypsin (A1AT), also referred to as SERPINA1, is a member of a family of serine protease inhibitors. A1AT inhibits trypsin, chymotrypsin, and plasminogen activator irreversibly. Serpins have several functions in the cell, including regulation of homeostasis, cellular survival, and blood clotting (Normandin et al. 2010). Urinary A1AT or at least some fragments of this protein in patients with FSGS have been reported by several studies. Candiano et al. used proteomic technique (two-dimensional electrophoresis followed by MALDI-TOF MS) to detect specific pattern of A1AT fragmentation in association with albumin in the urine of subject with nephrotic syndrome (FSGS, MCD, and MGN) (Candiano et al. 2006). Their findings were confirmed by Western blotting. Navarro-Muñoz et al. also reported two major peptides belonging to uromodulin and A1AT that distinguished between proliferative forms of glomerular kidney disease and nonproliferative forms (Navarro-Muñoz et al. 2012). According to their findings, nonproliferative forms correlated with higher A1AT peptide levels of which focal segmental glomerulosclerosis was linked more closely to high levels of the m/z 1945 peptide (one of the peptides of A1AT) than minimal change disease. They used magnetic bead peptide enrichment, MALDI-TOF MS analysis, and ClinProTools v2.0 to select differential peptides. The same group recently published another paper regarding urine peptide profiling of FSGS and MCD patients. Alpha-1-antitrypsin, in their study, showed lower peak area (unlike uromodulin with higher peak area) in FSGS patients compared with MCD patients and hence could be suggested as a potential diagnostic urinary marker for FSGS (Pérez et al. 2014). Elevated urinary excretion of A1AT is also reported in a proteomic study on FSGS and healthy controls using nano-LC-MS/MS technique (Nafar et al. 2014).

To sum up detection of A1AT by several advanced proteomic techniques in FSGS is enough to candidate this protein as a potential diagnostic biomarker; however, it does not seem to be highly specific.

Ribonucleic Acid Biomarkers

Fn14 and MCP-1 mRNA, miR-192, miR-205 and miRNA-186, miR-10a, and miR-30d and miR-200c are recent ribonucleic acid biomarkers reported for FSG (see Table 2). A comprehensive transcriptomic study on cultured podocytes and two distinct populations of patients with FSGS showed upregulation of Fn14 and monocyte chemotactic protein-1 (MCP-1) mRNA in glomeruli from patients with focal segmental glomerulosclerosis (Sanchez-Niño et al. 2013). Expression of both transcripts was also correlated. This finding also was confirmed in a second focal segmental glomerulosclerosis cohort. As NF-κB inhibitor parthenolide could prevent the activation of NF-κB signaling mediated by TWEAK activation and the following increase of MCP-1 mRNA and protein, a regulatory role was suggested for TWEAK receptor Fn14 in mediating glomerular inflammation. A significant difference observed in the urinary mRNA expression of MCP-1 (and a

Table 2 Diagnostic ribonucleic acid candidate biomarkers

Name of transcript	Name of expressed protein	Source	Analytical technique	Reference
Fn14 mRNA	Fibroblast growth factor-inducible immediate-early response protein 14	Cultured podocyte	RT-PCR	Sanchez-Niño et al. 2013
MCP-1 mRNA	Monocyte chemotactic protein-1	Cultured podocyte/ urine	RT-PCR	Sanchez-Niño et al. 2013; Szeto et al. 2005
miR-192, miR-205	–	Serum	RT-qPCR	Cai et al. 2013
miR-186	–	Serum	RT-qPCR	Zhang et al. 2014a
miR-200c	–	Urine	RT-qPCR	Wang et al. 2013
miR-10a, miR-30d	–	Urine	RT-qPCR	Wang et al. 2012

mRNA or microRNA diagnostic biomarkers detected in different sources by transcriptomic techniques

few other target genes) between disease groups (including IgA nephropathy and glomerulosclerotic patients) and controls in the other transcriptomic study is performed by real-time polymerase chain reaction (RT-PCR) (Szeto et al. 2005). In this report urinary MCP-1 expression correlated with the degree of glomerulosclerosis.

MicroRNAs (miRNAs, miRs) are involved in most physiological, developmental, and pathological processes and hence scientists devoted much attempts to investigate miRNAs recently. miR-192 and miR-205 are expressed preferentially in the renal cortex and believed to have a role in renal diseases. Serum levels of these two miRNAs in FSGS, MCD, and healthy control subjects have been evaluated by RT-qPCR technique (Cai et al. 2013). Accordingly patients with FSGS had higher serum levels of miR-192 and miR-205 than those with MCD. Positive correlation between the levels of miR-192 and miR-205 and proteinuria is also observed in FSGS patients.

Plasma miR-186 was proposed by Zhang et al. (2014a) as a biomarker for FSGS with nephrotic proteinuria (Zhang 2014a). They applied quantitative reverse transcription–polymerase chain reaction analysis for studying the plasma miRNAs in FSGS and healthy subjects.

Wang and colleagues in two distinct studies reported urinary miR-10a and miR-30d as differential candidate biomarkers for FSGS and healthy kidney donors and urinary miR-200c as differential between FSGS or MCD patients and those with other causes of nephrotic syndrome (Wang et al. 2012, 2013).

Since miRNAs are relatively stable and many of them can be found in extracellular fluid such as plasma, serum, and urine in addition to tissues and cells, we believe that they can be ideal source of biomarkers for disease. In this regard,

miRNAs beside protein biomarkers are promising in diagnosis of glomerular diseases such as FSGS.

Metabolite Biomarkers

Metabolomics is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al. 1999). A vast variety of molecules are considered as metabolites such as: lipids, sugars, amino asides, and small peptides. The major techniques for detection and investigation of these molecules are nuclear magnetic resonance (NMR), gas chromatography coupled with mass spectrometry (GC-MS), and liquid chromatography coupled with mass spectrometry. Serum and urinary metabolites are appropriate candidates for noninvasive diagnosis and could shed light on the pathogenesis of the diseases and especially renal diseases.

In this regard, Rosendale et al. analyzed urinary metabolites using NMR technique on patients with membranous lupus nephritis (LN), proliferative lupus nephritis, and FSGS (Romick-Rosendale et al. 2011). They suggested hippurate as a perfect discriminator for distinguishing between class V LN and FSGS patients with 100 % specificity, 100 % sensitivity, and 100 % accuracy.

Later, Hao et al. performed a comprehensive study on urinary metabolite biomarkers differentiating FSGS from other glomerular diseases with the same technique (Hao et al. 2013).

In FSGS patients they detected elevated urinary levels of glucose, dimethylamine, and trimethylamine compared with healthy controls and decreased level of pyruvate, valine, hippurate, isoleucine, phenylacetylglycine, citrate, tyrosine, 3-methylhistidine, and β-hydroxyisovalerate. Additionally, FSGS patients had lower urine N-methylnicotinamide levels compared with other glomerulopathies. The advantages of this study are (1) a relatively enough number of glomerular diseases as control disease, (2) enough clinical data with consideration of exclusion criteria, and (3) strong statistical analysis with validation using permutation tests.

In a recent study, urinary proteins and metabolites from pediatric idiopathic nephrotic syndrome (INS) are profiled via label-free mass spectrometry technique (Sedic et al. 2014). Hydroxyphenylacetate and uridine showed upregulation, while glutamine and phenylalanine levels showed downregulation in INS subjects. The data showed involvement of oxidative stress in pathogenesis of nephrotic syndrome. However, they revealed involvement of some specific metabolites for the first time; the study had some limitations including a relatively small cohort and lack of specification of nephrotic syndrome.

Serum and urinary malondialdehyde (MDA), an end product of lipid peroxidation, are suggested as a diagnostic biomarker discriminating FSGS from MCD patients by two research groups during 2005 and 2010 (Kuo et al. 2005; Nezhad et al. 2010). Since MDA is an important and known biomarker of oxidative stress, these findings emphasize the involvement of oxidative stress-induced lipid

Table 3 Diagnostic metabolite candidate biomarkers

Metabolite name	Source	Analytical technique	Reference
Hippurate	Urine	NMR	Romick-Rosendale et al. 2011
Glucose, dimethylamine, trimethylamine, pyruvate, valine, hippurate, isoleucine, phenylacetylglycine, citrate, tyrosine, 3-methylhistidine, β-hydroxyisovalerate, N-methylnicotinamide	Urine	NMR	Hao et al. 2013
Hydroxyphenylacetate, uridine, glutamine, phenylalanine	Urine	Label-free mass spectrometry	Sedic et al. 2014
Malondialdehyde	Serum/urine/glomeruli	Immunostaining	Kuo et al. 2005; Nezhad et al. 2010

Metabolites detected in urine, serum, or tissue of FSGS subjects using metabolomics techniques for diagnosis purposes

peroxidation in FSGS pathogenesis. Metabolite candidate biomarkers which are discussed above have been tabulated in Table 3.

Prognostic Biomarkers

We focus here on the important protein biomarkers reported in the literature.

There is no adequate evidence regarding ribonucleic acid or metabolite biomarkers for monitoring the disease progression. Future analysis using powerful high-throughput techniques that are complement of proteomics such as microarray in transcriptomic platform and NMR or GC-MS in metabolomics platform may clarify new candidates for prognosis of FSGS. Protein candidate biomarkers which are presented here have been tabulated in Table 4.

WT1

WT1, a transcription factor belonging to zinc finger superfamily, is associated with podocytes and essential for normal podocyte function (Zhou et al. 2008). It has a crucial role in development of kidney (maturation of podocytes) and genital system and regulates its target genes by binding to both DNA and messenger RNA (mRNA). Its mutation is associated with some diseases including Denys–Drash syndrome (mutation in exon 8 or 9), Frasier syndrome (mutation in intron 9), and WAGR syndrome (a deletion in WT1) (Niaudet and Gubler 2006; Fischbach et al. 2005). All of these mentioned syndromes are associated with FSGS. Since one of the target genes of this transcription factor is TGF-ß (Niaudet and Gubler

Table 4 Prognostic candidate biomarkers

Protein ID	Protein name	Source	Analytical technique	Reference
WT1	Wilms tumor protein	Urinary exosome/ podocyte-like cell line	Western blot	Zhou et al. 2013
AFAM	Afamin	Urine	LC-MS/MS, concanavalin A (ConA) enrichment	Zhao et al. 2014; Kalantari et al. 2014a
AMBP	α_1-Microglobulin	Urine	Nano-LC-MS/MS	Zhao et al. 2014; Kalantari et al. 2014a
OPN	Osteopontin	Glomeruli, urine	RT-PCR, Western blot, immunohistochemistry	Shui et al. 2007

Protein biomarkers useful for monitoring the disease severity

2006), one can hypothesize that one of the mechanisms of its involvement in glomerular diseases especially those with podocyte disorders could be via TGF-ß signaling. The relationship between TGF-ß and WT1 was described for the first time in a study in 2011 on human podocyte cell line treated with TGF-ß1 and kidneys in Alb/TGF-beta1-transgenic mice (Sakairi et al. 2011). Their findings showed TGF-beta1 reduced WT1 expression. It appears that there is a complex and mutual relationship between WT1 and TGF-ß and they can regulate each other. In the latter study, also WT1 is introduced as a potential useful marker of early podocyte injury.

Urinary exosomal WT1 is showed by another study to be a prognostic marker for monitoring the progression of podocyte injury in animal models as well as a diagnostic biomarker in FSGS patients (Zhou et al. 2008). Their findings showed the increased WT1 very early in the first and third day after puromycin injection, before albuminuria was detectable, and continued elevation at 7th day to 14th day in PAN-treated rats. Later, another study by the same author revealed urinary exosomal WT1 as a predictor of the onset of disease earlier than proteinuria in a mouse model of collapsing glomerulopathy (Zhou et al. 2013). Their findings also indicated the potential differentiating power of urinary exosomal WT1 in distinguishing active FSGS from active steroid-sensitive nephrotic syndrome (SSNS). WT1 in these studies was not detected in healthy subjects.

As several studies show the involvement of WT1 in FSGS and podocyte injury (elevated level with the increase of podocyte injury), it could be a useful candidate for diagnosis as well as prognosis. We suggest more investigations on the level of changes in a cohort consist of patients with well (lower proteinuria or higher GFR) and worse (higher proteinuria or lower GFR) prognosis using high-resolution techniques like single-reaction monitoring (SRM).

Afamin

The glycoprotein afamin, a member of the albumin gene family, is a vitamin E-binding protein which plays a crucial role in oxidative stress-related anti-apoptotic cellular processes (Heiser et al. 2002). It has been detected in kidney diseases and described as a candidate biomarker; nonetheless, very little is known about its physiological or pathological function. However, there is only a single report up to now regarding afamin as a prognostic biomarker for FSGS and involvement in disease progression; we brought it here because of reliable and valid data as well as the value of this study in terms of discovery of noninvasive biomarkers in urine. Proteomic technique such as LC-MS/MS (triple TOF mass spectrometer) accompanied by Western blot and concanavalin A (ConA) enrichment of urinary proteins are state-of-the-art techniques which authors applied (Zhao et al. 2014). They reported a 1.16-fold change in the rat urine on the third day after ADR injection with a continuous elevation of up to 5.63-fold on day 23 after the ADR injection. Despite this fact that afamin is an orthologous protein in human and also detection of this protein in a pilot study on FSGS patients used high-resolution proteomic tools (Kalantari et al. 2014a), further studies on human samples in a large cohort are essential.

AMBP

α_1-Microglobulin (AMBP), a 27–33 kDa glycoprotein, is synthesized by the liver and is a known marker for tubular proteinuria. It can pass freely through filtration barrier in glomerulus and reabsorbed by the proximal tubule (Devarajan et al. 2010). Some of the possible roles of α_1-microglobulin are immunoprotective/anti-inflammatory role, inhibition of IL-1-beta, and monocyte free radical production in a complex form with collagen (Santin and Cannas 1999). Nevertheless, the full biological function of this protein is not clear. It has been reported previously as a prognostic marker for nephrological disorders such as membranous nephropathy and diabetic nephropathy (Ponticelli and Passerini 2010; Marczewski et al. 1996) as well as a diagnostic marker for urological disorders (Everaert et al. 2000). AMBP was detected for the first time as a prognostic biomarker in the urine samples from FSGS patients with different prognoses using proteomic tools (Kalantari et al. 2014a). Soon later another research group reported AMBP as a useful candidate for early detection of FSGS, as it decreased during the first 7 days after ADR injection to the rat models (Zhao et al. 2014). They verified the findings using Western blot. Although changes of AMBP in both studies were detected during different phases of FSGS in the urine samples from rat models and human subjects, the trend was opposite. This protein with animal source showed a decrease during the early phase of ADR-induced FSGS; however, it indicated an increase with the ratio of 4.39 from the human source. Since both studies applied powerful analytical tools, the judgment is tough and further analysis with a large sample size may ensure us about the trend of changes of AMBP during the disease course.

Osteopontin

Osteopontin (OPN) is an acidic 70 kD glycoprotein that is classified as an extracellular matrix (ECM) protein. Various cell types can secrete OPN in the normal conditions including vascular smooth muscle cells, lymphocytes, macrophages, epithelial cells, cells of tubular system, and also podocyte cells in the pathogenic conditions (Schordan et al. 2013).

Overexpression of OPN is a well-known marker for tubulointerstitial diseases, FSGS, and diabetic nephropathy (Shui et al. 2007; Susztak et al. 2004). OPN mechanisms of action in glomerular injury have been investigated in several studies (Shui et al. 2007; Lorenzen et al. 2008; Endlich et al. 2002; Nakamura et al. 2005; Teramoto et al. 2005). It is suggested that it induces the expression of urokinase-type plasminogen activator (uPA), MMP-2, and MMP-9 through the NF-κB pathway which results in increased podocyte motility and proteinuria (Lorenzen et al. 2008). Contribution of OPN in F-actin reorganization involved in podocyte foot process effacement (Endlich et al. 2002), recruiting macrophage toward the compromised glomeruli (Shui et al. 2007), and binding of OPN to CD44 which induces the loosening of cell-matrix adhesion and cellular crescent formation are other possible mechanisms (Nakamura et al. 2005; Teramoto et al. 2005). However, some of the investigators believe that OPN is essential for preventing early damage of podocytes rather than promoting glomerulosclerosis or recruitment of macrophages (Schordan et al. 2013). Therefore, there is still controversy regarding inflammatory role of OPN in developing FSGS and its proinflammatory role in preventing podocyte injury.

Besides several attempts to elucidate the pathophysiological role of OPN, a well-designed study by Shui and colleagues (Shui et al. 2007) showed its potential capacity as a biomarker of disease progression in FSGS. They applied RT-PCR and Western blot to evaluate OPN in isolated glomeruli and urine from FSGS-induced rat models and immunohistochemistry technique to assess the OPN expression in epithelial lesions and macrophage infiltration around the glomeruli. They found that OPN mRNA and protein correlate with sclerosis and may be helpful in the prognosis of FSGS. The only weak point of their study was the lack of comparison of the results with human subjects.

Uncommon Prognostic Biomarkers

Serotransferrin, kininogen-1, fibronectin (FN), Rab23, and annexin A1 are some of the well-defined prognostic biomarkers in FSGS-induced rat models that are not validated in human subjects (Zhao et al. 2014; Shui et al. 2006, 2008) (see Table 5). Serotransferrin and kininogen were detected in the urine of rat models by two independent research groups that both applied proteomic techniques for identification (LC-MS/MS and two-dimensional electrophoresis followed by MALDI-TOF-MS). Other candidates that had dynamic changes in the urine of FSGS-induced rat

Table 5 Uncommon prognostic candidate biomarkers

Protein name	Source	Analytical technique	Reference
Kininogen, kallikrein, glutathione S-transferase, apoptosis-inducing factor-2, annexin A1, E-cadherin, collagen fragment, ECM protein 1, cerberus, tomoregulin fragment, ADAM 32	Urine	2DE-MALDI-TOF	Shui et al. 2008
Ceruloplasmin, cadherin-2, fetuin-B, beta-2-microglobulin, alpha-1-antiproteinase, alpha-2-HS-glycoprotein	Urine	LC-MS/MS	Zhao et al. 2014
Fibronectin	Serum, tissue	Immunoassay, immunohistochemistry, Western blot	Shui et al. 2006
Rab23	Serum, urine, tissue	Western blot, RT-PCR, immunohistochemistry	Huang et al. 2009
Annexin A1	Urine, tissue	2DE-MALDI-TOF , immunohistochemistry	Shui et al. 2008; Ka et al. 2014
Ribonuclease 2, CD59, prostaglandin-H2 D-isomerase, beta-2-microglobulin, extracellular sulfatase 2, corticosteroid-binding globulin, matrix-remodeling-associated protein 8, collagen alpha-1 (VI) chain, actin, and haptoglobin	Urine	Nano-LC-MS/MS	Kalantari et al. 2014a

Prognostic candidates detected in different sources which are reported in single study or only in animal models without validation in human subjects

models and are identified by 2DE-MALDI-TOF technique were kallikrein and kininogen precursor which caused hemodynamic problems, glutathione S-transferase, apoptosis-inducing factor-2 (AIF-2), annexin A1 which is involved in raised oxidative stress and apoptosis, E-cadherin which causes epithelial cell damage, collagen fragment, ECM protein 1, cerberus, tomoregulin fragment, and ADAM 32 which affects glomerular sclerosis (Shui et al. 2008). The urinary candidate proteins with dynamic changes during FSGS induction identified using LC-MS/MS were ceruloplasmin, cadherin-2, fetuin-B, and beta-2-microglobulin. The following proteins, identified using LC-MS/MS, are found to be orthologous with human proteins and verified by Western blot: alpha-1-antiproteinase, alpha-2-HS-glycoprotein, as well as afamin and AMBP which are mentioned earlier (Zhao et al. 2014). Fibronectin (FN) is suggested by Shui's research group in 2006 (Shui et al. 2006). They measured FN in the serum and urine using immunoassay technique and assayed in the tissue using Western blot, real-time PCR, and immunohistochemistry. As the increased trend of FN was first detected in the serum, then in the glomeruli and ultimately in the urine, one hypothesized that elevation of serum FN may contribute to FN deposition in glomeruli and final release to the urine. Verification of this valuable finding with human subjects has not been performed yet.

Rab23 was introduced in 2009 as a candidate biomarker that indicates the severity of FSGS in mice models (Huang et al. 2009). It was evaluated in the serum, urine, and mice kidneys by Western blot and monitored up to 20 days after FSGS induction using ADR injection to the mice models. Real-time PCR and immunohistochemistry are also applied to investigate its expression. The elevations of Rab23 were observed in the urine and glomeruli (mesangial cells) but not in the serum. The possible role of elevated Rab23 in FSGS state might be suppression of hedgehog signaling and/or influence collagen synthesis.

Urinary annexin A1 was detected in two independent studies on adriamycin-induced glomerulopathy mice models (Shui et al. 2008; Ka et al. 2014). However, in one of them, it was also measured in the urine of FSGS patients and other glomerulopathy human subjects; it was not specific for FSGS and hence we considered it as uncommon prognostic marker. Annexin A1 was also evaluated in the glomeruli by immunohistochemistry technique. The authors suggested that urinary ANXA1 comes from damaged and/or apoptotic renal tissues.

Prognostic urinary biomarker candidates recently detected in FSGS patients using nano-LC-MS/MS technique that are not confirmed by complementary techniques or in a larger population are ribonuclease 2, CD59, prostaglandin-H2 D-isomerase, beta-2-microglobulin, extracellular sulfatase 2, corticosteroid-binding globulin, matrix-remodeling-associated protein 8, collagen alpha-1 (VI) chain, actin, and haptoglobin (Kalantari et al. 2014a).

Because all of these candidates are detected in one single experiment without validation or only in animal models, we brought them under the title of "uncommon prognostic biomarkers."

Candidates for Both Diagnosis and Prognosis

Several biomarker candidates may serve as both diagnostic and prognostic including: urinary NGAL, urinary suPAR, and WT1.

These kinds of biomarkers appear to be more important and worth for more investigation and validation due to their capability of determining the type and severity of kidney damage simultaneously in a single test in the future clinical world.

uNGAL had a decrease in children with FSGS (in remission) in a 1-year follow-up study. Its level correlated negatively with creatinine clearance and correlated positively with both reduction of GFR and proteinuria (Youssef and El-Shal 2012). It should be noted that the elevated level of uNGAL compared with healthy subjects could be used as a diagnostic marker, while its decreased level is important to monitor the prognosis of FSGS.

In a comprehensive study on evaluating the urinary suPAR changes among FSGS patients and other nephropathies and normal controls, urinary suPAR could discriminate FSGS from other conditions as well as FSGS with complete remission after therapy (Huang et al. 2014). Accordingly, a decreasing trend was observed in

the median of urinary suPAR level in eight patients with complete remission but not partial remission after 80 weeks follow-up.

Several studies on exosomal WT1 on mice models as well as human subjects verified its value as a candidate biomarker for diagnosis and prognosis (Zhou et al. 2008, 2013; Orloff et al. 2005).

Predictive Biomarkers

Predictive biomarkers mostly refer to biomolecules that have different amounts in patients who are responder or nonresponder to a selective drug. In addition, these kinds of biomolecules can predict the state of relapse or remission before initiation therapy or predict the recurrence of FSGS after renal transplantation. Although predictive biomarkers are very applicable in saving time for choosing the appropriate therapy and preventing the side effects of noneffective therapy, the number of studies on these kinds of biomarkers for FSGS is very few. The noninvasive biomarkers detected in urine or serum have special value for prediction and most of the studies carried on these specimens. Because there are few overlaps between these studies and there is no validated biomarker for responsiveness or recurrence prediction, most of the following mentioned biomarkers may be considered as "uncommon predictive biomarkers." However, some of them might have diagnostic or prognostic value as well which were explained in sections "Diagnostic Biomarkers" and "Prognostic Biomarkers."

Apolipoprotein A-I

Apolipoprotein A-I (APOA-I) was detected in two independent studies on urinary predictive biomarkers on responder and nonresponder FSGS patients to steroid therapy and relapsing and non-relapsing FSGS patients (Lopez-Hellin et al. 2013; Kalantari et al. 2014b). Proteomic tools were applied in both studies. Two-dimensional electrophoresis followed by MALDI-TOF analysis showed a form of apolipoprotein A-I named APOA-Ib exclusively in the urine of relapsing FSGS. This study suggested the capacity of APOA-Ib for prediction of relapse before the clinical manifestation. This finding was verified using Western blot in the same patients as well as independent group of patients (Lopez-Hellin et al. 2013). Kalantari et al. applied nano-LC-MS/MS technique and detected 3.15-fold change of APOA-I in the urine of steroid-sensitive compared to steroid-resistant FSGS patients. The false discovery rate (FDR) <1 % in label-free quantification and introducing a predictive model with 100 % accuracy using multivariate statistical analysis for differentiating steroid-sensitive and steroid-resistant group are the strength aspects of this study (Kalantari et al. 2014b). They also reported 20 other candidates besides APOA-I as urinary predictive biomarkers for FSGS based on

multivariate predictive model: PGRP2, ACTG, FBLN3, PGBM, AACT, IGHG1, TRFE, YIPF3, A1AG2, THBG, A1BG, A2GL, ANAG, S10A9, CUBN, TITIN, AMPN, CLUS, IPSP, and MXRA8.

It is not well understood whether APOA-I is a consequence of relapsing FSGS or is related to its pathogenesis or how it is related to responsiveness, but there are some evidence about involvement of HDL or its components (including APOA-I and APOA-II) in FSGS. One possible hypothesis is the different sclerotic potential of SSNS patients in comparison with SRNS due to different excretions of APOA-I (Kalantari et al. 2014b).

Alpha 1-B Glycoprotein

Alpha 1-B glycoprotein (A1BG) is a member of the immunoglobulin superfamily with an unknown function. Detection of this protein in two independent studies on urine proteome profile of FSGS patients with different responsiveness enhances the possibility of being a specific biomarker of A1BG for differentiating SSNS from SRNS. Upregulation of this protein in the urine of steroid-resistant FSGS patients was consistent in both reports. Piyaphanee and colleagues identified an 11-fold upregulated 13.8 kDa fragment of α 1-B glycoprotein (A1BG) in urine of SRNS patients using SELDI-TOF and verified their finding by Western blot (Piyaphanee et al. 2011). A 1.2-fold upregulation of this protein is also detected recently using nano-LC-MS/MS (Kalantari et al. 2014b). It has worth to be validated in a large sample size in the future studies.

Uncommon Predictive Biomarkers

A pilot study in 1997 showed excretion of some nonspecific proteins with homogenous anionic charge (albumin and transferrin) in the urine of steroid-resistant nephrotic syndrome (SRNS) patients as well as some proteins with heterogeneity of electrical charge (IgG, beta2-microglobulin, and lysozyme) in FGS (Ramjee et al. 1997).

Few years later in 2006, SELDI-TOF technique was used to classify SSNS and SRNS patients, and beta-2-microglobulin was reported as a biomarker associated with SRNS (Khurana et al. 2006). Beta-2-microglobulin is not a specific biomarker and known as a common excreted protein in renal failure. In addition the low resolution and low reproducibility of SELDI-MS technique diminish the reliability of the results. Some special fragments of albumin and/or of alpha1-antitrypsin in the urine of a group of nephrotic syndrome (SSNS and SRNS) were reported in the same year (Candiano et al. 2006). In the later study, the versatile techniques including 2D-E followed by MALDI-TOF and Western blot were applied.

Haptoglobin was reported as a serum predictive biomarker for SSNS patients (Wen et al. 2012). A relatively high number of subjects ($n = 146$), detection, and identification of biomarkers using proteomic tools (2D-E/MALDI-TOF) and

verification using two antibody-based techniques (Western blot and ELISA) were the privileges of this study. However, haptoglobin was not reported in the other similar study on SSNS and SRNS.

Cardiotrophin-like cytokine factor 1 (CLCF1), one of the permeability circulating factors, was reported as a predictive candidate for recurrence of FSGS when its level can reach to 100 times higher than normal subjects (Savin et al. 2008).

Besides these candidates which were reported in single studies are not further evaluated; few other candidates for predicting responsiveness to steroid drugs are also reported which have diagnostic value as well including suPAR (Li et al. 2014), urinary miR-30a-5p (Zhang et al. 2014b), and uNGAL (Bennett et al. 2012). Uncommon predictive candidates have been tabulated in Table 6.

To sum up, it is worthy to note that the majority of studies on predictive biomarkers performed by proteomic tools and most of the specimens were urine. This indicates the prominence of high-throughput techniques such as SELDI-TOF, LC-MS/MS, and 2D-E in clinical researches and excellence of urine as a source of noninvasive biomarkers. Further studies for validation of these noninvasive biomarker candidates in the future for clinical usage will be promising.

Summary Points

- This review focuses on different candidate biomarkers detected by proteomics (2DE-MALDI-TOF-MS, LC-MS/MS, SELDI-MS/MS), metabolomics (NMR, MS), transcriptomic (RT-PCR) tools, and other immune-based assay techniques (such as Western blot and ELISA).
- Some of the candidates that have been reported by different research groups through independent studies and detected by different techniques have been discussed under a separate subheading and also explained its pathogenic importance. The other candidates which have been reported only by one group or detected in animal models without validation in human subjects are discussed under the title of “uncommon biomarkers.”
- suPAR, NGAL, and WT1 proteins which have been reported in numerous researches have special value for both diagnosis and prognosis of FSGS.
- High-throughput platforms especially proteomics, transcriptomics, and metabolomics besides the recent advances in mass spectrometry have shown exciting results in biomarker discovery fields and more confident identification of biomarkers.
- As urine is in direct contact with the injured kidney, its collection is noninvasive and less complex than serum and could be the best choice for biomarker discovery of glomerular diseases including FSGS. In addition some of the urinary biomarkers such as suPAR are less controversial among the scientists than their serum isoforms.
- Although lots of attempts have been devoted for identification of biomarkers which are specific and sensitive enough for FSGS, there is still not a consensus on the single ideal biomarker. Therefore, using a panel of biomarkers as a

Table 6 Uncommon predictive candidate biomarkers

Protein ID	Protein/miRNA name	Source	Analytical technique	Reference
APOA1, PGRP2, ACTG, FBLN3, PGBM, AACT, IGHG1, TRFE, YIPF3, A1AG2, THBG, A1BG, A2GL, ANAG, S10A9, CUBN, TITIN, AMPN, CLUS, IPSP, MXRA8	Apolipoprotein A-I , N-acetylmuramoyl-L-alanine amidase, gamma-actin, EGF-containing fibulin-like extracellular matrix protein 1, basement membrane-specific heparan sulfate proteoglycan core protein, alpha-1-antichymotrypsin, Ig gamma-1 chain C region, transferrin, killer lineage protein 1, alpha-1-acid glycoprotein 2, thyroxine-binding globulin, alpha-1B-glycoprotein, leucine-rich alpha-2-glycoprotein, alpha-N-acetylglucosaminidase, calgranulin-B, cubilin, connectin, aminopeptidase N, clusterin, plasma serine protease inhibitor, matrix-remodeling-associated protein 8	Urine	LC-MS/MS	Kalantari et al. 2014b
IgG, B2MG, LYZ	Immunoglobulin G, beta2-microglobulin, and lysozyme	Urine	2DE	Ramjee et al. 1997
B2MG	Beta-2-microglobulin	Urine	SELDI-TOF	Khurana et al. 2006
ALB, A1AT	Albumin, alpha1-antitrypsin	Urine	2DE-MALDI-TOF	Candiano et al. 2006
HPT	Haptoglobin	Serum	2D-E/MALDI-TOF	Wen et al. 2012
suPAR	Soluble urokinase plasminogen activator receptor	Serum	ELISA	Li et al. 2014
–	miR-30a-5p	Urine	Probe-based quantitative RT-PCR	Zhang et al. 2014b
NGAL	Neutrophil gelatinase-associated lipocalin	Urine	ELISA	Bennett et al. 2012

Candidates detected mostly by proteomic techniques are reported in single study or in animal models which are useful for predicting responsiveness to steroid therapy

diagnostic array can serve to be more reliable, efficient, and practical in the future clinical world.

- Besides the mentioned high-throughput platforms which are also part of systems biology, the other areas of this science including bioinformatics and network science are able to bright the dark sides of mechanism and pathogenesis of the diseases and suggest the possible candidate biomarkers hypothetically to be tested and evaluated in the wet lab.

References

Bennett MR, Piyaphanee N, Czech K, et al. NGAL distinguishes steroid sensitivity in idiopathic nephrotic syndrome. Pediatr Nephrol. 2012;27:807–12.

Bock ME, Price HE, Gallon L, et al. Serum soluble urokinase-type plasminogen activator receptor levels and idiopathic FSGS in children: a single-center report. Clin J Am Soc Nephrol. 2013;8:1304–11.

Cai X, Xia Z, Zhang C, et al. Serum microRNAs levels in primary focal segmental glomerulosclerosis. Pediatr Nephrol. 2013;28:1797–801.

Candiano G, Musante L, Bruschi M, et al. Repetitive fragmentation products of albumin and alpha 1 antitrypsin in glomerular disease associated with nephrotic syndrome. J Am Soc Nephrol. 2006;17:139–48.

Cara-Fuentes G, Araya C, Wei C, et al. CD80, suPAR and nephrotic syndrome in a case of NPHS2 mutation. Nefrologia. 2013;33:727–31.

Cara-Fuentes G, Wei C, Segarra A, et al. CD80 and suPAR in patients with minimal change disease and focal segmental glomerulosclerosis: diagnostic and pathogenic significance. Pediatr Nephrol. 2014;29:1361–71.

Carraro M, Zennaro C, Artero M, et al. The effect of proteinase inhibitors on glomerular permeability induced in vitro by serum from patients with idiopathic focal segmental glomerulosclerosis. Nephrol Dial Transplant. 2004;19:1969–75.

Chehade H, Parvex P, Poncet A, et al. Urinary low-molecular-weight protein excretion in pediatric idiopathic nephrotic syndrome. Pediatr Nephrol. 2013;28:2299–306.

Chun MJ, Korbet SM, Schwartz MM, et al. Focal segmental glomerulosclerosis in nephrotic adults: presentation, prognosis, and response to therapy of the histologic variants. J Am Soc Nephrol. 2004;15:2169–77.

Davin JC. CD80 and suPAR: diagnostic and pathogenic value in minimal change disease and focal segmental glomerulosclerosis? Pediatr Nephrol. 2014;29:1465–6.

Deegens JKJ, Steenbergen EJ, Borm GF, et al. Pathological variants of focal segmental glomerulosclerosis in an adult Dutch population—epidemiology and outcome. Nephrol Dial Transplant. 2008;23:186–92.

Devarajan P, Krawczeski CD, Nguyen MT, et al. Proteomic identification of early biomarkers of acute kidney injury after cardiac surgery in children. Am J Kidney Dis. 2010;56:632–42.

Devireddy LR, Gazin C, Zhu X, et al. A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. Cell. 2005;123:1293–305.

Endlich N, Sunohara M, Nietfeld W, et al. Analysis of differential gene expression in stretched podocytes: osteopontin enhances adaptation of podocytes to mechanical stress. FASEB J. 2002;16:1850–2.

Everaert K, Hoebeke P, Delanghe J. A review on urinary proteins in outflow disease of the upper urinary tract. Clin Chim Acta. 2000;297:183–9.

Falk RJ, Jennette JC, Nachman PH. Glomerular disease. In: Brenner B, editor. The kidney. 6th ed. Philadelphia: Saunders; 2000. p. 1283–349.

Fischbach BV, Trout KL, Lewis J, et al. WAGR syndrome: a clinical review of 54 cases. Pediatrics. 2005;116:984–8.

Flower DR. The lipocalin protein family: structure and function. Biochem J. 1996;318:1–14.

Goetz DH, Holmes MA, Borregaard N, et al. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell. 2002;10:1033–43.

Hao X, Liu X, Wang W, et al. Distinct metabolic profile of primary focal segmental glomerulosclerosis revealed by NMR-based metabolomics. PLoS One. 2013;8, e78531.

Hattori M, Akioka Y, Chikamoto H, et al. Increase of integrin-linked kinase activity in cultured podocytes upon stimulation with plasma from patients with recurrent FSGS. Am J Transplant. 2008;8:1550–6.

Heiser M, Hutter-Paier B, Jerkovic L, et al. Vitamin E binding protein afamin protects neuronal cells in vitro. J Neural Transm Suppl. 2002;62:337–45.

Henry J, Miller MM, Pontarotti P. Structure and evolution of the extended B7 family. Immunol Today. 1999;20:285–8.

Huang TH, Shui HA, Ka SM, et al. Rab 23 is expressed in the glomerulus and plays a role in the development of focal segmental glomerulosclerosis. Nephrol Dial Transplant. 2009;24:743–54.

Huang J, Liu G, Zhang YM, et al. Urinary soluble urokinase receptor levels are elevated and pathogenic in patients with primary focal segmental glomerulosclerosis. BMC Med. 2014;12:81.

Jefferson JA, Shankland SJ. Has the circulating permeability factor in primary FSGS been found? Kidney Int. 2013;84:235–8.

Ka SM, Tsai PY, Chao TK, et al. Urine annexin A1 as an index for glomerular injury in patients. Dis Markers. 2014;2014:1–12.

Kalantari S, Nafar M, Samavat S, et al. Urinary prognostic biomarkers in patients with focal segmental glomerulosclerosis. Nephrourol Mon. 2014a;6, e16806.

Kalantari S, Nafar M, Rutishauser D, et al. Predictive urinary biomarkers for steroid-resistant and steroid-sensitive focal segmental glomerulosclerosis using high resolution mass spectrometry and multivariate statistical analysis. BMC Nephrol. 2014b;15:141.

Kato H, Susztak K. Repair problems in podocytes: Wnt, notch and glomerulosclerosis. Semin Nephrol. 2012;32:350–6.

Khurana M, Traum AZ, Aivado M, et al. Urine proteomic profiling of pediatric nephrotic syndrome. Pediatr Nephrol. 2006;21:1257–65.

Kjeldsen L, Johnsen A, Sengeløv H, et al. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem. 1993;268:10425–32.

Korzeniecka-Kozerska A, Wasilewska A, Tenderenda E, et al. Urinary MMP-9/NGAL ratio as a potential marker of FSGS in nephrotic children. Dis Markers. 2013;34:357–62.

Kuo HT, Kuo MC, Chiu YW, et al. Increased glomerular and extracellular malondialdehyde levels in patients and rats with focal segmental glomerulosclerosis. Eur J Clin Invest. 2005;35:245–50.

Lee HS. Pathogenic role of TGF-ß in diabetic nephropathy. J Diabetes Metab. 2013;S9:008.

Li F, Zheng C, Zhong Y, et al. Relationship between serum soluble urokinase plasminogen activator receptor level and steroid responsiveness in FSGS. Clin J Am Soc Nephrol. 2014;9:1903–11.

Lopez-Hellin J, Cantarell C, Jimeno L, et al. A form of apolipoprotein a-I is found specifically in relapses of focal segmental glomerulosclerosis following transplantation. Am J Transplant. 2013;13:493–500.

Lorenzen J, Shah R, Biser A, et al. The role of osteopontin in the development of albuminuria. J Am Soc Nephrol. 2008;19:884–90.

Makris K, Markou N, Evodia E, et al. Urinary neutrophil gelatinase-associated lipocalin (NGAL) as an early marker of acute kidney injury in critically ill multiple trauma patients. Clin Chem Lab Med. 2009;47:79–82.

Marczewski K, Krawczyk W, Rozyc P, et al. Day/night ratio of microproteinuria and blood pressure rhythm in type II diabetes. Diabetes Res Clin Pract. 1996;33:169–72.
Matsumoto T, Hess S, Kajiyama H, et al. Proteomic analysis identifies insulin-like growth factor binding protein-related protein 1 as a podocyte product. Am J Physiol Renal Physiol. 2010;299 (4):F776–84.
McCarthy ET, Sharma M, Savin VJ. Circulating permeability factors in idiopathic nephrotic syndrome and focal segmental glomerulosclerosis. Clin J Am Soc Nephrol: Clin J Am Soc Nephrol. 2010;5:2115–21.
Meijers B, Maas RJ, Sprangers B, et al. The soluble urokinase receptor is not a clinical marker for focal segmental glomerulosclerosis. Kidney Int. 2014;85:636–40.
Nafar M, Kalantari S, Samavat S, et al. The novel diagnostic biomarkers for focal segmental glomerulosclerosis. Int J Nephrol. 2014;2014:574261.
Nakamura H, Kitazawa K, Honda H, et al. Roles of and correlation between alpha-smooth muscle actin, CD44, hyaluronic acid and osteopontin in crescent formation in human glomerulonephritis. Clin Nephrol. 2005;64:401–11.
Navarro-Muñoz M, Ibernon M, Bonet J, et al. Uromodulin and α(1)-antitrypsin urinary peptide analysis to differentiate glomerular kidney diseases. Kidney Blood Press Res. 2012;35:314–25.
Nezhad ST, Momeni B, Basiratnia M. Glomerular malondialdehyde levels in patients with focal and segmental glomerulosclerosis and minimal change disease. Saudi J Kidney Dis Transpl. 2010;21:886–91.
Niaudet P, Gubler MC. WT1 and glomerular diseases. Pediatr Nephrol. 2006;21:1653–60.
Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica. 1999;29:1181–9.
Normandin K, Peant B, Le Page C, et al. Protease inhibitor SERPINA1 expression in epithelial ovarian cancer. Clin Exp Metastasis. 2010;27:55.
Orloff MS, Iyengar SK, Winkler CA, et al. Variants in the Wilms' tumor gene are associated with focal segmental glomerulosclerosis in the African American population. Physiol Genomics. 2005;21:212–21.
Pérez V, Ibernón M, López D, et al. Urinary peptide profiling to differentiate between minimal change disease and focal segmental glomerulosclerosis. PLoS One. 2014;9, e87731.
Piyaphanee N, Ma Q, Kremen O, et al. Discovery and initial validation of α 1-B glycoprotein fragmentation as a differential urinary biomarker in pediatric steroid-resistant nephrotic syndrome. Proteomics Clin Appl. 2011;5:334–42.
Ponticelli C, Passerini P. Can prognostic factors assist therapeutic decisions in idiopathic membranous nephropathy? J Nephrol. 2010;23:156–63.
Proletov II, Saganova ES, Galkina OV, et al. Diagnostic value of cystatin C and neutrophil gelatinase-associated lipocalin in primary glomerulopathies. Ter Arkh. 2013;85:10–6.
Ramjee G, Coovadia HM, Adhikari M. Comparison of noninvasive methods for distinguishing steroid-sensitive nephrotic syndrome from focal glomerulosclerosis. J Lab Clin Med. 1997;129:47–52.
Reiser J, Mundel P. Danger signaling by glomerular podocytes defines a novel function of inducible B7-1 in the pathogenesis of nephrotic syndrome. J Am Soc Nephrol. 2004;15:2246.
Reiser J, von Gersdorff G, Loos M, et al. Induction of B7-1 in podocytes is associated with nephrotic syndrome. J Clin Invest. 2004;113:1390.
Rich AR. A hitherto undescribed vulnerability of the juxtamedullary glomeruli in lipoid nephrosis. Bull Johns Hopkins Hosp. 1959;100:173–86.
Romick-Rosendale LE, Brunner HI, Bennett MR, et al. Identification of urinary metabolites that distinguish membranous lupus nephritis from proliferative lupus nephritis and focal segmental glomerulosclerosis. Arthritis Res Ther. 2011;13, R199.
Sakairi T, Abe Y, Kopp JB. TGF-beta1 reduces Wilms' tumor suppressor gene expression in podocytes. Nephrol Dial Transplant. 2011;26:2746–52.

Sanchez-Niño MD, Poveda J, Sanz AB, et al. Fn14 in podocytes and proteinuric kidney disease. Biochim Biophys Acta. 2013;1832:2232–43.
Santin M, Cannas M. Collagen-bound alpha 1-microglobulin in normal and healed tissues and its effect on immunocompetent cells. Scand J Immunol. 1999;50:289–95.
Savin VJ, Sharma M, McCarthy ET, et al. Cardiotrophin-like cytokine-1: candidate for the focal glomerulosclerosis permeability factor (Abstract). J Am Soc Nephrol. 2008;19:59A.
Schordan S, Grisk O, Schordan E, et al. OPN deficiency results in severe glomerulosclerosis in uninephrectomized mice. Am J Physiol Renal Physiol. 2013;304:F1458–70.
Sedic M, Gethings LA, Vissers JPC, et al. Label-free mass spectrometric profiling of urinary proteins and metabolites from paediatric idiopathic nephrotic syndrome. Biochem Biophys Res Commun. 2014;452:21–6.
Sharma M, Sharma R, McCarthy ET, et al. The focal segmental glomerulosclerosis permeability factor: biochemical characteristics and biological effects. Exp Biol Med. 2004;229:85–98.
Shui HA, Ka SM, Lin JC, et al. Fibronectin in blood invokes the development of focal segmental glomerulosclerosis in mouse model. Nephrol Dial Transplant. 2006;21:1794–802.
Shui HA, Ka SM, Yang SM, et al. Osteopontin as an injury marker expressing in epithelial hyperplasia lesions helpful in prognosis of focal segmental glomerulosclerosis. Transl Res. 2007;150:216–22.
Shui HA, Huang TH, Ka SM, et al. Urinary proteome and potential biomarkers associated with serial pathogenesis steps of focal segmental glomerulosclerosis. Nephrol Dial Transplant. 2008;23:176–85.
Smith HW, Marshall CJ. Regulation of cell signaling by uPAR. Nat Rev Mol Cell Biol. 2010;11:23–36.
Susztak K, Bottinger E, Novetsky A, et al. Molecular profiling of diabetic mouse kidney reveals novel genes linked to glomerular disease. Diabetes. 2004;53:784–94.
Szeto CC, Chan RW, Lai KB, et al. Messenger RNA expression of target genes in the urinary sediment of patients with chronic kidney diseases. Nephrol Dial Transplant. 2005;20:105–13.
Teramoto H, Castellone MD, Malek RL, et al. Autocrine activation of an osteopontin-CD44-Rac pathway enhances invasion and transformation by H-RasV12. Oncogene. 2005;24:489–501.
Wang N, Zhou Y, Jiang L, et al. Urinary Micro-RNA 10a and Micro-RNA 30d serve as novel sensitive and specific biomarkers for kidney injury. PloS One. 2012;33:134–44.
Wang G, Kwan BC, Lai FM, et al. Urinary sediment miRNA levels in adult nephrotic syndrome. Clin Chim Acta. 2013;418:5–11.
Wei C, El Hindi S, Li J, et al. Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. Nat Med. 2011;17:952–60.
Wei C, Trachtman H, Li J, Dong C, et al. PodoNet and FSGS CT study consortia: circulating suPAR in two cohorts of primary FSGS. J Am Soc Nephrol. 2012;23:2051–9.
Wen Q, Huang LT, Luo N, et al. Proteomic profiling identifies haptoglobin as a potential serum biomarker for steroid-resistant nephrotic syndrome. Am J Nephrol. 2012;36:105–13.
Woroniecki RP, Kopp JB. Genetics of focal segmental glomerulosclerosis. Pediatr Nephrol. 2007;22:638–44.
Worthmann K, Peters I, Kümpers P, et al. Urinary excretion of IGFBP-1 and 3 correlate with disease activity and differentiate focal segmental glomerulosclerosis and minimal change disease. Growth Factors. 2010;28:129–38.
Yang J, Goetz D, Li JY, Wang W, et al. An iron delivery pathway mediated by a lipocalin. Mol Cell. 2002;10:1045–56.
Youssef DM, El-Shal AS. Urinary neutrophil gelatinase-associated lipocalin and kidney injury in children with focal segmental glomerulosclerosis. Iran J Kidney Dis. 2012;6:355–60.
Yu CC, Fornoni A, Weins A, et al. Abatacept in B7–1-positive proteinuric kidney disease. N Engl J Med. 2013;369:2416–23.
Zhang C, Zhang W, Chen HM, et al. Plasma microRNA-186 and proteinuria in focal segmental glomerulosclerosis. Am J Kidney Dis. 2014a;S0272-6386:01083-X.

Zhang W, Zhang C, Chen H, et al. Evaluation of microRNAs miR-196a, miR-30a-5P, and miR-490 as biomarkers of disease activity among patients with FSGS. Clin J Am Soc Nephrol. 2014b;9:1545–52.
Zhao M, Li M, Li X, et al. Dynamic changes of urinary proteins in a focal segmental glomerulosclerosis rat model. Proteome Sci. 2014;21:12–42.
Zhou H, Cheruvanky A, Hu X, et al. Urinary exosomal transcription factors, a new class of biomarkers for renal disease. Kidney Int. 2008;74:613–21.
Zhou H, Kajiyama H, Tsuji T, et al. Urinary exosomal Wilms' tumor-1 as a potential biomarker for podocyte injury. Am J Physiol Renal Physiol. 2013;305:F553–9.

Biomarkers of Renal Microthrombosis in Lupus Nephritis

35

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Abstract

The involvement of the kidneys, or lupus nephritis, develops in 30–50 % of patients with systemic lupus erythematosus and is a major cause of morbidity and mortality. Although some clinical variables have prognostic value, histological information obtained from biopsies continues to be indispensable for classification and outcome prediction, including the status of renal vascular lesions. In this chapter, we describe main histological patterns of vasculopathies by using routine methods for light microscopy, electron microscopy, and direct immunofluorescence. Lastly, we report more sensitive and specific methods for immunohistochemistry detection of platelet aggregates. These procedures could help to better identify vascular involvement in patients with lupus nephritis.

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Keywords
Lupus nephritis • Systemic lupus erythematosus • Thrombosis • Inflammation • Immunohistochemistry markers • Antiphospholipid antibodies • Platelet aggregates • Complement

Abbreviations

aCL antibodies	Anticardiolipin antibodies
ADAMTS-13	Disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
ANXA5	Annexin A5
aPL antibodies	Antiphospholipid antibodies
APS	Antiphospholipid syndrome
APSN	APS nephropathy
CEC	Circulating endothelial cell
CV	Cardiovascular
DIF	Direct immunofluorescence
EM	Electron microscopy
GN	Glomerulonephritis
GP	Glycoproteins
H&E	Hematoxylin and eosin
HUS	Hemolytic uremic syndrome
IC	Immunocomplex
IF	Immunofluorescence
IHC	Immunohistochemistry
LA	Lupus anticoagulant
LM	Light microscopy
LN	Lupus nephritis
MHA	Microangiopathic hemolytic anemia
PAS	Periodic acid-Schiff
RAS	Renal artery stenosis
SLE	Systemic lupus erythematosus
sTM	Soluble thrombomodulin
TMA	Thrombotic microangiopathy
TTP	Thrombotic thrombocytopenic purpura
VCAM-1	Vascular cell adhesion molecule-1
VWFCP	Von Willebrand factor-cleaving protease

Key Facts of Renal Biopsy

- The inflammation and involvement of kidneys in patients with lupus is known as lupus nephritis.
- Renal biopsy is necessary in patients with lupus and evidence of renal disease because the clinical presentation may not accurately reflect the histologic findings.

- There are various types of lupus-associated renal involvement although immunocomplex-mediated glomerular diseases are the commonest.
- The status of renal vascular lesions in lupus nephritis is also important as their presence can adversely affect the prognosis of the renal disease.
- In clinical practice, light microscopy, electron microscopy, and direct immunofluorescence are used to detect vascular renal lesions.
- Immunohistochemistry technique is able to detect thrombus formation from their earliest phases of the disease.

Definitions

Systemic lupus erythematosus Chronic inflammatory disease of unknown etiology that can affect the skin, joints, kidneys, lungs, nervous system, serous membranes, and/or other organs of the body, associated with immunologic abnormalities such as the production of autoantibodies.

Lupus nephritis Inflammatory disease affecting kidneys in patients with lupus, most commonly as an immunocomplex-mediated glomerular disease, which is usually differentiated with a renal biopsy.

Antiphospholipid syndrome Disease defined by the occurrence of venous or arterial thrombosis or of specific pregnancy morbidity, in the presence of laboratory evidence of antiphospholipid antibodies. These antibodies include lupus anticoagulant and/or anticardiolipin antibodies (IgG and/or IgM) and/or anti-beta-2-glycoprotein-I (IgG and/or IgM).

Immunocomplex Derived from complex interactions between antibody, antigens, complement, and various receptors as a part of adaptive immunity.

Microthrombi Small thrombus located in a capillary or other small blood vessels.

Thrombotic microangiopathy Is a descriptive name for the histologic presence of thrombi in the glomeruli and arterioles. Definition by electron microscopy includes the presence of subendothelial widening of the glomerular capillary wall due to the deposition of fibrin-like material.

Platelet glycoprotein The membrane glycoproteins of human platelets act as receptors that mediate two important functions, adhesion to the subendothelial matrix and platelet-platelet cohesion or aggregation.

Annexin A5 Protein that binds to phospholipid bilayers, forming two-dimensional crystals and blocking the phospholipids from availability for coagulation enzyme reactions.

Complement factor C4d C4d is a split product of C4 activation, without a biological function, that is considered as “a footprint” of antibody-mediated tissue injury.

Circulating endothelial cells Endothelial progenitor cells described in several pathologic conditions that have in common the presence of vascular injury.

Thrombomodulin Is a cell surface glycoprotein which is widely expressed in a variety of cell types and acts as a cofactor for thrombin binding that mediates protein C activation and inhibits thrombin activity.

Angiopoietin-2 Regulating factor of angiogenesis that exerts context-dependent effects on EC by binding the endothelial-specific receptor tyrosine kinase 2 (TIE2). Angiopoietin-2 acts as a negative regulator of ANG-1/TIE2 signaling during angiogenesis.

Vascular cell adhesion molecule-1 (VCAM-1) Or cell surface adhesion molecule involved in the recruitment of leukocytes to endothelial cells on arterial walls.

Immunofluorescence microscopy Technique that utilizes fluorescent-labeled antibodies to detect specific target antigens.

Electron microscopy Microscope that produces an electronically magnified image of a specimen with a greater resolving power than a light-powered optical microscope.

Introduction

Systemic lupus erythematosus (SLE) is a multisystem disease affecting many organs. The involvement of the kidneys, or lupus nephritis (LN), with proteinuria and hypertension as its most prominent features, develops in 30–50 % of patients with SLE (Cameron 1999) and is a major cause of morbidity and mortality (Faurschou et al. 2010). There are many variations in the prevalence and course of SLE-associated renal disease, and several factors, both clinical and demographic, have been shown to influence the outcome (Mok 2005). Most renal abnormalities emerge within 3–5 years after SLE diagnosis (Seligman et al. 2002). Although eventually up to one-third of SLE patients will develop elevated serum creatinine, evidence of significantly impaired renal function is an uncommon early manifestation. The standard clinical practice is to perform a renal biopsy if clinical or analytic parameters suggest renal involvement. Although some clinical variables, such as elevation of serum creatinine or persistent elevations of blood pressure, have prognostic value, histological information obtained from biopsies continues to be indispensable for classification and outcome prediction (Esdaile et al. 1991; Austin et al. 1995). Clinical findings alone can underestimate the true incidence of renal

involvement as some patients have significant pathological abnormalities without relevant clinical signs of renal involvement (silent LN) (Gonzalez-Crespo et al. 1996).

The renal biopsy is an invaluable method in the diagnosis of patients with renal disease. This procedure allows for an accurate diagnosis and gives information about the outcome and prognosis of renal disease, and it is a helpful tool in order to choose the best treatment.

Klemperer and his colleagues (Klemperer et al. 1984) were the first to describe the light microscopic (LM) renal pathology of SLE glomerulonephritis (GN) with cellular proliferation, wire loops, hematoxylin bodies, and fibrin thrombi in autopsies from untreated patients. There are various types of SLE-associated renal involvement although immunocomplex (IC)-mediated glomerular diseases are the commonest (Balow and Austin 1988). In addition to GN, the status of renal vascular lesions in LN is also important because their presence can adversely affect the prognosis of the renal disease (Banfi et al. 1991; Appel et al. 1994). Several studies report more frequent hypertension, fibrosis, and worse outcomes.

Histological Patterns of Renal Vascular Lesions in Patients with Lupus Nephritis

Renal vascular lesions in patients with SLE diagnosis may be inflammatory, thrombotic, or secondary to a podocytopathy. In vasculitic lesions, immune deposits are found in nearly every case of LN in the mesangium and less consistently in the capillary wall. Glomerular fibrin thrombi are a histologic feature of proliferative LN (Kant et al. 1981), and in the presence of severe glomerular inflammation, thrombus formation can result from endothelial activation or damage with activation of the coagulation system. Thrombi also occur without other signs of glomerular inflammation in the thrombotic thrombocytopenic purpura (TTP)-like lesion, a rare complication of SLE (Hamasaki et al. 2003). Autoantibody against ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), also known as von Willebrand factor-cleaving protease (VWFCP), is a common cause of acquired von Willebrand factor protease deficiency in patients with TTP. Although immunoglobulin G anti-ADAMTS-13 antibody is unusual in patients with SLE (Mannucci et al. 2003; Rieger et al. 2005), the association remains a potential cause of thrombosis. The formation of large multimers of von Willebrand factor leads to platelet aggregation and thrombosis (Gungor et al. 2001). Glomerular thrombosis also occurs in SLE in the absence of severe inflammation, suggesting that several additional pathogenic mechanisms could be implied. Lupus anticoagulant (LA) or antiphospholipid (aPL) antibodies are present in a significant proportion of patients with SLE, and these autoantibodies, directed against the phospholipid elements in the intrinsic clotting cascade, may arise by mechanisms analogous to antinuclear antibodies (Nzerue et al. 2002). Patients with diagnosis of antiphospholipid syndrome (APS) may develop thrombosis in the arteries, veins, and glomerular capillaries.

In some SLE patients, nephrotic syndrome may be also due to a podocytopathy with a non-immunocomplex-mediated injury to the glomerular podocyte, rather than immunocomplex-mediated glomerular injury.

In clinical practice, we use the following three methods to detect vascular renal lesions: LM, electron microscopy (EM), and direct immunofluorescence (DIF). The core for LM must be fixed and embedded in paraffin. Among the variety of fixatives that are recommended for the LM study, Zenker's fixative provides a good architectural and cytological detail. Hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Masson trichrome, and methenamine silver impregnation are the routine employed stains.

Nowadays, EM is an essential tool in the study of renal disease, as some disorders are still defined according to EM findings. After its fixation in glutaraldehyde, the specimen is embedded in an epoxy resin, to make a plastic-embedded tissue block, from which we get 1 μm thick section and we stain it with toluidine blue. Toluidine blue gives great information about the histology of the kidney and helps to choose the better place to do the thin section from EM study.

Finally, DIF is the best technique to detect immune deposits in renal parenchyma by using sera anti-IgG, anti-IgA, anti-IgM, anti-C1q, anti-C3, anti-C4, and anti-fibrinogen. For DIF study, renal tissue must be frozen in liquid nitrogen.

In the 1990s, Appel and Descombes published two extensive reviews where they described main histological subtypes of vascular lesions in patients with LN (Appel et al. 1994; Descombes et al. 1997). Lesions were categorized into the following types:

1. Lupus vasculopathy or noninflammatory necrotizing vascular lesion, defined as the presence of hyaline thrombi occluding the glomerular capillary and/or the arteriolar lumen. These hyaline thrombi are eosinophilic PAS positive and are not accompanied by inflammatory changes of the vascular wall (Fig. 1a,b). By using DIF microscopy, we can detect high content of immunoglobulins and complement complexes and fibrin. By EM, these deposits are electron dense and discrete and often have a granular texture, although occasionally a fingerprint or tactoid substructure is seen (Fig. 1c). They are most commonly located below an intact vascular endothelium or within the basement membranes surrounding the medial myocytes. Rarely, perivascular (adventitial) deposits are present. Such vascular deposits are commonly found with active glomerular proliferative forms of LN. They may be overlooked in biopsies because of their focal distribution and because greater attention is usually directed to the glomerular pathology. This type of lupus vasculopathy must be differentiated from hyalinosis, frequently associated with hypertension and arteriosclerosis, that commonly accompanies the more chronic and inactive forms of LN.
2. Thrombotic microangiopathy (TMA) is characterized by the extensive presence of thrombi in the arteries, arterioles, and glomerular capillaries, mainly containing fibrin, fragmented red blood cells, and leukocytes into the vascular lumen (Fig. 2a, b). Sometimes, plasmatic components infiltrate the vessel wall. This type of vasculopathy may involve the renal vessels of lupus patients with TTP or

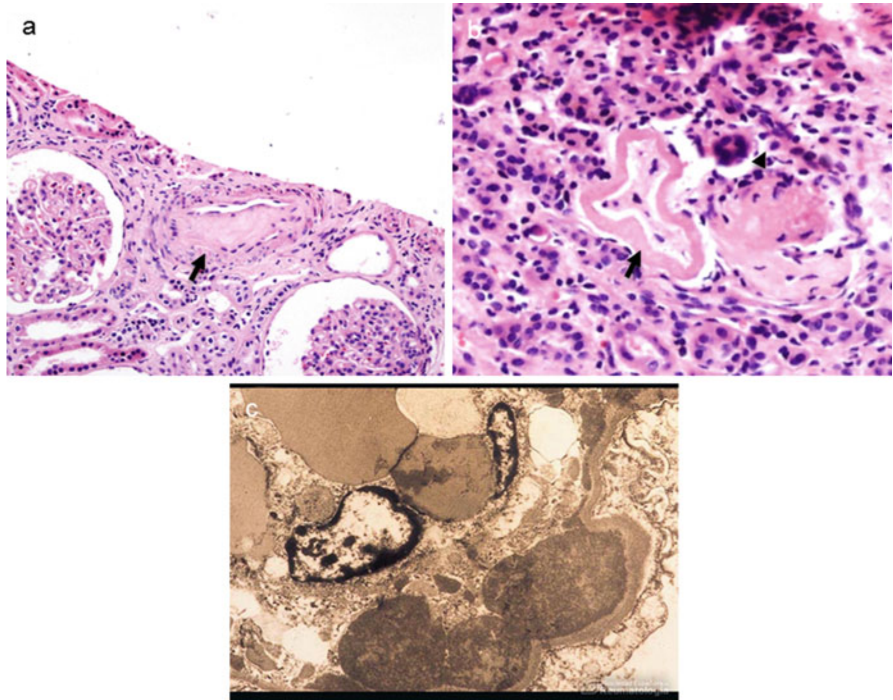

Fig. 1 Lupus vasculopathy or noninflammatory necrotizing vascular lesion. Renal tissue from a patient with lupus nephritis and noninflammatory necrotizing vasculopathy. (**a**, **b**) Hyalinization with vascular wall replaced by hyaline and eosinophilic PAS-positive material without inflammatory changes detected by hematoxylin and eosin (*H&E*) and periodic acid-Schiff (*PAS*) staining (*black arrows*). Fibrinoid necrosis is also observed (**b**, *arrow head*). Original magnification: ×200. (**c**) Electron microscopy with dense subendothelial deposits in a glomerular capillary (Image from "image collection" of the Spanish Rheumatology Society and courtesy of Dr. S. Castañeda)

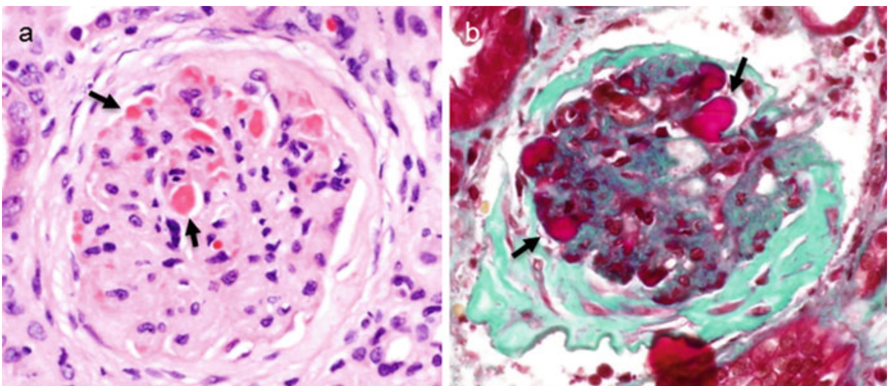

Fig. 2 Thrombotic microangiopathy. Detection of microthrombi (*black arrows*) in glomerular capillaries by H&E (**a**) and Masson trichrome (**b**) staining. Original magnification: ×400

APS or may occur without a recognizable thrombotic systemic process. Most commonly, it affects preglomerular arterioles and small interlobular arteries. Histologically, it appears identical to the vascular changes seen in non-lupus patients with hemolytic uremic syndrome (HUS), TTP, malignant hypertension, scleroderma, and other thrombotic microangiopathies (Tsai 2012). In the acute phase, there is marked luminal narrowing or total occlusion by intraluminal, subendothelial, or medial accumulation of eosinophilic, fuchsinophilic material with staining properties of fibrin, invariably associated with endothelial swelling, denudation, and sometimes fragmented and/or hemolyzed erythrocytes. It is distinguished from noninflammatory vasculopathy by the predominance of fibrin and the absence of discrete immune deposits on immunofluorescence (IF) and EM. In the chronic phase, mucoid edema of the intima and/or “onion skin” type of intimal fibroplasia may occur.

Microangiopathic hemolytic anemia (MHA), and specifically the presence of peripheral blood schistocytes, is a hallmark of TMA. Hu et al. described that MHA is present in 60 % of the patients with LN and TMA. Therefore, the detection of serum markers of MHA could be valuable for determining whether LN is complicated with TMA or not (Hu et al. 2010). They also described the presence of increased levels of circulating endothelial cells (CECs) in peripheral blood and soluble thrombomodulin (sTM) in patients with LN complicated by TMA (Yao et al. 2008a).

3. Vasculitis defined by the presence of fibrinoid necrosis of the arterial wall accompanied by an infiltration of inflammatory cells. This is the least frequent renal vascular lesion encountered in SLE. Its morphologic appearance is identical to that of microscopic polyangiitis. The affected vessels are usually intralobular small- and medium-sized arteries. There is prominent inflammatory cell infiltration of the arterial wall by neutrophils and mononuclear leukocytes, affecting the vessel eccentrically and circumferentially (Fig. 3). Acute mural inflammation is accompanied by fibrinoid necrosis, usually most severe in the intima and to a lesser extent in the media. IF discloses fibrin-related antigen sometimes associated with less intense staining for immunoglobulin and/or complement fractions. Although there are few EM descriptions, they have showed no electron-dense deposits of the immune type, being common the presence of fibrin deposition.
4. Fibrous intimal thickening and arteriosclerosis, considered nonspecific sclerotic vascular lesions and defined as fibrous thickening of the intima without necrosis, proliferation, or thrombosis and subendothelial hyaline deposits.
5. Uncomplicating vascular immune deposits, characterized by the presence of granular deposits of immunoglobulins and complement between the smooth muscle cells of the vascular wall or the intimal basement membrane. These deposits may appear either isolated or associated with one of the other preceding vascular lesions.

In patients with APS, renal manifestations include renal artery stenosis (RAS) and/or renovascular hypertension, renal infarction, APS nephropathy (APSN), renal vein thrombosis, and increased allograft vascular thrombosis.

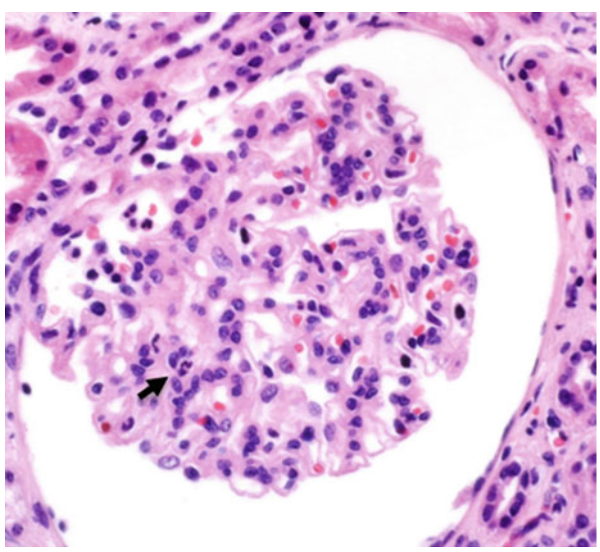

Fig. 3 Vasculitis. Inflammatory involvement of glomerular capillaries detected by H&E staining. Fibrinoid necrosis of the arterial wall accompanied by polymorphonuclear cell infiltration. Original magnification: ×400

Specifically, APSN is characterized by vascular involvement associated with hypertension, acute and/or chronic renal failure, and low-grade proteinuria. APSN vascular lesions may be acute (TMA) and/or chronic (arteriosclerosis, arterial fibrous intimal hyperplasia (FIH), tubular thyroidization, arteriolar occlusions, and focal cortical atrophy (FCA)) (Nochy et al. 1999). On repeated kidney biopsies, we can observe a progression of the acute lesions to chronic proliferative, obstructive, and fibrotic forms (Tektonidou et al. 2004). Biopsy-proven renal vascular lesions are statistically more related to LA than with anticardiolipin (aCL) antibodies, and their presence and severity represent independent risk factors to develop hypertension, elevated serum creatinine, and increased interstitial fibrosis (Fakhouri et al. 2003).

Acute APSN vascular lesions and TMA are identical to those previously reported in this text. Arteriosclerosis is characterized by fibrous intimal thickening and arteriolar hyalinosis that reduces the vascular lumen of arcuate and interlobular arteries.

FIH is different from that observed as a consequence of aging arteriosclerosis, because cellular proliferation is more intense. The myofibroblastic intimal cellular proliferation leads to intimal thickening, with tortuosity of interlobular arteries and their branches. The media shows two different patterns, proliferative with hypertrophic myocytes or, alternatively, atrophic and fibrous. Areas of fibrous thickening of intima (Fig. 4a) or recanalyzing thrombosis often reduce the lumen (Fig. 4b,c). Fibrocellular and fibrous arterial and arteriolar occlusions are often present in small interstitial arteries.

FCA involves superficial zones of the subcapsular cortex, associated with a depression of the contour of the renal capsule. With these areas involved by FCA, the glomeruli can appear small and sclerotic or, in contrast, pseudocystic and voluminous, often clustered in groups, sometimes in the same biopsy. This kind of

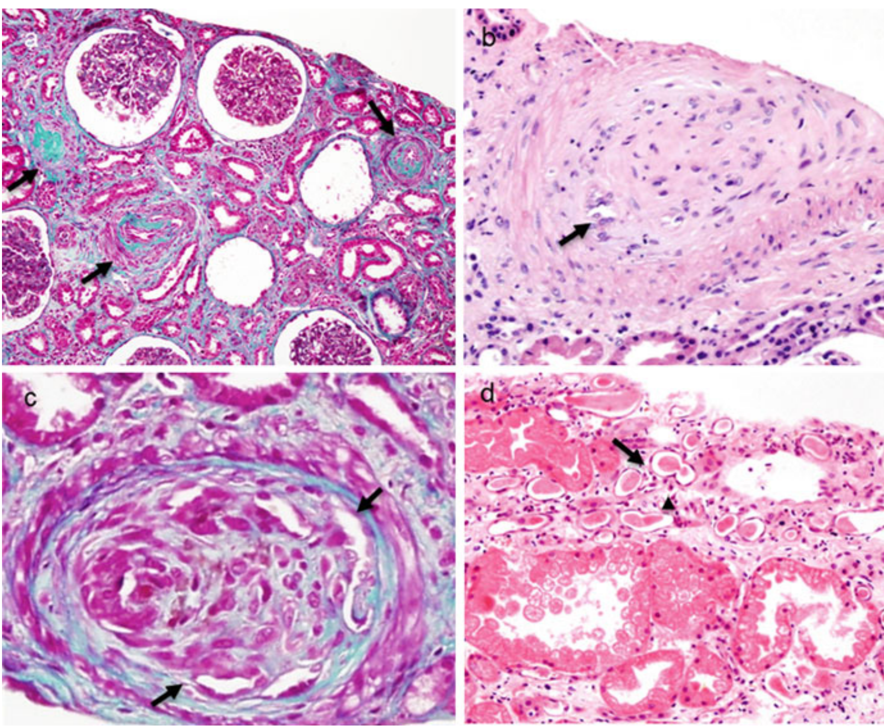

Fig. 4 Chronic lesions of antiphospholipid syndrome-associated nephropathy. (**a**) Fibrous intimal hyperplasia and thickening detected by Masson trichrome (*black arrows*). Original magnification: ×200. (**b**, **c**) Recanalyzing thrombi with reduced lumen (*black arrows*) by H&E (**b**) and Masson trichrome (**c**) staining. Original magnification: ×2200 and ×400, respectively. (**d**) Thyroidization of renal parenchyma (*black arrow*), tubular atrophy, and interstitial fibrosis (*arrow head*) by H&E. Original magnification: ×200

lesion is considered to be typical of APSN and leads to dense interstitial fibrosis, with thyroidization and tubular atrophy (Fig. 4d). Very often, FCA associates with vascular lesions of FIH. Immunofluorescence can reveal fibrin and more inconstantly C3 and IgM deposits in the vessels showing thickening, and, sometimes, renin deposits are observed in the juxtaglomerular apparatus. Tubular thyroidization is characterized by tubular atrophy, with eosinophilic casts, resembling thyroid tissue, often in the deep cortex or medulla.

Finally, both primary APS and aPL-positive patients with SLE nephritis are prone to develop thrombosis of the renal veins and inferior vena cava, associated with nephrotic-range proteinuria, especially in those with LA positivity.

APSN may occur independently of lesions attributable to lupus because its presence is not correlated to the WHO class of the lupus glomerulopathy (Daugas et al. 2002).

Immunohistochemistry Detection of Specific Markers of Microthrombosis

Current observation of renal vascular lesions mainly depends on renal biopsy examination, and biomarkers for clinical dynamic estimation of patients are greatly needed. The classification and clinicopathological correlations of vascular renal lesions are limited by two factors. Firstly, the sensitivity of routine histology to detect microthrombosis is limited by its relatively low presence and the small size of tissue samples obtained by renal biopsy. Moreover, vascular lesions include acute thrombosis but also less specific chronic vascular lesions such as arteriolar sclerosis or hyalinosis (Tektonidou et al. 2004).

Besides routine histological methods, recent data suggest that immunohistochemistry (IHC) detection of platelet aggregates on paraffin-embedded kidney sections increases the sensitivity to detect microthrombosis.

CD61 and CD41 are epitopes of the alpha-IIb-beta-3 integrin chain that is present in platelets, in microparticles arising from platelet activation, or in membrane fragments that are detectable by IHC in formalin-fixed tissues. Both markers provide a sensitive method to identify intravascular platelet aggregates. In fact, IHC studies focusing on cardiac and renal allograft rejection have shown the specific stain of small deposits of CD61/CD41 platelets to detect thrombus formation from their earliest phases (Arbustini et al. 2000; Meehan et al. 2003; Wierzbicki et al. 2006). In contrast, in mural layers or old thrombotic material, CD61/CD41 immunoreactivity is no longer detectable (Arbustini et al. 2000). Recently, it has been demonstrated that the detection of intravascular platelet microthrombi CD61+ analyzed by IHC is a more sensitive and specific marker for aPL-related microthrombi than histological criteria to detect the acute microthrombosis in patients with LN (Galindo et al. 2009) (Fig. 5a, b). In contrast, histological chronic vascular occlusions previously included as APS-associated kidney lesions were often negative by CD61 IHC. The presence of CD61+ aggregates was significantly associated with aPL and not to aging or cardiovascular risk factors. However, in our series, APS histological lesions were not associated with aPL but to an older age and to cardiovascular risk factors associated with arteriosclerotic disease. We also did not detect nonspecific immunostaining either in normal vessels of healthy kidney control sections or in extravascular structures of TMA control or SLE kidney sections, confirming the anti-CD61 antibody specificity.

Although CD41+ detection was found in both glomerular and extraglomerular sections, this marker is less specific and sensitive than CD61+ in patients with LN (Fig. 5c, d).

In cardiac allograft rejection, the amount of fibrin progressively increases with the increase of thrombus size. Fibrin is the only identifiable thrombus component in old mural thrombi embedded within the intimal lesions. Recent occlusive thrombi immunoreact with anti-CD41a and anti-CD61 and with anti-fibrin antibodies, whereas organized occlusive thrombi exclusively immunoreact with anti-fibrin

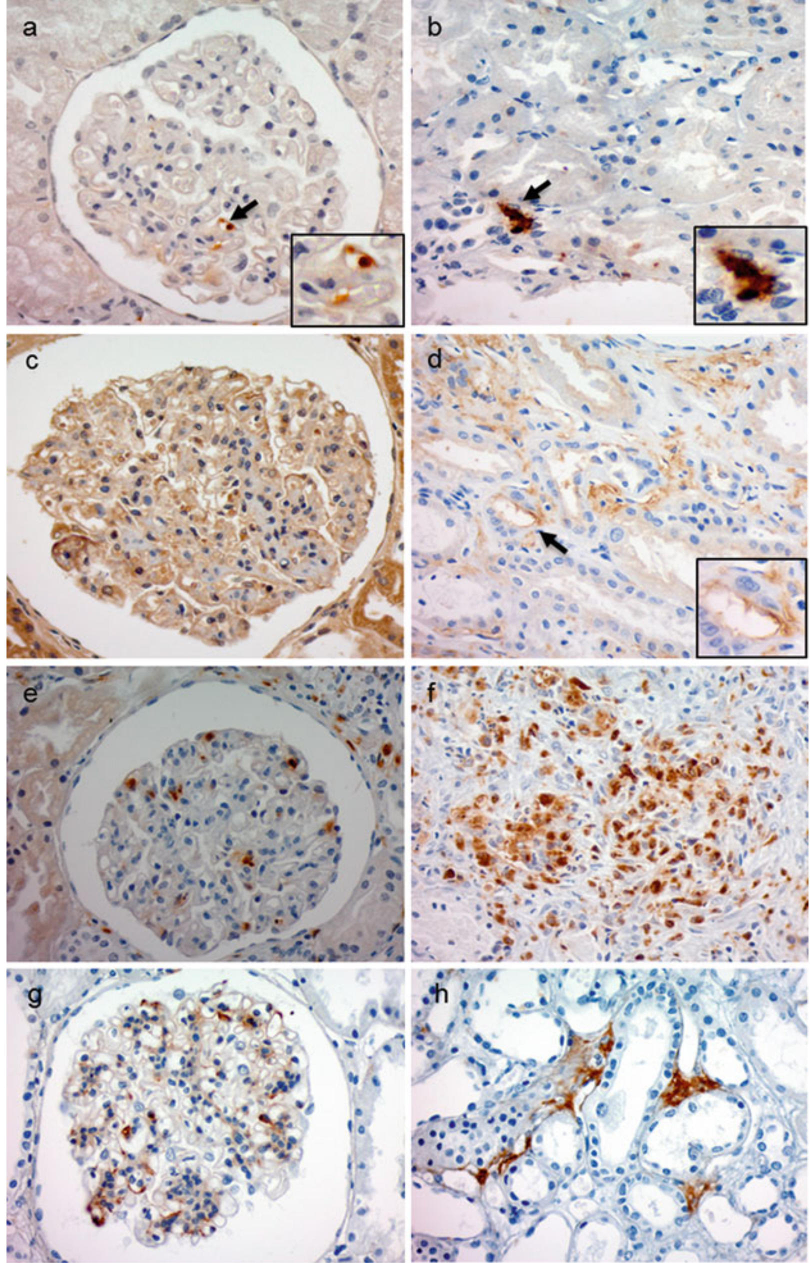

Fig. 5 Immunohistochemical detection of platelet microthrombi lesions, macrophagic infiltration, and activated complement deposition in kidney biopsies. CD61 (**a**, **b**), CD41 (**c**, **d**), CD68 (**e**, **f**), and C4d (**g**, **h**) antigens were detected with immunoperoxidase (*brown*) in serial sections of SLE nephritis tissues (**d**–**l**). SLE glomerular (**a**, **c**, **e**, **g**) or extraglomerular (**b**, **d**, **f**, **h**) markers are shown. Sections were counterstained with hematoxylin. Original magnification: ×400. One of the marked areas containing microthrombi is shown in inset with higher magnification (×630)

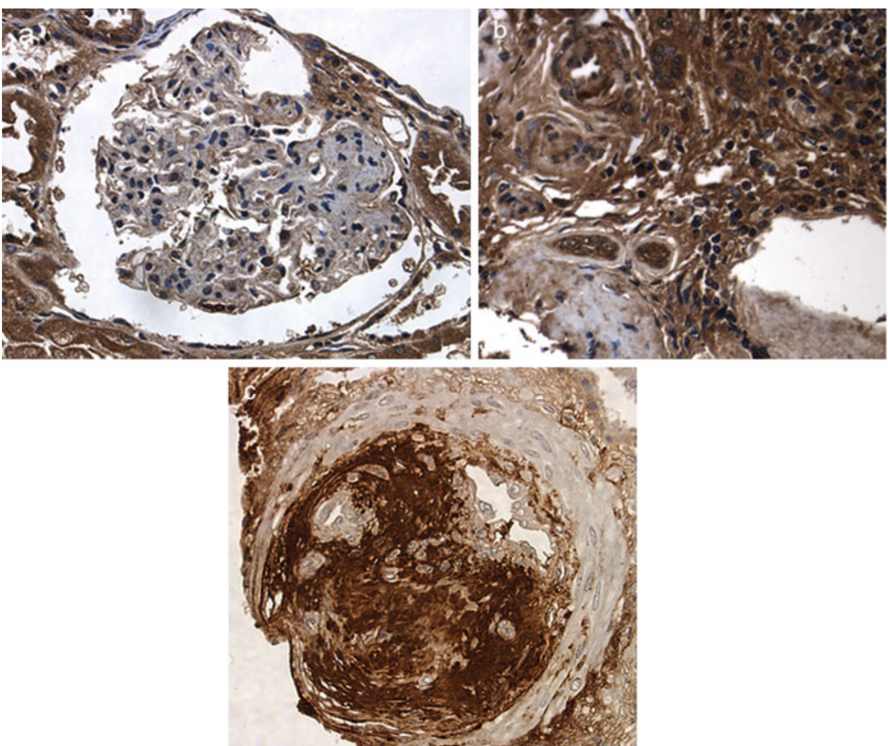

Fig. 6 Immunohistochemical detection of fibrinogen. Fibrinogen antigen is detected with immunoperoxidase (*brown*) in SLE nephritis tissue. SLE glomerular (**a**), extraglomerular (**b**), and a thrombus (**c**) marker are shown. Sections were counterstained with hematoxylin. Original magnification: ×400

antibodies (Arbustini et al. 2000). However, in renal tissue from patients with LN, immunoreaction with anti-fibrin antibodies leads to a diffuse and nonspecific pattern. This pattern of staining does not allow differentiating vascular lesions from glomerular and interstitial fibrosis (Fig. 6).

To identify the nature of the vascular intimal proliferation in patients with APS, Nochy et al. have used a monoclonal mouse antihuman muscle-specific actin antibody (HHF35) (Nochy et al. 1999). This antibody recognizes alpha and gamma smooth muscle actin on paraffin sections from smooth and striated muscle, stained using streptavidin-biotin method. It identifies FIH where the intima is thickened, primarily by an intense myofibroblastic intimal cellular proliferation.

Whether microthrombotic lesions are a consequence of renal inflammation or independently contribute to renal damage is unclear (Descombes et al. 1997; Nochy et al. 1999; Tektonidou et al. 2004). Glomerular and interstitial macrophagic accumulation is one of the individual variables that better correlates with clinical parameters and renal activity in SLE, and it is a feature of the most aggressive forms of human GN (Yang et al. 1998; Gonzalo et al. 2012). These cells, together with dendritic cells, are the major source of inflammatory cytokines, and their

interaction with resident T cells may amplify renal inflammation. In patients with LN, it has been confirmed that detection of intravascular microthrombi CD61+ associates with intra- and extraglomerular infiltration of CD68+ macrophages (Gonzalo et al. 2012), suggesting the importance of macrophagic infiltration as marker of SLE and renal activity in proliferative LN (Fig. 5e, f).

On the other hand, many studies have shown that complement activation may play an important role in thrombotic events. An association between the presence of GN and complement activation measured as glomerular C3 staining has been described. Complement-derived inflammatory mediators or anaphylatoxins such as C3a, C4a, and C5a increase vascular permeability, activate platelets and neutrophils, and promote the release of cytokines with induction of systemic inflammation and coagulation. Studies on murine models have highlighted how complement activation is essential for aPL-induced pregnancy morbidity, suggesting that tissue injury in APS may be caused by a complement-mediated inflammatory process, rather than by thrombosis alone (Salmon et al. 2007). In humans, a few studies also point to complement activation in aPL-mediated thrombosis (Distelmaier et al. 2009). C4d is a sensitive marker for the classical pathway of complement activation. In SLE patients, C4d deposition may be considered as a highly specific indicator of thrombotic and vascular complications (Navratil et al. 2006), and it has been suggested that intensity of staining correlates with the extent of IC deposition (Kim and Jeong 2003). Furthermore, immunodetection of glomerular C4d deposition in renal biopsy from LN patients could be a convenient method of identifying individuals at risk of TMA and vascular pathology associated with the presence of aPL (Cohen et al. 2008) and even with the absence of aPL antibodies. However, because both processes may be associated with proliferative LN and higher activity indexes, this may be an indirect association (Distelmaier et al. 2009). However, in patients with LN, no direct relationship has been demonstrated between C4d deposition and microthrombosis. Instead, C4d deposition correlates with the intensity of macrophagic infiltration, which in turn was associated with microthrombosis (Gonzalo et al. 2012) (Fig. 5g, h).

Annexin A5 (ANXA5) participates in the inhibition of blood coagulation by competing with prothrombin for phosphatidylserine binding sites. Also, ANXA5 inhibits the activity of phospholipase A1, and it has been proposed a role in large vessels as one of the mechanisms by which aPL might contribute to the increased risk of atherothrombosis in patients with SLE. This could be explained by causing decreased ANXA5 binding to endothelium in systemic circulation (Cederholm and Frostegard 2005). Other authors have revealed colocalization of ANXA1 with IgG2 in glomeruli of patients with LN by IHC techniques (Bruschi et al. 2014). In this sense, glomerular ANXA2, essential in the coagulation by recruiting plasminogen and tissue plasminogen activator, has been associated with IgG and C3 deposits in active LN and thrombosis (Yung et al. 2010). This could indicate the importance of ANX as glomerular target antigens in renal biopsy samples from patients with LN.

Serological Biomarkers of Vascular Involvement in Patients with LN

The presence of CEC has been suggested as a potential and useful marker for vasculopathy in patients with LN (Yao et al. 2008a). Actually, circulatory levels of thrombomodulin and vascular cell adhesion molecule-1 (VCAM-1) can be useful biomarkers of renal vascular in LN patients (Yao et al. 2008b). The increase of VCAM-1 levels suggests a state of endothelial cell activation or endothelial damage. Angiopoietin-2 activates CEC and increases vascular inflammation. Although an association between angiopoietin-2 and proliferative and nonproliferative lesions in LN has not been demonstrated, it may be used as a biomarker of disease activity and renal involvement in SLE patients (El-Banawy et al. 2012).

Other authors found that endothelial damage measured as CEC number was markedly elevated in patients with TMA and vasculitis (Erdbruegger et al. 2006). Even it has been suggested that the activated phenotype of CEC might be capable of further potentiating vascular injury by the production of inflammatory and pro-thrombotic immune mediators (Clancy et al. 2001).

Potential Applications to Prognosis, Other Diseases, or Conditions

The sensitivity of routine histological methods to detect microthrombosis in patients with LN and/or aPL is very low. The identification of intravascular platelet aggregates CD61+ by IHC is more sensitive and specific to detect acute microthrombosis but not chronic occlusive vascular lesions. Microthrombi detected with IHC may have a lower impact on renal function and outcome than do larger histological microthrombi. Further longitudinal prospective studies, including patients with LN and repeated biopsies after induction treatment, will help us to better define the real significance of this kind of histological and IHC lesions. Consequently, we will be able to better define correlation between histopathological and IHC findings, with serum and urinary data of renal function, the presence of aPL antibodies, and the degree of response to treatment.

CD61+ microthrombi were firstly described in heart transplant recipients with allograft vascular disease, the major cause of late graft failure. In allograft vascular disease, coronary thrombosis is a frequent and major complication. Whereas recent thin mural thrombi are mostly constituted of CD41- and CD61-positive platelets, the amount of fibrin increases with the increase of thrombus thickness and is the major component of occlusive thrombi. Fibrin is the only identifiable thrombus component in old mural thrombi embedded within the intimal lesions (Arbustini et al. 2000). Similar findings have been reported in renal acute humoral rejection, where CD61 has been able to detect intracapillary platelet activation in specimens without thrombi detectable by LM (Meehan et al. 2003).

C4d, a sensitive marker for the classical pathway of complement activation, has demonstrated to be a good marker of humoral rejection in renal transplant biopsy samples (Collins et al. 1999).

Most centers involved in the management of transplant recipients have incorporated routine C4d staining in diagnostic pathology evaluation of all renal, heart, and pancreas allograft biopsies. A solid base for regular C4d staining of biopsied allograft tissue is now established for heart transplantation and pancreas transplantation (Crespo-Leiro et al. 2005; de Kort et al. 2010). For other transplanted organs such as the lung, the usage of C4d staining is still controversial (Magro et al. 2003). In liver and short bowel transplantation, C4d seems to have no additional diagnostic value (Neil and Hubscher 2010).

Cohen et al. have demonstrated that complicated pregnancies of patients with SLE and APS share several pathophysiological aspects with antibody-mediated rejection. They describe that placental C4d is detectable in the majority of SLE and APS cases (>60 %) in a diffuse staining pattern at the fetal-maternal interface, whereas in normal pregnancies C4d is always negative. Excessive placental C4d relates to impaired fetal outcome due to fetal loss or due to prematurity in the setting of preeclampsia (Cohen et al. 2011).

Glomerular C4d deposition has been also found in some patients with antineutrophil autoantibody-negative pauci-immune glomerulonephritis, whereas in the antineutrophil autoantibody-positive patients, it is absent (Xing et al. 2010).

In the setting of thrombotic microangiopathies, independently of the underlying disease, performing a C4d stain might help clinicians understand the mechanisms of renal microvascular thrombosis. A positive C4d stain could indicate that complement is involved and could even guide future treatment, for instance, with complement inhibitors. However, this needs further basic study, and its clinical utility must await trials of complement inhibitory therapies.

Summary Points

- The major cause of morbidity and mortality in patients with systemic lupus erythematosus is lupus nephritis.
- Renal vascular lesions in patients with systemic lupus erythematosus may be inflammatory, thrombotic, or secondary to a podocytopathy and adversely affect the prognosis of the renal disease.
- Main histological subtypes of vascular lesions in patients with lupus nephritis include lupus vasculopathy, thrombotic microangiopathy, vasculitis, fibrous intimal thickening, and arteriosclerosis.
- Patients with antiphospholipid syndrome, in addition to acute vascular lesions or thrombotic microangiopathy, may develop chronic vasculopathies as arteriosclerosis, arterial fibrous intimal hyperplasia, tubular thyroidization, arteriolar occlusions, and focal cortical atrophy.

- The sensitivity of routine histology to detect microthrombosis is limited by its relatively low presence and the small size of tissue samples obtained by renal biopsy.
- Detection of intravascular platelet microthrombi CD61+ by immunohistochemistry is a more sensitive and specific marker for antiphospholipid antibody-related acute microthrombi than the routine methods.
- Detection of intravascular microthrombi CD61+ is associated with intra- and extraglomerular infiltration of CD68+ macrophages.
- C4d deposition in patients with systemic lupus erythematosus may be considered as a highly specific indicator of thrombotic and vascular complications.
- Serological markers as circulatory levels of thrombomodulin and vascular cell adhesion molecule-1 can be useful biomarkers of renal vascular disease in LN patients.

References

Appel GB, Pirani CL, D'Agati V. Renal vascular complications of systemic lupus erythematosus. J Am Soc Nephrol. 1994;4:1499–515.

Arbustini E, Dal Bello B, Morbini P, et al. Immunohistochemical characterization of coronary thrombi in allograft vascular disease. Transplantation. 2000;69:1095–101.

Austin 3rd HA, Boumpas DT, Vaughan EM, et al. High-risk features of lupus nephritis: importance of race and clinical and histological factors in 166 patients. Nephrol Dial Transplant. 1995;10:1620–8.

Balow JE, Austin 3rd HA. Renal disease in systemic lupus erythematosus. Rheum Dis Clin N Am. 1988;14:117–33.

Banfi G, Bertani T, Boeri V, et al. Renal vascular lesions as a marker of poor prognosis in patients with lupus nephritis. Gruppo Italiano per lo Studio della Nefrite Lupica (GISNEL). Am J Kidney Dis. 1991;18:240–8.

Bruschi M, Sinico RA, Moroni G, et al. Glomerular autoimmune multicomponents of human lupus nephritis in vivo: alpha-enolase and annexin AI. J Am Soc Nephrol. 2014;25:2483–98.

Cameron JS. Lupus nephritis. J Am Soc Nephrol. 1999;10:413–24.

Cederholm A, Frostegard J. Annexin A5 in cardiovascular disease and systemic lupus erythematosus. Immunobiology. 2005;210:761–8.

Clancy R, Marder G, Martin V, et al. Circulating activated endothelial cells in systemic lupus erythematosus: further evidence for diffuse vasculopathy. Arthritis Rheum. 2001;44:1203–8.

Cohen D, Koopmans M, Kremer Hovinga IC, et al. Potential for glomerular C4d as an indicator of thrombotic microangiopathy in lupus nephritis. Arthritis Rheum. 2008;58:2460–9.

Cohen D, Buurma A, Goemaere NN, et al. Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. J Pathol. 2011;225:502–11.

Collins AB, Schneeberger EE, Pascual MA, et al. Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. J Am Soc Nephrol. 1999;10:2208–14.

Crespo-Leiro MG, Veiga-Barreiro A, Domenech N, et al. Humoral heart rejection (severe allograft dysfunction with no signs of cellular rejection or ischemia): incidence, management, and the value of C4d for diagnosis. Am J Transplant. 2005;5:2560–4.

Daugas E, Nochy D, Huong DL, et al. Antiphospholipid syndrome nephropathy in systemic lupus erythematosus. J Am Soc Nephrol. 2002;13:42–52.
de Kort H, Munivenkatappa RB, Berger SP, et al. Pancreas allograft biopsies with positive c4d staining and anti-donor antibodies related to worse outcome for patients. Am J Transplant. 2010;10:1660–7.
Descombes E, Droz D, Drouet L, et al. Renal vascular lesions in lupus nephritis. Medicine (Baltimore). 1997;76:355–68.
Distelmaier K, Adlbrecht C, Jakowitsch J, et al. Local complement activation triggers neutrophil recruitment to the site of thrombus formation in acute myocardial infarction. Thromb Haemost. 2009;102:564–72.
El-Banawy HS, Gaber EW, Maharem DA, et al. Angiopoietin-2, endothelial dysfunction and renal involvement in patients with systemic lupus erythematosus. J Nephrol. 2012;25:541–50.
Erdbruegger U, Woywodt A, Kirsch T, et al. Circulating endothelial cells as a prognostic marker in thrombotic microangiopathy. Am J Kidney Dis. 2006;48:564–70.
Esdaile JM, Federgreen W, Quintal H, et al. Predictors of one year outcome in lupus nephritis: the importance of renal biopsy. Q J Med. 1991;81:907–18.
Fakhouri F, Noel LH, Zuber J, et al. The expanding spectrum of renal diseases associated with antiphospholipid syndrome. Am J Kidney Dis. 2003;41:1205–11.
Faurschou M, Dreyer L, Kamper AL, et al. Long-term mortality and renal outcome in a cohort of 100 patients with lupus nephritis. Arthritis Care Res (Hoboken). 2010;62:873–80.
Galindo M, Gonzalo E, Martinez-Vidal MP, et al. Immunohistochemical detection of intravascular platelet microthrombi in patients with lupus nephritis and anti-phospholipid antibodies. Rheumatology (Oxford). 2009;48:1003–7.
Gonzalez-Crespo MR, Lopez-Fernandez JI, Usera G, et al. Outcome of silent lupus nephritis. Semin Arthritis Rheum. 1996;26:468–76.
Gonzalo E, Toldos O, Martinez-Vidal MP, et al. Clinicopathologic correlations of renal microthrombosis and inflammatory markers in proliferative lupus nephritis. Arthritis Res Ther. 2012;14:R126.
Gungor T, Furlan M, Lammle B, et al. Acquired deficiency of von Willebrand factor-cleaving protease in a patient suffering from acute systemic lupus erythematosus. Rheumatology (Oxford). 2001;40:940–2.
Hamasaki K, Mimura T, Kanda H, et al. Systemic lupus erythematosus and thrombotic thrombocytopenic purpura: a case report and literature review. Clin Rheumatol. 2003;22:355–8.
Hu WX, Liu ZZ, Chen HP, et al. Clinical characteristics and prognosis of diffuse proliferative lupus nephritis with thrombotic microangiopathy. Lupus. 2010;19:1591–8.
Kant KS, Pollak VE, Weiss MA, et al. Glomerular thrombosis in systemic lupus erythematosus: prevalence and significance. Medicine (Baltimore). 1981;60:71–86.
Kim SH, Jeong HJ. Glomerular C4d deposition indicates in situ classic complement pathway activation, but is not a marker for lupus nephritis activity. Yonsei Med J. 2003;44:75–80.
Klemperer P, Pollack AD, Baehr G. Landmark article May 23, 1942: diffuse collagen disease. Acute disseminated lupus erythematosus and diffuse scleroderma. By Paul Klemperer, abou D. Pollack and George Baehr. JAMA. 1984;251:1593–4.
Magro CM, Pope Harman A, Klinger D, et al. Use of C4d as a diagnostic adjunct in lung allograft biopsies. Am J Transplant. 2003;3:1143–54.
Mannucci PM, Vanoli M, Forza I, et al. Von Willebrand factor cleaving protease (ADAMTS-13) in 123 patients with connective tissue diseases (systemic lupus erythematosus and systemic sclerosis). Haematologica. 2003;88:914–8.
Meehan SM, Limsrichamrern S, Manaligod JR, et al. Platelets and capillary injury in acute humoral rejection of renal allografts. Hum Pathol. 2003;34:533–40.
Mok CC. Prognostic factors in lupus nephritis. Lupus. 2005;14:39–44.
Navratil JS, Manzi S, Kao AH, et al. Platelet C4d is highly specific for systemic lupus erythematosus. Arthritis Rheum. 2006;54:670–4.

Neil DA, Hubscher SG. Current views on rejection pathology in liver transplantation. Transpl Int. 2010;23:971–83.
Nochy D, Daugas E, Droz D, et al. The intrarenal vascular lesions associated with primary antiphospholipid syndrome. J Am Soc Nephrol. 1999;10:507–18.
Nzerue CM, Hewan-Lowe K, Pierangeli S, et al. “Black swan in the kidney”: renal involvement in the antiphospholipid antibody syndrome. Kidney Int. 2002;62:733–44.
Rieger M, Mannucci PM, Kremer Hovinga JA, et al. ADAMTS13 autoantibodies in patients with thrombotic microangiopathies and other immunomediated diseases. Blood. 2005;106:1262–7.
Salmon JE, Girardi G, Lockshin MD. The antiphospholipid syndrome as a disorder initiated by inflammation: implications for the therapy of pregnant patients. Nat Clin Pract Rheumatol. 2007;3:140–7. quiz 141 p following 187.
Seligman VA, Lum RF, Olson JL, et al. Demographic differences in the development of lupus nephritis: a retrospective analysis. Am J Med. 2002;112:726–9.
Tektonidou MG, Sotsiou F, Nakopoulou L, et al. Antiphospholipid syndrome nephropathy in patients with systemic lupus erythematosus and antiphospholipid antibodies: prevalence, clinical associations, and long-term outcome. Arthritis Rheum. 2004;50:2569–79.
Tsai HM. Autoimmune thrombotic microangiopathy: advances in pathogenesis, diagnosis, and management. Semin Thromb Hemost. 2012;38:469–82.
Wierzbicki P, Klosowska D, Wyzgal J, et al. Beta 3 integrin expression on T cells from renal allograft recipients. Transplant Proc. 2006;38:338–9.
Xing GQ, Chen M, Liu G, Zheng X, E J, Zhao MH. Differential deposition of C4d and MBL in glomeruli of patients with ANCA-negative pauci-immune crescentic glomerulonephritis. J Clin Immunol. 2010;30:144–56.
Yang N, Isbel NM, Nikolic-Paterson DJ, et al. Local macrophage proliferation in human glomerulonephritis. Kidney Int. 1998;54:143–51.
Yao G, Liu ZH, Zheng C, et al. Evaluation of renal vascular lesions using circulating endothelial cells in patients with lupus nephritis. Rheumatology (Oxford). 2008a;47:432–6.
Yao GH, Liu ZH, Zhang X, et al. Circulating thrombomodulin and vascular cell adhesion molecule-1 and renal vascular lesion in patients with lupus nephritis. Lupus. 2008b;17:720–6.
Yung S, Cheung KF, Zhang Q, et al. Anti-dsDNA antibodies bind to mesangial annexin II in lupus nephritis. J Am Soc Nephrol. 2010;21:1912–27.

Planar Antibody Arrays for Biomarkers in Nephritis

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Christer Wingren

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Abstract

Affinity proteomics, represented by planar antibody arrays, is an established methodology for high-throughput disease proteomics. The technology can be used to generate multiplexed protein expression profiles of even crude proteomes. The antibodies are deposited one by one in an ordered pattern, an array, onto a planar, solid support, where they will act as specific catcher molecules. Next, the sample is added, and any specifically bound proteins are detected and quantify using mainly fluorescence as sensing technology. The observed binding pattern is then converted into a high-resolution protein expression map, or protein atlas,

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outlining the composition of the sample at the molecular level. Using state-of-the-art bioinformatics, candidate biomarker signatures are identified. Hence, the technology platforms provide unique opportunities for, e.g., biomarker discovery, disease diagnostics, monitoring, and evidence-based therapy selection, setting the stage for personalized medicine. Nephritis is inflammation of the kidney, a focal or diffuse proliferative or destructive disease, for which new panels of high-performing, blood-based biomarkers could have a clinical impact. In this chapter, we will describe the design and development of planar antibody microarrays for biomarker discovery and illustrate their use for delineating disease-associated biomarkers in nephritis.

Keywords
Recombinant antibodies • Antibody arrays • Protein expression profiling • Disease proteomics • Nephritis • SLE • Biomarkers

Abbreviations

GPS	Global proteome survey
scFv	Single-chain fragment variable
SLE	Systemic lupus erythematosus
TXP	Triple-X Proteomics

Key Facts of Planar Antibody Arrays and Nephritis

- Planar antibody arrays are miniaturized assays for multiplexed profiling of proteins in even crude samples, such as serum.
- Antibody arrays rely on the specific, sensitive, and selective binding properties of the arrayed antibodies for capture of the corresponding proteins (antigens).
- Planar antibody arrays can be used for protein expression profiling, resulting in biomarker discovery.
- Nephritis is a chronic or acute inflammatory condition of the kidneys.
- Nephritis-associated serum and urine biomarkers can provide the clinicians with actionable information (e.g., diagnosis and monitoring).

Definition

Array A miniaturized, ordered pattern of, e.g., dispensed antibodies.

Biomarker A measurable indicator of some biological state, condition, or disease.

Clinical proteomics A branch of proteomics, involving the application of proteomic technologies on clinical samples.

Microarray An array with micro-sized spot features.

Nanoarray An array with nano-sized spot features.

Nephritis An acute or chronic inflammatory condition of the kidneys.

Planar arrays Arrays printed on planar surfaces.

Proteome All proteins in a given sample, cellular system, or organism, at a given time point.

Proteomics Large-scale comprehensive study of all proteins (the proteome) in sample.

scFv The smallest fragment of an intact antibody containing the antigen-binding site.

Introduction

Nephritis is a chronic or acute inflammatory condition of the kidneys, involving the glomerulus, tubule, or interstitial tissue. The disease is due to a variety of causes, including kidney disease, infection, and autoimmune disease, and the treatment depends on the cause. In many cases, the damage is reversible when the cause is identified and removed, but can in severe cases progress to renal failure and fibrosis. Data indicates that this condition could be the ninth highest cause of death in humans across the world. There are several different types of nephritis, such as acute nephritis, chronic nephritis, glomerulonephritis, interstitial nephritis, pyelonephritis, autoimmune nephritis, and lupus nephritis.

Lupus nephritis is caused by systemic lupus erythematosus (SLE) and one of the most serious complications that can result from SLE (D'Cruz et al. 2007; Herbst et al. 2012; Mok 2010; Rovin et al. 2007). Data indicates that 35 % of the patients display signs of nephritis at the time of lupus diagnosis, and about 40–60 % of the patients will show kidney involvement during the course of this chronic autoimmune connective tissue disease. If not diagnosed and treated early enough, kidney nephritis could result in severe condition and even death. The clinical manifestations of SLE vary among the patients, and the signs and symptoms evolve over time and overlap with those of other autoimmune diseases, why SLE is often misdiagnosed and/or overlooked (Liu et al. 2010; Merrill 2005; Manzi 2009). In fact, SLE is often referred to as the "invisible disease." Hence, high-performing blood- and/or urine-based biomarkers would thus have a significant clinical impact, providing the clinicians with actionable information. However, deciphering disease-associated biomarker panels in crude samples, such as serum or plasma, has proven to be technologically very challenging.

Proteomics is the large-scale comprehensive study of all proteins in a given sample, cellular system, or organism, defined as the proteome. Clinical proteomics is a branch of proteomics, involving the application of proteomic technologies on clinical samples, such as blood. The aim is to decipher disease-associated biomarkers for, e.g., diagnosis, prognosis, classification, and therapeutic prediction, as well as for screening and/or monitoring how well the patient responds to a given treatment. In addition, and most importantly, the traditional approach of searching for a single, unique biomarker as the solution to an unmet clinical need (e.g., diagnosis) has been replaced by the concept of defining multiplexed biomarker panels. Such biomarker panels have been validated to provide a much more selective, specific, and robust disease classifier (Cordero et al. 2008; Hanash et al. 2008; Mischak et al. 2007; Borrebaeck and Wingren 2007) and will become the golden standard to aim for. In this process, the need for multiplexed, high-performing (e.g., resolution, specificity, sensitivity, and reproducibility) protein bioassays capable of handling also crude, complex samples (e.g., non-fractionated plasma) has become evident (Hanash 2003; Hanash et al. 2008). The challenging analytical nature of a proteome is well illustrated by plasma, containing thousands of individual proteins, ranging in concentration over more than nine orders of magnitude. This has been a major driving force in the development of a new line of proteomic technologies, denoted affinity proteomics, mainly represented by antibody microarrays (Borrebaeck and Wingren 2009a, 2011). The antibody microarray-based technology has rapidly evolved from early proof-of-concept setups to multiplexed, high-performing protein bioassays and today constitutes a key established approach within clinical proteomics at frontline laboratories (Borrebaeck and Wingren 2009a).

In 2000, the first set of papers was published, reporting focused efforts toward developing antibody microarrays (Haab et al. 2001; MacBeath and Schreiber 2000). In these publications, low-density (<10) antibody microarrays were generated by printing polyclonal and/or monoclonal antibodies one by one. The basic concepts of the antibody microarrays were demonstrated, but the work also highlighted some of the technical challenges that would have to be addressed and resolved before the technology would become an established proteomic approach. During the last 15 years, major efforts have therefore been launched to develop the technology further. As a result, a set of high-performing antibody micro- and nanoarray technology platforms are now at hand, providing novel opportunities for large-scale protein expression profiling of high- and low-abundant targets in crude, non-fractionated proteomes, such as serum (Borrebaeck and Wingren 2009a).

Antibody Micro- and Nanoarray: Basic Technological Concepts

An antibody array is a specific form of protein array that relies on the specific binding property of the antibody. More specifically, the antibodies are printed one by one onto a solid support in an ordered pattern, an array, where they are exploited as capture molecules, or probes, for the corresponding antigens, with the aim of detecting and quantifying the levels of the target proteins in the sample at hand

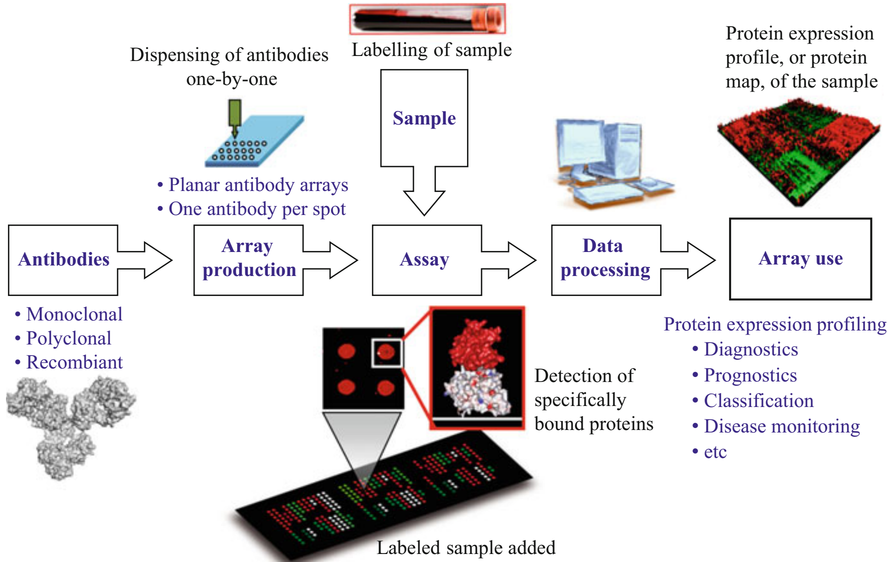

Fig. 1 Schematic illustration of planar antibody microarray setup

(Borrebaeck and Wingren 2009a; Wingren and Borrebaeck 2009) (Fig. 1). Producing such miniaturized, high-density arrays based on antibodies with a broad range of specificities enables the simultaneous screening of many protein targets, while consuming minute (μL range) amount of reagents. When the antibody microarray has been produced, the assay is run like a conventional immunoassay (e.g., ELISA). The observed signal intensities are then transformed into a protein expression map, or detailed protein atlas, revealing the composition of the sample at a molecular level. In other words, the antibody array technology provides unique opportunities for performing protein expression profiling of crude, non-fractionated proteomes that will enhance our fundamental knowledge of biological processes in both disease and health (Borrebaeck and Wingren 2007, 2009b; Haab 2006; Hartmann et al. 2009; Kingsmore 2006; Wingren and Borrebaeck 2006).

The current concept of generating miniaturized antibody arrays, ranging in size from mm^2 to cm^2, is based on either printing (pL scale or less) (Borrebaeck and Wingren 2007; MacBeath 2002; Wingren and Borrebaeck 2006), self-addressing (Wacker and Niemeyer 2004; Wacker et al. 2004), or self-assembling (He et al. 2008a, b; He and Taussig 2001; Ramachandran et al. 2004, 2008) small amounts (fmol range) of individual antibodies with the desired specificity onto a solid support (Fig. 2). Direct printing is by far the most commonly used approach and is based on using various dispensing methodologies, with non-contact ink-jet printers dominating the scene (Borrebaeck and Wingren 2007; Wingren and Borrebaeck 2007). The purified probes are printed one by one in the pL scale, generating ~150 μm sized spot features, depending on the printing buffer and the surface properties of the solid support. Self-addressing is a new method for

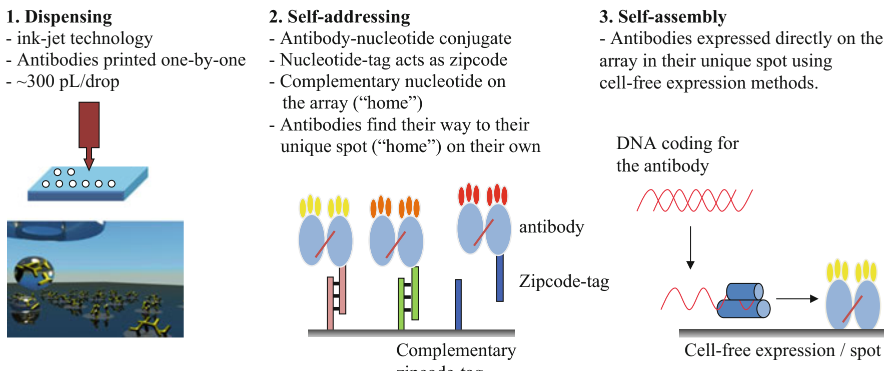

Fig. 2 Three main ways of producing planar recombinant antibody arrays

potentially producing truly high-density arrays, but still in its exploratory phase (Wacker and Niemeyer 2004; Wacker et al. 2004). In this approach, each individual antibody is tagged with a unique zip code tag, a short stretch of DNA. When added to the array in bulk, the antibodies will find their way on their own to their unique home (spot) on the array, composed of complementary DNA. Self-assembling antibody microarrays is also a new, exploratory approach to potentially generate high-density antibody arrays (He et al. 2008b). In this setup, the antibodies are produced directly on the chip in their unique position, using cell-free protein expression (He et al. 2008a, b; He and Taussig 2001; Ramachandran et al. 2004, 2008).

The size of the individually printed spots determines whether the array is denoted a microarray (spot diameter (Ø) in the μm range) or a nanoarray (Ø in the nm range) (Wingren and Borrebaeck 2007). Regarding antibody microarrays, arrays with an overall footprint of < 1 cm^2, based on 18×10^3 μm^2 (diameter (Ø) of ~150 μm) sized spots at a density of $\leq$ 2,000 spots/cm^2, have mainly been produced and applied (Hoheisel et al. 2013; Borrebaeck and Wingren 2009a; Kingsmore 2006; Sanchez-Carbayo 2010). Further, the multiplexity, i.e., the number of antibodies with different specificities per array, has been in the range of <900 different antibodies/array. Adopting ink-jet-based printers to produce the arrays, the antibodies have been sequentially spotted in parallel (1 to 4 antibodies at a time), and the multiplexity has been achieved by washing the nozzles and loading them with new antibodies.

In the case of nanoarrays, conceptual protein (antibody) nanoarrays displaying truly miniaturized (spot size; <0.8 μm^2, Ø < 1 μm) and high-density (spot density; >100,000 spots/cm^2) features have been designed and produced (Nettikadan et al. 2006; Lee et al. 2010; Hoff et al. 2004; Backmann et al. 2005; Arntz et al. 2003; Zheng et al. 2005; Ellmark et al. 2009; Ghatnekar-Nilsson et al. 2007; Bruckbauer et al. 2004; Tran et al. 2010) (for review see Wingren and Borrebaeck 2007). Despite the success, these nanoarray designs have been shown to be associated with three key technical bottlenecks. First, the production methodologies at

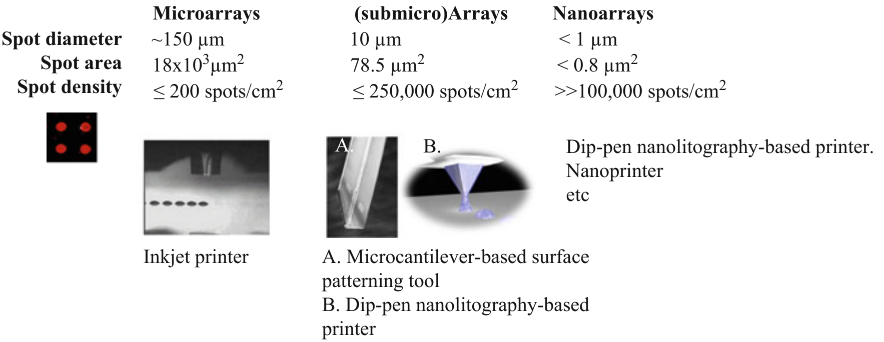

Fig. 3 Three main types of planar antibody arrays (with respect to size of the spots)

hand are currently limited to producing only 1-plex arrays (i.e., arrays composed of multiple spots of a single antibody), or in rare cases <5-plex designs (Wingren and Borrebaeck 2007; Nettikadan et al. 2006; Lee et al. 2002, 2010; Hoff et al. 2004; Backmann et al. 2005; Arntz et al. 2003; Zheng et al. 2005; Ellmark et al. 2009; Ghatnekar-Nilsson et al. 2007; Bruckbauer et al. 2004; Tran et al. 2010; Berthet-Duroure et al. 2008; Meister et al. 2004). Second, reducing the spot size < 1µm will lead to impaired rather than improved assay performance (e.g., sensitivity) (Ekins 1998). Third, hardware for sensitive (such as fluorescence-based) sensing of high-density nanoarrays remains to be established (Wingren and Borrebaeck 2007).

However, the density and multiplexity of antibody arrays are essential for large-scale protein expression profiling endeavors. In order to meet this demand without having to further develop the technologically challenging antibody nanoarray designs, miniaturized arrays based on submicron-sized (Ø 10 µm) rather than nano-sized (Ø < 1 µm) spot features have surfaced (Fig. 3) (Irvine et al. 2011; Jang et al. 2010; Lynch et al. 2004; Nettikadan et al. 2006; Petersson et al. 2014b). Using a nanoarrayer, based on dip-pen technology, the first 12- and 48-plex planar recombinant antibody arrays, based on 78.5 um^2 (Ø 10 µm) sized spots at a density of 38,000 spots/cm^2, interfaced with a fluorescent-based readout were recently produced (Petersson et al. 2014b, c). Importantly, their use for biomarker discovery in serum was also outlined, using systemic lupus erythematosus as showcase (Petersson et al. 2014c). Interestingly, adopting a microcantilever-based surface patterning tool, it was recently demonstrated that 16-plex recombinant antibody arrays, based on miniaturized spot features (78.5 um^2, Ø 10 µm) at a 7- to 125-times increased spot density (250,000 spots/cm^2 vs. 38,000 spots/cm^2 (Petersson et al. 2014b) or 2,000 spots/cm^2 (Borrebaeck and Wingren 2009a)), interfaced with a fluorescent-based readout could be produced (Petersson et al. 2014a). The feasibility of this conceptual array platform for serum protein profiling was also indicated (Petersson et al. 2014a).

In order to achieve high density, access to numerous renewable antibodies is a must. By using large antibody libraries, with, e.g., 10^{10} members (Söderlind et al. 2000), as a probe source, renewable antibodies displaying “any” specificity

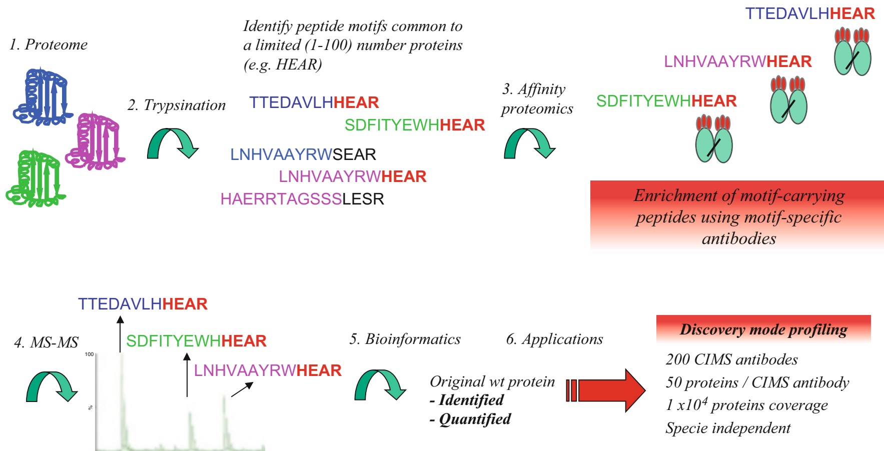

Fig. 4 Schematic illustration of the global proteome survey (*GPS*) setup, designed for global proteome profiling

can readily be selected and included on the arrays. The logistics behind large-scale selections could potentially constitute a logistical bottleneck. If so, two similar concepts were recently presented, demonstrating one solution to how to use a smaller set of antibodies while still targeting numerous proteins. The two concepts denoted Triple-X Proteomics (Poetz et al. 2009) (TXP) and global proteome survey (Wingren et al. 2009) (GPS) (Fig. 4) are based on the same fundamental principle and is based on combining antibody arrays with mass spectrometry. Briefly, antibodies are generated against short peptide motifs, four to six amino acid residues long, each motif being shared among 2 to 100 different proteins. These motif-specific antibodies could then be used to target motif-containing peptides. From a practical point of view, the proteome is first digested (e.g., trypsinated), and the peptide-specific antibodies are then used to specifically capture and enrich motif-containing peptides. Next, the motif-containing peptides are detected, identified, and quantified using tandem mass spectrometry, thereby allowing us to backtrack the original proteins in a quantitative manner. By using 200 such motif-specific antibodies, each targeting a motif shared among 50 unique proteins, would thus enable us to target about half the non-redundant proteome. As an example, a recent study showed that about 1400 tissue proteins could be profiled in a quantitative manner using only nine such motif-specific antibodies (Olsson et al. 2013).

Planar antibody arrays, printed on (microscope) slides (≤16 subarrays/slides; made of plastic, glass, or polymer) or on the bottom of ELISA plates, are the dominating format, although bead-based arrays, or arrays in solution, have also been manufactured (Borrebaeck and Wingren 2009a).

The assay is run like a traditional immunoassay (~4 h assay time), but consuming only µL scale volumes of the samples. It should be noted that crude, non-fractionated

proteomes, such as serum, plasma, urine, cell lysates, and tissue extracts, can, in contrast to many competing proteomic technologies, be directly used without having to pre-fractionate the sample (Belov et al. 2001, 2003; Campbell et al. 2006; Dexlin et al. 2006; Mischak et al. 2007; Ingvarsson et al. 2007; Haab 2003; Wingren et al. 2007; Haab et al. 2001; Wingren and Borrebaeck 2009).

In a majority of cases, the samples are labeled with a fluorescent dye, either directly or indirectly, and interfaced with a fluorescent-based readout (Kusnezow et al. 2007; Wingren and Borrebaeck 2008; Wingren et al. 2007). A dynamic range of at least four orders of magnitude and an assay sensitivity in the pM range can be obtained, thus allowing low-abundant (pg/ml) analytes to be targeted in crude proteomes. By quantifying the signal intensity of each spot in the array, the array images are transformed into protein expression profiles, outlining the protein composition of the sample. State-of-the-art bioinformatics is then applied in order to identify any disease-associated biomarker panels that can be explored and exploited for, e.g., diagnosis, prognosis, and classification (Borrebaeck and Wingren 2009a, b; Wingren and Borrebaeck 2009).

Potential Applications to Prognosis, Other Diseases, or Conditions

To date, planar antibody microarrays have been used for protein expression profiling of almost any kind of crude sample format, such as plasma and serum, with the aim of deciphering disease-associated biomarker signatures (for review see, e.g., Borrebaeck and Wingren 2007; 2009b; 2009a; Haab 2005; Haab 2006; Hartmann et al. 2009; Kingsmore 2006; Griffiths et al. 2005; Wingren and Borrebaeck 2009). The design of the applications ranges from small proof-of-concept studies to large semi-global protein expression profiling efforts. Reviewing the antibody array field, from early to recent applications, shows that the technology can be used in, but not limited to, the following areas: (1) autoimmunity, (2) allergy, (3) bladder proteomics, (4) cell proteomics, (5) drug abuse, (6) glycomics, (7) heart proteomics, (8) hereditary disease, (9) inflammatory conditions/infections, (10) liver proteomics, (11) lung proteomics, (12) medical microbiology, (13) neurology/psychiatry, (14) obstetrics/gynecology, (15) oncoproteomics, (16) periodontology, (17) phosphoproteomics, (18) protein expression, and (19) protein signaling (Table 1).

Cancer is by far the most targeted disease using this technology, and several publications have demonstrated the potential of the antibody microarray methodology for pin-pointing cancer-associated biomarkers for, e.g., diagnosis, prognosis, classification, predicting the risk for relapse, and evidence-based therapy selection, as illustrated by a few selected representative references (Sanchez-Carbayo 2010; Alhamdani et al. 2012; Wingren et al. 2012; Hoheisel et al. 2013). While planar antibody arrays have been frequently applied within the field of cancer, nephritis has so far only been addressed in a limited set of studies. Below, we have outlined the

Table 1 General overview of planar antibody array-based applications and area of use

A. Area of use (example disease, biological process)		B. Applications
A1. Autoimmunity	Systemic lupus erythematosus	B1. Protein expression profiling
A2. Allergy	Cytokine profiling	B2. (Early) Diagnosis
A3. Bladder proteomics	Smooth muscle hypertrophy	B3. Differential diagnosis
A4. Cell proteomics	Blood phenotyping	B4. Classification
A5. Drug abuse	Screening	B5. Phenotyping
A6. Glycomics	Pancreatic cancer	B6. Evidence-based therapy selection
A7. Heart proteomics	Myocardial infarction	B7. Predicting the risk for relapse
A8. Hereditary disease	Cystic fibrosis	B8. Drug abuse screening
A9. Inflammatory conditions/infections	Atherosclerosis, obesity	B9. Bacterial detection/ profiling
A10. Liver proteomics	APAP-induced liver disease	B10. Bacterial toxin detection
A11. Lung proteomics	Chromium(VI) treatment	
A12. Medical microbiology	Detection of bacteria/toxin	
A13. Neurology/psychiatry	Cerebral palsy	
A14. Obstetrics/gynecology	Preeclampsia	
A15. Oncoproteomics	Pancreatic cancer, breast cancer, lymphomas	
A16. Periodontology	Model system	
A17. Phosphoproteomics	Lung cancer	
A18. Protein expression	Posttranslational profiling	
A19. Protein signaling	Various model systems	

findings of some of those applications by selecting a set of representative publications (Table 2).

Resistin as a Potential Marker in Lupus Nephritis

In this study, the authors used commercially available planar antibody arrays to discover candidate biomarkers in serum and urine of patients suffering from SLE and lupus nephritis (Hutcheson et al. 2015). The hypothesis was to explore whether serum and urine levels of adipokines could act as biomarkers for lupus nephritis. In previous work, adipokines have been associated with SLE and cardiovascular disease. Based on the antibody array work, 15 adipokines, adiponectin, leptin, and resistin were selected. Next, ELISA was applied in an attempt to validate the biomarkers. Compared to matched controls, the results showed that the expression

Table 2 Overview of the selected applications, using (planar) antibody arrays for protein expression profiling and biomarker discovery in nephritis, used as representative examples

Study (aim/target disease/ reference)	Antibody array (design/ antibodies)	Key finding(s)
1. Serum and urine protein profiling for biomarker discovery 2. SLE and lupus nephritis 3. Hutcheson et al. 2015	Commercially available planar antibody arrays Mono-/ polyclonal antibodies	Resistin was indicated as a potential biomarker in lupus nephritis
1. Plasma protein profiling for biomarker discovery 2. Glomerulonephritis, diabetic nephropathy, obstructive uropathy, and analgesic abuse 3. Neiman et al. 2011	In-house designed bead-based arrays Polyclonal antibodies	Fibulin was outlined as a candidate biomarker for renal impairment, in particular for glomerulonephritis
1. Urine profiling for biomarker discovery 2. Lupus nephritis 3. Wu et al. 2013	Commercially available planar antibody arrays Mono-/ polyclonal antibodies	Angiostatin was outlined as a candidate urinary biomarker of renal disease in SLE
1. Design of miniaturized planar antibody arrays and serum protein profiling for biomarker discovery 2. SLE 3. Petersson et al. 2014c	In-house designed miniaturized, planar arrays Recombinant single-chain Fv antibodies	First generation of miniaturized planar antibody arrays. Three serum biomarkers associated with SLE were detected
1. Serum protein profiling for biomarker discovery 2. SLE (including lupus nephritis) and systemic sclerosis 3. Carlsson et al. 2011	In-house designed miniaturized, planar arrays Recombinant single-chain Fv antibodies	Multiple serum biomarker signatures for diagnosis, classification, and prognosis of SLE. SLE and systemic sclerosis could be differentiated

levels of adiponectin and resistin were increased in both serum and urine, while leptin was increased in lupus nephritis. Further, the levels of resistin in serum, but not in urine, were found to correlate with renal dysfunction in lupus nephritis. Taken together, resistin might thus prove useful as a biomarker of renal dysfunction in patients with lupus nephritis. Additional work targeting additional, independent patient cohorts will, however, be required to validate the data and to preferentially extend this single biomarker into a multiplex marker panel to increase the anticipated assay performance (specificity, sensitivity, and selectivity).

Plasma Profiling Reveals Candidate Biomarker for Renal Impairment

The ability to detect early signs of kidney toxicity and to monitor progression of disease represents essential unmet clinical needs. Spurred by this, the authors applied an in-house designed antibody suspension bead array to perform plasma protein expression profiling targeting four types of kidney disorders, including glomerulonephritis, diabetic nephropathy, obstructive uropathy, and analgesic abuse (Neiman et al. 2011). To this end, 129 polyclonal antibodies, targeting 94 unique proteins, were used to produce the bead-based array. In total, 200 clinical plasma samples, including renal-associated cases and controls, were profiled. Significantly higher expression levels were observed for 1 of 94 proteins, fibulin-1, in glomerulonephritis patients compared to all of the other patient cohorts, indicating a potential for differential diagnosis. Most importantly, using three different antibodies directed toward three separate, non-overlapping epitopes on fibulin-1 showed similar expression levels, further supporting the data. In addition, Western blot analysis of selected plasma samples confirmed the observations. Next, a novel, independent patient cohort, including glomerulonephritis and controls, was applied in an attempt to validate the findings in the discovery cohort. The data confirmed the indications, outlining fibulin-1 as a potential indicator to monitor kidney damage or kidney malfunction. The performance of the biomarker might be even further improved by finding additional markers, in the end resulting in a multiplexed panel.

Biomarker of Renal Pathology Chronicity in Lupus Nephritis

In this study, the authors used a commercially available, multiplexed antibody microarray to perform protein expression profiling of about 280 proteins in urine targeting lupus nephritis (Wu et al. 2013). The data indicated elevated levels of urine angiostatin. Angiostatin has been shown to have modulatory function in inflammation and angiogenesis. Using ELISA, the increased levels of urinary angiostatin were then validated in an independent cohort of SLE patients. Next, the authors investigated whether the levels of angiostatin also reflected the SLE disease activity. Indeed, the results showed that higher levels were observed in active SLE versus inactive SLE. In fact, the patients with the most severe form of SLE were found to have the highest levels of urinary angiostatin. The biomarker might also be used to differentiate SLE patients with active SLE versus inactive SLE, as illustrated by receiver operating curve analysis resulting in an area under the curve of 0.90. Finally, when analyzing lupus nephritis patients, urine-angiostatin levels were found to correlate with renal pathology chronicity index, but not with the activity index. Hence, angiostatin surfaced as a novel, candidate noninvasive biomarker of renal disease in SLE. Further studies will be required in order to validate these promising findings, targeting novel, independent patient cohorts.

Planar Antibody Arrays for Biomarker Discovery in Lupus Nephritis

In this exploratory work, the authors first developed and designed a 48-plex miniaturized recombinant single-chain Fv antibody array platform (Petersson et al. 2014c). In more detail, individual spot features with a diameter of 10 μm and an area of 78.5 μm^2 were printed at a density of 38,000 spots per cm^2 using dip-pen nanolithography. The setup was interfaced with a high-resolution scanner for fluorescence-based sensing. The performance and applicability of the in-house designed planar antibody arrays were demonstrated by performing protein expression profiling of lupus nephritis. To this end, the observed serum profiles of lupus nephritis ($n = 45$) versus healthy controls ($n = 30$) were compared, and differentially expressed proteins were defined. The results showed that differentially expressed serum levels of three proteins in lupus nephritis versus healthy controls were detected, including complement protein C1q (downregulated), interleukin 6 (upregulated), and low-density lipoprotein (upregulated). Of note, these data supported previous findings, based on using conventional recombinant antibody microarrays (Carlsson et al. 2011). Taken together, the data outlined that planar recombinant antibody arrays could be used to define lupus nephritis-associated serum biomarkers, while consuming minute amount of sample (<1 single drop of serum).

Planar Antibody Microarrays: Biomarker Discovery in Systemic Lupus Nephritis

In this discovery study, the authors used in-house designed 135-plex recombinant single-chain Fv antibody microarrays to perform protein expression profiling of systemic sclerosis, systemic lupus nephritis, and healthy controls (Carlsson et al. 2011). The 135 antibodies were directed against 60 different proteins, including mainly immunoregulatory proteins. The hypothesis was to explore (parts of) the immune system as an early, specific, and sensitive sensor for disease. The results showed that several candidate SLE-associated multiplexed serum biomarker panels were successfully deciphered, reflecting disease (with impact on diagnosis), disease severity (enabling phenotyping), and disease activity (indicating ability to detect, monitor, and potentially even predict flares). In addition, biomarker panels differentiating SLE and systemic sclerosis were detected, and the observed differences increased with severity of SLE. Hence, the study demonstrated that molecular portraits of systemic lupus nephritis (and systemic sclerosis) could be extracted from a crude serum sample. Of note, the assay was performed while consuming less than a single drop of serum, and low-abundant biomarkers (pg/ml) could readily be detected. In the end, the disease-associated marker panels might also enhance our fundamental understanding of these complex autoimmune diseases. Of note, the authors have a set of additional manuscripts in the pipeline, further validating the candidate serum biomarker signature for diagnosis and

outlining additional marker panels for classification and prognosis (Wingren et al., unpublished observations).

Summary Points

- This chapter focuses on the design of planar antibody arrays for protein expression profiling and biomarker discovery in nephritis.
- Planar antibody arrays have been developed for biomarker discovery in clinical proteomics.
- Miniaturized planar antibody arrays can be used to perform multiplexed protein expression profiling, targeting crude proteomes.
- Planar antibody arrays have been successfully used for biomarker discovery in nephritis.
- Nephritis-associated urine, serum, or plasma biomarkers have been deciphered using planar antibody arrays.
- Multiplexed biomarker panels can be deciphered in a single drop of blood, or less, using planar antibody arrays.

References

Alhamdani MS, Youns M, Buchholz M, Gress TM, Beckers MC, Marechal D, Bauer A, Schroder C, Hoheisel JD. Immunoassay-based proteome profiling of 24 pancreatic cancer cell lines. J Proteomics. 2012;75(12):3747–59.

Arntz Y, Seelig JD, Lang HP, Zhang J, Hunziker P, Ramseyer JP, Meyer E, Hegner M, Gerber C. Label-free protein assay based on a nanomechanical cantilever array. Nanotechnology. 2003;14:86–90.

Backmann N, Zahnd C, Huber F, Bietsch A, Pluckthun A, Lang HP, Guntherodt HJ, Hegner M, Gerber C. A label-free immunosensor array using single-chain antibody fragments. Proc Natl Acad Sci U S A. 2005;102(41):14587–92.

Belov L, De La Vega O, Dos Remedios CG, Mulligan SP, Christopherson RI. Immunophenotyping of leukemias using a cluster of differentiation antibody microarray. Cancer Res. 2001;61 (11):4483–9.

Belov L, Huang P, Barber N, Mulligan SP, Christopherson RI. Identification of repertoires of surface antigens on leukemias using an antibody microarray. Proteomics. 2003;3(11):2147–54.

Berthet-Duroure N, Leïchlé T, Pourciel J-B, Martin C, Bausells J, Lora-Tamayo E, Perez-Murano F, François J, Trévisiol E, Nicu L. Interaction of biomolecules sequentially deposited at the same location using a microcantilever-based spotter. Biomed Microdevices. 2008;10(4):479–87.

Borrebaeck CA, Wingren C. High-throughput proteomics using antibody microarrays: an update. Expert Rev Mol Diagn. 2007;7(5):673–86.

Borrebaeck CA, Wingren C. Design of high-density antibody microarrays for disease proteomics: key technological issues. J Proteomics. 2009a;72(6):928–35.

Borrebaeck CA, Wingren C. Transferring proteomic discoveries into clinical practice. Exp Rev Proteomics. 2009b;6(1):11–3.

Borrebaeck CK, Wingren C. Recombinant antibodies for the generation of antibody arrays. In: KORF U, editor. Protein microarrays. New York: Humana Press; 2011.

Bruckbauer A, Zhou D, Kang D-J, Korchev YE, Abell C, Klenerman D. An addressable antibody nanoarray produced on a nanostructured surface. J Am Chem Soc. 2004;126(21):6508–9.

Campbell CJ, O'looney N, Chong Kwan M, Robb JS, Ross AJ, Beattie JS, Petrik J, Ghazal P. Cell interaction microarray for blood phenotyping. Anal Chem. 2006;78(6):1930–8.
Carlsson A, Wuttge DM, Ingvarsson J, Bengtsson AA, Sturfelt G, Borrebaeck CA, Wingren C. Serum protein profiling of systemic lupus erythematosus and systemic sclerosis using recombinant antibody microarrays. Mol Cell Proteomics. 2011;10(5): M110 005033.
Cordero OJ, De Chiara L, Lemos-Gonzalez Y, Paez De La Cadena M, Rodriguez-Berrocal FJ. How the measurements of a few serum markers can be combined to enhance their clinical values in the management of cancer. Anticancer Res. 2008;28(4C):2333–41.
D'cruz DP, Khamashta MA, Hughes GR. Systemic lupus erythematosus. Lancet. 2007;369 (9561):587–96.
Dexlin L, Ingvarsson J, Jönsson M, Ellmark P, Frendeus B, Borrebaeck CA, Wingren C. Membrane protein profiling of intact cells using recombinant antibody microarrays. J Proteome Res. 2006;7 (1):319–27.
Ekins RP. Ligand assays: from electrophoresis to miniaturized microarrays. Clin Chem. 1998;44 (9):2015–30.
Ellmark P, Ghatnekar-Nilsson S, Meister A, Heinzelmann H, Montelius L, Wingren C, Borrebaeck CA. Attovial-based antibody nanoarrays. Proteomics. 2009;9(24):5406–13.
Ghatnekar-Nilsson S, Dexlin L, Wingren C, Montelius L, Borrebaeck CA. Design of atto-vial based recombinant antibody arrays combined with a planar wave-guide detection system. Proteomics. 2007;7(4):540–7.
Griffiths B, Mosca M, Gordon C. Assessment of patients with systemic lupus erythematosus and the use of lupus disease activity indices. Best Pract Res Clin Rheumatol. 2005;19(5):685–708.
Haab BB. Methods and applications of antibody microarrays in cancer research. Proteomics. 2003;3 (11):2116–22.
Haab BB. Antibody arrays in cancer research. Mol Cell Proteomics. 2005;4(4):377–83.
Haab BB. Applications of antibody array platforms. Curr Opin Biotechnol. 2006;17(4):415–21.
Haab, BB., Dunham MJ, Brown PO. Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. Genome Biol. 2001;2(2): RESEARCH0004.
Hanash S. Disease proteomics. Nature. 2003;422(6928):226–32.
Hanash SM, Pitteri SJ, Faca VM. Mining the plasma proteome for cancer biomarkers. Nature. 2008;452(7187):571–9.
Hartmann M, Roeraade J, Stoll D, Templin MF, Joos TO. Protein microarrays for diagnostic assays. Anal Bioanal Chem. 2009;393(5):1407–16.
He M, Stoevesandt O, Palmer EA, Khan F, Ericsson O, Taussig MJ. Printing protein arrays from DNA arrays. Nat Methods. 2008a;5(2):175–7.
He M, Stoevesandt O, Taussig MJ. In situ synthesis of protein arrays. Curr Opin Biotechnol. 2008b;19(1):4–9.
He M, Taussig MJ. Single step generation of protein arrays from DNA by cell-free expression and in situ immobilisation (PISA method). Nucleic Acids Res. 2001;29(15):E73.
Herbst R, Liu Z, Jallal B, Yao Y. Biomarkers for systemic lupus erythematosus. Int J Rheum Dis. 2012;15(5):433–44.
Hoff JD, Cheng LJ, Meyhofer E, Guo LJ, Hunt AJ. Nanoscale protein patterning by imprint lithography. Nano Lett. 2004;4(5):853–7.
Hoheisel JD, Alhamdani MSS, Schröder C. Affinity-based microarrays for proteomic analysis of cancer tissues. Proteomics Clin App. 2013;7(1-2):8–15.
Hutcheson J, Ye Y, Han J, Arriens C, Saxena R, Li QZ, Mohan C, Wu T. Resistin as a potential marker of renal disease in lupus nephritis. Clin Exp Immunol. 2015;179(3):435–43.
Ingvarsson J, Larsson A, Sjoholm AG, Truedsson L, Jansson B, Borrebaeck CA, Wingren C. Design of recombinant antibody microarrays for serum protein profiling: targeting of complement proteins. J Proteome Res. 2007;6(9):3527–36.
Irvine EJ, Hernandez-Santana A, Faulds K, Graham D. Fabricating protein immunoassay arrays on nitrocellulose using dip-pen lithography techniques. Analyst. 2011;136(14):2925–30.

Jang J-W, Smetana A, Stiles P. Multi-ink pattern generation by dip-pen nanolithography®. Scanning. 2010;32(1):24–9.
Kingsmore SF. Multiplexed protein measurement: technologies and applications of protein and antibody arrays. Nat Rev Drug Discov. 2006;5(4):310–21.
Kusnezow W, Banzon V, Schroder C, Schaal R, Hoheisel JD, Ruffer S, Luft P, Duschl A, Syagailo YV. Antibody microarray-based profiling of complex specimens: systematic evaluation of labeling strategies. Proteomics. 2007;7(11):1786–99.
Lee K-B, Park S-J, Mirkin CA, Smith JC, Mrksich M. Protein nanoarrays generated by Dip-Pen nanolithography. Science. 2002;295(5560):1702–5.
Lee K, Lee S, Yu H, Kang SH. Ultra-sensitive detection of tumor necrosis factor-alpha on gold nano-patterned protein chip formed via E-beam nanolithography by total internal reflection fluorescence microscopy. J Nanosci Nanotechnol. 2010;10(5):3228–31.
Liu Cc MS, Kao A, Js N, Jm A. Cell-bound complement biomarkers for systemic lupus erythematosus: from benchtop to bedside. Rheum Dis Clin North Am. 2010;36(1):161–72. x.
Lynch M, Mosher C, Huff J, Nettikadan S, Johnson J, Henderson E. Functional protein nanoarrays for biomarker profiling. Proteomics. 2004;4(6):1695–702.
Macbeath G. Protein microarrays and proteomics. Nat Genet. 2002;32(Suppl):526–32.
Macbeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. Science. 2000;289(5485):1760–3.
Meister A, Liley M, Brugger J, Pugin R, Heinzelmann H. Nanodispenser for attoliter volume deposition using atomic force microscopy probes modified by focused-ion-beam milling. Appl Phys Lett. 2004;85(25):6260–2.
Merrill Jt BJ. The role of biomarkers in the assessment of lupus. Best Pract Res Clin Rheumatol. 2005;19(5):709–26.
Mischak H, Apwiler R, Banks RE, Conaway M, Coon J, Dominiczak A, Ehrich JH, Fliser D, Girolami M, Hermjakob H, Hochstrasser D, Jankowski J, Julian BA, Kolch W, Massy ZA, Neusuess C, Novak J, Peter K, Rossing K, Schanstra JP, Semmes OJ, Theodorescu D, Thongboonkerd V, Weissinger EM, Van Eyk JE, Yamamoto T. Clinical proteomics: a need to define the field and begin to set adequate standards. Clin Proteomics. 2007;1:148–56.
Mok CC. Biomarkers for lupus nephritis: a critical appraisal. J Biomed Biotechnol. 2010;2010:638413.
Neiman M, Hedberg JJ, Donnes PR, Schuppe-Koistinen I, Hanschke S, Schindler R, Uhlen M, Schwenk JM, Nilsson P. Plasma profiling reveals human fibulin-1 as candidate marker for renal impairment. J Proteome Res. 2011;10(11):4925–34.
Nettikadan S, Radke K, Johnson J, Xu J, Lynch M, Mosher C, Henderson E. Detection and quantification of protein biomarkers from fewer than 10 cells. Mol Cell Proteomics. 2006;5(5):895–901.
Olsson N, Carlsson P, James P, Hansson K, Waldemarson S, Malmstrom P, Ferno M, Ryden L, Wingren C, Borrebaeck CA. Grading breast cancer tissues using molecular portraits. Mol Cell Proteomics. 2013;12(12):3612–23.
Petersson L, Berthet Duroure N, Auger A, Dexlin-Mellby L, Borrebaeck CA, Ait Ikhlef A, Wingren C. Generation of miniaturized planar recombinant antibody arrays using a microcantilever-based printer. Nanotechnol. 2014a;25(27):275104.
Petersson L, Coen M, Amro N, Truedsson L, Borrebaeck CA, Wingren C. Miniaturization of multiplexed planar recombinant antibody arrays for serum protein profiling. Bioanalysis. 2014b;6(9):1175–85.
Petersson L, Dexlin-Mellby L, Bengtsson AA, Sturfelt G, Borrebaeck CA, Wingren C. Multiplexing of miniaturized planar antibody arrays for serum protein profiling–a biomarker discovery in SLE nephritis. Lab Chip. 2014c;14(11):1931–42.
Poetz O, Hoeppe S, Templin MF, Stoll D, Joos TO. Proteome wide screening using peptide affinity capture. Proteomics. 2009;9(6):1518–23.
Ramachandran N, Hainsworth E, Bhullar B, Eisenstein S, Rosen B, Lau AY, Walter JC, Labaer J. Self-assembling protein microarrays. Science. 2004;305(5680):86–90.

Ramachandran N, Raphael JV, Hainsworth E, Demirkan G, Fuentes MG, Rolfs A, Hu Y, Labaer J. Next-generation high-density self-assembling functional protein arrays. Nat Methods. 2008;5 (6):535–8.
Rovin BH, Birmingham DJ, Nagaraja HN, Yu CY, Hebert LA. Biomarker discovery in human SLE nephritis. Bull NYU Hosp Jt Dis. 2007;65(3):187–93.
Manzi S. Lupus update: perspective and clinical pearls. Cleve Clin J Med. 2009;76(2):137–42.
Sanchez-Carbayo M. Antibody array-based technologies for cancer protein profiling and functional proteomic analyses using serum and tissue specimens. Tumor Biol. 2010;31(2):103–12.
Söderlind E, Strandberg L, Jirholt P, Kobayashi N, Alexeiva V, Aberg A-M, Nilsson A, Jansson B, Ohlin M, Wingren C, Danielsson L, Carlsson R, Borrebaeck CA. Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. Nat Biotechnol. 2000;18(8):852–6.
Tran PL, Gamboa JR, You DJ, Yoon JY. FRET detection of Octamer-4 on a protein nanoarray made by size-dependent self-assembly. Anal Bioanal Chem. 2010;398(2):759–68.
Wacker R, Niemeyer CM. DDI-microFIA–A readily configurable microarray-fluorescence immunoassay based on DNA-directed immobilization of proteins. Chembiochem. 2004;5(4):453–9.
Wacker R, Schroder H, Niemeyer CM. Performance of antibody microarrays fabricated by either DNA-directed immobilization, direct spotting, or streptavidin-biotin attachment: a comparative study. Anal Biochem. 2004;330(2):281–7.
Wingren C, Borrebaeck C. Antibody microarrays – current status and key technological advances. OMICS. 2006;10(3):411–27.
Wingren C, Borrebaeck CA. Progress in miniaturization of protein arrays–a step closer to high-density nanoarrays. Drug Discov Today. 2007;12(19–20):813–9.
Wingren C, Borrebaeck CA. Antibody microarray analysis of directly labelled complex proteomes. Curr Opin Biotechnol. 2008;19(1):55–61.
Wingren C, Borrebaeck CA. Antibody-based microarrays. Methods Mol Biol. 2009;509:57–84.
Wingren C, Ingvarsson J, Dexlin L, Szul D, Borrebaeck CA. Design of recombinant antibody microarrays for complex proteome analysis: choice of sample labeling-tag and solid support. Proteomics. 2007;7(17):3055–65.
Wingren C, James P, Borrebaeck CA. Strategy for surveying the proteome using affinity proteomics and mass spectrometry. Proteomics. 2009;9(6):1511–7.
Wingren C, Sandström A, Segersvärd R, Carlsson A, Andersson R, Löhr M, Borrebaeck CA. Identification of serum biomarker signatures associated with pancreatic cancer. Cancer Res. 2012;72(10):2481–90.
Wu T, Du Y, Han J, Singh S, Xie C, Guo Y, Zhou XJ, Ahn C, Saxena R, Mohan C. Urinary angiostatin–a novel putative marker of renal pathology chronicity in lupus nephritis. Mol Cell Proteomics. 2013;12(5):1170–9.
Zheng G, Patolsky F, Cui Y, Wang WU, Lieber CM. Multiplexed electrical detection of cancer markers with nanowire sensor arrays. Nat Biotechnol. 2005;23(10):1294–301.

Malondialdehyde as a Biomarker in Kidney Transplantation

37

Isabel Fonseca

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Abstract

Delayed graft function (DGF) is a clinical diagnosis that describes dysfunction of the kidney allograft immediately after kidney transplantation, which as a detrimental impact on kidney graft survival. In recent years, there is a considerable interest in identifying biomarkers indicative of early graft dysfunction and predictive of allograft survival. A variety of markers have been proposed as candidates to signal graft dysfunction earlier. Of these markers, malondialdehyde (MDA) appear to have a clinical relevance, given the results obtained in preliminary clinical studies and/or its biological properties that are directly related with its toxicity towards several cells and tissues.

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Almost 30 % of DGF following kidney transplantation is attributable to ischemia reperfusion injury, which is one of the most important non-specific and non-immunologic factor that affect early and long-term allograft function. The production of excessive quantities of reactive oxygen species (ROS) is an important mechanism of ischemia-reperfusion injury and lipid peroxidation is one of the most widespread hypotheses of ROS-mediated cell injury. Lipid oxidation gives rise to several aldehydes, including MDA, which are good indicators of nephrotoxicity and tissue damage. Malondialdehyde is a reliable diagnostic biomarker of initial graft injury and accurately detect graft dysfunction earlier than serum creatinine.

Moreover, levels of MDA in plasma within the first week after reperfusion of the graft predicts long-term graft outcome. As in every other domain in medicine, in organ transplantation early diagnosis and timely intervention will improve outcomes. Malondialdehyde is, therefore, more than a promising marker, but an alarm of cellular and tissue damage. And the approach of using MDA as a trigger to initiate and monitor immunosuppressive therapies, and as a safety biomarker when using potentially nephrotoxic agents, is also promising.

Keywords

Oxidative stress • Malondialdehyde • Lipid peroxidation • Kidney transplantation • Ischemia-reperfusion injury

Abbreviations

4-HNE	Dyhidroxynonel
AGEs	Advanced glycation end products
AOPP	Advanced oxidation protein products
DGF	Delayed graft function
DNA	Deoxyribonucleic acid
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
$HOO^{\bullet}$	Hydroperoxyl radical
HPLC	High-performance liquid chromatography
LOOHs	Lipid hydroperoxides
MDA	Malondialdehyde
MHC	Major histocompatibility complex
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
$O_2^{-\bullet}$	Superoxide
$OH^{\bullet}$	Hydroxyl radical
$OLOO^{\bullet}$	Epoxy-allylic peroxyl radicals
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species

SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances assay

Key Facts of Kidney Transplantation

- Kidney transplantation is the treatment of choice for almost all cases of renal failure due to better quality of life and survival than for chronic dialysis.
- The short-term outcome of renal transplantation has improved substantially in the past 20 years, however long-term survival has not paralleled that improvement.
- Delayed graft function is the most common complication in the immediate post-transplantation period mainly in deceased renal allografts, almost invariably in the non-heart beating and in some live donor transplants.
- Kidney DGF represents acute kidney injury in the immediate postoperative period after transplantation and continues to pose a significant challenge in kidney transplantation
- Several studies have found associations between DGF and increased risk for acute rejection, chronic allograft dysfunction, and worse graft survival.
- The association between DGF and worse outcomes has led to increased efforts to better understand the mechanisms of ischemia-reperfusion injury and to develop interventions to reduce its occurrence and impact.

Definitions

Antioxidants Antioxidants are substances, endogenous or exogenous, that, when present at low levels compared to an oxidizable compound (e.g. lipids), delay or inhibit oxidation of the substrate and the inherent oxidative damage.

Cold ischemia time Cold ischemia time is defined as the time between the chilling of a tissue or organ after its blood supply has been reduced or cut off and the time in which the tissue or organ reach physiological temperature after restoration of blood supply during implantation procedures.

Delayed graft function (DGF) Delayed graft function (DGF) represents renal failure persisting after transplantation and is usually defined as requirement for dialysis in the first week of transplantation (although several other definitions are used due to the difficulty of defining DGF precisely). Prolonged cold ischemia time is usually associated with a greater incidence of DGF, which as a detrimental impact on kidney graft survival.

Free radicals Free radicals are defined as molecules or molecular fragments containing one or more unpaired electron in atomic or molecular orbits, which gives a considerable reactivity to the free radical.

Ischemia Ischemia refers to reduction or cessation of arterial blood flow with immediate oxygen deprivation of cells.

Ischemia-reperfusion injury Ischemia-reperfusion injury refers to cellular/tissue damage that results damage that results from a period of ischemia that is followed by the reestablishment of the blood supply. The absence of blood and nutrients from blood creates a condition in which the restoration of circulation results in inflammation and oxidative damage.

Lipid peroxidation Lipid peroxidation is the metabolic process in which ROS attack and induce oxidative deterioration of lipids, especially polyunsaturated fatty acids (PUFAs), affecting cell membrane structure and function and causing tissue damage.

Malondialdehyde (MDA) Malondialdehyde (MDA) is a highly reactive three-carbon dialdehyde produced as an endproduct of lipid peroxidation, being considered a good marker of oxidative stress and of tissue damage.

Oxidative stress Oxidative stress can be defined as an imbalance favouring the oxidant (reactive oxygen species) generation over the ability of antioxidant mechanisms for detoxifying the reactive intermediates or for repairing the resulting damage that leads to macromolecular damage and dysfunction.

Pro-oxidants Pro-oxidants are species that causes or promotes oxidative stress, either by generating reactive oxygen species or by inhibiting antioxidant systems. Depending on the circumstances, a compound may exhibit pro- or antioxidant activity. Examples: polyphenols, thiols, α-tocopherol.

Reactive oxygen species (ROS) Reactive oxygen species (ROS) are highly reactive metabolites of molecular oxygen (O_2), such as oxygen-derived free radicals (superoxide, hydroxyl radical, nitric oxide) and non-radical oxygen derivatives of high reactivity (singlet oxygen, hydrogen peroxide, peroxynitrite, hypochlorite). ROS are constantly produced endogenously and under physiological conditions, ROS production and scavenging capacity are balanced. At high doses ROS become deleterious, exhibiting pathophysiological actions.

Introduction

Cellular damage caused by restoring the blood supply to previously viable ischemic tissues is defined as ischemia-reperfusion injury. This phenomenon is inevitable in organ transplantation. In kidney transplantation, decreased blood supply is associated with flow deviation from cortex to medulla, which preserves the oxygenation of the metabolically vulnerable medulla at the cost of cortical perfusion and glomerular filtration (Woolfson et al. 1994). Sensitivity to oxygen deprivation,

or ischemia, has been demonstrated in both the proximal tubules (Shanley et al. 1986) and their thick ascending limbs (Brezis et al. 1985). Thus, tubulointerstitial damage begins soon after transplantation due to ischemia-reperfusion injury. The resolution of this process is critical to obtain a more successful outcome for the kidney, because ischemia-reperfusion has been identified as a major risk factor for the development of delayed graft function (DGF), acute rejection and long-term chronic graft dysfunction (Woolfson et al. 1994; Koo et al. 1998; Kosieradzki and Rowinski 2008).

A number of mechanisms have been proposed to mediate the injury caused by the ischemia that occurs during the organ transfer from the donor to the recipient and the reperfusion once the vascular anastomosis in the recipient is complete. The most frequently cited mechanisms of ischemia-reperfusion damage include intracellular calcium overload, endothelial and microvascular dysfunction, leukocyte-endothelium interactions, altered kidney metabolism and perhaps most importantly the formation of oxygen free radicals (Kosieradzki and Rowinski 2008; Gulec 2011).

Oxidative Stress, Reactive Oxygen Species and Antioxidants

Oxygen free radicals or, more generally, reactive oxygen species (ROS) are products of normal cellular metabolism that exist in all aerobic cell, and their level is controlled by a balance between pro-oxidants and antioxidants. The average person has approximately 10,000–20,000 free radicals attacking each cell in his body every day (Halliwell and Gutteridge 1986; Valko et al. 2004). These well-known ROS, such as superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$), are derived from oxygen and are formed as intermediates in reduction-oxidation processes. They represent the most important class of reactive species generated in living systems (Valko et al. 2004; Small et al. 2012).

One to 3 % of inspired molecular oxygen (O_2) is converted to superoxide ($O_2^{-\bullet}$) rather than being reduced to water. Superoxide is the most common of the ROS and a precursor to other ROS generated in cells. In fact, the formation of this oxygen free radical leads to a cascade of other ROS. Superoxide has an unpaired electron that makes it highly reactive and renders it unstable and short-lived as an oxidising agent. Because of its charge, the superoxide anion is poorly permeable to cell membrane and remains mostly within the mitochondrial matrix, although it can also cross cell membranes via anion channels (Burton and Jauniaux 2011; Small et al. 2012). It can undergo several chemical reactions depending on the amount generated and the proximity to other radicals and enzymes. This free radical anion is a powerful precursor of hydrogen peroxide (H_2O_2), which is lipid soluble and freely crosses the cell membrane (Burton and Jauniaux 2011). Although cellular hydrogen peroxide is stable, it has the potential to interact with a variety of substrates and cause damage, particularly in the presence of the reduced metal ion Fe^{2+}. This interaction leads to the breakdown of H_2O_2 and the formation of the most highly reactive, toxic and damaging of the free radicals produced during normal metabolism and/or by exogenous sources, the hydroxyl radical ($OH^{\bullet}$) (Valko et al. 2004; Small et al. 2012).

This small, highly mobile, water-soluble, and short-lived molecule can be produced from molecular oxygen (O_2) in cell metabolism and under a variety of stress conditions.

A cell produces approximately 50 hydroxyl radicals every second. In a full day, each cell can generate four million hydroxyl radicals, which can either be neutralised or attack biomolecules (Ayala et al. 2014). Mitochondria constantly metabolize oxygen thereby producing ROS as a by-product. The estimated levels of ROS within the mitochondria are five to tenfold higher than other cytosolic and nuclear compartments because they are mostly generated by the mitochondria during oxidative phosphorylation (energy generation) and by the activation of cellular enzymes, including NADPH oxidase, cyclooxygenase, nitric oxide synthase and xanthine oxidase (Valko et al. 2004; Small et al. 2012). Under physiological conditions, ROS are produced specifically to serve in essential biological functions and play a physiological role in cellular responses to noxia, such as in defence against infectious agents and in the function of a number of cellular signalling systems (Rahman 2007). Under these conditions, ROS are the by-products of normal metabolic processes, and the rates of free radical production and elimination are similar, leading to a steady state that is presumably tolerated by the cell. In other cases, mostly pathological, ROS production exceeds the cellular antioxidant capacity and can be deleterious to biological systems, which causes oxidative stress. Traditionally, oxidative stress is defined as an imbalance between oxidant generation and antioxidant mechanisms for detoxifying the reactive intermediates or for repairing the resulting damage, which can lead to macromolecular damage and dysfunction (Halliwell and Gutteridge 1986; Rahman 2007).

The antioxidant defence mechanisms can be divided into two major groups: (i) endogenous, which are primarily enzymes, such as superoxide dismutase (SOD), catalase, glutathione reductase (GR) and glutathione peroxidase (GPx), (ii) and small molecules, which are primarily exogenous and act as free radical scavengers, such as reduced glutathione (GSH), vitamins A, C, and E, carotenoids and polyphenol (Valko et al. 2004; Lu 2009; Burton and Jauniaux 2011).

In aerobic organisms the antioxidant enzymes are widely involved in scavenging free radicals. The reactions catalysed by these defence enzymes are briefly presented in (Fig. 1). Superoxide dismutases, namely copper/zinc-SOD (CuZn-SOD) and manganese-SOD (Mn-SOD), play a key role in the oxidative stress process because

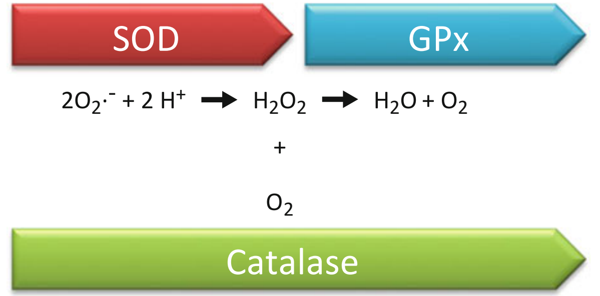

Fig. 1 Brief description of the reactions catalysed by the main antioxidant defence enzymes. *SOD* Superoxide dismutase, *GPx* Glutathione peroxidase

they accelerate the transformation of the highly reactive superoxide anion ($O_2^{-\bullet}$) into hydrogen peroxide (H_2O_2), a much more stable ROS. Hydrogen peroxide is then converted enzymatically into oxygen and water by catalase and glutathione peroxidase (Gutteridge 1995; Small et al. 2012). Hydrogen peroxide is not a free radical because it does not have an unpaired electron, but it is a ROS and must be promptly removed by catalase. In many tissues, catalase activity, which is largely localised to peroxisomes, is very low and frequently not available for decomposition of hydrogen peroxide (H_2O_2). Therefore, in most tissues, hydrogen peroxide is deactivated with GPx. Thus, high SOD activity, which results in increased H_2O_2 production, must be accompanied by increased GPx and/or catalase activity to limit injury. If this does not happen, the excess hydrogen peroxide can interact with superoxide in the presence of transition metal-containing molecules and yield a hydroxyl radical ($HO^{\bullet}$) (Haber-Weiss reaction), which is one of the most deleterious and potent oxidising agents known (Gutteridge 1995; Small et al. 2012). Glutathione peroxidase provides an effective mechanism against cytosolic injury because it reduces peroxides and hydroxyl radicals into nontoxic forms using GSH (Burton and Jauniaux 2011). Thus, the activity of GPx depends on the presence of reduced GSH as a hydrogen donor, which plays a critical role as antioxidant. In fact, GSH plays a critical role as an antioxidant because it participates in a large number of detoxifying reactions by forming glutathione disulphide. Glutathione disulphide is converted back to GSH by the action of glutathione reductase at the expense of NADPH, forming a redox cycle (Lu 2009; Burton and Jauniaux 2011).

When these antioxidant mechanisms cannot offset the generation of free radicals, oxidative stress-associated tissue injury occurs. Because free radicals are compounds highly unstable and reactive with a half-lives of only seconds, it is difficult to measure their levels directly, which makes understanding in vivo oxidative damage challenging. Oxyradical-modified lipids, proteins, carbohydrates and nucleic acids remain stable from hours to weeks, which makes them ideal surrogate markers of oxidative stress (Locatelli et al. 2003). Moreover, defects or low activity in the antioxidant defence system lead to the impaired clearance of ROS, which can also be used as indirect oxidative markers. A list of the common oxidative markers is found in (Table 1; Gutteridge 1995; Locatelli et al. 2003; Valko et al. 2004; Rahman 2007; Burton and Jauniaux 2011).

Lipid Damage by Reactive Oxygen Species

Reactive oxygen species may cause tissue injury via several mechanisms. Because they are potent oxidising and reducing agents, ROS directly damage cellular membranes and modify biological molecules, such as lipids, proteins, and nucleic acids. Of the many biological targets of oxidative stress, lipids are the most affected (Del Rio et al. 2005). Unsaturated phospholipids, glycolipids, and cholesterol in cell membranes and other organised systems are prominent targets of oxidant attack and lead to a process known as lipid peroxidation (Girotti 1985; Romero et al. 1998; Ayala et al. 2014). Lipid peroxidation is the most important source of free radical-

Table 1 Biomarkers of oxidative stress

Oxidants
1. Protein oxidation products
Protein carbonyls
Advanced oxidation protein products (AOPP)
Carboxymethyllysine and pentosidine
Pentosidine
2. Carbohydrate oxidation products
Advanced glycation end products (AGEs)
3. Lipid peroxidation products
Malondialdehyde
F_2-isoprostane
4-Hydroxynonenal
Acrolein
Thiobarbituric acid-reactive substances
4. DNA oxidation products
8-hydroxy-2′deoxyguanosine (8-OHdG)
8-hydroxyguanosine (8-oxoG)
Prooxidants
5. Prooxidant enzymes
Xanthine oxidase
NADPH oxidase
Antioxidants
6. Non-enzymatic antioxidants (*low molecular weight antioxidants*)
Antioxidant vitamins (from diet or pharmacological supplements)
Ascorbic acid (vitamin C)
Tocopherol (vitamin E)
Retinol (vitamin A)
Carotenoids
Innate compound
Glutathione (GSH)
7. Antioxidant enzymes
Superoxide dismutase
Catalase
Glutathione peroxidase
GSH/GSSG ratio in erythrocyte

NADPH Reduced nicotinamide adenine dinucleotide phosphate, *GSH* Glutathione, *GSSG* Oxidized glutathione, *DNA* deoxyribonucleic acid, *AOPP* Advanced oxidation protein products, *AGEs* Advanced glycation end products

mediated injury that directly damages cellular membranes and yields a number of secondary products responsible for extensive cellular damage (Romero et al. 1998).

The two most prevalent ROS that can profoundly affect lipids are the hydroxyl ($OH^•$) and hydroperoxyl ($HOO^•$) radicals. Polyunsaturated fatty acids (PUFAs) of the membrane phospholipids are the most susceptible molecules to attack by these

free radicals. Damage to PUFAs initiates self-propagating chain reactions that undergo peroxidation and form lipid hydroperoxides (LOOHs) and a variety of decomposition products, such as aldehydes or isoprostanes. In general, PUFAs are more susceptible to oxidation as the number of double bonds increase. Of the various biologically relevant PUFAs, long-chain n-3 fatty acids, such as eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), oxidise more readily than the less saturated fatty acids, such as linoleic acid (18:2n-6) (Girotti 1985).

Lipid peroxidation is naturally present in small amounts in the body because of the effect of ROS. However, because lipid peroxidation is a self-propagating chain-reaction, the initial oxidation of only a few lipid molecules can result in significant tissue damage. Any free radical with sufficient energy to extract a hydrogen atom from a methylene carbon (-CH2-) of an unsaturated fatty acid can initiate lipid peroxidation, which results in a carbon lipid radical ($L^\bullet$). The free-radical chain reaction of lipid peroxidation propagates until two free radicals annihilate each other to terminate the chain (Gutteridge 1995). The lipid carbon radical ($L^\bullet$) reacts with molecular oxygen to generate a reactive lipid peroxyl radical ($LOO^\bullet$), which may then extract a hydrogen from a nearby unsaturated fatty acid to form the non-radical intermediate lipid hydroperoxides (LOOHs). Thus, LOOHs are the primary products of fatty acid peroxidation and may undergo iron-mediated electron reduction and oxygenation to yield epoxy-allylic peroxyl radicals ($OLOO^\bullet$), which trigger the chain reaction of lipid peroxidation (Girotti 1985).

Under normal physiological conditions, these lipid hydroperoxides (LOOHs) produced in the course of normal conditions are completely inactivated by cellular and extracellular defence mechanisms. In certain pathological conditions, increased ROS-initiated peroxidation reactions and/or the depletion of antioxidant defence systems lead to the accumulation of lipid hydroperoxides (LOOHs). Accumulated LOOHs tend to perturb membrane structure/function and alterations in membrane integrity, fluidity, and permeability can occur, which results in cellular and tissue damage (Girotti 1985). The destruction of membrane lipids and the end-products from lipid peroxidation reactions are especially dangerous to the viability of cells and several tissues. Therefore, lipid oxidation is now known to be a crucial step in the pathogenesis of several disease states (Gutteridge 1995; Agarwal et al. 2004; Rahman 2007). Three of the major targets of the lipid peroxidation process are the brain, the kidney and the liver. Thus, the toxicity of lipid peroxidation products in mammals generally involves neurotoxicity, nephrotoxicity and hepatotoxicity (Repetto et al. 2012). The detection and measurement of lipid peroxidationis the evidence most frequently cited to support the involvement of free-radical reactions in diseases; thus, lipid peroxidation may be an excellent marker of tissue damage (Gutteridge 1995).

Malondialdehyde

Many lipid peroxidation products are formed during ROS attacks on the double bounds PUFAs, including lipid hydroperoxides (LOOHs) and conjugated dienes, the primary products of lipid peroxidation. A great number of aldehydes are among the

secondary products formed during lipid peroxidation, such as malondialdehyde (MDA) and 4-dyhidroxynonel (Fig. 2), as well as the prostaglandin F_2-like compounds (F_2-isoprostanes), the gold standard in lipid peroxidation studies (Janicka et al. 2010). All of these by-products can serve as biomarkers of lipid peroxidation. Choosing a convenient marker for evaluating the importance of lipid peroxidation in vivo is difficult because of the analytical problems of specificity and sensitivity. Isoprostanes are the most specific markers of lipid peroxidation but they are also most difficult to measure (Janicka et al. 2010). Thus, MDA is the principal and the most studied product of lipid peroxidation (Romero et al. 1998).

Malondialdehyde is a highly toxic and reactive bifunctional molecule that reacts with DNA and forms adducts with deoxyguanosine and deoxyadenosine. It can also modify RNA, proteins and other biomolecules (Siddique et al. 2012). The main source of MDA in biological samples is the peroxidation of PUFAs with two or more methylene-interrupted double bonds. Thus, MDA is widely used as a reliable biomarker for estimating the tissue damage from ROS and lipid peroxidation (Del Rio et al. 2005). In recent years, oxidative stress has been implicated in various diseases, and elevated serum or plasma MDA levels has been noted in different pathological conditions, including airway diseases, cancer, diabetes, renal disease, neurodegenerative disorders, cardiovascular diseases, among others (Table 2; Pucheu et al. 1995; Keith et al. 1998; Templar et al. 1999; Gonenc et al. 2001; Dib et al. 2002; Kolanjiappan et al. 2002; Dut et al. 2008; Bandeira et al. 2012; Antus et al. 2014).

Historically, the thiobarbituric acid reactive substances assay (TBARS) was the common method used to estimate MDA levels. However, this assay is notoriously

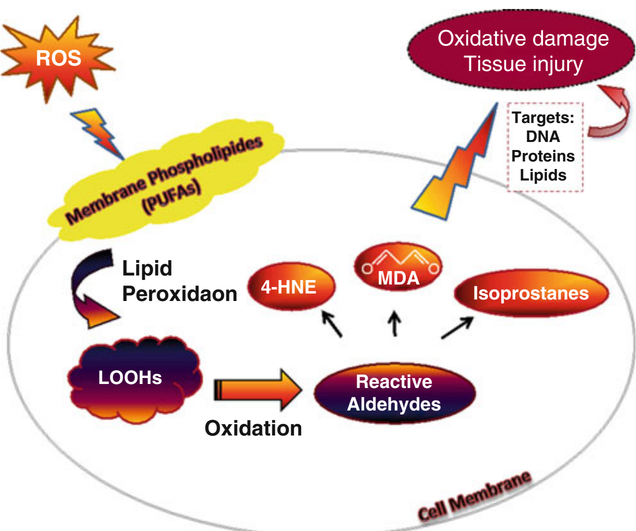

Fig. 2 Lipid peroxidation and MDA production. *ROS* Reactive oxygen species, *PUFAs* Polyunsaturated fatty acids, *LOOHs* Lipid hydroperoxides, *MDA* Malondialdehyde, *4-HNE* Dyhidroxynonel

Table 2 Malondialdehyde in some pathological conditions

Author	Disease or condition	Conclusions
(Pucheu et al. 1995)	Myocardial infarction	MDA is a good marker of reperfusion-related radical stress after thrombolysis, which might represent a simple and reliable test of reperfusion efficacy following thrombolysis, and it might enable one to test the effect of various antioxidant therapies associated with thrombolytic treatment
(Keith et al. 1998)	Congestive heart failure	A significant increase in MDA levels in patients with congestive heart failure was related to the functional severity of heart failure, with the highest levels being observed in patients in functional class III and IV. Increased MDA levels can be a objective marker of prognosis in congestive heart failure
(Gonenc et al. 2001)	Breast and lung cancer	MDA, a marker of lipid peroxidation in patients with breast and lung cancer
(Dib et al. 2002)	Neurodegenerative diseases	Plasma MDA levels are elevated in several neurodegenerative disorders, namely amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and Huntington's disease and there are indications that these levels correlate with clinical state. MDA can be considered as a marker for the evolution of these diseases
(Templar et al. 1999)	Chronic kidney disease and hemodialysis	Lipid peroxidation in patients with chronic renal failure is further exacerbated by hemodialysis, as evidenced by significant increased MDA levels. In the non-dialysis group, patients with glomerulonephritis presented the higher MDA levels, suggesting the possible involvement of ROS in the pathophysiology of glomerular disease
(Dut et al. 2008)	Asthma	Asthma is associated with an extremely powerful oxidative stress, expressed by higher levels of MDA
(Bandeira et al. 2012)	Diabetes mellitus	Increased level of MDA in diabetics suggests that peroxidative injury may be involved in the development of diabetic complications
(Antus et al. 2014)	Chronic obstructive pulmonary disease (COPD)	MDA useful marker for monitoring exacerbation-associated oxidative stress in patients with acute exacerbation of COPD

MDA Malondialdehyde, *ROS* Reactive oxygen species, *COPD* Chronic obstructive pulmonary disease

nonspecific because it also reacts with saturated and unsaturated non-functional aldehydes, carbohydrates and prostaglandins, which has led to controversy over its use for quantifying MDA from in vivo samples. The poor assay specificity associated with TBARS may lead to an overestimation of the levels of MDA in human plasma and other biological tissues and fluids, and this, in turn, may limit the likelihood of detecting the true differences in the levels of lipid peroxidation in clinical studies (Siddique et al. 2012; Moselhy et al. 2013; Ayala et al. 2014). Several technologies for determining free and total MDA, such as high-performance liquid

chromatography (HPLC) techniques, and several derivatisation-based strategies, have been developed during the last decade. These new assays have improved specificity and sensitivity of MDA determination. Consequently, due to the higher accuracy in the detection of the degree of lipid peroxidation and the indirect level of the free oxygen radicals, HPLC is currently recommended as the preferred MDA assay for human plasma samples. This is a remarkable advance in biomedical research community because MDA is becoming a very relevant molecule and a promising biomarker of tissue damage (Siddique et al. 2012; Moselhy et al. 2013; Ayala et al. 2014).

Malondialdehyde and the Kidney

Oxidative stress causes tissue damage by different mechanisms including lipid peroxidation, DNA damage, and protein modification. All of these processes have been implicated in the pathogenesis of several systemic diseases, including kidney disease. The kidney is very vulnerable to ROS damage because renal lipids are composed of an abundance of long-chain PUFAs. Though free radicals can attack many critical biological molecules, such as DNA and cellular proteins, the high content of unsaturated lipids marks lipid peroxidation as the central feature of oxidant injury in the kidney (Rodrigo and Rivera 2002; Chung and Perrella 2004; Ozbek 2012).

Chronic kidney disease is a pro-oxidant state and the degree of intracellular and extracellular oxidative stress is related to the severity of renal failure (Massy and Nguyen-Khoa 2002; Small et al. 2012). Formation of ROS is evident in many areas of the kidney, predominantly in the renal cortices. The renal medulla, however, is susceptible to hypoxia, but with less ROS production under physiological and pathological conditions (Bedard and Krause 2007). The structural characteristics of chronic kidney disease include increased tubular atrophy, interstitial fibrosis, glomerulosclerosis, renal vasculopathy and reduced renal regenerative capability. These characteristics may be caused, at least in part, by the gradual loss of renal energy through the development of mitochondrial dysfunction and resultant increase of oxidative stress (Small et al. 2012). Some studies report that ROS are involved in the pathogenic mechanisms of conditions such as glomerulosclerosis, renal tubular damage and tubulointerstitial fibrosis (Shah 1995; Rodrigo and Rivera 2002; Agarwal et al. 2004; Raju et al. 2013).

It has also been observed that free radical-induced lipid peroxidation tissue damage plays a significant role in the pathogenesis of various renal diseases (Raju et al. 2013). Elevated levels of MDA and decreased antioxidant activity have been reported in patients with renal disease, which is a reflection of the increased oxidative stress in these patients. This oxidative imbalance is exacerbated by dialysis procedure and by severe chronic inflammation (Boaz et al. 1999; Templar et al. 1999; Oberg et al. 2004; De Vecchi et al. 2009; Raju et al. 2013). Urinary MDA is considered to be a reliable indicator of enhanced lipid peroxidation in renal tubules and is thought to be directly proportional to renal damage in non-dialysis kidney patients (Draper et al. 1986). Although there are few studies in this field,

MDA levels seem to depend on type of renal disease; it is generally higher in patients with glomerular-associated reduced plasma clearance (Templar et al. 1999). Patients with chronic kidney disease on regular haemodialysis exhibit higher MDA levels than healthy controls. It has been suggested that enhanced lipid peroxidation might result from free radical activity generated by complement activation and the release of cytokines during the dialysis procedure. This was mostly attributed to the exposure of blood to bio-incompatible dialysis membranes and the diffusion of hydrophilic compounds to the dialysate and the influx of endotoxin from the dialysate (Templar et al. 1999; Locatelli et al. 2003). Increased lipid peroxidation, especially in haemodialysis patients, also results in excess consumption of antioxidant enzymes such as SOD. The loss of antioxidants, such as zinc and copper that act as cofactors of SOD activity, in the dialysate fluid also contribute to the further decrease in antioxidant activity of these patents (Locatelli et al. 2003).

Biomarkers of Oxidative Stress in Kidney Transplantation

There is substantial literature on oxidative stress and renal disease, but there is a lack of data on oxidative stress in kidney transplantation. Restoring kidney function after transplantation can lead to an improvement in oxidative stress, but some studies demonstrate increased systemic biomarkers of oxidative stress in kidney transplant recipients, (Simic-Ogrizovic et al. 1998; Campise et al. 2003) specifically in the early post-transplant phase (Zahmatkesh et al. 2010; Ardalan et al. 2013) and, thereafter, coexisting with chronic allograft tubular atrophy/interstitial fibrosis (Simic-Ogrizovic et al. 1998; Djamali 2007).

The first line of cellular defence against oxidative injury includes the antioxidant enzymes catalase, SOD, and GPx. There are conflicting results in the literature on the activities of antioxidant enzymes in kidney transplant patients. Glutathione compounds and SOD have been reported to increase (Whitin et al. 1998; Zachara et al. 2004), decrease (Campise et al. 2003), or not change (Vostalova et al. 2012) following renal transplantation. Whitin and colleagues reported a rapid increase in plasma GPx activity after transplantation (Whitin et al. 1998). Plasma GPx activity was two times higher 3 days after transplantation in adult patients who received a kidney transplant from a related donor and rapidly increased over the first 2 weeks post-transplant in adult recipients from a deceased-donor and in paediatric patients undergoing kidney transplantation from related donors (Whitin et al. 1998). Zachara et al. demonstrated that plasma GPx activity increases rapidly 3 days after renal transplantation and doubles 2 weeks later (Zachara et al. 2004). Both of these studies suggested that monitoring plasma GPx might be a useful biomarker for monitoring the transplanted kidney function and a valuable tool for the postoperative detection of early graft dysfunction and/or DGF (Whitin et al. 1998; Zachara et al. 2004).

In kidney transplantation, some processes including immunologic disorders of the kidneys, ischemic insults, and nephrotoxic drugs, lead to oxidative stress, subsequent renal injury and graft dysfunction (Chung and Perrella 2004). In the perioperative period, all solid organs used for transplantation undergo varying

degrees of ischemic damage and reperfusion injury after retrieval, storage, and transplantation into the recipient (Koo et al. 1998). Ischemic changes start at the time of a donor's brain death. Specifically, brain death is associated with generalised ischemia due to a hyperactivity of the sympathetic system, which aims to maintain the cerebral perfusion pressure. Subsequently, free radical-mediated injury induces the generation and release of proinflammatory cytokines and activates the innate immune response. The immune response against a transplanted organ may not solely involve a major histocompatibility complex (MHC)-specific alloimmune response, but instead, an immediate nonspecific inflammatory response caused by ischemia-reperfusion injury (Koo et al. 1998). It has been suggested that all of these changes (the early innate response and the ischemic tissue damage) play roles in the development of adaptive responses, which may in turn lead to early graft dysfunction and/or acute kidney rejection (Kosieradzki and Rowinski 2008). Hypothermic storage before transplantation add ischemic tissue damage to the organ and increase the susceptibility to injury upon reperfusion, which is the final stage of ischemic injury. The reperfusion injury is considered the effector phase of ischemic injury and develops hours or days after the initial insult. The whole process has been described as ischemia-reperfusion injury and it has a profound influence on not only the early but also the late function of a transplanted kidney (Koo et al. 1998; Kosieradzki and Rowinski 2008).

In the kidney, once the ischemia-reperfusion injury occurs, it causes a highly complex cascade of events (Fig. 3; Koo et al. 1998; Kosieradzki and Rowinski 2008; Gulec 2011). During ischemia, hypoxia is rapidly induced and followed by a sudden drop in intracellular levels of ATP and a rapid increase in ROS production, including superoxide (Edelstein et al. 1997; Dagher 2000; Lee et al. 2005). The kidney is highly energetic and, therefore, relies heavily on aerobic metabolism for the production of ATP by oxidative phosphorylation. The reduction of molecular oxygen along the electron transport chain within the mitochondria is vital for renal cellular function, and the interruption of blood supply results in an ischemic injury (Small et al. 2012). The tubulointerstitium is a section of the kidney that includes the tubules, which comprise approximately 80 % of the kidney volume, and the compartment of the kidney bounded by the vasculature and nephrons. This component of the kidney performs vital functions and is particularly vulnerable to oxygen depletion. Thus, renal cells and the endothelial and tubular epithelial cells have high energy requirements and are highly susceptible to injury in situations of relative oxygen and nutrient deprivation (Khand et al. 2002; Kim et al. 2002; Maenpaa et al. 2008; Nilakantan et al. 2008). In fact, the absence of blood supply causes a state in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress (Sydykov 2009).

Although crucial for tissue survival, oxygen can be injurious during the reperfusion of previously ischemic organ. Molecular oxygen, when reintroduced into a previously ischemic kidney, undergoes sequential reduction steps, leading to the generation of oxygen free radicals, which is a key process in the development of reperfusion injury (Ponticelli 2014). Even thought the restoration of circulation (reperfusion) is essential for the recovery of normal cellular function and the

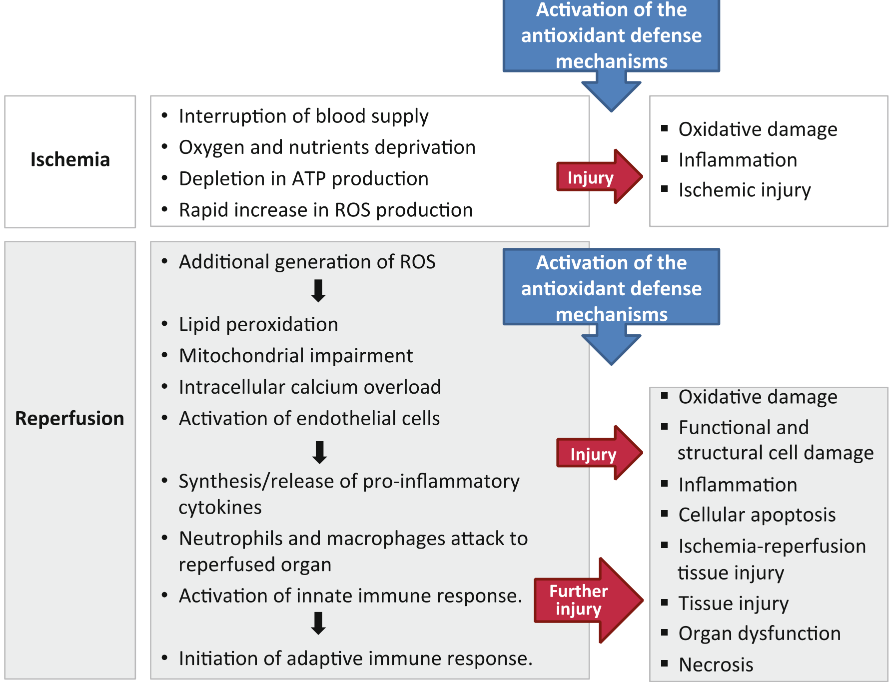

Fig. 3 Brief description and sequence of events in ischemia-reperfusion injury. *ATP* Adenosine triphosphate, *ROS* Reactive oxygen species

prevention of irreversible tissue injury, the reperfusion itself may initiate a series of pathophysiological alterations that can augment the tissue injury produced by ischemia alone (Sydykov 2009). The reperfusion of the ischemic kidney further worsens the state of oxidation by promoting the additional release of free radicals. In an study performed in animal model, Bolli and colleagues showed that potent oxidant radicals, such as the superoxide anion, hydroxyl radical, and peroxynitrite, are produced within the first few minutes of reflow and play a critical role in the development of reperfusion injury (Bolli et al. 1989). Hence, ROS are generated during both the ischemia phase and the reperfusion phase (Ponticelli 2014).

In kidney transplantation, not only do the ischemic and reperfusion periods required for the organ preservation and implantation procedures induce oxidative stress, but placing the kidney into an immune milieu can also act as an adjuvant for oxidative damage. The warm ischemia after the kidney vessels are clamped and the cold ischemia after refrigeration also reduce the oxygen and nutrients supply to the tissues. A wide range of protective substances, such as antioxidant enzymes, may protect the transplanted organ by limiting the production of ROS and the damage from oxidative stress following ischemia-reperfusion injury of the kidney graft. The kidney has naturally occurring antioxidant enzymes to counteract the effects of oxygen free radicals. Superoxide dismutase catalyses the conversion of superoxide

to the harmless hydrogen peroxide. Glutathione works in a similar manner, but it can also act on organic peroxides (Deneke and Fanburg 1989; Weinberg 1992; Lu 2009). The protective abilities of these scavengers are overwhelmed when oxygen free radical concentrations exceed the reducing capabilities of the scavengers (Granger 1988). There is some evidence that during the early phase of ATP depletion, which typically occurs in ischemia, manganese superoxide dismutase (MnSOD), a major mitochondrial antioxidant that eliminates superoxide, is inactivated (Parajuli and MacMillan-Crow 2013). Some studies performed on heart transplantation (animal model), have revealed a decrease in the antioxidant protein levels and a sequential loss in MnSOD, catalase and GPx activity in acutely rejecting cardiac allografts (Nilakantan et al. 2005a, b). To the best of our knowledge, there are no similar studies in humans.

Malondialdehyde and Kidney Transplantation

Delayed graft function (DGF) is a clinical diagnosis that describes dysfunction of the kidney allograft immediately after kidney transplantation, usually related to ischemic damage to the graft. The rate of DGF after kidney transplantation varies from 2 % to 50 %, depending on the definition used and the centre's transplant protocols. It is one of the most important risk factors for both acute rejection and impaired renal function at 1 year post-transplant. The has been a large amount of research devoted to the search for DGF predictors or biomarkers that would allow for an early assessment of allograft function and prediction of 1-year prognosis and long-term graft survival (Koo et al. 1998; Yarlagadda et al. 2008, 2009; Ponticelli 2014).

A range of factors could lead to DGF such as organ procurement, donor characteristics, prolonged ischemia time, recipient factors, renal toxicity, and ureteral obstruction, among others (Schroppel and Legendre 2014). Almost 30 % of DGF following kidney transplantation is attributable to ischemia reperfusion injury, which is one of the most important non-specific and non-immunologic factor that affect early and long-term allograft function (Gulec 2011). The production of excessive quantities of ROS is an important mechanism of ischemia-reperfusion injury. As previously discussed, these ROS cause tissue injury through lipid peroxidation and the activation of endothelial cells, resulting in functional and structural cell damage (Li and Jackson 2002; Eltzschig and Collard 2004; Dolegowska et al. 2012). Thus, the importance of oxidative stress in kidney transplantation is highlighted by the observation that increased oxidative stress exists in the presence of ischemia-reperfusion injury and possibly other risk factors for kidney graft dysfunction. Measurement of the circulating levels of oxidative stress markers are useful along the whole spectrum of the graft injury process and may be used for precise evaluation of oxidative stress status of these patients in vivo. Moreover, oxidative markers could be used for screening and risk assessment and may have may have a predictive value for early graft function and long-term graft function.

Because of considerable amount of long-chain PUFAs in the composition of renal lipids, lipid peroxidation is one of the most widespread hypotheses of ROS-mediated

cell injury (Ozbek 2012). Despite the controversy regarding whether lipid peroxidation is the cause or the result of injury, increased lipid peroxidation is observed in ischemia-reperfusion injury. Malondialdehyde is the principal by-product of PUFA peroxidation and can be an indicator of tissue damage, thus it would reflect the ischemia-reperfusion stress of the graft (Del Rio et al. 2005). Moreover, a recent study in animal model revealed that clearance and kidney levels of MDA could predict cyclosporine-induced nephrotoxicity (Sereno et al. 2015). Oxidants and antioxidants can be biomarkers of graft dysfunction with diagnostic accuracy, not only in the early post-transplant period but also in the longer-term. Increased plasma and intragraft levels of MDA and decreased antioxidant activity were found in kidney allografts with chronic tubular atrophy/interstitial fibrosis, which suggest the possibility of early detection even when graft dysfunction is undetectable with serum creatinine (Simic-Ogrizovic et al. 1998; Djamali et al. 2005; Djamali 2007).

Very few studies have investigated MDA levels in kidney transplantation as a marker of lipid peroxidation and as a biomarker of ischemia-reperfusion injury (Pincemail et al. 1993; Davenport et al. 1995; Fonseca et al. 2014). Although these studies have shown elevated levels of MDA in plasma after graft reperfusion, the potential correlations of MDA with subsequent graft function were examined in a recent study (Fonseca et al. 2014). This study was done in 40 kidney recipients from deceased and living donors and the mean levels of MDA were higher in the deceased donor recipients at all measured time points. However, the mean difference was only statistical significant on the second and fourth days (Fig. 4). Tubular and vascular damage in the donor organ during cold ischemia before transplantation is associated with subsequent ischemia-reperfusion injury and DGF. Thus, the kidneys from deceased donors are more susceptible to ischemia and reperfusion damages than from living donor, leading to higher production of ROS and subsequent lipid peroxidation. However, in this study the mean differences in MDA levels between deceased- and living-donors were not statistical significant on most of the days. In addition, cold ischemia time was not correlated with MDA values. More research is needed in this area to clarify these findings.

As the MDA content is an indicator of ischemia-reperfusion damage and as renal ischemia-reperfusion injury occurring after kidney transplantation contributes to kidney dysfunction and it is the most common cause of DGF, it seems rational that MDA levels could be a considered a marker of graft injury, and then a predictive biomarker of DGF. And effectively, the author observed that recipients who developed DGF presented increased MDA levels during the first week after kidney transplantation, which appear to reflect the postischemic tissue damage of DGF kidneys. Compared to pre-transplant, these patients presented higher MDA levels at 8–12-h following kidney transplantation, in contrast to recipients with prompt graft function whose MDA levels continuously decreased throughout the week (Fig. 5). Moreover, MDA on day-1 accurately predicted the need for dialysis within the first week (AUC = 0.90), with a diagnostic performance higher than serum creatinine (AUC = 0.73) and similar to that of cystatin C (AUC = 0.91), which is considered a marker with greater sensitivity for the detection of impaired renal function. In addition, the independent association of high levels of plasma MDA with poorer

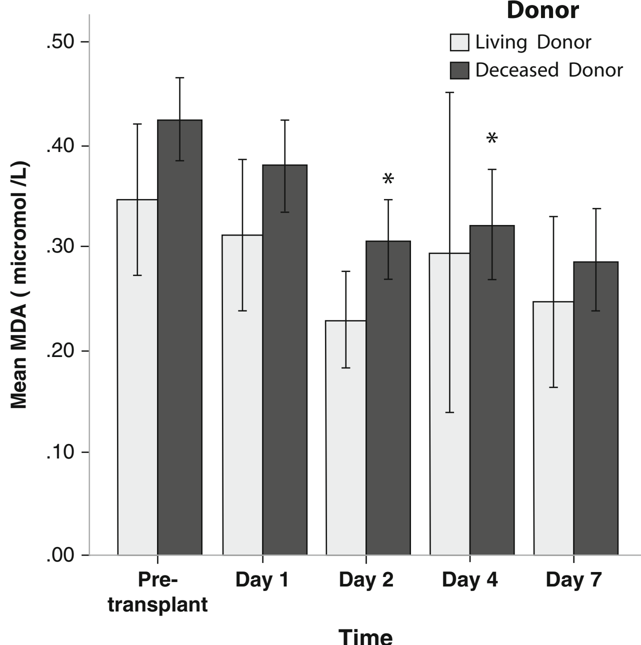

Fig. 4 Longitudinal changes of the pre-transplant and 1 week post-transplant circulating levels of MDA regarding donor status. Evolution of mean values of MDA with 95 % confidence intervals according to donor status; measurements were performed preoperatively (pre-transplant), and then at first (day-1), second (day-2), fourth (day-4) and seventh (day-7) days after kidney transplantation. *Statistical significant ($P < 0.05$)

1-year allograft function was also reported (Fonseca et al. 2014). This suggests that the products of oxygen free radical damage can be measured during kidney transplantation, and that they may have an adverse effect on early and long-term graft function.

A wide range of protective substances, such as antioxidant enzymes, may potentially exert a protective influence by limiting the production of ROS and the damage of oxidative stress following an ischemia-reperfusion injury of the kidney graft. The antioxidant activity was also evaluated in this study. Compared with healthy controls, kidney patients had significantly higher levels of SOD and GR prior to kidney transplantation, which were, most likely, in response to the increased oxidative stress in end-stage renal disease patients. However, no significant changes were observed following kidney transplantation, even when stratified by graft function. Kidney DGF is a manifestation of acute kidney injury; thus, for an existing or acute injury, it is natural for higher quantities of ROS to be released. Under normal conditions, endogenous antioxidant enzymes neutralise these radicals and the enzyme activity subsequently decreases. Another possibility is that the capacity of the antioxidant defence system increases as a response to a

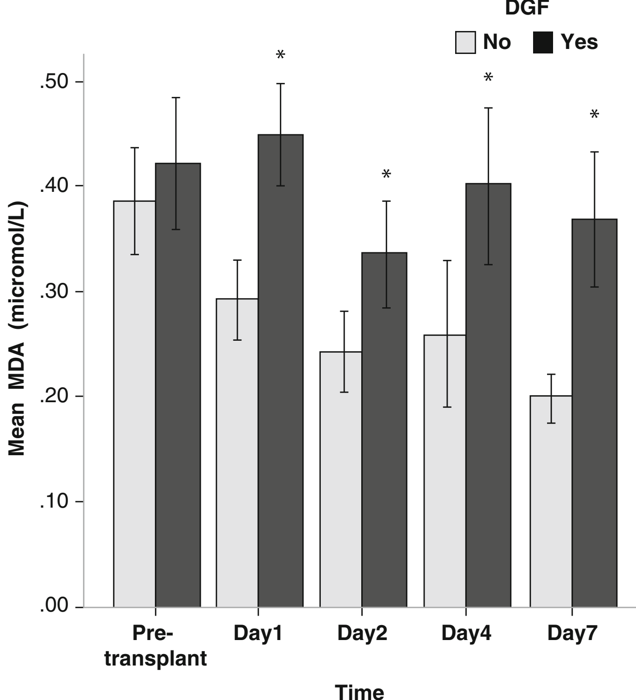

Fig. 5 Longitudinal changes of the pre-transplant and 1 week post-transplant circulating levels of MDA regarding graft function. Evolution of mean values of MDA with 95 % confidence intervals according to the development of delayed graft function (DGF) or not; measurements were performed preoperatively (pre-transplant), and then at first (day-1), second (day-2), fourth (day 4) and seventh (day-7) days after kidney transplantation. *Statistical significant ($P < 0.05$)

higher production of oxidant radicals. Because neither of these two situations occurred, it was hypothesised that in the first week after kidney transplantation, the antioxidant defence system does not effectively respond to the higher levels of oxidative stress detected in DGF.

Oxidative stress is believed to be a common pathway that leads to both immunological and nonimmunological stress in the setting of kidney transplantation and to the development or progression of chronic allograft nephropathy. Our understanding of oxidative stress has significantly advanced in the last decade, but these experimentally derived ideas have yet to be fully integrated into clinical practice. Current hypotheses of renal oxidative stress caused by ischemia-reperfusion injury include the direct impact of free radicals; and the ability of ischemia-reperfusion generate ROS, which are able to start lipid peroxidation either directly or by exhausting antioxidant defence substances. It is also possible that, similar to MnSOD, the major enzymes of the antioxidant system become inactivated during the early phase of ATP depletion in ischemia

(Parajuli and MacMillan-Crow 2013). Maybe, one way of combating this increased oxidative stress and the following damages would be to increase antioxidant defences, namely through the supplementation of non-enzymatic antioxidant molecules.

Potential Applications to Prognosis, Other Diseases or Conditions

Reliable biomarkers enabling early discrimination of DGF in kidney transplantation are lacking, which impairs timely therapeutic interventions. There are many new approaches devoted to the search for new DGF biomarkers that would allow for early determination of allograft dysfunction and of prognoses for 1-year and long-term graft survival. Elevated MDA levels reflected kidney dysfunction and predicted the need for renal replacement therapy within the first week following kidney transplantation with more precisions and efficiency than monitoring serum creatinine, which is a promising finding because it will possibly enable to target any intervention to those patients who will benefit most.

With the understanding of the mechanisms of the pathophysiology of kidney ischemia-reperfusion having evolved, many possible interventions suggest themselves. Oxidative stress is one of the most important components of the ischemia-reperfusion process, which is an inevitable phenomenon in kidney transplantation (Paller 1992; Kim et al. 2009; Hariharan et al. 2011; Dolegowska et al. 2012). Given the important role that ROS play in ischemia-reperfusion injury, it is not surprising that there is growing interest in the possibility that supplementation with nutritional antioxidants will provide renoprotection. In other organs, such as the myocardium and cerebrum, some research using animal models reveals that the free radical-associated ischemia-reperfusion injury can be prevented or reduced by a pre- or post-insult treatment with nutritional antioxidants such as vitamin E, selenium, vitamin C, and beta carotene (Yoshida 1989; Poltronieri et al. 1992; Coombes et al. 2000). Although animal studies suggest that dietary supplementation with antioxidants provides protection against ischemia-reperfusion induced injury, it is unclear if antioxidant supplementation can provide the same protection in humans and specifically in kidney transplantation against ischemia-reperfusion injury. Moreover, the discovery of novel biomarkers that are able to predict the clinical response to antioxidant interventions is a crucial challenge to overcome to allow for the personalization and timely intervention of kidney transplant therapies. Will MDA be able to evaluate the effectiveness of antioxidant therapy? Although researchers already have gained substantial insight into the mechanisms and consequences of ischemia-reperfusion injury and tissue damage induced by oxidative stress, there is also almost a complete absence of studies which address the MDA molecule and kidney transplantation. Thus, additional studies are required to further clarify the ability of MDA to monitor the progression (or regression) of the graft damage after ischemia-reperfusion injury, particularly in patients who experienced DGF.

Summary Points

- The production of excessive quantities of ROS is an important mechanism of reperfusion injury. In kidney transplantation, oxygen free radicals are the most likely agents responsible for initiating the damage associated with reperfusion injury.
- Oxidative stress, and specifically lipid peroxidation, is a vicious cycle: the disease causes increased lipid peroxidation, which is then responsible for toxicity and more cellular and tissue damage
- Lipid peroxidation is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from PUFAs, are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds, which is the most abundant is MDA. Therefore, measurement of MDA is widely used as a biomarker of lipid peroxidation.
- Lipid peroxidation and MDA content are good indicators of ischemia-reperfusion damage.
- Malondialdehyde is a reliable diagnostic biomarker of initial graft injury and accurately detect graft dysfunction earlier than serum creatinine.
- Malondialdehyde is a predictor of dialysis requirement during the first week post-transplantation and of kidney graft function within the first year post-transplantation
- The endogenous antioxidant defence system does not effectively respond to the higher levels of oxidative stress detected in DGF.

References

Agarwal R, Vasavada N, Sachs NG, Chase S. Oxidative stress and renal injury with intravenous iron in patients with chronic kidney disease. Kidney Int. 2004;65(6):2279–89.

Antus B, Harnasi G, Drozdovszky O, Barta I. Monitoring oxidative stress during chronic obstructive pulmonary disease exacerbations using malondialdehyde. Respirology. 2014;19(1):74–9.

Ardalan MR, Estakhri R, Hajipour B, Ansarin K, Asl NA, Nasirizade MR, Azar AN, Ghorbanihaghjou A, Vatankhah AM, Esmaili HA. Erythropoietin ameliorates oxidative stress and tissue injury following renal ischemia/reperfusion in rat kidney and lung. Med Princ Pract. 2013;22(1):70–4.

Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014;2014:360438.

Bandeira SM, Guedes GS, da Fonseca LJ, Pires AS, Gelain DP, Moreira JC, Rabelo LA, Vasconcelos SM, Goulart MO. Characterization of blood oxidative stress in type 2 diabetes mellitus patients: increase in lipid peroxidation and SOD activity. Oxid Med Cell Longev. 2012;2012:819310.

Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev. 2007;87(1):245–313.

Boaz M, Matas Z, Biro A, Katzir Z, Green M, Fainaru M, Smetana S. Serum malondialdehyde and prevalent cardiovascular disease in hemodialysis. Kidney Int. 1999;56(3):1078–83.

Bolli R, Jeroudi MO, Patel BS, DuBose CM, Lai EK, Roberts R, McCay PB. Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. Proc Natl Acad Sci U S A. 1989;86(12):4695–9.

Brezis M, Shanley P, Silva P, Spokes K, Lear S, Epstein FH, Rosen S. Disparate mechanisms for hypoxic cell injury in different nephron segments. Studies in the isolated perfused rat kidney. J Clin Invest. 1985;76(5):1796–806.

Burton GJ, Jauniaux E. Oxidative stress. Best Pract Res Clin Obstet Gynaecol. 2011;25(3):287–99.

Campise M, Bamonti F, Novembrino C, Ippolito S, Tarantino A, Cornelli U, Lonati S, Cesana BM, Ponticelli C. Oxidative stress in kidney transplant patients. Transplantation. 2003;76 (10):1474–8.

Chung SW, Perrella MA. Role of HO-1 in renoprotection: location, location, location. Kidney Int. 2004;65(5):1968–9.

Coombes JS, Powers SK, Demirel HA, Jessup J, Vincent HK, Hamilton KL, Naito H, Shanely RA, Sen CK, Packer L, Ji LL. Effect of combined supplementation with vitamin E and alpha-lipoic acid on myocardial performance during in vivo ischaemia-reperfusion. Acta Physiol Scand. 2000;169(4):261–9.

Dagher PC. Modeling ischemia in vitro: selective depletion of adenine and guanine nucleotide pools. Am J Physiol Cell Physiol. 2000;279(4):C1270–7.

Davenport A, Hopton M, Bolton C. Measurement of malondialdehyde as a marker of oxygen free radical production during renal allograft transplantation and the effect on early graft function. Clin Transplant. 1995;9(3 Pt 1):171–5.

De Vecchi AF, Bamonti F, Novembrino C, Ippolito S, Guerra L, Lonati S, Salini S, Aman CS, Scurati-Manzoni E, Cighetti G. Free and total plasma malondialdehyde in chronic renal insufficiency and in dialysis patients. Nephrol Dial Transplant. 2009;24(8):2524–9.

Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis. 2005;15 (4):316–28.

Deneke SM, Fanburg BL. Regulation of cellular glutathione. Am J Physiol. 1989;257(4 Pt 1): L163–73.

Dib M, Garrel C, Favier A, Robin V, Desnuelle C. Can malondialdehyde be used as a biological marker of progression in neurodegenerative disease? J Neurol. 2002;249(4):367–74.

Djamali A. Oxidative stress as a common pathway to chronic tubulointerstitial injury in kidney allografts. Am J Physiol Renal Physiol. 2007;293(2):F445–55.

Djamali A, Reese S, Yracheta J, Oberley T, Hullett D, Becker B. Epithelial-to-mesenchymal transition and oxidative stress in chronic allograft nephropathy. Am J Transplant. 2005;5 (3):500–9.

Dolegowska B, Blogowski W, Domanski L. Association between the perioperative antioxidative ability of platelets and early post-transplant function of kidney allografts: a pilot study. PLoS One. 2012;7(1):e29779.

Draper HH, McGirr LG, Hadley M. The metabolism of malondialdehyde. Lipids. 1986;21 (4):305–7.

Dut R, Dizdar EA, Birben E, Sackesen C, Soyer OU, Besler T, Kalayci O. Oxidative stress and its determinants in the airways of children with asthma. Allergy. 2008;63(12):1605–9.

Edelstein CL, Ling H, Schrier RW. The nature of renal cell injury. Kidney Int. 1997;51(5):1341–51.

Eltzschig HK, Collard CD. Vascular ischaemia and reperfusion injury. Br Med Bull. 2004;70:71–86.

Fonseca I, Reguengo H, Almeida M, Dias L, Martins LS, Pedroso S, Santos J, Lobato L, Henriques AC, Mendonca D. Oxidative stress in kidney transplantation: malondialdehyde is an early predictive marker of graft dysfunction. Transplantation. 2014;97(10):1058–65.

Girotti AW. Mechanisms of lipid peroxidation. J Free Radic Biol Med. 1985;1(2):87–95.

Gonenc A, Ozkan Y, Torun M, Simsek B. Plasma malondialdehyde (MDA) levels in breast and lung cancer patients. J Clin Pharm Ther. 2001;26(2):141–4.

Granger DN. Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. Am J Physiol. 1988;255(6 Pt 2):H1269–75.

Gulec B. Ischemia reperfusion injury in kidney transplantation. In: Trzcinska DM, editor. Kidney transplantation – new perspectives. InTech; 2011. 356 pp. ISBN 978-953-307-684-3. doi:10.5772/721.

Gutteridge JM. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem. 1995;41(12 Pt 2):1819–28.

Halliwell B, Gutteridge JM. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch Biochem Biophys. 1986;246(2):501–14.

Hariharan N, Zhai P, Sadoshima J. Oxidative stress stimulates autophagic flux during ischemia/reperfusion. Antioxid Redox Signal. 2011;14(11):2179–90.

Janicka M, Kot-Wasik A, Kot J, Namiesnik J. Isoprostanes-biomarkers of lipid peroxidation: their utility in evaluating oxidative stress and analysis. Int J Mol Sci. 2010;11(11):4631–59.

Keith M, Geranmayegan A, Sole MJ, Kurian R, Robinson A, Omran AS, Jeejeebhoy KN. Increased oxidative stress in patients with congestive heart failure. J Am Coll Cardiol. 1998;31(6):1352–6.

Khand FD, Gordge MP, Robertson WG, Noronha-Dutra AA, Hothersall JS. Mitochondrial superoxide production during oxalate-mediated oxidative stress in renal epithelial cells. Free Radic Biol Med. 2002;32(12):1339–50.

Kim YK, Lee SK, Ha MS, Woo JS, Jung JS. Differential role of reactive oxygen species in chemical hypoxia-induced cell injury in opossum kidney cells and rabbit renal cortical slices. Exp Nephrol. 2002;10(4):275–84.

Kim J, Seok YM, Jung KJ, Park KM. Reactive oxygen species/oxidative stress contributes to progression of kidney fibrosis following transient ischemic injury in mice. Am J Physiol Renal Physiol. 2009;297(2):F461–70.

Kolanjiappan K, Manoharan S, Kayalvizhi M. Measurement of erythrocyte lipids, lipid peroxidation, antioxidants and osmotic fragility in cervical cancer patients. Clin Chim Acta. 2002;326 (1–2):143–9.

Koo DD, Welsh KI, Roake JA, Morris PJ, Fuggle SV. Ischemia/reperfusion injury in human kidney transplantation: an immunohistochemical analysis of changes after reperfusion. Am J Pathol. 1998;153(2):557–66.

Kosieradzki M, Rowinski W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. Transplant Proc. 2008;40(10):3279–88.

Lee YJ, Park SH, Jeung TO, Kim KW, Lee JH, Han HJ. Effect of adenosine triphosphate on phosphate uptake in renal proximal tubule cells: involvement of PKC and p38 MAPK. J Cell Physiol. 2005;205(1):68–76.

Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am J Physiol Cell Physiol. 2002;282(2):C227–41.

Locatelli F, Canaud B, Eckardt KU, Stenvinkel P, Wanner C, Zoccali C. Oxidative stress in end-stage renal disease: an emerging threat to patient outcome. Nephrol Dial Transplant. 2003;18(7):1272–80.

Lu SC. Regulation of glutathione synthesis. Mol Aspects Med. 2009;30(1–2):42–59.

Maenpaa CJ, Shames BD, Van Why SK, Johnson CP, Nilakantan V. Oxidant-mediated apoptosis in proximal tubular epithelial cells following ATP depletion and recovery. Free Radic Biol Med. 2008;44(4):518–26.

Massy ZA, Nguyen-Khoa T. Oxidative stress and chronic renal failure: markers and management. J Nephrol. 2002;15(4):336–41.

Moselhy HF, Reid RG, Yousef S, Boyle SP. A specific, accurate, and sensitive measure of total plasma malondialdehyde by HPLC. J Lipid Res. 2013;54(3):852–8.

Nilakantan V, Halligan NL, Nguyen TK, Hilton G, Khanna AK, Roza AM, Johnson CP, Adams MB, Griffith OW, Pieper GM. Post-translational modification of manganese superoxide dismutase in acutely rejecting cardiac transplants: role of inducible nitric oxide synthase. J Heart Lung Transplant. 2005a;24(10):1591–9.

Nilakantan V, Zhou X, Hilton G, Roza AM, Adams MB, Johnson CP, Pieper GM. Hierarchical change in antioxidant enzyme gene expression and activity in acute cardiac rejection: role of inducible nitric oxide synthase. Mol Cell Biochem. 2005b;270(1–2):39–47.
Nilakantan V, Liang H, Maenpaa CJ, Johnson CP. Differential patterns of peroxynitrite mediated apoptosis in proximal tubular epithelial cells following ATP depletion recovery. Apoptosis. 2008;13(5):621–33.
Oberg BP, McMenamin E, Lucas FL, McMonagle E, Morrow J, Ikizler TA, Himmelfarb J. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. Kidney Int. 2004;65(3):1009–16.
Ozbek E. Induction of oxidative stress in kidney. Int J Nephrol. 2012;2012:465897.
Paller MS. Free radical-mediated postischemic injury in renal transplantation. Ren Fail. 1992;14 (3):257–60.
Parajuli N, MacMillan-Crow LA. Role of reduced manganese superoxide dismutase in ischemia-reperfusion injury: a possible trigger for autophagy and mitochondrial biogenesis? Am J Physiol Renal Physiol. 2013;304(3):F257–67.
Pincemail J, Defraigne JO, Franssen C, Bonnet P, Deby-Dupont G, Pirenne J, Deby C, Lamy M, Limet M, Meurisse M. Evidence for free radical formation during human kidney transplantation. Free Radic Biol Med. 1993;15(3):343–8.
Poltronieri R, Cevese A, Sbarbati A. Protective effect of selenium in cardiac ischemia and reperfusion. Cardioscience. 1992;3(3):155–60.
Ponticelli C. Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. Nephrol Dial Transplant. 2014;29(6):1134–40.
Pucheu S, Coudray C, Vanzetto G, Favier A, Machecourt J, de Leiris J. Assessment of radical activity during the acute phase of myocardial infarction following fibrinolysis: utility of assaying plasma malondialdehyde. Free Radic Biol Med. 1995;19(6):873–81.
Rahman K. Studies on free radicals, antioxidants, and co-factors. Clin Interv Aging. 2007;2 (2):219–36.
Raju DS, Lalitha DL, Kiranmayi P. A study of lipid profile and lipid peroxidation in chronic kidney disease with special reference to hemodialysis. J Clin Res Bioeth. 2013;4(1):1–5.
Repetto MG, Semprine J, Boveris A. Lipid peroxidation: chemical mechanism, biological implications and analytical determination. In: Catala A, editor. Lipid peroxidation. InTech; 2012. 546 pp. ISBN 978-953-51-0716-3. doi: 10.5772/2929.
Rodrigo R, Rivera G. Renal damage mediated by oxidative stress: a hypothesis of protective effects of red wine. Free Radic Biol Med. 2002;33(3):409–22.
Romero FJ, Bosch-Morell F, Romero MJ, Jareno EJ, Romero B, Marin N, Roma J. Lipid peroxidation products and antioxidants in human disease. Environ Health Perspect. 1998;106 Suppl 5:1229–34.
Schroppel B, Legendre C. Delayed kidney graft function: from mechanism to translation. Kidney Int. 2014;86(2):251–8.
Sereno J, Vala H, Nunes S, Rocha-Pereira P, Carvalho E, Alves R, Teixeira F, Reis F. Cyclosporine A-induced nephrotoxicity is ameliorated by dose reduction and conversion to sirolimus in the rat. J Physiol Pharmacol. 2015;66(2):285–99.
Shah SV. The role of reactive oxygen metabolites in glomerular disease. Annu Rev Physiol. 1995;57:245–62.
Shanley PF, Brezis M, Spokes K, Silva P, Epstein FH, Rosen S. Hypoxic injury in the proximal tubule of the isolated perfused rat kidney. Kidney Int. 1986;29(5):1021–32.
Siddique YH, Ara G, Afzal M. Estimation of lipid peroxidation induced by hydrogen peroxide in cultured human lymphocytes. Dose Response. 2012;10(1):1–10.
Simic-Ogrizovic S, Simic T, Reljic Z, Markovic S, Blagojevic R, Radivojevic D, Lezaic V, Djukanovic L, Mimic-Oka J. Markers of oxidative stress after renal transplantation. Transpl Int. 1998;11 Suppl 1:S125–9.
Small DM, Coombes JS, Bennett N, Johnson DW, Gobe GC. Oxidative stress, anti-oxidant therapies and chronic kidney disease. Nephrology (Carlton). 2012;17(4):311–21.

Sydykov A. Characterization of the ROS production in ischemia/reperfusion-induced lung injury. PhD Electronic version, Justus Liebig University Giessen. 2009.

Templar J, Kon SP, Milligan TP, Newman DJ, Raftery MJ. Increased plasma malondialdehyde levels in glomerular disease as determined by a fully validated HPLC method. Nephrol Dial Transplant. 1999;14(4):946–51.

Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem. 2004;266(1–2):37–56.

Vostalova J, Galandakova A, Svobodova AR, Orolinova E, Kajabova M, Schneiderka P, Zapletalova J, Strebl P, Zadrazil J. Time-course evaluation of oxidative stress-related biomarkers after renal transplantation. Ren Fail. 2012;34(4):413–9.

Weinberg JM. Glutathione and glycine in acute renal failure. Ren Fail. 1992;14(3):311–9.

Whitin JC, Tham DM, Bhamre S, Ornt DB, Scandling JD, Tune BM, Salvatierra O, Avissar N, Cohen HJ. Plasma glutathione peroxidase and its relationship to renal proximal tubule function. Mol Genet Metab. 1998;65(3):238–45.

Woolfson RG, Millar CG, Neild GH. Ischaemia and reperfusion injury in the kidney: current status and future direction. Nephrol Dial Transplant. 1994;9(11):1529–31.

Yarlagadda SG, Coca SG, Garg AX, Doshi M, Poggio E, Marcus RJ, Parikh CR. Marked variation in the definition and diagnosis of delayed graft function: a systematic review. Nephrol Dial Transplant. 2008;23(9):2995–3003.

Yarlagadda SG, Coca SG, Formica Jr RN, Poggio ED, Parikh CR. Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. Nephrol Dial Transplant. 2009;24(3):1039–47.

Yoshida S. Brain injury after ischemia and trauma. The role of vitamin E. Ann N Y Acad Sci. 1989;570:219–36.

Zachara BA, Wlodarczyk Z, Masztalerz M, Adamowicz A, Gromadzinska J, Wasowicz W. Selenium concentrations and glutathione peroxidase activities in blood of patients before and after allogenic kidney transplantation. Biol Trace Elem Res. 2004;97(1):1–13.

Zahmatkesh M, Kadkhodaee M, Mahdavi-Mazdeh M, Ghaznavi R, Hemati M, Seifi B, Golab F, Hasani K, Lessan-Pezeshki M, Einollahi B. Oxidative stress status in renal transplant recipients. Exp Clin Transplant. 2010;8(1):38–44.

Utility of Neutrophil Gelatinase-Associated Lipocalin in Kidney Transplantation: Detailed Review

38

Juan C. Ramirez-Sandoval, William Herrington, and Luis E. Morales-Buenrostro

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Abstract

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein expressed by kidney tubular cells in response to ischemia but may also be an early indicator of immunological rejection, calcineurin inhibitor toxicity, obstructive nephropathy, subclinical tubulitis, or infection. Although there is currently no evidence to support the routine serial measurement of blood or urinary NGAL to detect subclinical acute tubular injury, NGAL has the potential to provide useful information to those that care for kidney transplant recipients (KTRs). First, high

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urinary or serum NGAL concentrations shortly after transplantation are a predictor of delayed graft function and are associated with reduced graft function at 1 year. Secondly, among KTRs with previously stable graft function who then suffer acute graft dysfunction, a high urinary NGAL predicts graft loss at 1 year. If further refined, diagnostic tests based on NGAL levels may provide future useful clinical tools.

Keywords
NGAL • Biomarkers • Kidney transplantation • Acute kidney injury • Delayed graft function • Immunological rejection • Graft survival • Early diagnosis • Sensitivity and specificity • ROC curve

Abbreviations

AKI	Acute kidney injury
AUC	Area under the curve
DGF	Delayed graft function
eGFR	Estimated glomerular filtration rate
KTR	Kidney transplant recipient
NGAL	Neutrophil gelatinase-associated lipocalin
ROC	Receiver-operating characteristic curve
Se	Sensitivity
sNGAL	Serum neutrophil gelatinase-associated lipocalin
Sp	Specificity
uNGAL	Urinary neutrophil gelatinase-associated lipocalin

Key Facts of Urinary Neutrophil Gelatinase and Graft Loss in Kidney Transplant

- Kidney transplantation replaces normal kidney function and should (in most situations) be regarded as the optimum treatment of end-stage kidney disease.
- Patients who are fit for transplantation survive longer and have a better quality of life.
- According to 2009 and 2005 SRTR National Report and 2002 UNOS report, kidney graft survival for adult recipients after 10 years of transplant is 51 % and 68 % for deceased and living donor, respectively.
- Living donor transplantations had better graft and KTR survival than deceased transplantation, probably explained by less ischemia-reperfusion damage to graft and closer major histocompatibility complex (MHC) matching before surgery.
- Deceased donor transplantation is the most common form of transplant in developed countries, but there is a significant and growing organ shortfall.
- Retrieving of kidneys from deceased donors is exposed to ischemia, between circulatory arrest and the start of cold storage (warm ischemia) and during the time that graft is on cold storage before transplantation (cold ischemia).

- Current means of graft evaluation after transplantation, particularly using creatinine, show multiple flows, especially when the patient is in an unstable state.
- A biomarker identifying the quality of donor kidneys at the time of donor evaluation would therefore be very useful.
- For now, the ideal noninvasive biomarker of graft dysfunction does not exist.

Definitions

Area under receiver operating characteristic curve (AUC/ROC) A curve plots the true positives (sensitivity) vs false positives (1 − specificity) at each "cutoff" value for any diagnostic test that uses a continuous variable. An area under curve of 100 % (1.0) defines a perfect test. An area value of 0.5 indicates no discriminative value.

Calcineurin inhibitors Drugs that suppress the immune system by inhibiting interleukin production in T cells and are associated with acute or chronic graft dysfunction when blood concentrations are in high levels (i.e., tacrolimus or cyclosporine).

Delayed graft function Normally, kidney transplant recipients have progressive diuresis and gradual decrease in serum creatinine levels in the following hours after kidney transplant surgery. If dialysis is required within 7 days after transplantation, it is classified as delayed graft function.

Immunological rejection Adaptive immune response against the graft caused by cellular and/or antibody recognition of proteins perceived as foreign by the recipient.

Estimated glomerular filtration rate Calculation based generally in serum or urinary creatinine that describes the milliliters of blood filtered through kidneys in 1 min. It depends from several factors including age, gender, and race, among others. As a rule, its value in young people must be higher than 90 mL/min/1.73 m^2.

Introduction

In appropriately selected patients with end-stage kidney disease, kidney transplantation offers improved long-term survival when compared to continuing dialysis (Suthanthiran and Strom 1994).

Nevertheless, those caring for kidney transplant recipient (KTRs) still face many challenges. First, even in countries with well-developed kidney services, the number of patients on dialysis eligible for a kidney transplant exceeds supply of donor organs (Pussell et al. 2012). Secondly, although in recent years there have been significant gains in the prevention of immunological graft loss during the first year after transplantation, there has been little to no improvement in long-term graft

survival (Meier-kriesche et al. 2004a). Thirdly, despite apparently increasingly effective treatment of acute rejection, in about two-fifths of cases, graft function does not return to its pre-rejection baseline, which is associated with an incremental increase in death-censored graft loss (Meier-Kriesche et al. 2004b). Lastly, the currently available, inexpensive, noninvasive diagnostic tests (such as measurement of serum creatinine) are poor predictors of early histopathological changes associated with rejection (Yilmaz et al. 2007). Therefore, in addition to increasing the donor pool, novel early diagnostic and prognostic biomarkers are needed to detect those at risk of future graft injury and loss.

Several proteins that are shed by (or are failed to be resorbed by) injured kidney tubule epithelial cells have been described and may have the potential to identify ongoing kidney injury and perhaps even distinguish the etiology of the injury (Tesch 2010).

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, is a 25 kDa single disulfide-bridged polypeptide covalently bound to a gelatinase (Kjeldsen et al. 1993). As has been discussed in other chapter of this book, NGAL has several forms. The dimeric form of NGAL is expressed in neutrophils and functions as part of the innate immune system by sequestering iron siderophores (Flo et al. 2004). These processes reduce iron availability, limiting bacterial proliferation (Goetz et al. 2002; Paragas et al. 2012).

The dimeric form of NGAL is detectable in the urine of those with urinary tract infections (Mårtensson et al. 2012) but is also induced by aseptic injury. It is proposed that, in addition to bacteriostasis, NGAL may, by inhibiting apoptosis and enhancing cellular proliferation/differentiation (Kashiwagi et al. 2014; Schmidt-Ott et al. 2007), also have a reparatory role in injured epithelial tubular cells.

NGAL is secreted in low levels from several cell types and, due to its size and charge, is freely filtered through the glomerulus. Filtered NGAL is then almost completely resorbed by healthy tubular cells. The average urinary NGAL (uNGAL) concentration in healthy adults is also about 20 ng/mL (Kuwabara et al. 2009). Similar average concentrations of NGAL (unadjusted for urine concentration) have been observed in KTRs with stable good graft function. In a sample of seven stable KTR with an estimated glomerular filtration rate of 96 mL/min/1.73 m^2 and a normal graft biopsy, median uNGAL concentration was 5 (interquartile range 1–7) ng/mL or, corrected for grams of urinary creatinine, 0.212 (interquartile range 55–289) ng/mg creatinine. These biomarker values are similar to those obtained from healthy donors (Ramirez-Sandoval et al. 2014).

Lower creatinine excretion in the setting of acute kidney injury may amplify a tubular injury biomarker signal, thereby increasing its clinical utility. However, one report demonstrated marked variability in creatinine excretion across and within individuals in the setting of AKI or kidney transplantation (Lebkowska et al. 2009).

Response to acute kidney injury in non-KTRs is an early rise in uNGAL and serum NGAL (sNGAL) concentration, especially in its monomeric form (Mårtensson et al. 2012). Some of the increased uNGAL represents impaired proximal tubular resorption, but the main source of the raised sNGAL and uNGAL is secretion from the distal nephron (Singer et al. 2013). This rapid change in NGAL concentration is

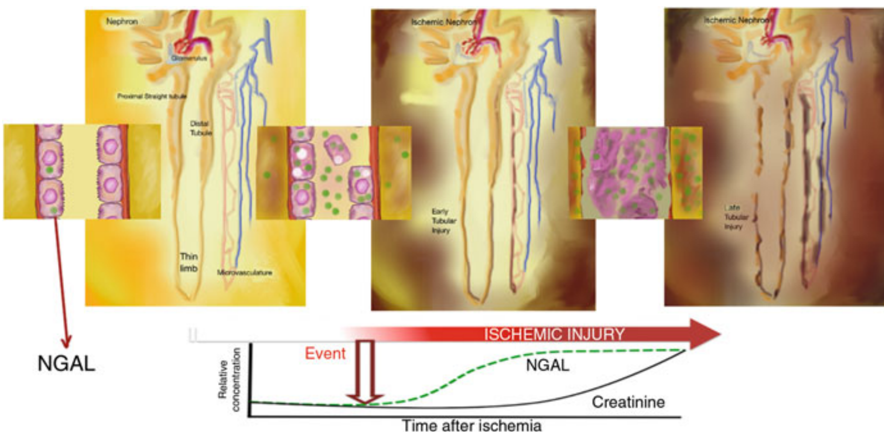

Fig. 1 NGAL is released from ischemic kidney tubules and may serve as an early diagnostic biomarker better than serum creatinine. Raised urinary or serum NGAL levels are useful for early diagnosis of kidney injury

detectable far in advance of changes in serum creatinine (Fig. 1) (Devarajan 2010; Barrera-Chimal and Bobadilla 2012).

Serum creatinine is also an imperfect prognostic biomarker of long-term graft survival in KTRs, as although a raised creatinine at 1 year (or decline in creatinine between 6 months and 1 year from transplantation) predicts those that are likely to progress to graft loss (i.e., the test is highly sensitive), a large proportion of those who go on to have graft failure have a normal creatinine at 1 year (i.e., the test has poor specificity) (Kaplan et al. 2003).

Although the gold standard assay to certify NGAL concentration is immunoblot based (which is a time-consuming and impractical assay for routine clinical use), an ELISA, a chemiluminescent microparticle immunoassay (ARCHITECT®, Abbott), and a point-of-care fluorescence-based immunoassay (Triage®, Biosite) are all available and suited for clinical use (with acceptable correlation with ELISA) (Hollmen et al. 2014). In this chapter, the potential for NGAL as a biomarker for clinical use in KTR is considered.

Utility of NGAL Assessment During the Early Posttransplantation Period

Delayed graft function (DGF) is commonly defined as the necessity for dialysis within the first week after kidney transplantation (Mallon et al. 2013). DGF occurs less frequently in grafts from living donor (4–10 %) compared with deceased donor (5–50 %) (Irish et al. 2003; Troppmann et al. 1995; Sharif and Borrows 2013). This complication is associated with an increased risk of late graft loss, acute

immunological rejection, and KTR death even after recovery of delayed graft function (Yarlagadda et al. 2009; Tapiawala et al. 2010).

The principal causes of delayed graft function are derived from donor ischemic injury prior to retrieval or during preservation and recipient-derived ischemia-reperfusion injury after implantation (Siedlecki et al. 2011).

In kidney biopsies taken approximately 1 h after reperfusion, NGAL expression is observed in proximal and distal tubular epithelial cells. Intensity of NGAL staining is greatest in deceased donor kidneys and is strongly correlated with cold ischemic time ($R = 0.86$) and peak postoperative creatinine (Mishra et al. 2006). Measurement of uNGAL and/or sNGAL in the immediate posttransplant period would be expected to predict DGF.

Early Diagnosis of Delayed Graft Function (Fig. 2)

Indeed, a systematic review of 1,079 KTR from 14 studies has demonstrated that a uNGAL or sNGAL measurement 6–12 h after transplantation is highly predictive of DGF. Aggregation of results suggested that a "raised" NGAL concentration was both sensitive (82 %) and specific (82 %) for predicting DGF (aggregated area under receiver operating characteristic curve [AUC/ROC] was 0.87) (Haase et al. 2009).

Ten of the twelve studies that have investigated the role of uNGAL posttransplantation have found that it predicts DGF (these studies are summarized in Table 1). However, although in theory urinary NGAL might be expected to be more specific for kidney injury than sNGAL (as NGAL can be secreted into circulation from other organs), this hypothesis is not borne out in practice due to the high frequency of oliguria and anuria postoperatively (between 12 % and 38 % of KTRs of deceased donors are anuric in the postoperative period) (Pajek et al. 2014; Fonseca et al. 2013). Furthermore, differences in glomerular filtration of sNGAL derived from extrarenal tissues and urine from residual functioning native kidneys

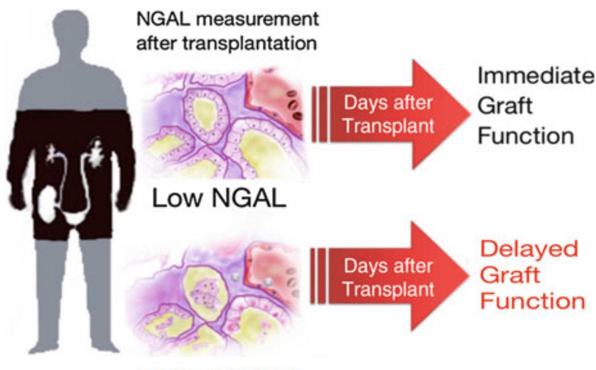

Fig. 2 NGAL is a short-term prognosis biomarker in kidney transplantation. The serum or urinary NGAL levels 6–12 h after transplantation predict delayed graft function

Table 1 Predictive capacity for delayed graft function of urinary NGAL

First author	Year	Number of DGF[a] or SGF[b]/total KTR	Hours after transplantation	Test NGAL	AUC/ROC	Optimal threshold value (sensitivity, specificity) and comments
Hollmen	2011a, b	66/176	Before surgery	Chemiluminescent microparticle immunoassay (ARCHITECT, Abbott Diagnostics)	0.595	4 ng/mL (Se 77 %, Sp 28 %)
						20 ng/mL (Se 35 %, Sp 79 %)
Hall	2011	34[a]/91	6 h	ELISA (Antibody Shop, Gentofte, Denmark)	0.81	350 ng/mL (Se 77 %, Sp 74 %)
			24 h		0.82	
Hollmen	2011a, b	66[a]/176	24 h	Chemiluminescent microparticle immunoassay (ARCHITECT, Abbott Diagnostics)	0.75	560 ng/mL (Se 68 %, Sp 73 %)
Salamzadeh	2012	11/68	24 h	ELISA kits were utilized for measuring urine NGAL (Antibody Shop, Gentofte, Denmark)	Not reported	3.77 ng/mL in group with DGF vs 1.82 ng/mL
Rostami	2013	31[c]/64	2 h	ELISA test kit (Antibody Shop, Gentofte, Denmark)	0.71	204 ng/mL (Se 72 %, Sp 67 %)
			24 h		0.68	77 ng/mL (Se 72 %, Sp 64 %)
Fonseca	2013	18/40	8–12 h after transplant	Chemiluminescent microparticle immunoassay (ARCHITECT, Abbott Diagnostics)	0.88	286 ng/mL (Se 100 %, Sp 76 %)
			48 h after transplant		0.96	
Choi	2013	14[b]/69	48 h	ELISA (BioPorto Diagnostics, Gentofte, Denmark)	0.778	uNGAL 254.7 ng/mg UCr (Se 85.7 %, Sp 66.7 %)
Field	2014a, b	49[a]/182	Before surgery	ELISA using Luminex (R&D systems, Minneapolis, MN, USA)	No differences	uNGAL was similar in donors giving immediate or aberrant functioning kidneys (92.17 ng/mL versus 92.63 ng/mL, respectively)

(*continued*)

Table 1 (continued)

First author	Year	Number of DGF[a] or SGF[b]/ total KTR	Hours after transplantation	Test NGAL	AUC/ ROC	Optimal threshold value (sensitivity, specificity) and comments
Qurashi	2014	11/67	6 h	Chemiluminescent microparticle immunoassay (ARCHITECT, Abbott Diagnostics)	–	DGF had 950 ± 750 ng/mL vs 375 ± 387 ng/mL in group without DGF
Buemi	2014	20[a]/97	6 h	Automated two-site sandwich immunoassay (Abbott Laboratories, Abbott Park, IL) and fluorescence immunoassay Triage NGAL test (Biosite–Inverness Medical, Waltham, MA)	Not reported	uNGAL no differences compared in KTR with DGF versus KTR without DGF
			24 h			
			48 h			
Pajek	2014	40[b]/71	24 h	ELISA (BioPorto Diagnostics, Gentofte, Denmark)	0.82	33.1 μg/mmol UCr (Se 68 %, Sp 93 %)
Pianta	2015	23/81	4 h	ELISA (R&D DuoSets, R&D systems, Minneapolis, MN)	0.77	

KTR kidney transplant recipients, *DGF* delayed graft function, *AUC/ROC* area under receiver operating characteristic curve, *Se* sensitivity, *Sp* specificity, *UCr* urinary creatinine

[a]DGF was defined as the need for at least one dialysis course in the first 7 days following transplantation

[b]SGF was defined as a serum creatinine (Scr) reduction ratio (difference between Scr at 0 h and the Scr on day 7 divided by Scr at 0 h) less than 0.75

[c]Includes DGF and defined as Cr level more than 1.5 mg/dL on the second postoperative day

may modify the uNGAL signal from graft tubules. As an example of relationship between uNGAL and sNGAL, Buemi et al. has demonstrated that uNGAL concentrations may be half that of sNGAL at 6 h posttransplant. Median values of sNGAL 6 h after transplant were 620 ng/mL and 538 ng/mL in KTR who received living and deceased donor kidneys, respectively, while the corresponding uNGAL measurements were 92.5 ng/mL and 212.3 ng/mL, respectively (Buemi et al. 2014).

Moreover, evidence to date suggests that uNGAL does not improve the prediction of DGF when urine output and urinary creatinine excretion are already known. Pajek et al. found that a urinary creatinine excretion rate of less than 0.56 mmol/h 10 h posttransplant predicted DGF and slow graft function with 94 % sensitivity and 84 % specificity (AUC/ROC of 0.90, 95 % confidence interval [CI] 0.80–0.96), while a uNGAL-to-creatinine ratio >33.1 mcg/mmol had 68 % sensitivity and 93 % specificity and similar AUC/ROC of 0.82, 95 % CI 0.70–0.91 (Pajek et al. 2014).

Nine of the ten studies have found that sNGAL predicts DGF (Table 2). sNGAL measurement is easier than uNGAL and negatively correlates with urine output. Hollmen et al. reported that the mean posttransplant sNGAL was 822 ng/mL if the patient is anuric, 697 ng/ml if the diuresis was between 100 and 1,000 ml in 24 h, and 561 ng/ml if the diuresis was more than 1,000 ml in 24 h; a day 1 post-op sNGAL concentration of over 426 mg/mL is a predictor of DGF with sensitivity of over 90 % and specificity of 83 % (AUC/ROC 0.91, 95 % CI 0.86–0.96). sNGAL was also an independent predictor of DGF even when other factors had been taken account of (including urine output and donor characteristics) (Hollmen et al. 2014).

There are several difficulties with interpreting the studies of NGAL: notable limitations include variability in the performance of NGAL assays, heterogeneity in DGF definition, heterogeneous kidney transplant patient populations, and the lack of uniformly applicable cutoff values (see Tables 1 and 2). Nevertheless, the current data suggest that sNGAL may offer additional diagnostic information with regard to which kidney transplants are likely to develop delayed graft function.

Prognosis of Graft Loss Months or Years After Transplantation (Fig. 3)

It has also been suggested that sNGAL concentration measured at 24 and 48 h after transplantation predicts long-term prognosis after transplantation. Four studies have tested this hypothesis (Table 3).

Interestingly, after excluding those KTR with DGF, uNGAL predicts different long-term outcomes in patients with early normal graft function. Fonseca et al. found that uNGAL measured on the fourth and seventh days postsurgery was independently associated with 1-year graft function, independent of donor characteristics, acute rejection episodes, and rehospitalizations (Fonseca et al. 2013). Similarly, Choi et al. found that a uNGAL-to-creatinine ratio cutoff value of ≥153 ng/mgCr had a sensitivity and specificity of 95 % and 65 %, respectively (AUC/ROC 0.83), for

Table 2 Predictive capacity for delayed graft function of serum/plasma NGAL

Author	Year	Number of DGF[a] or SGF***/total KTR	Hours after transplantation	Test NGAL	ROC/AUC	Optimal threshold value (sensitivity, specificity)
Parikh (include adults and children)	2006	10/60	24 h	ELISA (HYB211–01B, Antibody Shop)	0.90	450 ng/mg UCr (Se 90 %, Sp 48 %); 1,000 ng/mg UCr (Se 90 % Sp 83 %)
Kusaka	2008	7/67	24	Chemiluminescent microparticle immunoassay (ARCHITECT, Abbott Diagnostics)	0.99	500 ng/mL (Se 91 %, Sp 97 %)
Lebkowska	2009	4/41	24 h	ELISA (Antibody Shop, Gentofte, Denmark)	Not reported	Four KTR with DGF did not show a fall in sNGAL
Hall	2011	26/78	0, 24, 48	ELISA (Antibody Shop)	No differences	sNGAL was not different between KTR with DGF and others
Bataille	2011	15/41	24	Triage meters (NGAL test Triage; Biosite Inc., Inverness Medical, San Diego, CA)	0.97	400 ng/mL (Se 93.3 %, Sp 88.5 %)
Lee	2012	14/59	24 h	ELISA (BioPorto Diagnostics, Gentofte, Denmark)	0.86	411.4 ng/mL (Se 57 %, Sp 98 %)
Rahimzadeh (include only children <18 years)	2012	2[b]/27	24 h	ELISA (?)	0.95	174 ng/mL (Se 100 %, Sp 95.5 %)

Buemi	2014	20/97	6 h	Automated two-site sandwich immunoassay (Abbott Laboratories, Abbott Park, IL) and fluorescence immunoassay Triage NGAL test (Biosite–Inverness Medical, Waltham, MA)	0.73	
			24 h		0.80	
			48 h		0.85	
Hollmen	2014	66/176	24 h	ELISA kit (BioPorto Diagnostics, Gentofte, Denmark) and point-of-care (POC) fluorescence immunoassay NGAL kit and device (Triage Biosite, San Diego, California, USA)	0.85	423 ng/mL (Se 87 %, Sp 77 %)
Pajek	2014	40[b]/71	24 h	ELISA (BioPorto Diagnostics, Gentofte, Denmark)	0.82	33.1 μg/mmol UCr (Se 68 %, Sp 93 %)

KTR kidney transplant recipients, *DGF* delayed graft function (the need for hemodialysis procedure in the first week), *SGF* slow graft function (less than 70 % reduction of serum creatinine in the first week), *AUC/ROC* area under receiver operating characteristic curve, *Se* sensitivity, *Sp* specificity, *UCr* urinary creatinine

[a]DGF was defined as the need for at least one dialysis course in the first 7 days following transplantation

[b]DGF or SGF

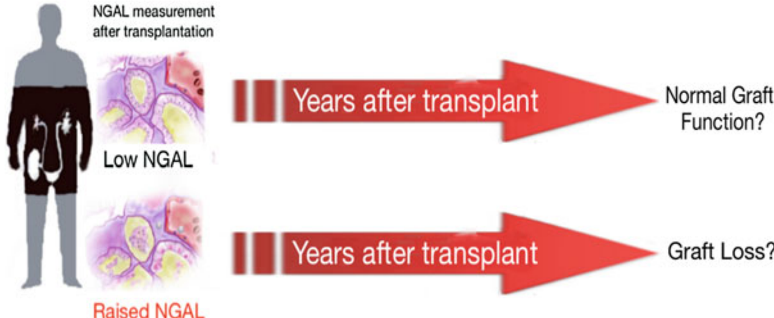

Fig. 3 NGAL might be a long-term prognosis biomarker in kidney transplantation. In some studies, the serum or urinary NGAL levels assessed the first week after transplantation predict long-term graft function or graft loss at 1 year

Table 3 Predictive capacity for long-term graft prognosis of urinary NGAL assessed early after transplantation

Author	Year	Outcome	Assessment of NGAL	Comments
Hall	2011	eGFR at 3 months after transplantation	At time 0, 1st day and 2nd day after transplant	No association
Hollmen	2011a, b	Graft survival after 1 year between donor with serum NGAL $\geq$214 ng/mL vs donors with NGAL $<$214 ng/mL	Before donor operation	No differences between groups
Choi	2013	eGFR $<$60 mL/min/1.73 m^2 at 1 year after transplantation	2nd day after transplant	AUC/ROC of 0.832
				Cutoff value 152.9 ng/mL (Se 95 %, Sp 65 %)
Fonseca	2013	Multivariate analysis for prediction of graft function	4th day after transplant	Regression coefficient adjusted Ln uNGAL at 4th day = 0.067
			7th day after transplant	Regression coefficient adjusted Ln uNGAL at 7th day = 0.0138
				Urinary NGAL was associated with 1-year serum creatinine
Yang	2014	eGFR $<$60 mL/min/1.73 m^2 at 2 years after transplantation	At time 0, 2nd day and 6th day	No association

eGFR estimated glomerular filtration rate, *AUC/ROC* area under receiver operating characteristic curve

predicting "poor 1-year outcome" (defined as a eGFR <60 mL/min/1.73 m^2) in 62 KTR (Choi et al. 2013).

Interestingly, raised sNGAL measured 2 days after transplant surgery was not associated graft function 3 months after transplant (Hall et al. 2010, 2011), raising the hypothesis that uNGAL is a more useful prognostic for subclinical tubular ischemic injury postsurgery as significant extrarenal NGAL secretion in the recovery phase from surgery may mask any increased kidney secretion of NGAL into circulating blood.

Utility of NGAL Assessment After AKI in Late Posttransplantation Period (Fig. 4)

KTR have an increased risk of severe AKI which carries with it high risk of graft failure (Nakamura et al. 2012). Paradoxically, AKI might be more frequent in KTRs with higher eGFR (Mehrotra et al. 2012). uNGAL concentrations during AKI are an independent predictor of graft loss. In a study of 67 KTRs with AKI occurring on average nearly 3 years since successful transplantation, uNGAL predicted graft loss with sensitivity and specificity of 84 % and 91 %, respectively (AUC/ROC 0.89, 95 % CI 0.81–0.97), using a cutoff value of 210 ng/mL (Fig. 6) (Ramirez-Sandoval et al. 2014). Similar urinary NGAL cutoffs predict the development of AKI or need for renal replacement therapy initiation/death in non-KTR populations at high risk of AKI (Haase et al. 2009).

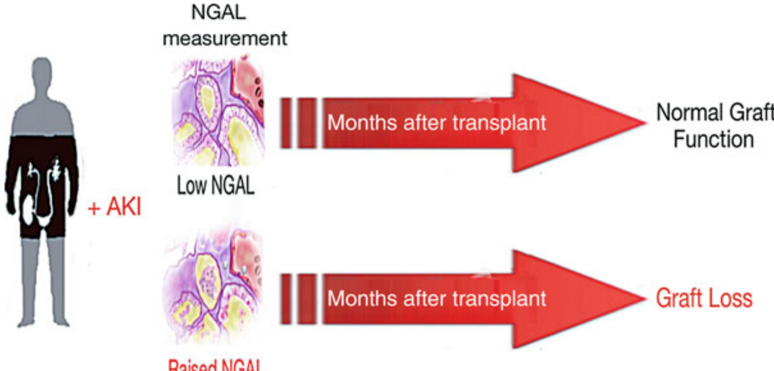

Fig. 4 NGAL might be a long-term prognosis biomarker after acute graft injury. In kidney transplant recipients with stable graft function, NGAL assessed during an episode of acute kidney injury is useful to predict graft loss

Utility of NGAL Assessment During KTR Surveillance

The available evidence suggests that there is no utility in routinely measuring NGAL during routine KTR surveillance. Kaufeld et al. compared protocol transplant biopsies with uNGAL results over 6 months in 140 KTR. Median urinary NGAL-to-creatinine ratios did not discriminate KTRs with the presence or absence of acute tubular injury on biopsy. NGAL-to-creatinine ratios were also not useful at discriminating those with serial evidence of acute tubular injury (Kaufeld et al. 2012).

However, there is some evidence among high-risk KTRs that NGAL surveillance may have a use. Field et al. studied sNGAL in 94 highly sensitized patients following HLA-incompatible kidney transplantation, among who 44 experienced a rejection episode within 30 days. A sNGAL concentration $\geq$6,865 ng/mL at day 1 postsurgery had a sensitivity of 73 % and a specificity of 60 % for predicting immunological rejection (AUC 0.67) (Field et al. 2014a).

Utility of NGAL in Differentiating Etiology of Graft Failure (Fig. 5)

uNGAL's potential to distinguish rejection from other causes of AKI in KTRs was hypothesized in data from 44 KTR with AKI. Compared to 138 stable allografts, uNGAL was significantly higher in the 35 individuals with non-rejection AKI (median uNGAL concentration 8 [IQR 4–17] ng/mL compared to 59 [IQR 33–136] ng/mL). Moreover, compared to non-rejection AKI, nine KTRs with

Fig. 5 NGAL and long-term graft loss after acute kidney injury. This survival graft curve shows that kidney transplant recipients with acute graft dysfunction and urinary NGAL higher than 163 ng/mL at diagnosis have a worst prognosis of graft survival compared with lower levels

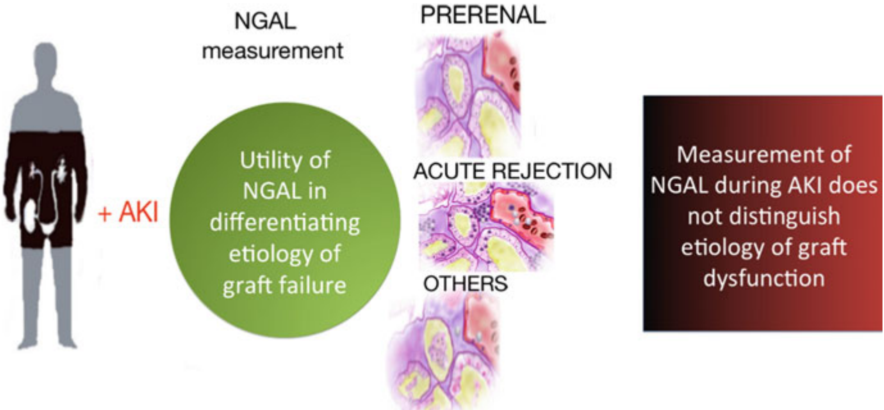

Fig. 6 Utility of NGAL for differential diagnosis of acute kidney injury etiology in kidney transplant recipients. At this time, NGAL seems not to be a useful biomarker for etiology diagnosis in acute graft injury, especially to identifying immunological rejection

biopsy-proven acute rejection had substantially elevated uNGAL (median 339 [165–499] ng/mL). A cutoff of 100 ng/mL represented 100 % sensitivity and 93 % sensitivity (AUC of ROC 0.98) (Heyne et al. 2012). However, these promising results have not been replicated in other studies. Ramirez-Sandoval et al. did not find utility in uNGAL when 20 KTR with immunological rejection were compared with 47 non-immunological causes of AKI. uNGAL-to-creatinine ratio with a cutoff point of 59 ng/mg had 60 % sensitivity and 58 % specificity at differentiating rejection from other causes of AKI (AUC/ROC 0.75, 95 % CI 0.61–0.88) (Ramirez-Sandoval et al. 2014). Furthermore in a study of KTRs by Zhang et al., no significant difference was observed between uNGAL measurements in 41 KTR with biopsy-confirmed acute rejection, 29 with biopsy-proven acute tubular necrosis, and 15 stable allograft controls (Zhang et al. 2013) (Fig. 6).

Table 4 summarizes other studies that have assessed NGAL as a biomarker to differentiate calcineurin inhibitor toxicity, obstructive nephropathy, or subclinical tubulitis. Overall, the current evidence suggests that measuring NGAL, at least in isolation, does not help distinguish etiology of acute graft dysfunction.

Conclusions

NGAL is a promising biomarker of graft injury and may have clinical utility in predicting early and long-term prognosis after kidney transplantation, for long-term prognosis after an acute kidney injury and, possibly, for early diagnosis of graft dysfunction. Appropriate randomized clinical trials on important outcomes in grafts and recipients comparing the use of NGAL versus the current standard of clinical

Table 4 Different causes of urinary NGAL elevation in graft dysfunction

Cause	Author/year	Example
Reperfusion injury	See Table 1	See Table 1
Calcineurin inhibitors	Wasilewska et al. (2010)	A group of children who receive cyclosporine and uNGAL were determined before and 3, 6, and 12 months after transplantation. uNGAL increase from basal 4.74 to 15.6 ng/mL (uNGAL/uCr 4.2–18.3)
	Tsuchimoto et al. (2014)	Twenty liver transplant recipients developed AKI by tacrolimus ($n = 31$). The AUC/ROC of urinary NGAL was 0.876
Obstructive nephropathy	Lucarelli et al. (2014)	Not study in kidney transplant but evidence in other kidney diseases
IgA nephropathy	Ding et al. (2007)	Not study in kidney transplant but evidence in other kidney diseases
Urinary tract infection	Kaufeld et al. (2012)	Urinary tract infection has a linear correlation with urinary NGAL
BK virus nephropathy	Rau et al. (2013)	One report did not find significant difference in plasma NGAL expression between BKV+ patients and BKV negative patients

AUC/ROC area under receiver operating characteristic curve, *UCr* urinary creatinine

care are required (with economic assessments) in order to promote more widespread use of NGAL in kidney transplantation.

Changes in NGAL concentration however are not specific for a particular mechanism of kidney injury, and therefore, NGAL will not replace the need of graft biopsy in order to distinguish different etiologies of graft dysfunction.

Potential Applications to Prognosis, Other Disease, or Conditions

Evidence-Based Applications of NGAL

- Early diagnosis of delayed graft function
- Prognostic tools to predict graft loss after acute kidney injury

Possible Applications of NGAL

- Screening of patients with increased risk of graft dysfunction in order to early therapeutic interventions.
- Assisting clinical decision-making in the management of transplant-related complications or during the follow-up of kidney transplant recipients.
- The ability of monomeric kidney-derived NGAL to detect specific graft damage needs to be further explored.

Summary Points

- Urine and serum NGAL concentration in kidney transplant recipients with normal graft function appear to be similar to the concentration observed in health individuals.
- Studies of NGAL in kidney transplant recipients have focused on its utility for predicting short- and long-term graft failure.

Utility of NGAL in Transplantation
- Higher sNGAL or uNGAL levels 6–72 h after transplantation predict delayed graft function (i.e., the necessity for dialysis within the first week after kidney transplantation). For example, uNGAL concentrations >150 ng/mL assessed a few hours after transplantation predict reduced graft function at 1 year.
- In kidney transplant recipients with acute graft dysfunction and previously stable graft function, uNGAL concentrations >210 ng/mL predict graft loss.

Nonutility of NGAL in Kidney Transplantation
- Routine serial measurements of NGAL in grafts with stable function do not identify subclinical acute tubular injury.
- Measurement of NGAL during acute kidney injury does not distinguish etiology of the cause of graft dysfunction and may rise because of ischemic injury, calcineurin inhibitors toxicity, obstructive nephropathy, subclinical tubulitis, or infection.

References

Barrera-Chimal J, Bobadilla NA. Are recently reported biomarkers helpful for early and accurate diagnosis of acute kidney injury? Biomarkers. 2012;17:385–93.

Bataille A, Abbas S, Semoun O, et al. Plasma neutrophil gelatinase-associated lipocalin in kidney transplantation and early renal function prediction. Transplantation. 2011;92:1024–30.

Buemi A, Musuamba F, Frederic S, et al. Is plasma and urine neutrophil gelatinase-associated lipocalin (NGAL) determination in donors and recipients predictive of renal function after kidney transplantation? Clin Biochem. 2014;47:68–72.

Choi HM, Park KT, Lee JW, et al. Urine neutrophil gelatinase-associated lipocalin predicts graft outcome up to 1 year after kidney transplantation. Transplant Proc. 2013;45:122–8.

Devarajan P. Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury. Biomark Med. 2010;4:265–80.

Ding H, He Y, Li K, et al. Urinary neutrophil gelatinase-associated lipocalin (NGAL) is an early biomarker for renal tubulointerstitial injury in IgA nephropathy. Clin Immunol. 2007;123:227–34.

Field M, Dronavalli V, Mistry P, et al. Urinary biomarkers of acute kidney injury in deceased organ donors – kidney injury molecule-1 as an adjunct to predicting outcome. Clin Transplant. 2014a;28:808–15.

Field M, Lowe D, Cobbold M, et al. The use of NGAL and IP-10 in the prediction of early acute rejection in highly sensitized patients following HLA-incompatible renal transplantation. Transpl Int. 2014b;27:362–70.

Flo TH, Smith KD, Sato S, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature. 2004;432:917–21.

Fonseca I, Oliveira JC, Almeida M, et al. Neutrophil gelatinase-associated lipocalin in kidney transplantation is an early marker of graft dysfunction and is associated with one-year renal function. J Transplant. 2013;2013:650123.

Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell. 2002;10:1033–43.

Haase M, Bellomo R, Devarajan P, Schlattmann P, Haase-Fielitz A, NGAL Meta-analysis Investigator Group. Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. Am J Kidney Dis. 2009;54:1012–24.

Hall IE, Yarlagadda SG, Coca SG, et al. IL-18 and urinary NGAL predict dialysis and graft recovery after kidney transplantation. J Am Soc Nephrol. 2010;21:189–97.

Hall IE, Doshi MD, Poggio ED, Parikh CR. A comparison of alternative serum biomarkers with creatinine for predicting allograft function after kidney transplantation. Transplantation. 2011;91:48–56.

Heyne N, Kemmner S, Schneider C, Nadalin S, Königsrainer A, Häring HU. Urinary neutrophil gelatinase-associated lipocalin accurately detects acute allograft rejection among other causes of acute kidney injury in renal allograft recipients. Transplantation. 2012;93:1252–7.

Hollmen ME, Kyllönen LE, Inkinen KA, Lalla ML, Salmela KT. Urine neutrophil gelatinase-associated lipocalin is a marker of graft recovery after kidney transplantation. Kidney Int. 2011a;79:89–98.

Hollmen ME, Kyllönen LE, Inkinen KA, Lalla ML, Merenmies J, Salmela KT. Deceased donor neutrophil gelatinase-associated lipocalin and delayed graft function after kidney transplantation: a prospective study. Crit Care. 2011b;15:R121.

Hollmen ME, Kyllönen LE, Merenmies J, Salmela KT. Serum neutrophil gelatinase-associated lipocalin and recovery of kidney graft function after transplantation. BMC Nephrol. 2014;15:123.

Irish WD, McCollum DA, Tesi RJ, Owen AB, Brennan DC, Bailly JE, Schnitzler MA. Nomogram for predicting the likelihood of delayed graft function in adult cadaveric renal transplant recipients. J Am Soc Nephrol. 2003;14:2967–74.

Kaplan B, Schold J, Meier-Kriesche HU. Poor predictive value of serum creatinine for renal allograft loss. Am J Transplant. 2003;3:1560–5.

Kashiwagi E, Tonomura Y, Kondo C, et al. Involvement of neutrophil gelatinase-associated lipocalin and osteopontin in renal tubular regeneration and interstitial fibrosis after cisplatin-induced renal failure. Exp Toxicol Pathol. 2014;66:301–11.

Kaufeld JK, Gwinner W, Scheffner I, Haller HG, Schiffer M. Urinary NGAL ratio is not a sensitive biomarker for monitoring acute tubular injury in kidney transplant patients: NGAL and ATI in renal transplant patients. J Transplant. 2012;2012:563404.

Kjeldsen L, Johnsen AH, Sengeløv H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem. 1993;268:10425–32.

Kusaka M, Kuroyanagi Y, Mori T, et al. Serum neutrophil gelatinase-associated lipocalin as a predictor of organ recovery from delayed graft function after kidney transplantation from donors after cardiac death. Cell Transplant. 2008;17:129–34.

Kuwabara T, Mori K, Mukoyama M, et al. Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. Kidney Int. 2009;75:285–94.

Lebkowska U, Malyszko J, Lebkowska A, et al. Neutrophil gelatinase-associated lipocalin and cystatin C could predict renal outcome in patients undergoing kidney allograft transplantation: a prospective study. Transplant Proc. 2009;41(1):154–7.

Lee EY, Kim MS, Park Y, Kim HS. Serum neutrophil gelatinase-associated lipocalin and interleukin-18 as predictive biomarkers for delayed graft function after kidney transplantation. J Clin Lab Anal. 2012;26:295–301.

Lucarelli G, Mancini V, Galleggiante V, et al. Emerging urinary markers of renal injury in obstructive nephropathy. Biomed Res Int. 2014;2014:303298.

Mallon DH, Summers DM, Bradley JA, Pettigrew GJ. Defining delayed graft function after renal transplantation: simplest is best. Transplantation. 2013;96:885–9.

Mårtensson J, Xu S, Bell M, Martling CR, Venge P. Immunoassays distinguishing between HNL/NGAL released in urine from kidney epithelial cells and neutrophils. Clin Chim Acta. 2012;413:1661–7.

Mehrotra A, Rose C, Pannu N, Gill J, Tonelli M, Gill JS. Incidence and consequences of acute kidney injury in kidney transplant recipients. Am J Kidney Dis. 2012;59:558–65.

Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. Am J Transplant. 2004a;4:378–83.

Meier-Kriesche HU, Schold JD, Kaplan B. Long-term renal allograft survival: have we made significant progress or is it time to rethink our analytic and therapeutic strategies? Am J Transplant. 2004b;4:1289–95.

Mishra J, Ma Q, Kelly C, et al. Kidney NGAL is a novel early marker of acute injury following transplantation. Pediatr Nephrol. 2006;21:856–63.

Nakamura M, Seki G, Iwadoh K, et al. Acute kidney injury as defined by the RIFLE criteria is a risk factor for kidney transplant graft failure. Clin Transplant. 2012;26:520–8.

Pajek J, Škoberne A, Šosterič K, et al. Non-inferiority of creatinine excretion rate to urinary L-FABP and NGAL as predictors of early renal allograft function. BMC Nephrol. 2014;15:117.

Paragas N, Qiu A, Hollmen M, Nickolas TL, Devarajan P, Barasch J. NGAL-Siderocalin in kidney disease. Biochim Biophys Acta. 2012;1823:1451–8.

Parikh CR, Jani A, Mishra J, et al. Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. Am J Transplant. 2006;6:1639–45.

Pianta TJ, Peake PW, Pickering JW, et al. Clusterin in kidney transplantation: novel biomarkers versus serum creatinine for early prediction of delayed graft function. Transplantation. 2015;99:171–9.

Pussell BA, Bendorf A, Kerridge IH. Access to the kidney transplant waiting list: a time for reflection. Intern Med J. 2012;42:360–3.

Qurashi S, Ghamdi G, Jaradat M, et al. Urinary neutrophil gelatinase-associated lipocalin and the occurrence of delayed graft function after kidney transplant. Exp Clin Transplant. 2014;12:396–400.

Rahimzadeh N, Otukesh H, Hoseini R, et al. Are serum and urine neutrophil gelatinase-associated lipocalin predictive of renal graft function in short term? Pediatr Transplant. 2012;16:796–802.

Ramirez-Sandoval JC, Barrera-Chimal J, Simancas PE, Correa-Rotter R, Bobadilla NA, Morales-Buenrostro LE. Tubular urinary biomarkers do not identify aetiology of acute kidney injury in kidney transplant recipients. Nephrology (Carlton). 2014a;19:352–8.

Ramirez-Sandoval JC, Barrera-Chimal J, Simancas PE, et al. Urinary neutrophil gelatinase-associated lipocalin predicts graft loss after acute kidney injury in kidney transplant. Biomarkers. 2014b;19:63–9.

Rau S, Schönermarck U, Jäger G, et al. BK virus-associated nephropathy: neutrophil gelatinase-associated lipocalin as a new diagnostic tool? Clin Transplant. 2013;27:E184–91.

Rostami Z, Nikpoor M, Einollahi B. Urinary neutrophil gelatinase associated lipocalin (NGAL) for early diagnosis of acute kidney injury in renal transplant recipients. Nephro Urol Mon. 2013;5:745–52.

Salamzadeh J, Sahraee Z, Nafar M, Parvin M. Delayed graft function (DGF) after living donor kidney transplantation: a study of possible explanatory factors. Ann Transplant. 2012;17:69–76.

Schmidt-Ott KM, Mori K, Li JY, et al. Dual action of neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol. 2007;18:407–13.
Sharif A, Borrows R. Delayed graft function after kidney transplantation: the clinical perspective. Am J Kidney Dis. 2013;62:150–8.
Siedlecki A, Irish W, Brennan DC. Delayed graft function in the kidney transplant. Am J Transplant. 2011;11:2279–96.
Singer E, Markó L, Paragas N, et al. Neutrophil gelatinase-associated lipocalin: pathophysiology and clinical applications. Acta Physiol (Oxf). 2013;207:663–72.
Suthanthiran M, Strom TB. Renal transplantation. N Engl J Med. 1994;331:365–76.
Tapiawala SN, Tinckam KJ, Cardella CJ, et al. Delayed graft function and the risk for death with a functioning graft. J Am Soc Nephrol. 2010;21:153–61.
Tesch GH. Review: serum and urine biomarkers of kidney disease: a pathophysiological perspective. Nephrology (Carlton). 2010;15:609–16.
Troppmann C, Gillingham KJ, Benedetti E, et al. Delayed graft function, acute rejection, and outcome after cadaver renal transplantation. The multivariate analysis. Transplantation. 1995;59:962–8.
Tsuchimoto A, Shinke H, Uesugi M, et al. Urinary neutrophil gelatinase-associated lipocalin: a useful biomarker for tacrolimus-induced acute kidney injury in liver transplant patients. PLoS One. 2014;9:e110527.
Wasilewska A, Zoch-Zwierz W, Taranta-Janusz K, Michaluk-Skutnik J. Neutrophil gelatinase-associated lipocalin (NGAL): a new marker of cyclosporine nephrotoxicity? Pediatr Nephrol. 2010;25:889–97.
Yang J, Choi HM, Seo MY, et al. Urine liver-type fatty acid-binding protein predicts graft outcome up to 2 years after kidney transplantation. Transplant Proc. 2014;46:376–80.
Yarlagadda SG, Coca SG, Formica Jr RN, Poggio ED, Parikh CR. Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. Nephrol Dial Transplant. 2009;24:1039–47.
Yilmaz S, Isik I, Afrouzian M, et al. Evaluating the accuracy of functional biomarkers for detecting histological changes in chronic allograft nephropathy. Transpl Int. 2007;20(7):608–15.
Zhang R, Li M, Chouhan KK, Simon EE, Hamm LL, Batuman V. Urine free light chains as a novel biomarker of acute kidney allograft injury. Clin Transplant. 2013;27:953–60.

miR-210 as a Biomarker in Renal Carcinoma 39

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Abstract

Renal cell carcinoma (RCC) is the most common urological neoplasm of the kidney in adults. The worldwide incidence and mortality rate is in the order of 270,000 cases and 120,000 deaths, respectively. Despite the fact that the diagnostic modalities and therapeutic techniques for RCC continue to improve, the overall incidence and mortality of RCC has increased in the last 20 years. The 5-year survival rate is approximately 98 % for stage I disease and approximately 50 % for stage III or higher disease. These data underscore the importance of early detection and adequate prediction of prognosis of RCC.

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However, both early detection and adequate prediction are often difficult in the clinical setting. Renal tumor in the early stage is often asymptomatic and non-palpable, and it is impossible to correctly distinguish between RCC and BRT, even with the full use of diagnostic modalities such as imaging examinations, and determination of the adequate frequency of radiological imaging examinations as a screen for tumor recurrence is confusing. These problems are caused by the lack of an accurate biomarker for diagnosis and prognosis in RCC.

Recent studies suggest that tissue microRNAs (miRNAs), which are non-protein-coding small RNAs, are involved in carcinogenesis and cancer progression. Further studies suggest that microRNAs are highly stable and abundant in the serum, urine, and other body fluids. Therefore, both tissue miRNAs and circulating miRNAs might be good biomarkers for diagnostic and prognostic considerations in a variety of cancers.

Of all the miRNAs, miR-210, a well-known hypoxia-inducible miRNA, is one of the best studied miRNA in various cancers. Some research groups have reported that miR-210 was induced under hypoxic conditions via hypoxia-inducible factors (HIFs) in various cancer cell lines. In the case of RCC, it is well known that HIF1α and HIF2α accumulate in clear cell carcinoma (ccRCC), which is the largest subtype of RCC, as a result of the deficiency of the von Hippel-Lindau (VHL) tumor suppressor gene. Therefore, a number of research groups have attempted to determine the relationship between miR-210 expression and the VHL-HIF pathway and to use miR-210 as a new biomarker in ccRCC.

Regarding the possibility of using miR-210 as a diagnostic biomarker in RCC, two studies have reported that serum miR-210 expression was significantly higher in ccRCC patients than in healthy controls. These studies found no significant correlation between serum miR-210 levels and clinicopathological parameters of ccRCC patients. However, there has been no report regarding the utility of urine miR-210 as a diagnostic biomarker in RCC, and there has been no study of the difference between miR-210 expression levels of RCC and benign renal tumor.

Regarding the use of miR-210 as a prognostic biomarker in RCC, some studies have demonstrated that high miR-210 expression in ccRCC tissue was statistically related to poor prognosis. On the other hand, several other studies have demonstrated precisely the opposite results regarding miR-210 levels and prognosis. Thus, there is no consensus regarding the utility of miR-210 as a prognostic biomarker. Further studies with a larger number of patients are warranted to validate these results.

Keywords

Renal cell carcinoma • MicroRNA-210 (miR-210) • Circulating miRNA in body fluids • Hypoxia-inducible factors (HIFs) • Von Hippel-Lindau tumor suppressor gene (VHL tumor suppressor gene) • Diagnostic biomarker • Prognostic biomarker

Abbreviations

BRT	Benign renal tumor
CAIX	Carbonic anhydrase IX

ccRCC	Clear cell carcinoma
chRCC	Chromophobe RCC
DFS	Disease-free survival
HCs	Healthy controls
HIFs	Hypoxia-inducible factors
ISCU1/2	Iron-sulfur cluster assembly protein
MIBC	Muscle-invasive bladder cancer
miRNAs	MicroRNAs
NMIBC	Non-muscle-invasive bladder cancer
OS	Overall survival
pRCC	Papillary RCC
RCC	Renal cell carcinoma
VHL	Von Hippel-Lindau

Key Facts of Diagnosis and Management of Renal Cell Carcinoma (RCC)

- Diagnosis of RCC is generally performed based on imaging examination, including abdominal ultrasonography, computed tomography, magnetic resonance imaging, and so on.
- Since histopathological diagnosis represented by tissue biopsy is not basically performed to avoid dissemination of cancer cell, it is impossible to distinguish between RCC and BRT correctly.
- Most patients with renal tumor have been applied to surgical treatment represented by nephrectomy and partial nephrectomy.
- It is known that approximately 20 % of small renal tumors less than 4 cm are BRT.
- Surgical treatment is associated with a risk of deterioration in renal function, even if partial nephrectomy is performed to spare normal renal parenchyma.
- It is known that RCC is basically characterized by low response rate to chemotherapy, radiation therapy, and so on.
- In recent years, although efficacy of targeted therapy for progressive RCC such as metastatic RCC has been reported, the response rate is still definitive.
- To this day, progressive RCC indicates a poor prognosis.
- At this time, there are no accurate biomarkers for diagnosis and prognosis in RCC.

Definitions

TNM stage The TNM staging is one of the most popular systems used in cancer staging. The TNM system is based on the size and/or extensity of the primary tumor (T), the size and/or number of regional lymph nodes metastasis (N), and the existence of distant metastasis (M). For many cancers, TNM combinations correspond to one of five stages. Criteria for stages differ for different types of cancer. For example, bladder cancer T3N0M0 is stage III, whereas colon cancer T3N0M0 is

stage II. This system is useful for estimating a prognosis of cancer patients and helps the doctor plan the appropriate treatment.

Exosome Exosomes are membrane-bound particles, 50 to 90 nm, formed via invagination of the early endosome and released upon fusion of late endosome with plasma membrane.

RNase RNase is a general term for enzyme that catalyzes the degradation of RNA into smaller components. RNase is also contained in the body fluid such as blood, urine, saliva and so on.

Hypoxia-inducible factors (HIFs) HIF is a protein which has been shown to control mitochondrial function and is essential for this repression of mitochondrial respiration during hypoxia. HIFs are known familiarly as a master regulator of the cellular hypoxic response.

Von Hippel-Lindau (VHL) tumor suppressor gene VHL gene is located on the short arm of chromosome 3. Deficiency of VHL gene leads to HIF accumulation; this accumulation induces upregulation of their transcriptional target genes, such as vascular endothelial growth factor (VEGF) and glucose transporter type 1 (GLUT1), thus promoting angiogenesis and metabolic changes associated with carcinogenesis. Since VHL gene deficiency is observed in 70 % or more of ccRCCs, it is suggested that its deficiency occurs in the early stage of carcinogenesis in ccRCC.

Carbonic anhydrase IX (CA IX) Carbonic anhydrase catalyzes the reversible hydration of carbon dioxide to carbonic acid and plays a role in pH regulation. To date, 14 human isoforms have been reported. CA IX is strongly induced by hypoxia in a range of tumor cell lines.

Iron-sulfur cluster assembly protein (ISCU1/2) Prosthetic groups are critical for electron transport and mitochondrial oxidation-reduction reactions. ISCU1/2 facilitates the assembly of iron-sulfur clusters.

Introduction

Renal cell carcinoma (RCC) is the most common urological neoplasm of the kidney in adults, representing over 90 % of primary renal neoplasms and accounting for approximately 3–5 % of all adult malignancies in western countries. In Japan, the results of a 2002 survey revealed that the crude incidence rates of RCC were 8.2 and 3.6 per 100,000 population for men and women, respectively (Marumo et al. 2007). In the United States, 58,000 new cases were diagnosed in 2010, with approximately 13,000 deaths (Jemal et al. 2010). The worldwide incidence and mortality rate is in the order of 270,000 cases and 120,000 deaths, respectively (Ferlay et al. 2010). Moreover, RCC represents a spectrum of histologic subtypes that are

morphologically and cytogenetically distinct. The most common subtype is clear cell RCC (ccRCC), which accounts for approximately 75–80 % of cases. Other subtypes include papillary RCC (pRCC) (10–15 %), chromophobe RCC (chRCC) (5 %), and collecting duct carcinoma (Meloni-Ehrig 2002). It is known that these different histological subtypes of RCC vary in their clinical courses and their prognosis and different clinical strategies have been developed for their management. Oncocytoma, papillary adenoma, mesonephric adenoma, and angiomyolipoma are the main benign renal tumors (BRT).

Despite the fact that the diagnostic modalities and therapeutic techniques for RCC continue to improve, the overall incidence and mortality of RCC has increased in the last 20 years (Hollingsworth et al. 2006). At the time of initial diagnosis, approximately 60 % of the patients have a localized carcinoma and nearly 20 % are identified with regional or distant metastases (Howlader et al. 2009). Approximately 30 % of RCC cases develop metastatic disease following surgery with curative intent. It is known that RCC is basically characterized by low response rate to chemotherapy, radiation therapy, and so on. Therefore, the 5-year survival rate is approximately 98 % for stage I disease and approximately 50 % for stage III or higher disease (Devita et al. 2008). These data underscore the importance of early detection of renal tumor before metastasis and of adequate prediction of tumor recurrence and distant metastases after surgery.

However, both early detection and adequate prediction are often difficult in the clinical setting. Renal tumor in the early stage is often asymptomatic and non-palpable. Recent data show that approximately 70–80 % of renal tumors are detected incidentally as a result of scanning for another medical problem. It is impossible to correctly distinguish between RCC and BRT, even with the full use of diagnostic modalities such as imaging examinations, abdominal ultrasonography, computed tomography, and magnetic resonance imaging. Additionally, scanning for tumor recurrence and distant metastases must rely mainly on radiological imaging examinations in the outpatient clinic after surgery. However, determination of the adequate frequency of radiological imaging examinations is confused by differences in factors including T stage and tumor histopathology (Sejima et al. 2013). These problems are caused by the lack of an accurate biomarker for diagnosis and prognosis in RCC. Therefore, identification of novel biomarkers is urgently needed.

Recent studies demonstrated that microRNAs (miRNAs), which are non-protein-coding small RNAs, are involved in carcinogenesis, cancer progression, and metastasis. MicroRNAs are approximately 22 nucleotides in length and regulate gene expression at the posttranscriptional level by binding to the untranslated region (3′UTR) of target mRNAs, leading to translational inhibition and/or mRNA degradation (Slaby et al. 2010). Specific expression profiles of miRNAs in tissue have been reported in a variety of cancers, including in RCC (Volinia et al. 2006). Early studies suggested that miRNAs were strictly intracellular molecules. However, more recent studies have reported that miRNAs are highly stable and abundant in the serum, urine, and other body fluids, which is likely due to exosome protection of

miRNAs against degradation by RNase (Mitchell et al. 2008; Chim et al. 2008). Interestingly, serum miRNA levels are similar in men and women and do not vary with patient age (Hunter et al. 2008). Thus, both tissue miRNAs and circulating miRNAs might be good biomarkers for diagnostic and prognostic considerations in a variety of cancers. Indeed, several studies have demonstrated that specific tissue and circulating miRNAs were valuable for distinguishing patients with cancer from healthy controls (HCs).

miR-210 Investigation in RCC

Of all of the miRNAs, miR-210, a well-known hypoxia-inducible miRNA, is one of the best studied miRNA in cancer. MiR-210 is upregulated in various types of human cancer (Camps et al. 2008; Ho et al. 2010), suggesting that it has an important role in carcinogenesis. Previous studies reported that miR-210 is induced under hypoxic conditions via hypoxia-inducible factors (HIFs) in various cancer cell lines and that miR-210 is an important factor in cell proliferation, mitochondrial respiration, DNA repair, vascular biology, and angiogenesis in various types of cancer (Jung et al. 2012; Chan et al. 2012).

In the case of RCC, most of the studies have investigated miR-210 expression in clear cell carcinoma (ccRCC), which is the largest subtype of RCC. It is well known that HIF1α and HIF2α accumulate in ccRCC as a result of abrogated ubiquitin-mediated degradation due to loss or deficiency of the von Hippel-Lindau (VHL) tumor suppressor gene (Eble et al. 2004). Therefore, a number of research groups have attempted to determine the relationship between miR-210 expression and the VHL-HIF pathway in RCC. Nakada et al. (2011) demonstrated that miR-210 expression clearly correlated with the accumulation of HIF1α under normoxia as well as under hypoxia, suggesting that miR-210 upregulation in ccRCC was most likely due to the accumulation of HIF1α. They further confirmed that restoration of VHL expression in a VHL-deficient cell line led to the degradation of HIF1α and suppressed the expression of miR-210, suggesting that miR-210 expression is regulated via the VHL-HIF1α pathway. Neal et al. (2010) reported that miR-210 levels showed a correlation with a HIF-regulated mRNA, carbonic anhydrase IX (CAIX), and VHL mutation or promoter methylation. They also reported that the inverse correlation between miR-210 levels and iron-sulfur cluster assembly protein (ISCU1/2) provides support for the hypothesis that ISCU1/2 is a target of miR-210 and that it may contribute to the anaerobic respiration seen in renal tumors. Along with these studies, research aimed at using miR-210 as a new biomarker in RCC, especially in ccRCC, has also been advancing.

miR-210 as a Diagnostic Biomarker in RCC

Circulating miRNA is suitable for analysis as a diagnostic biomarker. In particular, serum and urinary miRNAs have been frequently used for this analysis.

Previous studies have described the potential use of serum miRNA as a diagnostic biomarker for various cancers (e.g., miR-29a and miR-92 in colorectal cancer (Huang et al. 2010); miR-195 in breast cancer (Heneghan 2010); miR-17-5p, miR-21, miR-106a, and miR-106b in gastric cancer (Tsujiura et al. 2010); and miR-141 and miR-26a in prostate cancer (Mitchell 2008; Mahn et al. 2011)).

These two studies have investigated the utility of serum miR-210 as a diagnostic biomarker for RCC. Zhao et al. (2013) reported that tissue miR-210 levels in 33 ccRCC patients were significantly higher in tumor tissue than in adjacent non-tumoral renal parenchyma tissue ($P = 0.004$) (Fig. 1). Serum miR-210 levels were also significantly higher in 68 ccRCC patients than in 42 HCs ($P < 0.001$) (AUC, 0.87; sensitivity, 81.0 %; specificity, 79.4 %) (Fig. 2). Furthermore, they confirmed that serum miR-210 was significantly elevated in patients with TNM stage I ccRCC as well as in patients with other stages compared with HCs, although there was no difference between the different stages and serum miR-210 levels in patients with ccRCC that was decreased 1 week after surgical resection of the tumor. Iwamoto et al. (2014) demonstrated a similar result; tissue miR-210 levels in 34 ccRCC patients were significantly higher in tumor tissue than in adjacent non-tumoral renal parenchyma ($P < 0.001$). In 31 cases (92 %), the miR-210 level in tumor tissues was increased by > twofold when compared with that in normal tissues. Serum miR-210 levels were also significantly higher in ccRCC patients than in 24 HCs ($P = 0.001$) (AUC, 0.77; sensitivity, 65 %; specificity, 83 %). Moreover, there was no significant correlation between serum miR-210 levels and age, gender, tumor size, or the existence of metastasis at diagnosis (Fig. 3). The results of these two studies suggest that at least some of the serum miR-210 arises from release from the primary renal tumors and that upregulation of serum miR-210 may occur in the early stage of ccRCC. There have been a few studies regarding the utility of serum miRNAs as a diagnostic biomarker in RCC using a similar study methodology (e.g., miR-1233, miR-378, miR-451, and miR-378; refer to the next section).

Some studies using urinary miRNA as a diagnostic biomarker in other cancers have been published (e.g., miR-143, miR-222, miR-452, miR-145, and miR-200a in bladder cancer; refer to the next section). Lorenzen et al. (2011) reported that urinary miR-210 levels identified patients with acute T-cell rejection and predicted long-term kidney function in renal allograft recipients. However, there have been no reports of the availability of urinary miR-210 as a diagnostic biomarker in RCC.

In clinical practice, it is also important to distinguish the subtypes of renal tumor, in particular, to distinguish between RCC and BRT in order to avoid invasive overtreatment. Munari et al. (2014) analyzed the miRNA expression profile of a set of 15 tissue samples of clear cell papillary renal cell carcinoma (ccpRCC) and evaluated similarities and differences between ccRCC and pRCC. They reported that miR-210 was upregulated in both ccpRCC and ccRCC compared with normal tissue. As an alternative approach, although it was not a study using miR-210 alone, Fridman et al. (2010) used expression levels of six microRNAs in tissue samples including 17 ccRCC, 20 pRCC, 13 chRCC, and 21 oncocytoma, to demonstrate a two-step decision-tree classifier that could distinguish four common subtypes of

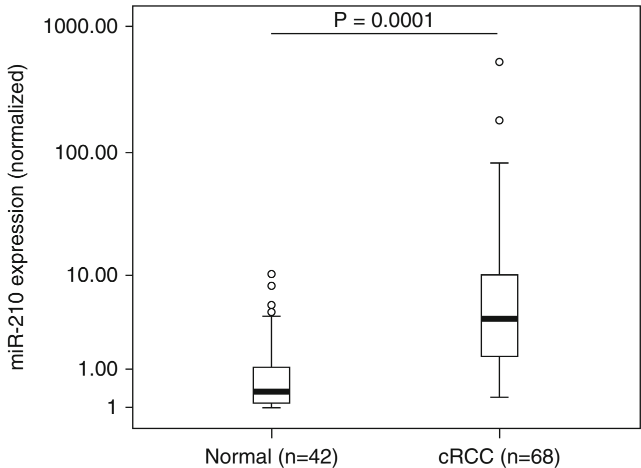

Fig. 1 **Serum miR-210 level in ccRCC patients and HCs**. Serum miR-210 level was significantly higher in the ccRCC patients than in HCs. The *lines inside the boxes* represent the median value (Data are from Zhao et al. (2013), with permission from the Publishers)

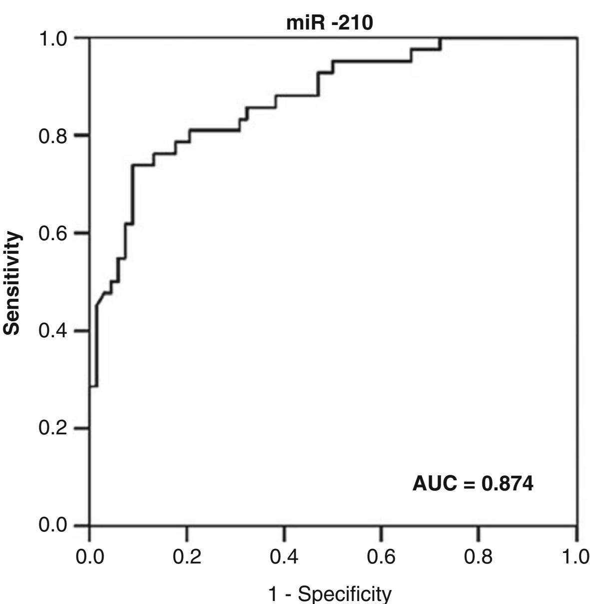

Fig. 2 **Receiver operating characteristic (ROC) curve analysis using serum miR-210 for discriminating ccRCC patients**. Serum miR-210 yielded an AUC (the areas under the ROC curve) of 0.874 (95 % Cl: 0.806–0.941) with a sensitivity of 81.0 % and a specificity of 79.4 % for discrimination between the ccRCC patients and HCs (Data are from Zhao et al. (2013), with permission from the Publishers)

renal tumor (Fig. 4). The first step used expression levels of miR-210 and miR-221 to distinguish between the two pairs of subtypes: ccRCC and pRCC vs. oncocytoma and chRCC. The second step used either miR-200c with miR-139-5p to distinguish oncocytoma from chRCC or miR-31 with miR-126 to distinguish ccRCC from pRCC. Using this classifier, these four subtypes of RCC could be distinguished

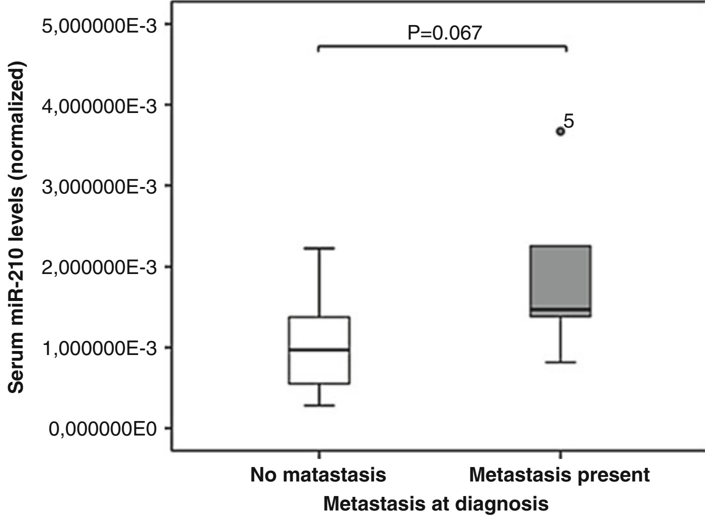

Fig. 3 Analysis of the correlation between serum miR-210 level and existence of metastasis at diagnosis. There was no significant association between serum miR-210 level and existence of metastasis at diagnosis (Data are from Iwamoto et al. (2014), with permission from the Publishers)

more than 90 % of the time. Youssef et al. (2011) also developed a classifier that could distinguish the different RCC subtypes using multiple miRNAs from 70 tissue samples, although miR-210 was not included in their analysis. Although the diagnostic accuracy of their analysis was very high, the use of multiple miRNAs was somewhat confusing. These studies were innovative. However, preoperative diagnosis using tissue miRNAs is difficult. There have been no similar studies using circulating miRNAs to distinguish the different RCC or BRT subtypes.

Studies regarding the significance of miR-210 as a diagnostic biomarker in RCC are summarized as follows. It is clear that miR-210 levels are upregulated in tissue and serum in the early stage of ccRCC. However, some problems remain to be solved in preparation for clinical application. Examples of such problems are the problem of organ specificity, i.e., distinguishing between ccRCC and other organ cancers, and the problem of cancer specificity, i.e., distinguishing between ccRCC and BRT. Moreover, the use of urine samples as a less invasive sample for analysis of biomarkers will be expected in the future.

miR-210 as a Prognostic Biomarker in RCC

A number of studies have reported the effect of miR-210 on the prognosis of different cancers, including breast cancer (Camps et al. 2008), epithelial ovarian cancer (Giannakakis et al. 2008), diffuse large B-cell lymphoma (Lawrie et al. 2008), lung cancers (Duncavage et al. 2009), and pancreatic adenocarcinomas

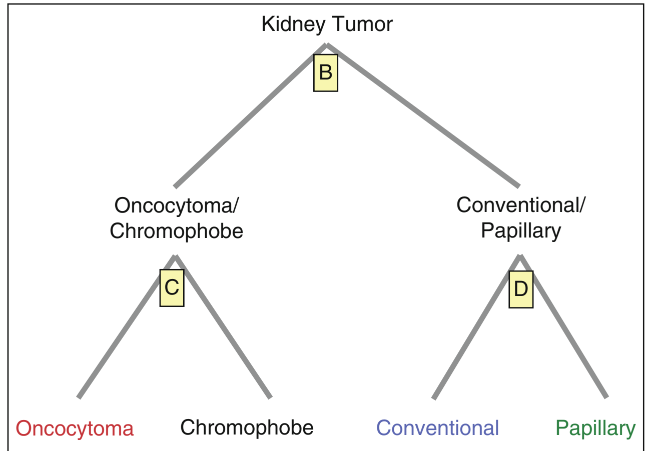

Fig. 4 Two-step decision-tree classifier to distinguish four common subtypes of renal tumor. First, samples are classified into either the oncocytoma/chromophobe pair or the conventional/ papillary pair, using expression levels of hsa-miR-210 and hsa-miR-221 (*B*). In the second step, oncocytoma is differentiated from chromophobe using expression levels of hsa-miR-200c and hsa-miR-139-5p (*C*), and conventional is differentiated from papillary using expression levels of hsa-miR-31 and hsa-miR-126 (*D*) (Data are from Fridman et al. (2010), with permission from the Publishers)

(Ho et al. 2010). Most of these studies found upregulation of miR-210 levels in cancer tissues or blood of cancer patients compared with the levels in normal tissues or blood of HCs, which was related to a poor survival outcome (Camps et al. 2008; Duncavage et al. 2009; Gee et al. 2010; Ho et al. 2010; Rothe et al. 2011; Toyama et al. 2012; Qiu et al. 2013).

In the case of RCC, several studies that investigated the correlation between miR-210 levels and the prognosis of patients with RCC have been published (Table 1). Neal et al. (2010) found a significant inverse correlation between miR-210 expression in the tissue samples of 31 clear cell RCC patients (Fig. 5) and 5-year overall survival ($r = -0.481$, $P = 0.006$). Samaan et al. (2015) analyzed tissue samples of 276 primary clear cell RCC patients and confirmed that patients with high miR-210 expression had a statistically higher chance of disease recurrence (hazard ratio, 1.82; $P = 0.018$) and shorter overall survival (HR, 2.46; $P = 0.014$). However, in multivariate analysis, miR-210 lost its statistically significant association with shorter disease-free survival and overall survival after adjusting for tumor size and for tumor, node, and metastasis stage. These results are in agreement with most of the previous studies in other cancers.

On the other hand, Wotschofsky et al. (2013) suggested that there was no significant difference between miR-210 expression in tissue samples of primary RCC and that of metastatic RCC and that miR-210 expression was not statistically related to recurrence-free survival. Iwamoto et al. (2014) reported similar results using serum samples. However, McCormick et al. (2013) demonstrated

Table 1 Summary table of studies regarding miR-210 as a biomarker. This table indicates specimen and intended purpose of miR-210 as a biomarker for each study

Authors	Year	Specimen	Purpose
Zhao	2013	Serum	Early diagnosis of ccRCC
Iwamoto	2014	Serum	Early diagnosis of ccRCC
Lorenzen	2011	Urine	Prediction of acute rejection and kidney function in renal allograft recipients
Munari	2014	Tissue	Differential diagnosis of RCC subtypes
Fridman	2010	Tissue	Differential diagnosis of RCC subtypes
Youssef	2011	Tissue	Differential diagnosis of RCC subtypes
Neal	2010	Tissue	Prediction of poor prognosis in ccRCC
Samaan	2015	Tissue	Prediction of poor prognosis in ccRCC
Wotschofsky	2013	Tissue	Prediction of poor prognosis in ccRCC
McCormick	2013	Tissue	Prediction of good prognosis in ccRCC

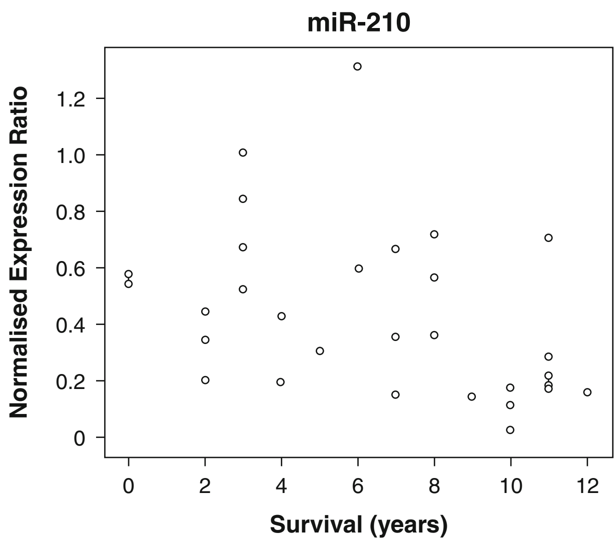

Fig. 5 Analysis of the correlation between tissue miR-210 level and patient survival. A significant correlation was found between miR-210 level and 5-year overall survival ($r = -0.481$, $P = 0.006$) (Data are from Neal et al. (2010), with permission from the Publishers)

precisely the opposite results; high miR-210 expression in clear cell RCCs was associated with lower stage and grade, and high miR-210 expression was associated with improved survival post nephrectomy, compared with medium and low levels of miR-210. As an explanation for these results, they suggested that, although VHL inactivation in ccRCC should lead to constitutive HIF activation and miR-210 upregulation, further loss of cell differentiation coupled with

ongoing mutations may manifest with reduction in expression of genes such as miR-210 and reduced miR-210 expression reflects a shift from HIF-1 to HIF-2 predominance.

Those studies regarding the significance of miR-210 as a prognostic biomarker in RCC are summarized as follows. Unfortunately, at the present time, there does not appear to be a consensus as to whether miR-210 expression is associated with good prognosis or poor prognosis. Further studies with a larger number of patients' clinical specimens are warranted to validate correctly these results.

Potential Applications to Prognosis and Other Diseases or Conditions

A few studies have investigated the utility of serum miRNAs as a diagnostic biomarker for RCC. Wulfken et al. (2011) were the first to report that the serum miR-1233 level was increased in RCC patients. They found miR-1233 levels were increased in 84 patients with RCC from a multicenter cohort (AUC, 0.588; sensitivity, 77.4 %; specificity, 37.6 %). They included in this investigation 13 samples from patients with angiomyolipoma or oncocytoma whose serum miR-1233 levels were similar to those of patients with RCC. Redova et al. (2012) demonstrated that serum miR-378 and miR-451 were potential biomarkers for RCC. When the utility of miR-378 plus miR-451 as a biomarker was evaluated in an independent cohort of 90 patients with RCC and 35 HCs, the combination of serum miR-378 and miR-451 enabled identification of RCC with a relatively high accuracy rate (AUC, 0.86; sensitivity, 81 %; specificity, 83 %). Hauser et al. (2012) confirmed that serum miR-378 was significantly increased in ccRCC (25 patients, $P = 0.006$), but they did not detect a difference in the level of this biomarker when they compared 117 patients with RCC versus 123 HCs. Comparison of these miRNAs with miR-210 in terms of diagnostic accuracy indicated that miR-210 had the highest diagnostic accuracy as a single miRNA marker.

Many studies have reported the significance of miR-210 as a diagnostic and prognostic biomarker in different cancers, especially in breast cancer. Jung et al. (2012) reported that high expression levels of plasma miR-210 before trastuzumab-based neoadjuvant chemotherapy were associated with resistance to treatment in 29 patients ($P = 0.035$) and that expression levels of plasma miR-210 were significantly lower in postoperative samples than in preoperative samples of 39 patients ($P = 0.029$). Camps et al. (2008) studied 219 early breast cancer patients with long-term follow-up and reported that miR-210 expression levels showed an inverse correlation with disease-free survival (DFS) and overall survival (OS), which was significant in both univariate and multivariate analyses (DFS, $p = 0.003$ and OS, $P < 0.001$, respectively). Toyama et al. (2012) demonstrated a similar relationship between high miR-210 levels and poor prognosis of breast cancer in 161 Asian patients. They found that triple-negative breast cancer patients with low miR-210 expression experienced significantly better disease-free and overall survival than those with high miR-210 expression ($P = 0.02$ and $P = 0.05$, respectively).

Furthermore, higher expression of miR-210 in triple-negative breast cancer patients was an independent prognostic factor, indicating a worse prognosis than lower miR-210 expression.

Additionally, the utility of urinary miRNA as a diagnostic and prognostic biomarker has been reported in other cancers, especially in bladder cancer. Yun et al. (2012) demonstrated that the levels of miR-145 were significantly decreased in 138 non-muscle-invasive bladder cancer (NMIBC) patients and 69 muscle-invasive bladder cancer (MIBC) patients compared to 144 healthy controls ($P < 0.001$). Also, MIBC patients had significantly lower miR-145 levels than NMIBC patients ($P = 0.036$). Furthermore, they confirmed in multivariate analysis that miR-200a was an independent predictor of NMIBC recurrence ($P = 0.013$). Puerta-Gil et al. (2012) also reported that urinary miR-452 (AUC, 0.848) and miR-222 (AUC, 0.718) provided high accuracies for bladder cancer diagnosis. In the case of RCC, only one study has been carried out that focused on urinary miRNAs. Brandenstein et al. (2012) found that urinary miR-15a levels showed a marked decrease postoperatively in ten ccRCC patients ($P < 0.005$) and that there was significant upregulation of miR-15a levels in the urine of 10 ccRCC patients compared to 35 patients with other medical conditions including conditions such as oncocytoma, urothelial carcinoma, colon carcinoma, and urinary bladder infection (Fig. 6). Even though there are a number of studies that have reported an association between miRNAs and RCC in tissue and serum samples, the number of studies that have analyzed urinary miRNAs is extremely small. This small number of reports indicates that some unconventional mechanism may make it difficult to detect miRNAs associated with RCC in urine samples. However, urine is the most minimally invasive sample in clinical examination. Therefore, if urinary miR-210 can be used as a biomarker in RCC, it will be of practical value.

Summary Points

- This chapter focuses on the significance of miR-210 as a diagnostic and prognostic biomarker in RCC.
- miR-210, a well-known hypoxia-inducible miRNA, is one of the best studied miRNAs in cancer.
- miR-210 is upregulated in various types of human malignancies, suggesting it has an important role in carcinogenesis.
- In the case of RCC, it is well known that HIF1α and HIF2α accumulate in ccRCC as a result of a deficiency of the VHL tumor suppressor gene.
- A number of research groups have attempted to reveal the relationship between miR-210 expression and the VHL-HIF pathway.
- Research regarding the utility of miR-210 as a new biomarker in RCC, especially in ccRCC, has also been advancing.
- In terms of its use as a diagnostic biomarker, previous studies have found that serum miR-210 levels were significantly higher in ccRCC patients than in HCs.

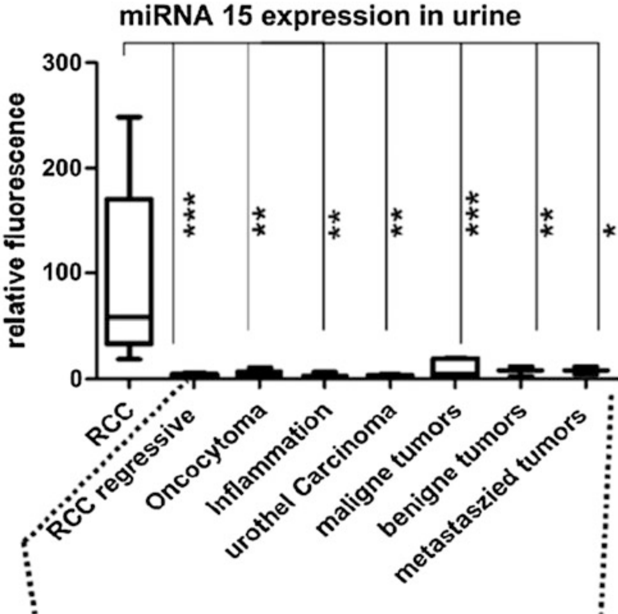

Fig. 6 **Box plot analyses from the urine of patients with different disease entities in the urogenital tract**. Carcinoma of the urogenital tract and other tumors, such as colon cancer and hepatic cell carcinoma, failed to show increased miRNA 15a levels. Furthermore, inflammatory conditions were equally incapable of increasing miRNA 15a levels. The increased expression of miRNA 15a in patients' urine with RCCs compared with all other collected urine samples was significant, as indicated by asterisks (Data are from von Brandenstein et al. (2012), with permission from the Publishers)

- No studies of the investigation of the utility of urine miR-210 as a diagnostic biomarker have been published.
- In terms of its use as a prognostic biomarker, some studies have suggested that high miR-210 expression in ccRCC tissue is statistically related to poor prognosis.
- Several studies have demonstrated precisely the opposite results, i.e., that low miR-210 levels correlate with RCC prognosis.
- A consensus has not yet been reached regarding the significance of miR-210 as a prognostic biomarker.

References

Camps C, Buffa FM, Colella S, et al. hsa-miR-210 is induced by hypoxia and is an independent prognostic factor in breast cancer. Clin Cancer Res. 2008;14:1340–8.

Chan YC, Banerjee J, Choi SY, et al. miR-210: the master hypoxamir. Microcirculation. 2012;19:215–23.

Chim SS, Shing TK, Hung EC, et al. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem. 2008;54:482–90.
Devita Jr VT, Hellman S, Rosenberg SA. Cancer principles and practice of oncology. 8th ed. Philadelphia: Lippincott Williams & Wilkins; 2008. p. 1332–53.
Duncavage EJ, Goodgame B, Govindin R, et al. Evaluation of microrna markers to predict outcome in low stage lung cancer. Lab Invest. 2009;89:352A–3.
Eble JN, Sauter G, Epstein JI, et al. World Health Organization classification of tumours. Pathology and genetics of tumours of the urinary system and male genital organs. Lyon: IARC Press; 2004. p. 9–87.
Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127:2893–917.
Fridman E, Dotan Z, Barshack I, et al. Accurate molecular classification of renal tumors using microRNA expression. J Mol Diagn. 2010;12:687–96.
Gee HE, Camps C, Buffa FM, et al. hsa-mir-210 is a marker of tumor hypoxia and a prognostic factor in head and neck cancer. Cancer. 2010;116:2148–58.
Giannakakis A, Sandaltzopoulos R, Greshock J, et al. miR-210 links hypoxia with cell cycle regulation and is deleted in human epithelial ovarian cancer. Cancer Biol Ther. 2008;7:255–64.
Hauser S, Wulfken LM, Holdenrieder S, et al. Analysis of serum microRNAs (miR-26a-2*, miR-191, miR-337-3p and miR-378) as potential biomarkers in renal cell carcinoma. Cancer Epidemiol. 2012;36:391–4.
Heneghan HM, Miller N, Lowery AJ, et al. Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. Ann Surg. 2010;251:499–505.
Ho AS, Huang X, Cao H, et al. Circulating miR-210 as a novel hypoxia marker in pancreatic cancer. Transl Oncol. 2010;3:109–13.
Hollingsworth JM, Miller DC, Daignault S, et al. Rising incidence of small renal masses: a need to reassess treatment effect. J Natl Cancer Inst. 2006;98:1331–4.
Howlader N, Noone AM, Krapcho M, et al. SEER cancer statistics review, 1975–2009. Bethesda: National Cancer Institute; 2009. http://seer.cancer.gov/csr/1975_2009_pops09/.
Huang Z, Huang D, Ni S, et al. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int J Cancer. 2010;127:118–26.
Hunter MP, Ismail N, Zhang X, et al. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One. 2008;3:e3694.
Iwamoto H, Kanda Y, Sejima T, et al. Serum miR-210 as a potential biomarker of early clear cell renal cell carcinoma. Int J Oncol. 2014;44:53–8.
Jemal A, Siegel R, Xu J, et al. Cancer statistics, 2010. CA Cancer J Clin. 2010;60:277–300.
Jung EJ, Santarpia L, Kim J, et al. Plasma microRNA 210 levels correlate with sensitivity to trastuzumab and tumor presence in breast cancer patients. Cancer. 2012;118:2603–14.
Lawrie CH, Gal S, Dunlop HM, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol. 2008;141:672–5.
Lorenzen JM, Volkmann I, Fiedler J, et al. Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft recipients. Am J Transplant. 2011;11:2221–7.
Mahn R, Heukamp LC, Rogenhofer S, et al. Circulating microRNAs (miRNA) in serum of patients with prostate cancer. Urology. 2011;77:1265.e9–16.
Marumo K, Kanayama H, Miyao N, et al. Prevalence of renal cell carcinoma: a nation-wide survey in Japan, 2002. Int J Urol. 2007;14:479–82.
McCormick RI, Blick C, Ragoussis J, et al. miR-210 is a target of hypoxia-inducible factors 1 and 2 in renal cancer, regulates ISCU and correlates with good prognosis. Br J Cancer. 2013;108:1133–42.
Meloni-Ehrig AM. Renal cancer: cytogenetic and molecular genetic aspects. Am J Med Genet. 2002;115:164–72.
Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105:10513–8.

Munari E, Marchionni L, Chitre A, et al. Clear cell papillary renal cell carcinoma: micro-RNA expression profiling and comparison with clear cell renal cell carcinoma and papillary renal cell carcinoma. Hum Pathol. 2014;45:1130–8.
Nakada C, Tsukamoto Y, Matsuura K, et al. Overexpression of miR-210, a downstream target of HIF1α, causes centrosome amplification in renal carcinoma cells. J Pathol. 2011;224:280–8.
Neal CS, Michael MZ, Rawlings LH, et al. The VHL-dependent regulation of microRNAs in renal cancer. BMC Med. 2010;8:64.
Puerta-Gil P, García-Baquero R, Jia AY, et al. miR-143, miR-222, and miR-452 are useful as tumor stratification and noninvasive diagnostic biomarkers for bladder cancer. Am J Pathol. 2012;180:1808–15.
Qiu S, Lin S, Hu D, et al. Interactions of miR-323/miR-326/miR-329 and miR-130a/miR-155/miR-210 as prognostic indicators for clinical outcome of glioblastoma patients. J Transl Med. 2013;11:10.
Redova M, Poprach A, Nekvindova J, et al. Circulating miR-378 and miR-451 in serum are potential biomarkers for renal cell carcinoma. J Transl Med. 2012;10:55.
Rothe F, Ignatiadis M, Chaboteaux C, et al. Global microRNA expression profiling identifies MiR-210 associated with tumor proliferation, invasion and poor clinical outcome in breast cancer. PLoS One. 2011;6:e20980.
Samaan S, Khella HW, Girgis A, et al. miR-210 is a prognostic marker in clear cell renal cell carcinoma. J Mol Diagn. 2015;17:136–44.
Sejima T, Iwamoto H, Masago T, et al. Oncological and functional outcomes after radical nephrectomy for renal cell carcinoma: a comprehensive analysis of prognostic factors. Int J Urol. 2013;20:382–9.
Slaby O, Jancovicova J, Lakomy R, et al. Expression of miRNA-106b in conventional renal cell carcinoma is a potential marker for prediction of early metastasis after nephrectomy. J Exp Clin Cancer Res. 2010;29:90.
Toyama T, Kondo N, Endo Y, et al. High expression of microRNA-210 is an independent factor indicating a poor prognosis in Japanese triple-negative breast cancer patients. Jpn J Clin Oncol. 2012;42:256–63.
Tsujiura M, Ichikawa D, Komatsu S, et al. Circulating microRNAs in plasma of patients with gastric cancers. Br J Cancer. 2010;102:1174–9.
Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103:2257–61.
von Brandenstein M, Pandarakalam JJ, Kroon L, et al. MicroRNA 15a, inversely correlated to PKCα, is a potential marker to differentiate between benign and malignant renal tumors in biopsy and urine samples. Am J Pathol. 2012;180:1787–97.
Wotschofsky Z, Busch J, Jung M, et al. Diagnostic and prognostic potential of differentially expressed miRNAs between metastatic and non-metastatic renal cell carcinoma at the time of nephrectomy. Clin Chim Acta. 2013;416:5–10.
Wulfken LM, Moritz R, Ohlmann C, et al. MicroRNAs in renal cell carcinoma: diagnostic implications of serum miR-1233 levels. PLoS One. 2011;6:e25787.
Youssef YM, White NM, Grigull J, et al. Accurate molecular classification of kidney cancer subtypes using microRNA signature. Eur Urol. 2011;59:721–30.
Yun SJ, Jeong P, Kim WT, et al. Cell-free microRNAs in urine as diagnostic and prognostic biomarkers of bladder cancer. Int J Oncol. 2012;41:1871–8.
Zhao A, Li G, Péoc'h M, et al. Serum miR-210 as a novel biomarker for molecular diagnosis of clear cell renal cell carcinoma. Exp Mol Pathol. 2013;94:115–20.

Matrix Metalloproteinase-2 (MMP-2) and Plasminogen Activator Inhibitor-1 (PAI-1) in Peritoneal Dialysis: Biological Implications and Clinical Utility

40

Deirisa Lopes Barreto and Raymond T. Krediet

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Abstract

Peritoneal dialysis (PD) represents an important renal replacement therapy for end-stage renal disease patients. Besides several advantages, long-term PD therapy is associated with functional and anatomical modifications that may lead to peritoneal membrane dysfunction. The clinical presentation of these complications is not always reflected in underlying morphological abnormalities. Unfortunately, it is not feasible to gain insight into intraperitoneal events that affect the structure of the peritoneal membrane without invasive procedures. The peritoneal

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effluent after drainage is the most clinically relevant specimen in PD. As proxies, peritoneal effluent biomarkers could be utilized as noninvasive tools for monitoring and diagnostic purposes. To date, no biomarkers have yet been identified to assess the degree of tissue remodeling and fibrosis in chronic PD patients.

In this chapter a review is given on matrix metalloproteinase-2 (MMP-2) and plasminogen activator inhibitor-1 (PAI-1) as biomarkers for the clinical practice of PD therapy. MMP-2 is a 72 kDa gelatinase that is involved in tissue remodeling. PAI-1 is a 50 kDa member of the serine protease inhibitors (SERPINs) that inhibits fibrinolysis and proteolysis. Peritoneal effluent MMP-2 and PAI-1 have recently been gauged as biomarkers for peritoneal membrane alterations. Their biological functions during physiological and pathological conditions are discussed as well as the expression in cells and tissue. This is followed by a brief synopsis of the current literature on circulatory MMP-2 and PAI-1 in PD therapy. Investigations on the levels of peritoneal effluent MMP-2 and PAI-1 are discussed more extensively with respect to their appearance in the peritoneal cavity, associations with parameters of peritoneal transport, and potential clinical applications. Finally, the clinical relevance of effluent MMP-2 and PAI-1 will be addressed.

Keywords

Adhesions • Biomarker • Effluent biomarker • Fibrosis • Matrix metalloproteinase-2 • Peritoneal dialysis • Peritoneal effluent • Peritoneal membrane dysfunction • Plasminogen activator inhibitor-1 • Tissue remodeling

Abbreviations

CA125	Cancer antigen 125
ECM	Extracellular matrix
eGFR	Estimated glomerular filtration rate
EMT	Epithelial-to-mesenchymal transition
EPS	Encapsulation peritoneal sclerosis
HD	Hemodialysis
IgG	Immunoglobulin G
IL-6	Interleukin-6
kDa	Kilodalton
MMP-2	Matrix metalloproteinase-2
MTAC	Mass transfer area coefficients
PAI-1	Plasminogen activator inhibitor-1
PD	Peritoneal dialysis
SERPINs	Serine protease inhibitors
TGF-β1	Transforming growth factor-beta1
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumor necrosis factor-α
t-PA	Tissue plasminogen activator
UFF	Ultrafiltration failure
u-PA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor

Definitions

Encapsulation peritoneal sclerosis (EPS) EPS is the most severe complication of PD therapy that is accompanied by high mortality rates and severe morbidity. Anatomically, EPS is characterized by a dense cocoon of fibrous tissue covering the abdomen.

Epithelial-to-mesenchymal transition (EMT) Transdifferentiation of mesothelial cell into mesenchymal cell phenotype. EMT is a short-term consequence of PD therapy due to the continuous exposure of PD solutions to the mesothelium.

Free water transport (FWT) Aquaporin-1 mediated transport of water, without dissolved solutes and electrolytes. FWT is one of the peritoneal transport parameter that becomes impaired in long-term PD patients.

Mass transfer area coefficients (MTAC) A peritoneal transport parameter of small solutes that reflects the peritoneal vascular surface area. In general, the MTAC of low molecular weight solutes increase with duration of PD therapy.

Peritoneal function test A standardized peritoneal test to assess the function status of the peritoneal membrane. Several forms of peritoneal function tests are available; however, all are characterized by a predefined dwell time with or without intermediate sampling of the peritoneal effluent. Depending on the duration and complexity of the test, more peritoneal transport parameters are gained.

Peritoneal membrane The peritoneal membrane is described to be a semipermeable membrane that consists of three main layers. Firstly, the mesothelial cell layer is encountered, followed by the interstitium in which the peritoneal capillaries are imbedded. Long-term and continuous exposure of PD solutions to this membrane may lead to functional and morphological alterations.

Introduction

For the past five decades, peritoneal dialysis (PD) has been used as a well-established renal replacement therapy for end-stage renal disease patients (Table 1). Other than hemodialysis (HD), PD is an intracorporeal dialysis system where the peritoneum is responsible for the removal of excess fluid and toxic waste products from the circulation into the peritoneal cavity. The peritoneum is a continuous membrane that consists of mesothelial cells and underlying tissues, lining the anterior abdominal wall (parietal peritoneum) and the intraperitoneal organs (visceral peritoneum). The peritoneal cavity is the space between these two opposing surfaces (Fig. 1). A permanent catheter is inserted into the peritoneal cavity through which 1.5–2.5 l of a hyperosmolar dialysis solution can be infused per dialysis

Table 1 Key facts of peritoneal dialysis

The invention of continuous ambulatory PD occurred in 1976 by Popovich and Moncrief. However, PD was already applied in patients, but solely suitable as a treatment option for acute renal failure since the 1940s. In the following decades, the technique has been optimized by undertaking prevention measures for PD-related peritonitis and exit-site infections. Additionally, novel dialysis solutions were introduced and referred as the more biocompatible PD solutions. All these precautions resulted in a diminished rate of PD therapy discontinuation
For almost half of a century, PD therapy belongs to one of the renal replacement therapy that is offered to end-stage renal disease patients. The proportion of PD patients in the worldwide dialysis population covers 11 %. The mortality rate remains high, but the 5-year patient survival has improved from 29 % to 41 %. In many countries, PD therapy is presented as the primary dialysis modality choice, as there is a survival benefit over hemodialysis in the first 3 years. Nevertheless, a global decrease in the proportion of PD patient is observed except for developing countries
In PD therapy the peritoneum serves as a biological dialysis membrane. Through this semipermeable serous membrane, toxins and excess fluid are removed from the circulation into the peritoneal cavity. For proper adequacy, three to five daily exchanges of PD solutions take place varying in dose and dwell duration. The efficacy of PD therapy is measured by means of peritoneal function tests. These tests provide insight into the magnitude of small solute removal, fluid transport, and ultrafiltration capacity
Chronic exposure to PD solutions may affect the peritoneal membrane negatively. Especially, in patients who are treated with PD for more than 2 years. The observed modifications are reflected by the function peritoneal transport parameters. Moreover, effluent biomarkers as proxies are capable to reveal anatomical alterations. However, attempts are being made to reduce or delay the development of peritoneal membrane dysfunction

PD peritoneal dialysis

session. Several exchanges are performed throughout the day or night with drainage of the peritoneal dialysate after prespecified time intervals.

In the worldwide dialysis population, the prevalence of PD therapy approximates 11 % (Jain et al. 2012). Paradoxically, PD is underutilized as a primary dialysis therapy despite its initial survival benefit over a 3-year period when compared with incident hemodialysis patients (van de Luijtgaarden et al. 2011; Weinhandl et al. 2010). Moreover, a European registry indicated prolongation of this survival benefit for up to 5 years after commencing dialysis treatment (van de Luijtgaarden 2014). Discontinuation of PD therapy occurs because of catheter-related complications, psychosocial features, peritoneal membrane dysfunction, or other clinical factors. On a continuum (Fig. 2), peritoneal membrane dysfunction may be partitioned into two time frames, namely, short-term and long-term consequences of PD therapy. Peritoneal membrane dysfunction is the cause of modifications that may arise on a functional as well as anatomical level. Functionally, early discontinuation is merely due to inherent ultrafiltration failure, i.e., a rapid dissipation of the osmotic gradient that is observed in approximately 15 % of incident PD patients. The global identification strategy of ultrafiltration failure (UFF) is by performing a 4-h peritoneal function test with a 3.86 % or 4.25 % glucose-containing dialysis solution. The ultrafiltration volume is thereafter measured from the drained dialysate, and when this amount is below the threshold of 400 ml, the diagnosis of UFF is made (Mujais et al. 2000). The actual or acquired peritoneal membrane dysfunction is usually observed after more than 2 years of PD therapy (Kolesnyk et al. 2010). At this stage, anatomical modifications, such as neoangiogenesis, are becoming more overt as

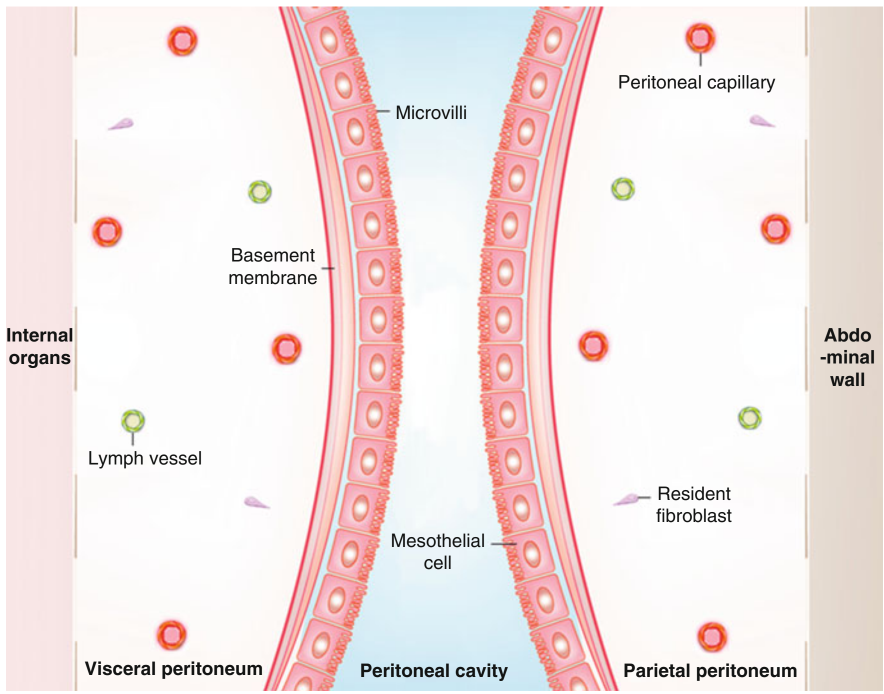

Fig. 1 Anatomy of the peritoneum. The peritoneum lines the inner abdominal wall and external surface of the internal organs in a continuous manner with the peritoneal cavity as space between the facing surfaces. The peritoneal membrane consists of three main layers: mesothelium, interstitium, and peritoneal capillaries

reflected by some parameters of peritoneal membrane transport. In the initial phase of PD therapy, the peritoneal membrane experiences low-grade inflammation due to the instillation of the nonbiological PD solutions. Epithelial-to-mesenchymal transition (EMT) has also been detected in the first 2 years of PD therapy (Del Peso et al. 2008). Further alterations in the anatomy of the peritoneal membrane comprise loss of mesothelial cell mass and neoangiogenesis, and after a treatment period of more than 4 years, adhesions or extensive fibrosis may be detected. However, these structural modifications are difficult to assess, as peritoneal biopsies are required which may lead to discontinuation of PD therapy. Moreover, classical imaging techniques have shown to be insufficient in the timely detection of anatomical alterations. Nevertheless, as PD therapy requires drainage of the peritoneal cavity in order to discard toxins and excess fluid, the drained dialysate, i.e., peritoneal effluent, is an important diagnostic tool, because it contains a large proportion of proteins and substances that are readily available for analysis. Therefore, the peritoneal effluent should be regarded as the most clinically relevant diagnostic specimen in PD. Over the years several candidate effluent biomarkers have been investigated (Lopes Barreto and Krediet 2013). The purpose of these effluent biomarkers is to gain insight into pathophysiological events within the peritoneal cavity and indirectly assess morphological alterations without

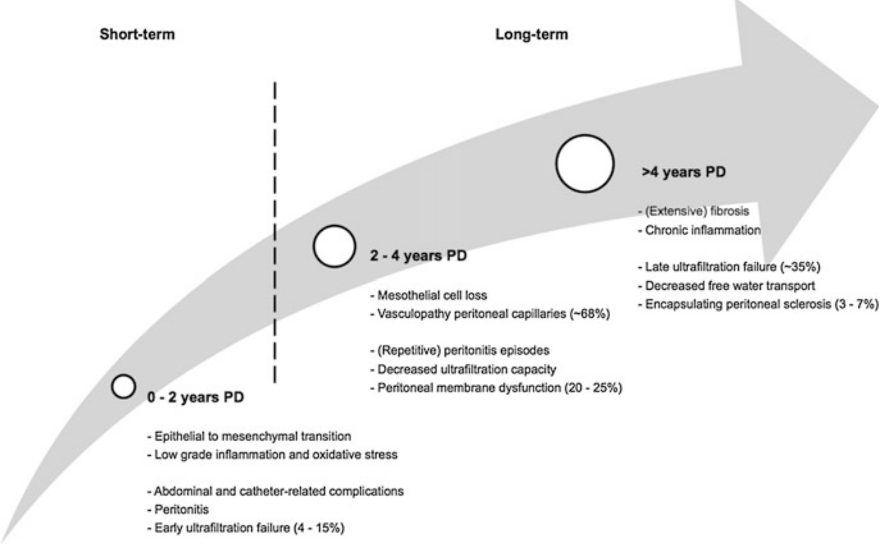

Fig. 2 Continuum PD therapy: Possible modifications and consequences. Depending on the duration of PD therapy, several functional and morphological peritoneal membrane alterations are induced. However, the degree of peritoneal impairment is patient specific, and therefore, not all PD patients encounter and develop certain PD-related complications

causing harm to the peritoneal membrane. More importantly, acknowledged effluent biomarkers are expected to aid in early detection of severe complications or to determine the right time/moment to discontinue PD treatment.

The anatomical alterations that mainly affect the peritoneal membrane are the increase in peritoneal capillaries, loss of mesothelial cells, and tissue remodeling and fibrosis. Surrogate markers have been identified to monitor the increase of peritoneal capillaries and loss of mesothelial cells, like vascular endothelial growth factor (VEGF) and cancer antigen 125 (CA125), respectively. Unfortunately, no markers have yet been identified or acknowledged to monitor the degree of peritoneal adhesions and fibrosis. However, matrix metalloproteinase-2 (MMP-2) and plasminogen activator inhibitor-1 (PAI-1) have been promoted recently as emerging effluent biomarkers for peritoneal tissue remodeling and fibrosis. Besides their biological functions and implications, this chapter will focus on PD-related investigations of systemic as well as peritoneal effluent MMP-2 and PAI-1. Furthermore, their potential clinical applications and utility as effluent biomarkers in PD will be discussed.

Structure and Biological Functions of MMP-2 and PAI-1

MMPs have been associated with several physiological and pathological processes including cell differentiation, migration, angiogenesis, apoptosis, and growth. However, their main function remains the degradation of the extracellular matrix (ECM)

components. Primarily MMPs are synthesized as latent zymogens and their activation is contingent upon the cellular environment (Nagase et al. 1991; Khasigov et al. 2001). This zinc-dependent matrix-degrading endopeptidase family is furthermore subdivided into five main classes: interstitial collagenases, stromelysins, metalloelastases, membrane-type MMPs, and gelatinases. MMP-2 (molecular weight 72 kDa) is classified into the latter one and is therefore also referred as gelatinase A. The common trait of MMPs is a pro-peptide domain, which structures the inactive form for the majority of matrixins. The distinctive characteristic of MMP-2 is that it also exhibits a sequence of fibronectin type II-resembling modules in the catalytic domain for collagen- and gelatin-binding purposes (Bode et al. 1999).

MMP-2 is the only gelatinase that is constitutively secreted by mesothelial cells on the apical side (Martin et al. 2000; Marshall et al. 1993). Initially, the expression of its gene, which is located on human chromosome 16q13, is regulated by various cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Borden and Heller 1997; Kossakowska et al. 1999). Furthermore, in vitro experiments in endothelial, mesenchymal, and vascular smooth muscle cells suggest tissue-specific regulation of MMP-2 (Zucker et al. 1995; Kaizuka et al. 1999; Galis et al. 1997). Other regulators of MMP-2 function encompass oxidative stress and a decreased bioavailability of nitric oxide (Wang et al. 2005). MMP-2 susceptibility to oxidative modification diminishes its activity, potentially leading to an altered turnover of the extracellular matrix (Mattana et al. 1998). Membrane-type MMPs, TIMP-2, and plasmin and serine proteases are a few enzymes through which pro-MMP-2 may be activated. MMP-2 substrates cover a broad range of ECM components including collagens, elastin, fibronectin, gelatins, and laminin (Klein and Bischoff 2011). Overall, evidence has been found for MMP-2 in angiogenesis, cell growth and migration, invasion, and metastasis (Chang and Werb 2001).

PAI-1 is a single-chain glycoprotein that is a member of the serine protease inhibitors (SERPINs) with a molecular weight of 50 kDa. The encoding gene is located at chromosome 7 and approximates 12.2 kilobases. In general, SERPINs are known to control proteolytic pathways and to date over 36 human SERPINs are acknowledged (Law et al. 2006). In comparison to the other SERPINs, PAI-1 is exceptional as it appears in four distinct conformations: (1) an active form that interacts with plasminogen activator, (2) a nonreactive latent form, (3) a non-inhibitory cleaved substrate, and (4) a cleaved form. Due to these entities, PAI-1 is able to respond rapidly in changing microenvironments. Hence, the stability of PAI-1 is affected merely by cellular acidity and thermal dynamics. The active form is considered to be the least stable conformation and the cleaved form as the most stable (Sancho et al. 1995). At neutral pH the half-life of active PAI-1 is nearly 2 h at 37 °C (Lindahl et al. 1989). The latent form may be converted into the active form as well by negatively charged phospholipids or denaturants (Lambers et al. 1987; Hekman and Loskutoff 1985). However, PAI-1 in plasma or extracellular matrix is stabilized when bound to vitronectin. In this conformation the activity of PAI-1 decays with a 12-fold increased half-life (Lijnen 2005; Mimuro et al. 1987).

The main sites of production are endothelium, mesothelium, and vascular smooth muscle cells (Loskutoff et al. 1989; Holmdahl et al. 1997). The enzymatic activities of tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA)

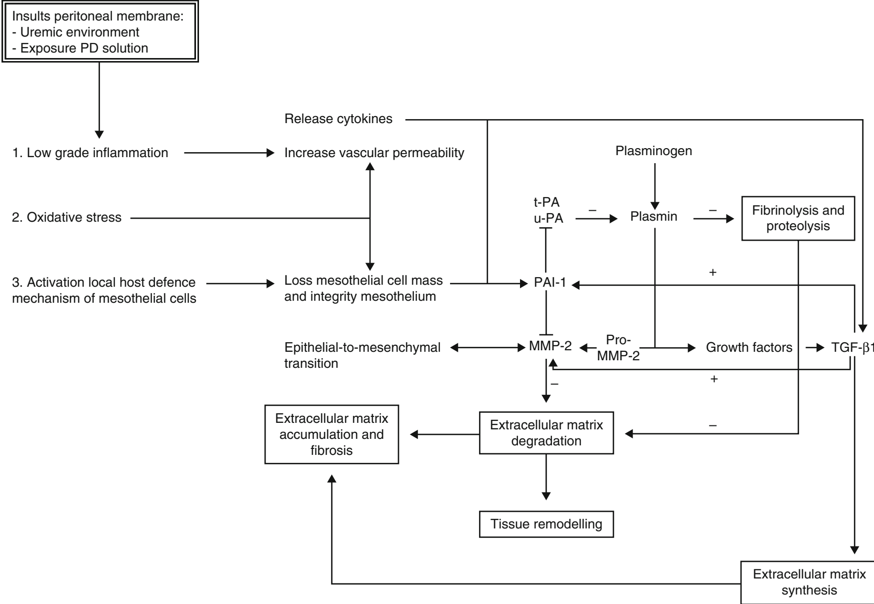

Fig. 3 Schematic diagram of MMP-2 and PAI-1 pathways. Depicted in this figure are the factors and mediators of MMP-2 and PAI-1. Both markers encompass pleiotropic entities, which may result in excessive deposition of extracellular matrix constituents and fibrosis when impaired

are mainly restricted by PAI-1. For this reason PAI-1 is regarded as the predominant inhibitor of fibrinolysis. Additionally, PAI-1 interacts with integrins and ECM components (Dellas and Loskutoff 2005). PAI-1 release occurs through cytokines, growth and inflammatory factors, and hormones. At a transcriptional level, transforming growth factor-β1 (TGF-β1) is a major regulator of PAI-1, and in cultured human peritoneal mesothelial cells, PAI-1 antigen was increased after stimulation with TGF-β1 (Rougier et al. 1998). The biological processes to which PAI-1 has been related are angiogenesis, cell migration, fibrosis, inflammation, metastasis, and tissue remodeling (Dellas and Loskutoff 2005). The development of abundant interstitial fibrosis and peritoneal membrane thickening is likely a consequence of long-term PD therapy. A disbalance in proteolytic activity may result in accumulation of ECM in basement membranes and interstitium. It is here where MMP-2 and PAI-1 are intertwined due to their individual pleiotropic characteristics. Figure 3 is a schematic diagram in which the pathways are depicted.

Cellular and Peritoneal Tissue Expression of MMP-2 and PAI-1

The efficacy of PD therapy relies on the integrity of the peritoneal membrane through which the exchange of fluid and solutes occurs. However, anatomical and morphological studies describing consecutive peritoneal tissue modifications in PD patients

are seldom performed, as the procedure forms a direct threat to the continuation of PD therapy. Even though the mesothelium is not considered as a prominent barrier for peritoneal solute transport, it is an important constituent as it is the source from which several substances are secreted and excreted including MMP-2 and PAI-1. Unfortunately, the mesothelium is also difficult to assess due to its susceptibility to artifactual damage during a peritoneal biopsy. From the abovementioned reasons, it can be deduced that in vitro experiments using human peritoneal mesothelial cell lines or in vivo rodent models are valuable proxies despite discordant results on some aspects when compared with the dialyzed human peritoneal membrane condition.

So far, no histological studies have been performed to examine MMP-2 in human peritoneal tissue. However, in a chronic PD rodent model, the degree of fibrosis was correlated with effluent levels of MMP-2 (Lopes Barreto 2013). MMP-2 has been investigated in harvested arteries from hemodialysis and PD kidney transplant recipients. The densitometric measurements indicated a higher level of MMP-2 activity and expression in these previously dialyzed patients, compared to chronic renal failure patients or healthy living kidney donors, suggestive for altered vascular remodeling (Chung et al. 2009). More recently, immunohistochemical analysis of renal allograft biopsies from patients with chronic humoral rejection revealed the presence of MMP-2 in podocytes, whereas this was less common or absent in patients experiencing other renal complications (Wong et al. 2010). Involvement of MMP-2 in EMT of peritoneal mesothelial cells has been demonstrated. During TGF-β1-induced EMT, MMP-2 gene and protein expression was upregulated, coinciding with demolition of the basement membrane (Margetts et al. 2005). Additionally, in human peritoneal mesothelial cells stimulated by >1 U/mL thrombin, the activity of MMP-2 indicated a maximum reduction of 56 % in cell supernatants. Moreover, the effect of thrombin was associated with a decline in MMP-2 production and an enhanced tissue inhibitor of metalloproteinases (TIMP)-1 synthesis (Haslinger et al. 2000). If prolonged, a potential consequence as a result of this loss in homeostasis is peritoneal tissue remodeling where the decrease in MMP-2 leads to accumulation of ECM constituents.

The association between glucose-induced PAI-1 activation contributes to the interest of in vitro experiments investigating the effect of glucose in PD solutions on PAI-1 expression. For this purpose, isolated human peritoneal mesothelial cell has been exposed to conventional, biocompatible, and icodextrin (a glucose polymer)-based PD solutions varying in pH, glucose concentration, or duration of exposure. Common findings comprise elevated PAI-1 antigen levels for PD solutions high in glucose (>2.5 %) versus low-glucose PD solutions (<1.5 %). Icodextrin-based PD fluids indicated a consistently lower PAI-1 activity. Additionally, a time-dependent increase in PAI-1 release has been detected (Katsutani et al. 2007; Mandl-Weber et al. 2001a; Breborowicz et al. 1997). Regulators of PAI-1 activity have also received great attention. In vitro, the expression of PAI-1 is enhanced by TGF-β1 and thrombin, but no significant impact seems to be present for hyaluronan (Rougier et al. 1998; Mandl-Weber et al. 1999; Sitter et al. 2003). Depending on the strain, bacterial peritonitis augments mesothelial PAI-1 synthesis, hence diminishing the intraperitoneal fibrinolytic activity (Mandl-Weber et al. 2001b).

The formation of adhesions is a physiological response to peritoneal tissue repair. The capacity to restore peritoneal insults in a controlled manner is therefore highly dependent on the balance between fibrinogenesis and fibrinolysis. As a consequence, PAI-1 has been studied extensively in patients undergoing laparotomy due to peritoneal inflammation or trauma, or elective surgery. Nevertheless, studies on the peritoneal distribution and localization of PAI-1 have not yet been performed in patients receiving PD therapy. When comparing homogenates from normal peritoneal tissue to an inflamed peritoneum, it was clear that the latter exhibited elevated levels of PAI-1 (Vipond et al. 1990). The authors stated that a decrease in functional fibrinolytic activity under inflammatory conditions is mediated by PAI-1. Later, the expression of PAI-1 in the human peritoneum was localized. Immunohistochemical analyses revealed that PAI-1 is present in the mesothelium and capillary vascular walls of the peritoneum. However, in inflamed peritoneal tissue, PAI-1 is extensively distributed in the submesothelium and partly co-localized with immunoreactivity of macrophages (Holmdahl et al. 1997).

Systemic Levels of MMP-2 and PAI-1

As the critical importance of tissue remodeling and fibrosis in PD is centered at the integrity of the peritoneal membrane, a limited number of PD study groups have investigated circulatory MMP-2 and PAI-1. Nevertheless, these investigations have related the systemic levels of these markers to major causes of morbidity and mortality in PD such as cardiovascular risk and inflammation. The reported systemic levels of MMP-2 and PAI-1 in PD patients are summarized in Table 2.

In a cross-sectional study, similar serum values between HD and PD patients, and between pre-end-stage renal disease patients and transplant patients, were found for MMP-2. The findings from this study indicated inferior MMP-2 levels of dialysis patients suggesting a dialysis-related suppression of MMP-2 activity (Preston et al. 2002). Within the PD population, the median serum level of MMP-2 approximates 243 ng/mL when measured the first year after commencing PD therapy in incident PD patients (Lopes Barreto et al. 2013). These serum levels are in line with a second cross-sectional study, which additionally reported MMP-2 levels of healthy controls without diabetes mellitus, hypertension, and renal or vascular diseases (Pawlak et al. 2010). Moreover, in the same study, it was demonstrated that PD patients with a previous diagnosis of cardiovascular diseases exhibit a significantly augmented serum level of MMP-2 with concentrations ranging from 200 to 625 ng/mL. However, it is ambiguous whether elevated serum MMP-2 resides in the causal pathway or reflects a consequence of cardiovascular diseases in PD patients.

Normal values of PAI-1 activity and antigen have been found in PD patients (Irish 1997; Kim et al. 1997). In PD patients with atherosclerosis, serum PAI-1 antigen is increased and averages 25 ng/mL, which points toward an impaired fibrinolytic activity (Kim et al. 1997). Comparative investigations on the effect of PAI-1 activity in chronic renal failure and dialysis patients have also been executed. Throughout the subdivided study population, a significantly lower activity of PAI-1 appeared to be present for HD

Table 2 Systemic levels of MMP-2 and PAI-1

			Serum level in study population (ng/mL)	
Author and year	Sample size	Detection method	Healthy controls	PD patients
MMP-2				
Preston et al. (2002)	21	ELISA	542.0	850.0
Pawlak et al. (2010)	56	ELISA	192.4	258.2
Lopes Barreto et al. (2013)	86	ELISA	–	242.9
PAI-1				
Kim et al. (1997)	82	ELISA	18.0	17.0
Pawlak et al. (2006)	34	ELISA	24.5	13.5
Arikan et al. (2009)	46	ELISA	–	50.4
Lopes Barreto et al. (2013)	86	ELISA	–	20.9

Systemic levels of the biomarkers are expressed as average of study population
PD peritoneal dialysis, *MMP-2* matrix metalloproteinase-2, *ELISA* enzyme-linked immunosorbent assay, *PAI-1* plasminogen activator inhibitor-1

patients, compared to patients with low and high proteinuria, and continuous ambulatory PD patients (Irish 1997). The opposite was found in a study focused at assessing the risk of cardiovascular diseases between HD and PD patients (Tomura et al. 1996). Conflicting evidence encompasses PAI-1 antigen as well, where enhanced or impaired fibrinolysis is described (Pawlak et al. 2006). In summary, data on the impact of PAI-1 activity between dialysis modalities are inconclusive and likely to be attributed to the circadian rhythm of PAI-1 that peaks in the morning (Angleton et al. 1989) and analytical procedures. Recently, a cohort study with a minimal follow-up duration of 5 years reported a worse survival rate for all-cause and cardiovascular mortality in prevalent PD patients with PAI-1 plasma levels exceeding 41 ng/mL (Arikan et al. 2009).

Potential Applications to Prognosis, Other Diseases, or Conditions

MMP-2 has been associated with arterial stiffness (Yasmin et al. 2005). Additionally, in patients with an acute coronary syndrome, MMP-2 was considered as a predictive factor for all-cause mortality (Dhillon et al. 2010). In renal diseases elevated serum and plasma levels of MMP-2 have been related to progressive glomerulosclerosis. Systemic levels of MMP-2 in chronic kidney disease patients are negatively correlated with kidney function as measured by the estimated glomerular filtration rate (eGFR) and increase linearly with the degree of proteinuria (Nagano et al. 2009). Moreover, a recent finding indicated that plasma MMP-2 may be an independent predictor of rapid decline in eGFR, accompanied by a hazard ratio of 2.47 for progression of kidney disease in patients with MMP-2 levels above >861 ng/mL (Hsu et al. 2013).

As a consequence of PAI-1 main function, i.e., an inhibitor of fibrinolysis, the primary use in clinical practice is usually to detect deficiencies in the fibrinolytic system. Elevated plasma levels of PAI-1 are therefore merely associated with coronary diseases. Renal cell carcinomas stain positive for cytoplasmatic PAI-1 (Zubac et al. 2010). Furthermore, in a large multiethnic cohort, patients with an eGFR <60 mL/min/1.73 m2 had 6.5 % higher levels of PAI-1 in comparison to those with better kidney function. Additionally, PAI-1 was associated with eGFR by cystatin C calculations, but not with creatinine-based eGFR (Dubin et al. 2011). PAI-1 has been proposed as a potential therapeutic target in renal fibrogenesis (Rerolle et al. 2000).

Peritoneal Effluent MMP-2 and PAI-1

The identification of effluent biomarkers in PD is merely from a hypothesis-driven perspective rather than a discovery-based approach. The exact sequence of pathophysiological events with the peritoneal cavity is unknown. However, since the inception of PD therapy, numerous study groups have revealed molecular mechanisms indicating short- and long-term peritoneal membrane alterations. From these data, abundant peritoneal adhesions and fibrosis are considered as final events in the continuum, which sometimes ends with the clinical presentation of encapsulating peritoneal sclerosis (EPS). EPS is a rare (prevalence 3–7 %), but devastating complication of PD therapy that is high in morbidity and mortality. Several attempts have been made to timely identify PD patients at risk. However, no early detection strategy is yet available.

The lead time of peritoneal tissue remodeling and interstitial fibrosis encompasses years of chronic exposure to PD solutions. The biological functions of MMP-2 and PAI-1, including remodeling of the ECM and prevention of excessive collagen and fibrin depositions, led to investigations on their presence, potential significance, and local effects of impaired homeostasis in PD therapy. Additionally, these markers have been related to peritoneal transport parameters, PD-related peritonitis, and effects of various PD solutions.

Appearance of MMP-2 and PAI-1 in the Peritoneal Cavity

Peritoneal effluent MMP-2 has been studied in incident as well as prevalent PD patients. These studies reported average values of effluent MMP-2 and PAI-1, revealing an overall increasing tendency in a time-dependent manner (Table 3). Preferably, effluent levels are presented in appearance rates, which are an adjusted value that is corrected for the drained effluent volume. However, this can only be done when a marker has demonstrated to increase linearly during a predefined dwell time. PAI-1 is known to increase linearly during a 4-h dwell regardless of the PD

Table 3 Peritoneal effluent levels of MMP-2 and PAI-1

Author and year	Study design	Sample size	Detection method	PD duration (months)[a]	Effluent level (ng/mL)[b]
MMP-2					
Nishina et al. (2004)	Cohort	13	Zymography	7–137	126[a]
Hirahara et al. (2007)	Cross-sectional	444	ELISA	13–92	167
Minami et al. (2007)	Cross-sectional	16	EIA	10–97	319
Hirahara et al. (2011)	Cross-sectional	215	ELISA	22–69	167
Lopes Barreto et al. (2013)	Cohort	86	ELISA	0.5–12	21.4[c]
Cho et al. (2016)	Cohort	178	ECL	0–3	34.2[c]
PAI-1					
Selgas et al. (1992)	Cross-sectional	20	ELISA	6–121	7.3
Goedde et al. (1997)	Cross-sectional	16	ELISA	Unknown	2.6
Opatrný et al. (1998)	Cross-sectional	31	ELISA	4–35	1.45
Lopes Barreto et al. (2013)	Cohort	86	ELISA	0.5–12	0.9[c]

PD peritoneal dialysis, *MMP-2* matrix metalloproteinase-2, *ECL* electrochemiluminescence immunoassay, *EIA* enzyme immunoassay, *ELISA* enzyme-linked immunosorbent assay, *PAI-1* plasminogen activator inhibitor-1. Peritoneal effluent levels of the biomarkers expressed as average of study population

[a]Range PD duration study population

[b]Biomarker levels represent baseline measurement of study

[c]Median biomarker level

solution glucose concentration (Lin et al. 1995; Opatrný et al. 1998). The release pattern of MMP-2 has not yet been determined.

One of the main criteria for an effluent biomarker is the presence of local peritoneal production. This is established by computing a peritoneal transport line based on dialysate to plasma ratios of β2-microglobulin, albumin, IgG, and α2-macroglobulin when plotted against their molecular weight. By interpolation of the candidate marker, a positive discrepancy is attributed to local production. In the case of MMP-2, local production accounts for 90 % of the effluent concentration. Intraperitoneal release of PAI-1 has been established in both pediatric and adult PD patients (de Boer et al. 1999; Selgas et al. 1992; Lopes Barreto et al. 2013). Currently, the biological variability of effluent MMP-2 and PAI-1 between and within PD patients is not known.

Associations with PD Duration, Peritoneal Transport Parameters, and Peritoneal Effluent Markers

With regard to PD therapy duration, transversal analyses report positive relationships with MMP-2 and PAI-1 (Lopes Barreto et al. 2013; Hirahara et al. 2007). However, time-trend analyses of MMP-2 report conflicting results. A possible reason could be the difference in follow-up duration (Lopes Barreto et al. 2013; Cho et al. 2016). In contrast, time courses of PAI-1 indicate a significant elevation in effluent levels with duration of PD therapy alongside an explained variance of 5 % in cross-sectional analyses.

Peritoneal transport parameters reflect the functional integrity of the peritoneal membrane and are necessary in order to determine the efficacy of PD therapy. The functional parameters are derived from standardized peritoneal membrane function tests, such as the standard peritoneal permeability analysis (SPA), and include components of solute and fluid transport. The net ultrafiltration, mass transfer area coefficients (MTAC) of small solutes, and free water transport are the most essential parameters. High levels of MMP-2 and PAI-1 are accompanied by high MTAC of creatinine and low free water transport. Also dialysate to plasma ratios of creatinine and the maximal initial dialysate sodium dip have indicated moderate to strong correlation coefficients with both effluent markers (Hirahara et al. 2007, 2011; Lopes Barreto 2013). A relationship between net ultrafiltration and MMP-2 or PAI-1 is absent (Lopes Barreto 2013).

Besides correlating peritoneal transport parameters with MMP-2 and PAI-1, linear regression analyses have also been executed with other peritoneal effluent markers. Research findings suggested no or less active participation of the mesothelium in the production of MMP-2 or PAI-1 in long-term incident PD patients. Even though moderate, the total variance of 22 % and 32 % for, respectively, MMP-2 and PAI-1 can be explained by differences in IL-6 levels (Lopes Barreto 2013). This is in line with pathophysiological findings reporting on inflammatory conditions and loss in mesothelial cell function preceding fibrotic processes.

Effect of PD Solutions on Levels of MMP-2 and PAI-1

With the introduction of novel PD solutions, the aim has also been to objectify the effect of conventional dialysis solutions high in glucose versus the more biocompatible PD solutions in terms of peritoneal membrane integrity. However, these studies have only been performed with established effluent biomarkers and currently with effluent MMP-2 as well. Unfortunately, effluent PAI-1 has not yet been incorporated. The first study addressing this question was performed a decade ago in a number of 13 prevalent PD patients with a follow-up duration of 21 months. The authors concluded that MMP-2 levels were decreased in patients treated with the more biocompatible PD solutions (Nishina et al. 2004). A second study, characterized by a cross-sectional design, compared a glucose-based PD solution with icodextrin. The study population consisted of 16 prevalent

PD patients who received both solutions in an 8-h overnight dwell divided over 2 days. The dialysate exchange with icodextrin had significantly higher levels of MMP-2 in comparison to the 2.27 % glucose-based dwell (Minami 2007). More recently, the BalANZ trial evaluated the influence of conventional dialysis solution on the time course of effluent MMP-2 versus neutral pH PD solutions low in glucose degradation products. Solely incident PD patients with archived effluent specimens were included in the study ($n = 178$). Their findings indicated increasing levels of effluent MMP-2, irrespective of the type of PD solution (Cho et al. 2016).

Peritoneal Effluent Levels of MMP-2 and PAI-1 During PD-Related Complications

Even though peritonitis has a cure rate of more than 88 %, it still remains a frequently encountered complication in PD (van Esch et al. 2014). In general it is advocated to measure peritoneal effluent biomarkers after resolution of a peritonitis episode as the laboratory results in the acute phase are usually elevated. Nevertheless, the behavior of effluent markers has been investigated right before the onset of peritonitis as well as during a peritonitis episode. During inflammation the vascular permeability is increased and the fibrinolytic activity is altered (Sitter et al. 1995). Measurements of effluent PAI-1 on PD-related peritonitis have been performed in adult as well as pediatric PD patients. Still, no data is available describing MMP-2 in pediatric PD-related peritonitis.

In pediatric PD patients, the role of the fibrinolytic system has been investigated as well. It indicated that stable pediatric and adult PD patients have similar concentrations of effluent PAI-1 (Lin et al. 1995; Goedde 1997). To the same extent both populations have significantly elevated effluent PAI-1 levels when experiencing a peritonitis episode (Goedde 1997; de Boer et al. 1999). Concerning effluent MMP-2, median values approximate 250 ng/mL and 160 ng/mL for, respectively, PD patients with and without infectious peritonitis when measured by gelatin zymography (Hirahara et al. 2007). However, contrary results are found in the activity of MMP-2 where no differences were detected between peritonitis-free PD patients and those with peritonitis. Moreover, an alteration in effluent MMP-2 activity between the onset and recovery of peritonitis was absent (Fukudome et al. 2001).

Frequent peritonitis episodes are believed to be one of the major risk factors for the development of EPS. Since effluent MMP-2 and PAI-1 are recently gauged to reflect the degree of intraperitoneal fibrosis and due to the rarity of EPS, current literature relating these candidate markers to EPS is limited to two investigations: a single- and multicenter study. A Japanese multicenter study aimed to assess the potential of MMP-2 as indicator of the progression to EPS. For this purpose, the authors stratified adult PD patients with peritoneal injury into four categories, including patients identified as EPS cases, and added a control group. Subsequently, they measured MMP-2 by means of an enzyme-linked immunosorbent assay. The cross-sectional analysis demonstrated elevated levels of effluent MMP-2 in those with overt peritoneal damage (Hirahara et al. 2007). The objective of the single-

center study was to describe time trends and to evaluate the clinical validity of effluent MMP-2 and PAI-1 in 4 years preceding EPS diagnosis. This study followed a nested case-control design including 10 patients diagnosed with EPS and 34 long-term controls. The same detection method as in the multicenter study was used for both effluent markers. Interestingly, the time course of MMP-2 illustrated no difference between the two groups, whereas EPS patients exhibited persistent elevated levels of PAI-1 with an increasing tendency when compared with long-term PD patients. In addition, diagnostic accuracy measures indicated the potential for effluent PAI-1 to detect preclinical EPS. The discriminative capacity for effluent MMP-2 was restricted to 1 year prior to the diagnosis of EPS (Lopes Barreto et al. 2014).

Clinical Relevance of Peritoneal Effluent MMP-2 and PAI-1

From the preliminary literature of MMP-2 in EPS follows that MMP-2 is unlikely to be significant as a biomarker that can be used for the early detection of EPS. However, as all physiological and pathological processes require remodeling of the ECM for cell migration, a significant application for MMP-2 in short-term consequences of PD should not be ruled out. This is supported by its involvement in EMT illustrating that MMP-2 is a necessary component during the transdifferentiation of mesothelial cells. For this reason it would be of interest to assess the association of effluent MMP-2 with PD technique failure due to peritoneal membrane dysfunction.

The potential application of effluent PAI-1 resides more in the purpose of a screening or diagnostic tool for EPS. The capacity of effluent PAI-1 to discriminate between long-term PD patients and those who will develop EPS is not of neglectable magnitude. A histological study on the distribution and localization of PAI-1 in peritoneal tissue within the PD population would be of great relevance. Furthermore, such explorations would have the ability to objectify the degree of concordance between the actual measured effluent levels of PAI-1 and its expression in peritoneal tissue. Effluent PAI-1 could also be utilized as an effluent biomarker to monitor progression of PD therapy, as the development of interstitial fibrosis is process of large lead time.

Conclusions

Intraperitoneal events that occur as a consequence of PD therapy are multifactorial processes. The use of effluent biomarkers would facilitate a noninvasive strategy to monitor PD therapy and support the early identification of PD-related outcomes. This chapter has given insight into the biological implications and clinical utility of MMP-2 and PAI-1 as biomarkers in PD. It is obvious that beforehand implementation in routine patient care external validation is necessary with respect to their clinical validity and utility. Nevertheless, a panel of effluent biomarkers including

MMP-2 or PAI-1 in conjunction with a routine peritoneal function test should be utilized for optimal chronic PD patient care.

Summary Points

- This chapter focuses on the biological implications and clinical utility of matrix metalloproteinase-2 (MMP-2) and plasminogen activator inhibitor-1 (PAI-1) in peritoneal dialysis (PD) therapy.
- The peritoneal effluent is the most clinically relevant specimen in PD.
- MMP-2 is a 72 kDa gelatinase that is involved in tissue remodeling.
- PAI-1 is a 50 kDa member of the serine protease inhibitors (SERPINs) that inhibits fibrinolysis and proteolysis.
- Peritoneal effluent MMP-2 and PAI-1 have recently been gauged as biomarkers for peritoneal membrane alterations.
- Current literature is limited with respect to the assessment of MMP-2 and PAI-1 clinical validity and utility.
- Incorporating peritoneal effluent biomarkers in routine PD patient care, alongside peritoneal function tests, will lead to a more personalized medicine.

References

Angleton P, Chandler WL, Schmer G. Diurnal variation of tissue-type plasminogen activator and its rapid inhibitor (PAI-1). Circulation. 1989;79:101–6.

Arikan H, Koc M, Tuglular S, Ozener C, Akoglu O. Elevated plasma levels of PAI-1 predicts cardiovascular events and cardiovascular mortality in prevalent peritoneal dialysis patients. Ren Fail. 2009;31:438–45.

Bode W, Fernandez-Catalan C, Tschesche H, et al. Structural properties of matrix metalloproteinases. Cell Mol Life Sci. 1999;55:639–52.

Borden P, Heller RA. Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. Crit Rev Eukaryot Gene Expr. 1997;7:159–78.

Breborowicz A, Karon RJ, Martis L, et al. In vitro stimulation of the effect of peritoneal dialysis solution on mesothelial cells. Am J Kidney Dis. 1997;29:404–9.

Chang C, Werb Z. The many faces of metalloproteinases: cell growth, invasion, angiogenesis and metastasis. Trends Cell Biol. 2001;11:S37–43.

Cho Y, Johnson DW, Vesey DA, Hawley CM, Pascoe EM, Clarke M, Topley N; balANZ Trial Investigators. Higher Dialysate Matrix Metalloproteinase-2 Levels Are Associated with Peritoneal Membrane Dysfunction. Perit Dial Int. 2016;36:16–25.

Chung AW, Yang HH, Kim JM, et al. Upregulation of matrix metalloproteinase-2 in the arterial vasculature contributes to stiffening and vasomotor dysfunction in patients with chronic kidney disease. Circulation. 2009;120:792–801.

de Boer AW, Levi M, Reddingius RE, et al. Intraperitoneal hypercoagulation and hypofibrinolysis is present in childhood peritonitis. Pediatr Nephrol. 1999;13:284–7.

Del Peso G, Jiménez-Heffernan JA, Bajo MA, et al. Epithelial-to-mesenchymal transition of mesothelial cells is an early event during peritoneal dialysis and is associated with high peritoneal transport. Kidney Int. 2008;73:S26–33.

Dellas C, Loskutoff DJ. Historical analysis of PAI-1 from its discovery to its potential role in cell motility and disease. Thromb Haemost. 2005;93:631–40.

Dhillon OS, Khan SQ, Narayan HK, et al. Matrix metalloproteinase-2 predicts mortality in patients with acute coronary syndrome. Clin Sci (Lond). 2010;118:249–57.
Dubin R, Cushman M, Folsom AR, et al. Kidney function and multiple hemostatic markers: cross sectional associations in the multi-ethnic study of atherosclerosis. BMC Nephrol. 2011;12:3.
Fukudome K, Fujimoto S, Sato Y, Hisanaga S, Eto T. Peritonitis increases MMP-9 activity in peritoneal effluent from CAPD patients. Nephron. 2001;87:35–41.
Galis ZS, Kranzhofer R, Fenton JW, et al. Thrombin promotes activation of matrix metalloproteinase-2 produced by cultured vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 1997;17:483–9.
Goedde M, Sitter T, Schiffl H, et al. Coagulation and fibrinolysis-related antigens in plasma and dialysate of CAPD patients. Perit Dial Int. 1997;17:162–6.
Haslinger B, Mandl-Weber S, Sitter T. Thrombin suppresses matrix metalloproteinase-2 activity and increases tissue inhibitor of metalloproteinase-1 synthesis in cultured human peritoneal mesothelial cells. Perit Dial Int. 2000;20:778–83.
Hekman CM, Loskutoff DJ. Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. J Biol Chem. 1985;260:11581–7.
Hekman CM, Loskutoff DJ. Kinetic analysis of the interactions between plasminogen activator inhibitor 1 and both urokinase and tissue plasminogen activator. Arch Biochem Biophys. 1988;262:199–210.
Hirahara I, Inoue M, Okuda K, et al. The potential of matrix metalloproteinase-2 as a marker of peritoneal injury, increased solute transport, or progression to encapsulating peritoneal sclerosis during peritoneal dialysis – a multicentre study in Japan. Nephrol DIal Transplant. 2007;22:560–7.
Hirahara I, Inoue M, Umino T, et al. Matrix metalloproteinase levels in the drained dialysate reflect the peritoneal solute transport rate: a multicentre study in Japan. Nephrol Dial Transplant. 2011;26:1695–701.
Holmdahl S, Falkenberg M, Ivarsson M, et al. Plasminogen activators and inhibitors in peritoneal tissue. APMIS. 1997;105:25–30.
Hsu TW, Kuo KL, Hung SC, et al. Progression of kidney disease in non-diabetic patients with coronary artery disease: predictive role of circulating matrix metalloproteinase-2, -3, and -9. PLoS One. 2013;8:e70132.
Irish AB. Plasminogen activator inhibitor-1 activity in chronic renal disease and dialysis. Metabolism. 1997;46:36–40.
Jain AK, Blake P, Cordy P. Global trends in rates of peritoneal dialysis. J Am Soc Nephrol. 2012;23:533–44.
Kaizuka M, Yamabe H, Osawa H, et al. Thrombin stimulates synthesis of type IV collagen and tissue inhibitor of metalloproteinase-1 by cultured mesangial cells. J Am Soc Nephrol. 1999;10:1516–23.
Katsutani M, Ito K, Masaki T, et al. Glucose-based PD solution, but not Icodextrin-based PD solution, induces plasminogen activator inhibitor-1 and tissue-type plasminogen activator in human peritoneal mesothelial cells via ERK1/2. Ther Apher Dial. 2007;9:173–81.
Khasigov PZ, Podobed OV, Ktzoeva SA, et al. Matrix metalloproteinases of normal human tissues. Biochemistry (Mosc). 2001;66:130–40.
Kim KJ, Yang WS, Kim SB, et al. Fibrinogen and fibrinolytic activity in CAPD patients with atheroslerosis and its correlation with serum albumin. Perit Dial Int. 1997;17:157–61.
Klein T, Bischoff R. Physiology and pathophysiology of matrix metalloproteases. Amino Acids. 2011;41:271–90.
Kolesnyk I, Dekker FW, Boeschoten EW, et al. Tine-dependent reasons for peritoneal dialysis technique failure and mortality. Perit Dial Int. 2010;30:170–7.
Kossakowska AE, Edwards DR, Prusinkiewicz C, et al. Interleukin-6 regulation of matrix metalloproteinase (MMP-2 and MMP-9) and tissue inhibitors of matrix metalloproteinase (TIMP-1) expression in malignant non-Hodgkin's lymphomas. Blood. 1999;94:2080–9.
Lambers JW, Cammenga M, Konig BW, et al. Activation of human endothelial cell-type plasminogen activator inhibitor (PAI-1) by negatively charged phospholipids. J Biol Chem. 1987;262:17492–6.

Law RH, Zhang Q, McGowan S, et al. An overview of the serpin superfamily. Genome Biol. 2006;7:216.
Lijnen HR. Pleiotropic functions of plasminogen activator inhibitor-1. J Thromb Haemost. 2005;3:35–45.
Lin JJ, Singhal K, Parton L, et al. Correlations between plasminogen activator inhibitor-1 and peritoneal transport in paediatric CCPD patients. Perit Dial Int. 1995;15:246–51.
Lindahl TL, Sigurdardottir O, Wiman B. Stability of plasminogen activator inhibitor 1 (PAI-1). Thromb Haemost. 1989;62:748–51.
Lopes Barreto D, Krediet RT. Current status and practical use of effluent biomarkers in peritoneal dialysis. Am J Kidney Dis. 2013;62:823–33.
Lopes Barreto D, Coester AM, Struijk DG, et al. Can effluent matrix metalloproteinase and plasminogen activator inhibitor-1 be used as biomarkers of peritoneal membrane alterations in peritoneal dialysis patients? Perit Dial Int. 2013;33:529–37.
Lopes Barreto D, Struijk DG, Krediet RT. Peritoneal effluent MMP-2 and PAI-1 in encapsulating peritoneal sclerosis. Am J Kidney Dis. 2015;65:748–53.
Loskutoff DJ, Sawdey M, Mimuro J. Type 1 plasminogen activator inhibitor. Prog Hemost Thromb. 1989;9:87–115.
Mandl-Weber S, Haslinger S, Sitter T. Thrombin upregulates production of plasminogen activator inhibitor type 1 in human peritoneal mesothelial cells. Perit Dial Int. 1999;19:319–24.
Mandl-Weber S, Haslinger S, Schalkwijk CG, et al. Early glycated albumin, but not advanced glycated albumin, methylglyoxal, or 3-deoxyglucosone increases the expression of PAI-1 in human peritoneal mesothelial cells. Perit Dial Int. 2001a;21:487–94.
Mandl-Weber S, Haslinger S, Lederer SR, et al. Heat-killed microorganisms induce PAI-1 expression in human peritoneal mesothelial cells: role of interleukin-1α. Am J Kidney Dis. 2001b;37:815–9.
Margetts PJ, Bonniaud P, Liu L, et al. Transient overexpression of TGF-beta1 induces epithelial mesenchymal transition in the rodent peritoneum. J Am Soc Nephrol. 2005;16:425–36.
Marshall BC, Santana A, Xu QP, et al. Metalloproteinases and tissue inhibitor of metalloproteinases in mesothelial cells. J Clin Invest. 1993;91:1792–9.
Martin J, Yung S, Robson RL, et al. Production and regulation of matrix metalloproteinases and their inhibitors by human peritoneal mesothelial cells. Perit Dial Int. 2000;20:524–33.
Mattana J, Margiloff L, Sharma P, et al. Oxidation of the mesangial matrix metalloproteinase-2 impairs gelatinolytic activity. Inflammation. 1998;22:269–76.
Mimuro J, Schleef RR, Luskutoff DJ. Extracellular matrix of cultured bovine aortic endothelial cells contains functionally active type 1 plasminogen activator inhibitor. Blood. 1987;70:721–8.
Minami S, Hora K, Kamijo Y, et al. Relationship between effluent levels of b2m and peritoneal injury markers in 7.5% icodextrin-based peritoneal dialysis solution. Ther Apher Dial. 2007;11:296–300.
Mujais S, Nolph K, Gokal R, et al. Evaluation and management of ultrafiltration problems in peritoneal dialysis. Perit Dial Int. 2000;20:S5–21.
Nagano M, Fukami K, Yamagishi S, et al. Circulating matrix metalloproteinase-2 is an independent correlate of proteinuria in patients with chronic kidney disease. Am J Nephrol. 2009;29:109–15.
Nagase H, Ogata Y, Suzuki K, et al. Substrate specificities and activation mechanisms of matrix metalloproteinases. Biochem Soc Trans. 1991;19:717–8.
Nishina M, Endoh M, Suzuki D, et al. Neutral-pH peritoneal dialysis solution improves peritoneal function and decreases matrix metalloproteinase-2 (MMP-2) in patients undergoing continuous ambulatory peritoneal dialysis (CAPD). Clin Exp Nephrol. 2004;8:339–43.
Opatrný K Jr, Opatrná S, Vít L, Opatrný K. Tissue-type plasminogen activator (tPA) and its inhibitor (PAI-1) in patients treated with continuous ambulatory peritoneal dialysis. Am J Nephrol. 1998;18:186–92.
Pawlak K, Pawlak D, Mysilwiec M. Oxidative stress effects fibrinolytic system in dialysis ureamic patients. Thromb Res. 2006;117:517–22.

Pawlak K, Tankiewicz J, Mysliwiec M, et al. Systemic Levels of MMP-2/TIMP2 and cardiovascular risk in CAPD patients. Nephron Clin Pract. 2010;115:c251–8.
Preston GA, Barrett CV, Alcorta DA, et al. Serum matrix metalloproteinases MMP-2 and MMP-3 levels in dialysis patients vary independently of CRP and IL-6 levels. Nephron. 2002;92:817–23.
Rerolle JP, Hertig A, Nguyen G, et al. Plasminogen activation inhibitor-1 is a potential target in renal fibrogenesis. Kidney Int. 2000;58:1841–50.
Rougier JP, Guia S, Hagege J, et al. PAI-1 secretion and matrix deposition in human peritoneal mesothelial cell cultures: transcriptional regulation by TGF-beta 1. Kidney Int. 1998;54:87–98.
Sancho E, Declerck PJ, Price NC, et al. Conformational studies on plasminogen activator inhibitor (PAI-1) in active, latent, substrate, and cleaved forms. Biochemistry. 1995;34:1064–9.
Selgas R, Cuesta MV, Riñon C, Romero JR, de Alvaro F, Bajo MA, Sanchez-Sicilia L. Tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-I (PAI-I) levels in plasma and peritoneal effluent in patients on CAPD. Adv Perit Dial. 1992;8:160–5.
Sitter T, Spannagl M, Schiffl H, et al. Imbalance between intraperitoneal coagulation and fibrinolysis during peritonitis of CAPD patients: the role of mesothelial cells. Nephrol Dial Transplant. 1995;10:677–83.
Sitter T, Sauter M, Haslinger B. Modulation of fibrinolytic system components in mesothelial cells by hyaluronan. Perit Dial Int. 2003;23:222–7.
Tomura S, Nakamura Y, Doi M, et al. Fibrinogen, coagulation factor VII, tissue plasminogen activator, plasminogen activator inhibitor-1 and lipid as cardiovascular risk factors in chronic hemodialysis and continuous ambulatory peritoneal dialysis patients. Am J Kidney Dis. 1996;27:848–54.
van de Luijtgaarden MW. Starting renal replacement therapy in end-stage renal disease patients [PhD thesis]. Amsterdam: University of Amsterdam; 2014.
van de Luijtgaarden MW, Noordzij M, Stel VS, et al. Effects of comorbid and demographic factors on dialysis modality choice and related patient survival in Europe. Nephrol Dial Transplant. 2011;26:2940–7.
van Esch S, Krediet RT, Struijk DG. 32 years' experience of peritoneal dialysis-related peritonitis in a university hospital. Perit Dial Int. 2014;34:162–70.
Vipond MN, Whawell SA, Thompson JN, et al. Peritoneal fibrinolytic activity and intra-abdominal adhesions. Lancet. 1990;335:1120–2.
Wang W, Viappiani S, Sawicka J, et al. Inhibition of endogenous NO in the heart enhances MMP-2 release. Br J Pharmacol. 2005;145:43–9.
Weinhandl ED, Foley RN, Gilbertson DT, et al. Propensity-matched mortality comparison of incident hemodialysis and peritoneal dialysis patients. J Am Soc Nephrol. 2010;21:499–506.
Wong W, DeVito J, Nguyen H, et al. Chronic humoral rejection of human kidney allografts is associated with MMP-2 accumulation in podocytes and its release in the urine. Am J Transplant. 2010;10:2463–71.
Yasmin, Wallace S, McEniery CM, et al. MMP-9, MMP-2, and serum elastase activity are associated with systolic hypertension and arterial stiffness. Arterioscler Thromb Vasc Biol. 2005;25:372–8.
Zubac DP, Wentzel-Larsen T, Seidal T, et al. Type 1 plasminogen activator inhibitor (PAI-1) in clear cell renal cell carcinoma (CCRCC) and its impact on angiogenesis, progression and patient survival after radical nephrectomy. BMC Urol. 2010;10:20. doi:10.1186/1471-2490-10-20.
Zucker S, Conner C, DiMassmo BI, et al. Thrombin induces the activation of progelatinase A in vascular endothelial cells. Physiologic regulation of angiogenesis. J Biol Chem. 1995;270:23730–2378.

Part IV

Molecular, Cellular, and Histological Variables

Assessing Fibrosis in Kidney Biopsies

41

Behtash Ghazi Nezami and Alton B. Farris

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Abstract

Renal interstitial fibrosis and tubular atrophy (IFTA) is one of the primary end points of kidney injury, and accurate IFTA quantitation in biopsy samples is crucial in establishing the diagnosis and assessing disease severity. Therefore, knowing the basic procedures in the preparation of biopsy for IFTA and specific staining techniques available for IFTA is pivotal to the pathologists' practice. This

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chapter reviews the mechanisms of IFTA pathogenesis pertinent to biopsy evaluation and the common techniques used to evaluate biopsies for IFTA. The challenges facing IFTA evaluation in biopsies and recent technical developments in this field are discussed.

Keywords

Kidney/renal fibrosis • Epithelial–mesenchymal transition • Myofibroblast • Morphometry • Masson's trichrome stain • Picrosirius red stain

Abbreviations

BMP	Bone morphogenic protein
CCR-2	C-C chemokine receptor type 2
CKD	Chronic kidney disease
DC	Dendritic cell
ECM	Extracellular matrix
EMR-1	EGF-like module-containing mucin-like hormone receptor-like 1
EMT	Epithelial-mesenchymal transition
EndoMT	Endothelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ERKs	Extracellular-signal-regulated kinases
FGF-2	Fibroblast growth factor 2
FSP-1	Fibroblast-specific protein 1
GFR	Glomerular filtration rate
GSK	Glycogen synthase kinase
HGF	Hepatocyte growth factor
HIF	Hypoxia-induced factor
IF	Interstitial fibrosis
IFTA	Interstitial fibrosis/tubular atrophy
IHC	Immunohistochemical
ILK	Integrin-linked kinase
JAK/STAT	Janus kinase/signal transducer and activator of transcription
LTBP	Latent TGF-β binding protein
miRNA	MicroRNA
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor κB
PAPMS	Pathogen-associated molecular patterns
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
SMA	Smooth muscle actin
TA	Tubular atrophy
TEC	Tubular epithelial cell
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitors of metalloproteinases

TLR	Toll-like receptor
tPA	Tissue plasminogen activator
TSP-1	Thrombospondin-1
tTG	Tissue transglutaminase
USAG-1	Uterine sensitization-associated gene 1 (also known as sclerostin domain-containing protein 1)
VEGF	Vascular endothelial growth factor

Definitions

Birefringent Birefringence in the context of this article refers to colors that are somewhat iridescent or anomalous (Howie et al. 2008). This anomalous quality is imparted to tissue when viewed under polarized light due to a particular interaction of the polarized light with the tissue or a combination of the stain's interaction with the tissue. For example, a Sirius red stain of fibrotic tissue, particularly the collagen in this fibrotic tissue, has a yellowish birefringence when viewed under polarized light.

Computerized morphometry Tissue is imaged and then analyzed with computerized programs called algorithms to obtain quantitative measures of different parameters. For example, a positive pixel count algorithm tuned to detect the color of fibrotic tissue can be used to quantitate fibrotic areas.

Endothelial/mesenchymal transition (EMT) The change in endothelial phenotype during renal injury leading to fibrosis, in which endothelial cells lose their specific markers and acquire the mesenchymal phenotype, becoming more invasive with increased migratory abilities.

Epithelial/mesenchymal phenotype (EMP) The putative phenotypic change of epithelial cells leading to the loss of their cell polarity and adhesion surface proteins and gain of migratory properties to become mesenchymal stem cells.

Extracellular matrix (ECM) Connective tissue material that essentially serves as the framework for tissue and that is not contained within cells.

Fibrosis This term refers to the deposition of excess fibrous tissue within an organ and is derived from the Latin *fibra*, meaning "fiber," and the Greek -*osis*, meaning a condition or state [derived from Dictionary.com].

Genomics The analysis of the genetic state of a biologic specimen or set of biologic specimens. Genomics is one of the main "-*omics*" disciplines in which the suffix "-ics" derived from the Latin "-*ica*" and Greek "-*ika*" [derived from Dictionary.com].

IFTA Parenchymal contraction in which intervening tubulointerstitial parenchyma between obsolescent glomeruli have been lost and occupied by fibrotic matrix through interstitial fibrosis (IF), which is usually accompanied by tubular atrophy (TA).

microRNA Short single-stranded RNA molecules that regulate a wide range of genes posttranscriptionally by targeting mRNAs and are stable in tissues for extending periods of time.

Proteomics Large-scale study of structures and functions of proteins using protein purification techniques and mass spectrometry. Referring to proteome as the entire set of proteins of an organism or tissue. This is an example of another "-*omics*" discipline.

Sclerosis Refers to hardening or stiffening of tissue or organ, often due to a pathologic growth of fibrous tissue or chronic inflammation [derived from Dictionary.com]. The word is derived from the Greek *sclerosis*, which means "hardening."

Introduction

Interstitial extracellular matrix (ECM) accumulation is common to many chronic kidney diseases and contributes to loss of kidney function. After renal damage, a constellation of intra- and extrarenal cells are activated to limit the extent of tissue damage and start the healing process. Interstitial fibrosis is the result of such processes (Table 1), when a complete regeneration of the renal tissue cannot be achieved. Interstitial fibrosis (IF) mostly refers to the excessive and pathological deposition of ECM, which is often accompanied by tubular atrophy (TA) and is collectively termed IFTA (Liu 2006; Boor et al. 2010; Zeisberg and Neilson 2010;

Table 1 Key facts of renal fibrosis pathogenesis

A variety of cells contribute to interstitial fibrosis, including fibroblasts, dendritic cells, lymphocytes, and monocyte/macrophages
Tubular epithelial cells possibly contribute to fibrosis as they are damaged and undergo a putative epithelial-mesenchymal transition (EMT)/phenotype (EMP). Endothelial cells also possibly contribute to fibrosis through an endothelial to mesenchymal transition (EndoMT). However, despite evidence for these processes, some question EMT and EndoMT
Cellular damage is thought to contribute to the release of molecular mediators, many of which are pro-fibrotic, including tumor growth factor beta (TGF-β), a major fibrogenic and inflammatory cytokine. Other growth factors include bone morphogenic protein (BMP), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF)
Fibrosis ultimately occurs through the deposition of extracellular matrix (ECM), including collagen, proteoglycans, and admixed ECM molecules such as fibronectin, biglycan, decorin, tissue transglutaminase (tTG), matrix metalloproteinase (MMP), tissue plasminogen activator (tPA), and laminin

This table lists the key facts of the cellular and molecular contributors to renal fibrosis

Farris and Colvin 2012; Farris and Alpers 2014). TA is characterized by the presence of small dilated and thin tubules with pale cytoplasm and thickened and irregularly contoured basement membranes. TA probably has distinct mechanisms than IF, related to blood flow restriction, glomerular filtration rate (GFR), or loss of tubular continuity. Many studies show a reciprocal correlation between kidney function and the IF extent. IF has been shown to predict the outcome of renal allograft (Choi et al. 2005; Farris et al. 2010; Meas-Yedid et al. 2011) and native kidney diseases such as IgA nephropathy (Working Group of the International IgA Nephropathy Network and the Renal Pathology Society et al. 2009).

Renal biopsies play major role in clinical diagnosis of IFTA. However, there is still controversy over the best qualitative and quantitative techniques for IFTA (Farris et al. 2014). Accurate IFTA measurement is required in numerous applications, including in comparison of renal allograft protocol biopsies, helping in the determination of prognosis in glomerular disease (e.g., IgA nephropathy and lupus nephritis), and pharmaceutical studies on therapeutic inhibition of IF (Liu 2006; Vilayur and Harris 2009). Furthermore, as stem cell therapy is gaining attention in renal fibrosis research, the need for establishing accurate and valid assessment techniques for fibrosis is more pronounced (Reinders et al. 2014; Choi et al. 2015).

Mechanisms of Renal Fibrosis

Normal renal interstitium consists of sparse cells, mainly fibroblasts and dendritic cells (DCs), embedded in ECM network. During and after renal injury (infection, ischemia, diabetes, allograft rejection, etc.), a constellation of mechanisms are activated to protect the damaged tissue and speed up regeneration (such as reepithelialization, epithelial barrier repair, regeneration after vascular injury, construction of the ECM skeleton, and recovering from mesangial damage). A variety of cells (Fig. 1) and molecular mechanisms (Fig. 2) contribute to IF.

IF is most likely driven by a lack of highly differentiated cells, which are replaced by scarring connective tissue. Tumor growth factor beta (TGF-β) is a major fibrogenic and inflammatory cytokine, produced by damaged native and inflammatory cells (Farris and Colvin 2012; Friedman et al. 2013). Tubular epithelial cells unable to regenerate get arrested in the G2/M phase and produce TGF-β (Yang et al. 2010), which causes augmented deposition of ECM proteins and renal fibrosis (Bottinger 2007). TGF-β-inducible integrins (e.g., αVβ6) ultimately act through integrin-linked kinases (ILK) and other mediators to produce collagen, contributing to the production of the ECM (Farris and Colvin 2012). ECM production is also affected by other systemic physiologic states, such as the renin/angiotensin system (Naito et al. 2010).

ECM accumulation usually occurs prior to chronic kidney disease (CKD) and contains sulfated and non-sulfated proteoglycans and glycosaminoglycans involved in IF and other major molecules such as types I and III collagen, fibronectin, biglycan, decorin (Boor et al. 2010; Zeisberg and Neilson 2010), tissue transglutaminase (tTG) (Huang et al. 2009), matrix metalloproteinase (MMP) (Wang

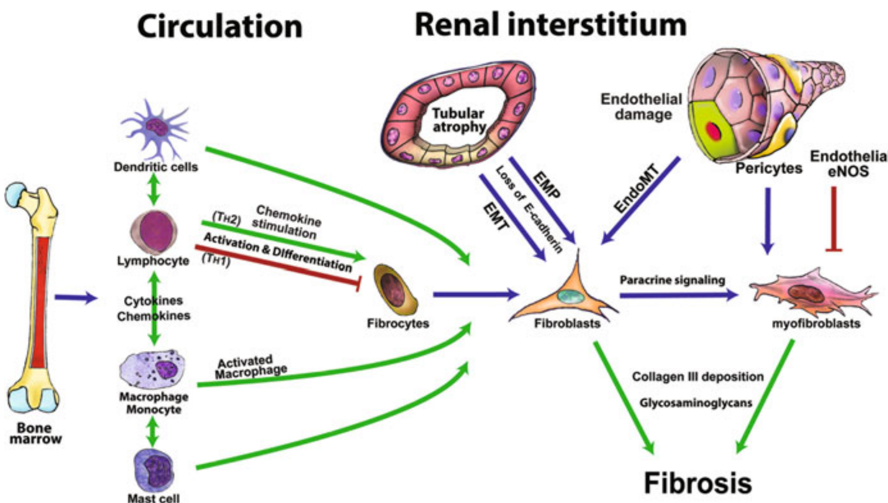

Fig. 1 Cells contributing to interstitial fibrosis: A variety of cells contribute to interstitial fibrosis, including renal vasculature, tubular cells, and inflammatory cells such as lymphocytes, monocyte/macrophages, mast cells, and dendritic cells. The renal tubules undergo changes to acquire epithelial–mesenchymal phenotype (EMP) and may undergo the process of epithelial–mesenchymal transition (EMT). The endothelium is possibly involved in a process of endothelial–mesenchymal transition (EndoMT). Inflammatory cells have important role in both the processes of EMT/EMP and EndoMT. Fibrocytes and pericytes can transform to fibroblasts and myofibroblasts. Fibroblasts/mesenchymal cells mediate the production of fibrosis and extracellular matrix (ECM) deposition and also may undergo a transition to a myofibroblastic phenotype through paracrine signaling from inflammatory cells, further increasing ECM deposition and fibrosis

et al. 2010), tissue plasminogen activator (tPA) (Yang et al. 2002), and laminin (Abrass et al. 2010). Proteoglycans fill the majority of renal extracellular interstitial space and act as a reservoir of pro-fibrotic growth factors, such as the latent forms of TGF-β or fibroblast growth factor 2 (FGF-2). Fibronectin and thrombospondin-1 (TSP-1) are adhesive glycoproteins involved in IF. Fibronectin accumulation is one of the first events during renal fibrosis (Eddy 1996).

Other mediators important in IF include Smads, bone morphogenic proteins (BMPs), particularly BMP-7, sclerostin domain-containing protein 1 (also known as uterine sensitization-associated gene 1 (USAG-1)), protein kinase C (PKC), extracellular-signal-regulated kinases (ERKs) (Sun et al. 2014), platelet-derived growth factor (PDGF), PDGF-β (Wilkinson et al. 2009), hepatocyte growth factor (HGF), the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, fibrinogen (Sorensen et al. 2011), and possibly toll-like receptors (TLRs). Smads act on ILK, stimulating glycogen synthase kinase (GSK) to produce β-catenin, which traverses into the nucleus to induce transcription that ultimately leads to fibrosis (Farris and Colvin 2012).

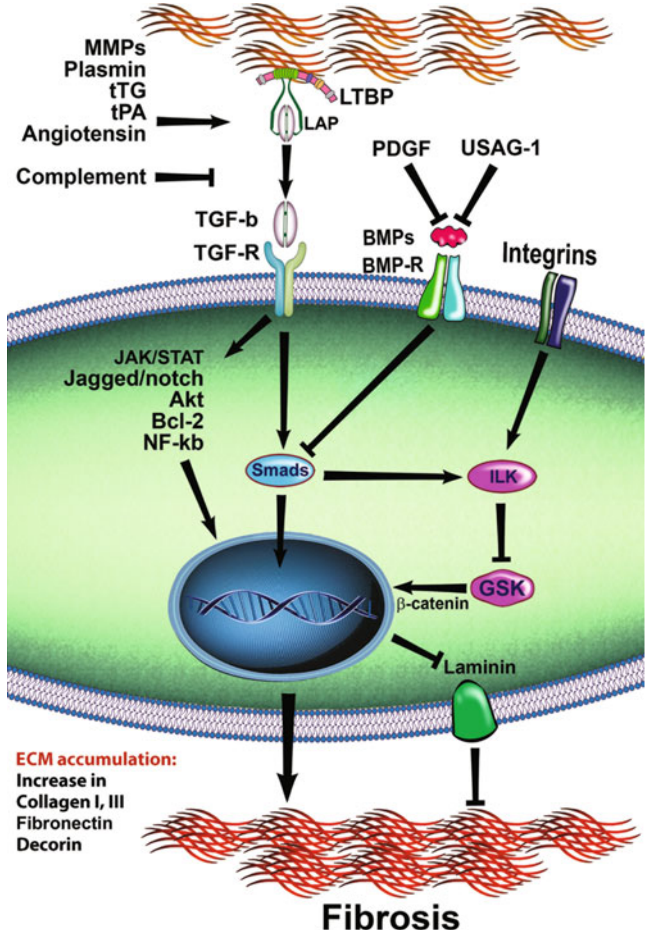

Fig. 2 Key molecular mechanisms contributing to interstitial fibrosis: A variety of molecular mechanisms contribute to interstitial fibrosis. Transforming growth factor (TGF-β) is released from activated inflammatory and damaged mesangial cells through interactions with matrix metalloproteinases (MMPs), plasmin, integrin, angiotensin, tissue plasminogen activator (tPA), and tissue transglutaminase (tTG). When it is released from inhibition by latent TGF-β-binding protein (LTBP) and latency-associated peptide (LAP), TGF-β binds the transforming growth factor receptor (TGF-R), activating intracellular signals such as the Smads, jagged/notch, Akt, Bcl-2, and NF-κB pathways. These lead to nuclear transcription, ultimately culminating in collagen and ECM production. In epithelial cells it may lead to epithelial-mesenchymal transition (EMT). Smads also increase fibrosis via integrin-linked kinase (ILK). ILK acts through glycogen synthase kinase (GSK) to produce β-catenin, which traverses into the nucleus to induce transcription. Integrins can also act through ILK in a similar manner. Bone morphogenic proteins (BMPs) generally restrict fibrosis. BMPs bind to their receptor (BMPR) to inhibit Smads, a process inhibited by uterine sensitization-associated gene 1 (USAG-1) and platelet-derived growth factor (PDGF)

Renal fibroblasts are the major constituent cells in ECM and are in charge of producing excessive collagen (Boor et al. 2010). Thrombospondin-1 stimulates fibroblast proliferation and migration in CKD and is correlated with the degree of tubulointerstitial fibrosis in rat models of renal fibrosis (Mason and Wahab 2003). Fibroblasts stain for vimentin (intermediate filament protein) and stain weakly for alpha smooth muscle actin (α-SMA). Activated fibroblasts stain for fibroblast-specific protein 1 (FSP-1). However, there are not any completely specific markers for fibroblasts that are widely utilized, and this makes the study of fibroblasts quite difficult (Xia et al. 2013; Farris and Alpers 2014).

Myofibroblasts secrete collagen and glycosaminoglycans and are activated by various mechanisms such as paracrine signals derived from lymphocytes and macrophages, autocrine factors, and pathogen-associated molecular patterns (PAMPS). Myofibroblasts have multiple potential origins with candidates being fibroblasts, fibrocytes, pericytes, and epithelial or endothelial cells (Humphreys et al. 2010). These cells share features with smooth muscle cells, including the expression of α-SMA, and contain vimentin, fibronectin, and S100A4 (also known as FSP-1) (Lin et al. 2008). Pericytes contribute to vascular reconstruction via tissue inhibitors of metalloproteinases (TIMPs) and ADAMTS1 (Schrimpf et al. 2012). Fibrocytes are derived from peripheral blood leukocytes (Pilling et al. 2009) producing ECM and expressing both hematopoietic (e.g., CD45) and stromal cell markers (e.g., type I collagen). Recent in vitro studies suggest that fibrocytes develop outside the kidney independent of infiltrating monocytes and rely on CCR2 for migration into target organs (Reich et al. 2013).

Endothelial cells and tubular epithelial cells (TECs) participate in the recruitment of circulating leukocyte populations and facilitating the inflammatory response in the injured kidney. TECs play major role in recruiting macrophages and lymphocytes via NF-κB and pro-inflammatory chemokines (Mezzano et al. 2004). Epithelial and endothelial cells increase the IF through a process of differentiation to myofibroblasts, in which they undergo a phenotypic conversion termed epithelial–mesenchymal or endothelial–mesenchymal transition (EMT/EndoMT) (LeBleu et al. 2013). In this process they lose their markers, such as E-cadherin, and acquire mesenchymal markers, such as vimentin and α-SMA (Zeisberg and Neilson 2010; Friedman et al. 2013). These markers can be used to determine EMT process in the tissue (Wang et al. 2015). However, many experts have questioned this migration feature of EMT (Kriz et al. 2011). Endothelial cells can suppress inflammation via endothelial nitric oxide synthase (eNOS) production in injured sites. VEGF alleviates fibrosis (Lian et al. 2011), and hypoxia promotes fibrosis through multiple mediators including hypoxia-inducible factor-1α (HIF-1α) (Higgins et al. 2008).

A wide range of mononuclear inflammatory cells can be identified in normal renal interstitium, including DCs, macrophages, and lymphocytes (T cells, B cells, and natural killer cells) (Paust et al. 2011). Lymphocytes play a variety of roles in the development of IFTA (Tapmeier et al. 2010). Type 2 T-helper cells (T_H2) produce mostly pro-fibrotic cytokines, inducing differentiation of fibrocytes, triggering macrophage recruitment and inflammatory response (Liu et al. 2012), and in contrast, type 1 T-helper cells (T_H1) inhibit differentiation of fibrocytes (Niedermeier

et al. 2009). Microarray analysis of renal allografts has shown increased T cell and natural killer gene sets in IFTA development (Scian et al. 2011). High T cell and macrophage but not B cell infiltration is associated with low IL-10 expression, which confers susceptibility to IFTA (Khan et al. 2010).

Monocyte/macrophages are heterogeneous, consisting of both infiltrating and resident cells (Anders and Ryu 2011). Renal DCs seem to mediate the recruitment of other cell types. During exposure to bacteria, DCs generate chemokines to attract effector cells, such as neutrophils (Rogers et al. 2014). Macrophages or DCs can be identified by F4/80 (also known as EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1), CD11b+ (integrin α_M), and the DC marker CD11c+ (integrin α_x). However, these markers are not specific (Rogers et al. 2014). The degree of macrophage infiltration correlates with both the severity of damage and extent of IF (Eardley et al. 2008). Some subsets of bone marrow-derived monocytes such as CD11b+ cells may attenuate fibrosis (Semedo et al. 2010).

Mast cells express immune-related surface receptors and store inflammatory cytokines, which give them the ability to immediately release pro-fibrosis mediators such as TGF-β and MMPs (Snelgrove et al. 2011). There is a correlation between the accumulation of mast cells and the degree of renal interstitial fibrosis (Mack and Rosenkranz 2009).

Interstitial Fibrosis Assessment

Interstitial fibrosis is typically assessed in a qualitative and a quantitative manner. A variety of methods can be used (Fig. 3) and are discussed below. Briefly, microscopy is one of the primary methods of this assessment since it assists in the qualitative measurement of fibrosis, and it also allows quantitation of the degree of fibrosis. Furthermore, molecular methods are becoming more widespread and will also likely aid in both qualitative and quantitative assessment of fibrosis (Table 2).

Fibrosis Patterns in Biopsies

It is important to note that depending on the pathology there are different patterns of IFTA. Table 3 highlights the features of IFTA in different pathologic conditions.

Despite these associations, there is often an essentially nonspecific pattern of fibrosis in renal biopsies of patients with chronic kidney disease, including diffuse or patchy fine IF surrounding tubules, which can be either normal or atrophic. This is associated with either diffuse or focal disease of glomeruli, tubules, or vessels (Farris and Colvin 2012; Farris and Alpers 2014; Haas et al. 2014). It is important to note that even though the clinical emphasis is usually put on cortical IF, medullary IF is likely happening in parallel to cortical IF and epithelial loss (Farris et al. 2013).

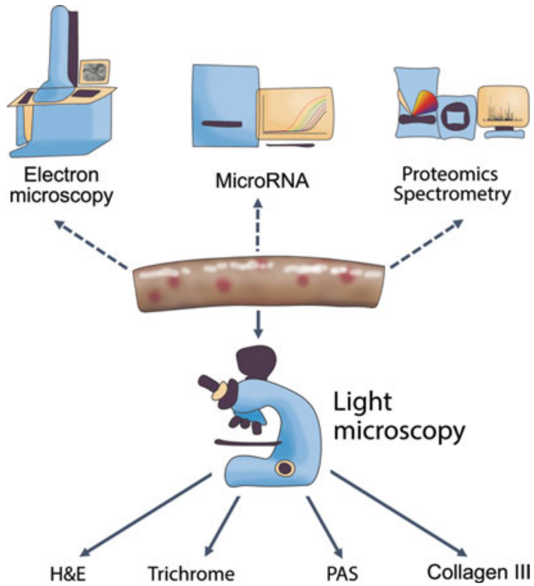

Fig. 3 Methods for interstitial fibrosis assessment: Various methods are available for assessing interstitial fibrosis. Renal biopsy samples are routinely prepared for light, immunofluorescent, and electron microscopic studies. For clinical evaluation of renal fibrosis, H&E, trichrome, Periodic acid–Schiff (PAS), picrosirius red, and collagen III immunohistochemical (IHC) stainings are commonly used. Other methods have been used for research only purposes to study renal fibrosis, such as electron microscopy, microRNA analysis, or transcriptomics or proteomics. However, these methods are not yet used for routine clinical evaluation of renal fibrosis

Staining Techniques and Light Microscopy

The preferred approach in staining for IFTA is using multiple complementary techniques to confirm the degree of fibrosis, because each technique has shortcomings and advantages. The routine techniques to obtain sample and assess renal fibrosis and tubular atrophy may be different between laboratories. Hence, a generally accepted algorithm is usually applied in each laboratory (Agarwal et al. 2013).

In the assessment of IFTA, paraffin embedded kidney sections are often stained for hematoxylin and eosin (H&E), trichrome, periodic acid–Schiff (PAS), and methenamine silver. Other methods such as picrosirius red or immunohistochemistry for collagen (particularly collagen types I and III) or SMA can give more information depending on the specimen. Table 4 summarizes the characteristics, advantages, and disadvantages of currently popular stains for IFTA in routine clinical diagnosis and research. Trichrome highlights fibrosis by color, typically with a blue or green hue, depending on the trichrome method employed (e.g., Mallory’s, Masson’s, etc.). For quantitation, visual assessment of slides is the standard practice at many institutions (Moreso et al. 2001). Picrosirius red stain is also a commonly used method to detect

Table 2 Key facts of renal fibrosis assessment

Key facts
Biopsies are often performed to assess interstitial fibrosis, which is often accompanied by tubular atrophy (IFTA)
A variety of stains can be used to highlight interstitial fibrosis, including trichrome, Sirius red, and collagen III immunohistochemistry
Assessment of histologic stains can be combined with image analysis to provide an objective method for IFTA assessment
Numerous IFTA assessment methods have shown relationships with renal function measures (e.g., glomerular filtration rate). In this sense, IFTA assessment can act as a biomarker of renal disease
Molecular methods such as transcriptional profiling and proteomic approaches have also been used to assess renal fibrosis
Knowing the degree of fibrosis and its relationship to genomic, proteomic, and other *-omic* data helps an investigator go from an understanding of the disease state ("-osis") to understanding on a variety of *-omic* levels

This table lists the key facts of renal fibrosis assessment

Table 3 Interstitial fibrosis patterns in different pathologic conditions

Etiology	Fibrosis pattern
Aging	Subcapsular fibrosis due to marginal supply of the peripheral cortex
	Often accompanied by thickening of capillary loop basement membrane
	Secondary to the ischemia associated with peritubular capillary injury
Pyelonephritis	Diffuse fibrosis with severe loss of tubules
	In acute phase is associated with inflammatory infiltrates
	Relative preservation of the glomerular structure
Infarct	Broad with loss of tubules
Calcineurin inhibitor	Patchy and striped corresponding with TA
	Hyaline arteriopathy and glomerulosclerosis
	Preferential involvement of medullary rays – toxic injury to discrete segments
Chronic allograft injury	Loss of peritubular capillaries in parallel to IF
	Diminished supply of nutrients to the tubulointerstitium
Chronic obstruction	IFTA with relative glomerular sparing, atubular glomeruli, dilated tubules, and intratubular Tamm–Horsfall protein casts with extravasation into the interstitium
Diabetic nephropathy	Diffuse and homogeneous

Interstitial fibrosis and tubular atrophy (IFTA) occurs in specific patterns depending on the etiology of the IFTA, as shown above

fibrosis in tissues (Fig. 5). Picrosirius red is typically considered to be specific for collagen types I and III under polarized light in which the collagen has a birefringent yellow hue (Sund et al. 2004). Movat's pentachrome stain allows the assessment of collagen, proteoglycan, and elastic tissue content with a single-staining procedure. It imparts different colors to collagen fibers, glycosaminoglycans, elastic fiber, fibrin, nuclei, and muscle. There have been modifications for this staining to increase

Table 4 Staining techniques

Method	Description	Advantages	Disadvantages
Hematoxylin and eosin (H&E)	Performed for routine diagnosis	Routinely performed by most laboratories on nearly every specimen	Does not provide much contrast for tissue with IF
Periodic acid–Schiff (PAS)	Imparts a pinkish hue to glomerular and tubular basement membranes	Routinely performed by many laboratories on renal biopsies	Does not provide much contrast for tissue with IF
		Relatively inexpensive	
		Useful for assessing TA	
		Useful for tubulitis	
Masson's trichrome	Imparts a blue or green hue to ECM, including collagen	Routinely performed by many laboratories on renal biopsies	Reproducibility sometimes limited
		Relatively inexpensive Useful for assessing extracellular matrix	Possibly less sensitive in mild fibrosis
			Sensitive to the length of formalin fixation
Picrosirius red	Stains ECM, particularly collagen	Specific for collagen type I and III	Not widely used
	Examined with polarized and unpolarized light	High signal-to-noise ratio	Subject to polarized vs. unpolarized measurement discrepancies
	Polarization color may reflect the extent of cross-linking and the age of fibrosis (fine vs. large bundles) or density of fibers	Computerized image analysis possible	Dependent on operator performance
		Shown correlates with the interstitial volume fraction of the cortex.	Time consuming and expensive
			Difficult to standardize, lacks cross institutional validation
			Cannot differentiate between collagen type
Movat's pentachrome	Five-colored stain, imparting different colors to collagen fibers, glycosaminoglycans, elastic fibers, fibrin, nuclei, and muscle	Collagen content, proteoglycan content, and elastic tissue content with a single-staining procedure	Not routinely performed on renal biopsies and may not be available in all laboratories
		Reveals early and subtle changes in the interrelationships of many of the tissues	

(*continued*)

Table 4 (continued)

Method	Description	Advantages	Disadvantages
Collagen type III IHC	IHC utilizing antibody for collagen III	High signal-to-noise ratio	Not widely available
		Computerized image analysis possible	Difficult to standardize
			Dependent on operator's performance
α –SMA IHC	IHC utilizing antibody for smooth muscle actin (SMA)	Interstitial SMA can be detected in some cases of IFTA	May not be present in all types of IFTA

This table describes stains used for the assessment of interstitial fibrosis (IF) and tubular atrophy (TA). Most of these stains are considered to be either "special" histochemical stains or immunohistochemistry (IHC). For the assessment of IF, stains are typically considered to be useful if they highlight extracellular matrix (ECM) or collagen in particular

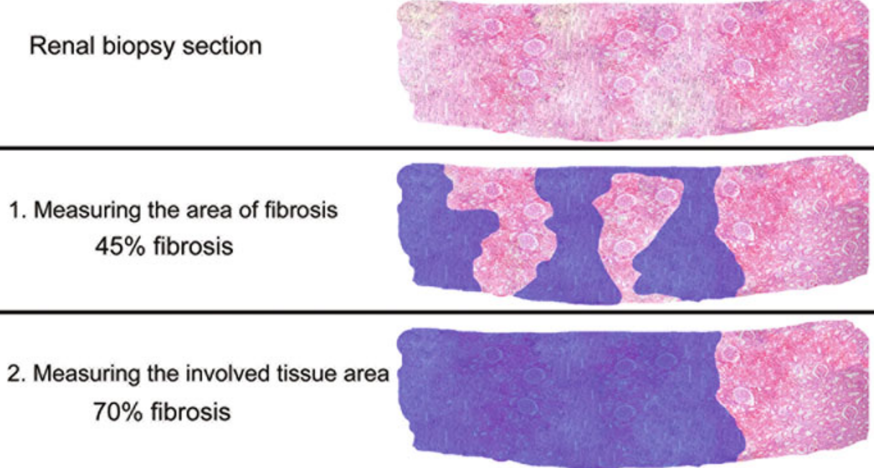

Fig. 4 Morphologic approaches for interstitial fibrosis assessment: Interstitial fibrosis can be approached in two basic ways by pathologists. (1) The fibrosis percentage taken as the percentage of tissue occupied by fibrosis excluding tubules and glomeruli and healthy islands of tissue, (2) assessing the percentage of the tissue that is abnormal

consistency and reliability and decrease preparation time (Doello 2014). Immunohistochemistry (IHC) staining can be used to identify particular protein or cell population in the biopsy sample, such as collagen I, III, and IV, smooth muscle

actin (e.g., α-SMA) (Choi et al. 2015), Smad7, E-cadherin (Liu et al. 2013), and CD11c+ cells (Kruger et al. 2004). Among the wide range of IHC stains available, collagen III (Satoh et al. 2001) is most often used to assess fibrosis (Fig. 5).

Computer-Based Morphometric Study

Morphometry techniques use computerized image analysis to measure the surface area affected by fibrosis. These computer-based methods include morphometry of slides stained with trichrome (Farris et al. 2011, 2014; Meas-Yedid et al. 2011), picrosirius red (Sund et al. 2004; Lattouf et al. 2014), and collagen III IHC (Farris et al. 2011, 2014; Farris and Colvin 2012) or a combination of staining techniques and automated analysis (Street et al. 2014). Analysis in some of these studies has shown correlation with GFR (Farris et al. 2011, 2014; Meas-Yedid et al. 2011); however, as shown in the studies by Farris et al. (2011), this may not improve upon the assessment made by the unaided human eye (Farris et al. 2011). As computerized scanners gain more widespread use in research and clinical settings, it is possible that computerized morphometry will integrate more in clinical standard practice. Commonly used software programs include NIH ImageJ (available at http://imagej.nih.

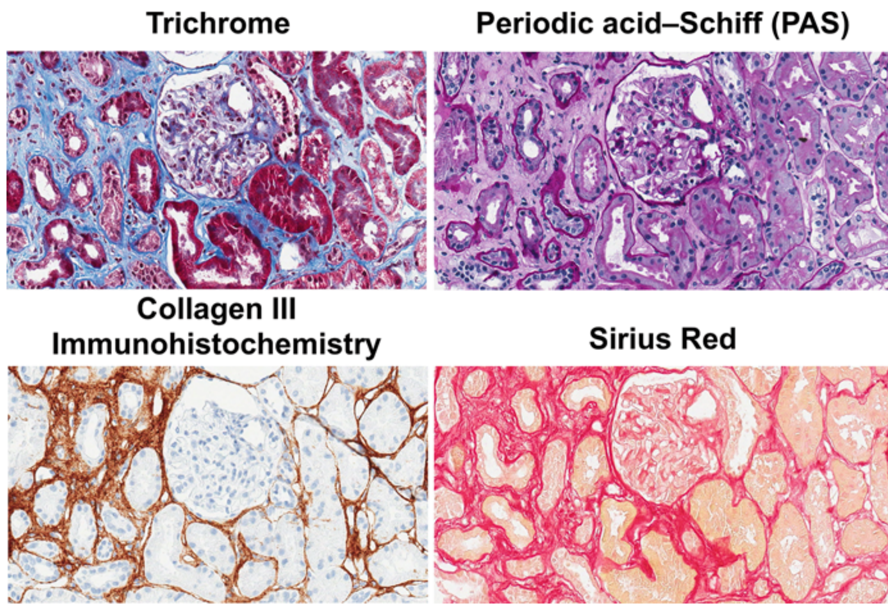

Fig. 5 Commonly used stains to assess renal fibrosis are shown: Fibrosis is visualized by different colors in each staining: *Blue* in trichrome, *light pink/purple* in PAS (vs. *dark pink/purple* for basement membranes), *dark brown* in collagen III immunohistochemistry, and *red* in picrosirius red stain. A glomerulus can be seen in the center of the images, and an area of fibrosis can be seen to the left of the glomerulus in each of the images (all at an original magnification of 200×)

gov/ij/), Fiji (available at http://fiji.sc/Fiji), Icy (available at http://icy.bioimageanalysis.org/), and proprietary programs that are stand-alone and/or are provided by microscope manufacturers (e.g., Aperio [Leica], Olympus, NIS-Elements [Nikon], MetaMorph, etc.). Whole slide scanners allow scanning of slides, often in an automated batch mode, which further facilitates the ease of this analysis (Farris et al. 2011, 2014).

Molecular Methods

A variety of molecular pathways can be probed through transcriptional profiling of gene expression (Maluf et al. 2008; Bunnag et al. 2009; Scian et al. 2011). Many approaches involve examining genes from set pathways together to determine which pathways predominate in IFTA, showing, for example, the importance of chronic inflammation in a study that examined immune response, cell-to-cell interaction, and inflammation pathways (Maluf et al. 2008). Other studies show the importance of specific derangements in the development of IFTA, showing that tissue injury and dedifferentiation are linked with functional deterioration (Bunnag et al. 2009). Older studies have focused on the analysis of DNA and RNA; however, newer studies have evaluated microRNAs (miRNAs), short single-stranded RNA molecules that regulate wide ranges of genes posttranscriptionally by targeting mRNAs (Zununi Vahed et al. 2014). MicroRNAs are stable in tissues for extended periods of time. So far a few miRNAs are the center of clinical attention, which may find implications in renal fibrosis assessment, especially in renal allograft tissue (Zununi Vahed et al. 2014; Chung and Lan 2015) with some miRNAs being pro-fibrotic and upregulated in renal fibrotic tissue and some miRNAs showing a decrease in IFTA (miR-107, 211, 204, 324, and 30a-3p) (Chung and Lan 2015).

Proteomic Approaches, Including Mass Spectrometry

There are a growing number of studies on fibrotic pathophysiological mechanisms using proteomic approaches in cell cultures, animal models, and human tissues (Klein et al. 2011; Prunotto et al. 2011). Many proteomic methods utilize techniques for protein fractionation and mass spectrometry (Konvalinka et al. 2010; Sethi et al. 2013). Proteomic methods can potentially be used on a variety of specimens, including urine, to provide noninvasive assessment of renal fibrosis. Current proteomic work has mainly explored in vitro systems. Databases, specific for normal and pathological human kidney proteomes, are available including normal glomeruli and medulla (e.g., http://www.hkupp.org/ and http://www.proteinatlas.org/humanproteome/kidney) (Habuka et al. 2014). However, despite the recent technical advancements and studies, proteomic biomarkers have not yet found direct implications in clinic (Konvalinka et al. 2010; Sethi et al. 2013).

Current techniques have specific shortcomings when it comes to IFTA evaluation as the whole kidney proteomic analysis does not provide accurate information

regarding localization (Walker et al. 2004), and, therefore, each kidney compartment should be separated prior to proteomic analysis (such as with laser capture microdissection). Still, the minute amount of available tissue severely limits exploration.

Fibrosis Measurement and Methodologies: Challenges and Promises

The measurement of IF has limitations including sampling issues and variable quality and quantity of the IF. Overall, there is no consensus regarding the best way to assess IF (Farris et al. 2014; Haas et al. 2014). Unfortunately, there is a lack of consistency in measuring fibrosis among pathologists, which stems from different conceptual ways of considering the percentage of fibrosis. Some pathologists consider IF percentage as the percent of overall tissue occupied by fibrous matrix, whereas others consider the area containing both fibrotic matrix and intact glomeruli and tubular structures. This is depicted in the Fig. 4. This discrepancy was highlighted in an interobserver variability study among numerous pathologists in multiple countries by Furness et al. (2003), and a recent study prompted by the Banff Allograft Pathology Conference also showed a great deal of variability in fibrosis assessment among pathologists (Farris et al. 2014). Discrepancies among pathologists involve the threshold of matrix needed in order to identify a region as being involved by fibrosis. Therefore, special effort should be made to overcome this caveat by constant education and unifying the definition.

Potential Applications to Prognosis and Other Diseases or Conditions

Fibrosis assessment is important in the kidney and also other organs because it provides a surrogate marker of chronic injury. IFTA assessment is important in the kidney in particular because it shows correlations with renal function (Farris et al. 2014; Farris and Alpers 2014). Some investigators distinguish conventional fibrosis from “sclerosis” since sclerosis may represent a late “hardened” stage at which fibrosis is more chronic in nature and thus be pathologically distinct. It is likely that future method refinements will help recognize later stages of fibrosis and also identify stages at which fibrosis may be reversible (Farris and Colvin 2012; Farris and Alpers 2014), and intervention may be possible at earlier stages of fibrosis (Friedman et al. 2013). Furthermore, fibrosis assessment is useful in a variety of other diseases including pulmonary fibrosis, liver fibrosis (cirrhosis), nephrogenic systemic fibrosis, systemic sclerosis, wound healing, and cancer (Farris and Colvin 2012; Friedman et al. 2013; Farris and Alpers 2014; Rybinski et al. 2014). Therefore, although this review has focused mostly on the kidney, it is likely that the kidney can serve as a window to view other organs and disease processes and thus improve the lives for patients (Friedman et al. 2013).

Conclusion

IFTA is brought about through complex molecular mechanisms of renal injury. Fibrosis can be assessed in a number of ways. Efforts to improve these methods could lead to improvements in the surrogate measures provided by pathologists, making pathologic assessment a better biomarker of renal disease, and molecular approaches including transcriptomics and proteomics will further complement these assessment methods. Assessing fibrosis, a type of *-osis* or state of the biologic system, can eventually help provide an *-omic* measure that can be correlated with other *-omic* measures (e.g., genomics/transcriptomics, proteomics, metabolomics, etc.). Going from the assessment of the *-osis* can eventually lead to better assessment of *-omics*. In this sense, fibrosis assessment can act as a biomarker. Ultimately, it is hoped that better assessment will facilitate the development of methods to ameliorate fibrosis and improve patient outcomes (Friedman et al. 2013).

Summary Points

- This chapter focuses on renal fibrosis, which is the pathologic accumulation of ECM proteins following tissue injury.
- IF is accompanied by TA, and these are collectively referred to as interstitial fibrosis and tubular atrophy (IFTA).
- IFTA is shown to have prognostic value in chronic kidney diseases.
- Renal biopsy can be used to diagnose and quantitate IFTA and further prognosticate renal function.
- A variety of staining techniques are useful in assessing the severity of IF, including trichrome, picrosirius red, and collagen III immunohistochemistry.
- Molecular methods such as transcriptional profiling and proteomics can provide surrogate measures of IFTA.

References

Abrass CK, Hansen KM, Patton BL. Laminin alpha4-null mutant mice develop chronic kidney disease with persistent overexpression of platelet-derived growth factor. Am J Pathol. 2010;176 (2):839–49.

Agarwal SK, Sethi S, Dinda AK. Basics of kidney biopsy: a nephrologist's perspective. Indian J Nephrol. 2013;23(4):243–52.

Anders HJ, Ryu M. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. Kidney Int. 2011;80(9):915–25.

Boor P, Ostendorf T, Floege J. Renal fibrosis: novel insights into mechanisms and therapeutic targets. Nat Rev Nephrol. 2010;6(11):643–56.

Bottinger EP. TGF-beta in renal injury and disease. Semin Nephrol. 2007;27(3):309–20.

Bunnag S, Einecke G, Reeve J, Jhangri GS, Mueller TF, Sis B, Hidalgo LG, Mengel M, Kayser D, Kaplan B, Halloran PF. Molecular correlates of renal function in kidney transplant biopsies. J Am Soc Nephrol. 2009;20(5):1149–60.

Choi BS, Shin MJ, Shin SJ, Kim YS, Choi YJ, Kim YS, Moon IS, Kim SY, Koh YB, Bang BK, Yang CW. Clinical significance of an early protocol biopsy in living-donor renal transplantation: ten-year experience at a single center. Am J Transplant. 2005;5(6):1354–60.

Choi HY, Lee HG, Kim BS, Ahn SH, Jung A, Lee M, Lee JE, Kim HJ, Ha SK, Park HC. Mesenchymal stem cell-derived microparticles ameliorate peritubular capillary rarefaction via inhibition of endothelial-mesenchymal transition and decrease tubulointerstitial fibrosis in unilateral ureteral obstruction. Stem Cell Res Ther. 2015;6:18.

Chung AC, Lan HY. MicroRNAs in renal fibrosis. Front Physiol. 2015;6:50.

Doello K. A new pentachrome method for the simultaneous staining of collagen and sulfated mucopolysaccharides. Yale J Biol Med. 2014;87(3):341–7.

Eardley KS, Kubal C, Zehnder D, Quinkler M, Lepenies J, Savage CO, Howie AJ, Kaur K, Cooper MS, Adu D, Cockwell P. The role of capillary density, macrophage infiltration and interstitial scarring in the pathogenesis of human chronic kidney disease. Kidney Int. 2008;74(4):495–504.

Eddy AA. Molecular insights into renal interstitial fibrosis. J Am Soc Nephrol. 1996;7 (12):2495–508.

Farris AB, Alpers CE. What is the best way to measure renal fibrosis?: A pathologist's perspective. Kidney Int Suppl. 2014;4(1):9–15.

Farris AB, Colvin RB. Renal interstitial fibrosis: mechanisms and evaluation. Curr Opin Nephrol Hypertens. 2012;21(3):289–300.

Farris AB, Adams CD, Brousaides N, Della Pelle PA, Collins AB, Moradi E, Smith RN, Grimm PC, Colvin RB. Morphometric and visual evaluation of fibrosis in renal biopsies. J Am Soc Nephrol. 2010;22(1):176-86.

Farris AB, Adams CD, Brousaides N, Della Pelle PA, Collins AB, Moradi E, Smith RN, Grimm PC, Colvin RB. Morphometric and visual evaluation of fibrosis in renal biopsies. J Am Soc Nephrol. 2011;22(1):176–86.

Farris AB, Lawson D, Cohen C, Rosen S. Medullary injury in the human renal biopsy: fibrosis assessment. Lab Investig. 2013;93(Supplement 1):387A–8.

Farris AB, Chan S, Climenhaga J, Adam B, Bellamy CO, Seron D, Colvin RB, Reeve J, Mengel M. Banff fibrosis study: multicenter visual assessment and computerized analysis of interstitial fibrosis in kidney biopsies. Am J Transplant. 2014;14(4):897–907.

Friedman SL, Sheppard D, Duffield JS, Violette S. Therapy for fibrotic diseases: nearing the starting line. Sci Transl Med. 2013;5(167), 167sr161.

Furness PN, Taub N, Assmann KJ, Banfi G, Cosyns JP, Dorman AM, Hill CM, Kapper SK, Waldherr R, Laurinavicius A, Marcussen N, Martins AP, Nogueira M, Regele H, Seron D, Carrera M, Sund S, Taskinen EI, Paavonen T, Tihomirova T, Rosenthal R. International variation in histologic grading is large, and persistent feedback does not improve reproducibility. Am J Surg Pathol. 2003;27(6):805–10.

Haas M, Sis B, Racusen LC, Solez K, Glotz D, Colvin RB, Castro MC, David DS, David-Neto E, Bagnasco SM, Cendales LC, Cornell LD, Demetris AJ, Drachenberg CB, Farver CF, Farris 3rd AB, Gibson IW, Kraus E, Liapis H, Loupy A, Nickeleit V, Randhawa P, Rodriguez ER, Rush D, Smith RN, Tan CD, Wallace WD, Mengel M, Banff meeting report writing, committee. Banff 2013 meeting report: inclusion of C4d-negative antibody-mediated rejection and antibody-associated arterial lesions. Am J Transplant. 2014;14(2):272–83.

Habuka M, Fagerberg L, Hallstrom BM, Kampf C, Edlund K, Sivertsson A, Yamamoto T, Ponten F, Uhlen M, Odeberg J. The kidney transcriptome and proteome defined by transcriptomics and antibody-based profiling. PLoS One. 2014;9(12), e116125.

Higgins DF, Kimura K, Iwano M, Haase VH. Hypoxia-inducible factor signaling in the development of tissue fibrosis. Cell Cycle. 2008;7(9):1128–32.

Howie AJ, Brewer DB, Howell D, Jones AP. Physical basis of colors seen in Congo red-stained amyloid in polarized light. Lab Invest. 2008;88(3):232–42.

Huang L, Haylor JL, Hau Z, Jones RA, Vickers ME, Wagner B, Griffin M, Saint RE, Coutts IG, El Nahas AM, Johnson TS. Transglutaminase inhibition ameliorates experimental diabetic nephropathy. Kidney Int. 2009;76(4):383–94.

Humphreys BD, Lin SL, Kobayashi A, Hudson TE, Nowlin BT, Bonventre JV, Valerius MT, McMahon AP, Duffield JS. Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. Am J Pathol. 2010;176(1):85–97.

Khan F, Sar A, Gonul I, Benediktsson H, Doulla J, Yilmaz S, Berka N. Graft inflammation and histologic indicators of kidney chronic allograft failure: low-expressing interleukin-10 genotypes cannot be ignored. Transplantation. 2010;90(6):630–8.

Klein J, Kavvadas P, Prakoura N, Karagianni F, Schanstra JP, Bascands JL, Charonis A. Renal fibrosis: insight from proteomics in animal models and human disease. Proteomics. 2011;11 (4):805–15.

Konvalinka A, Scholey JW, Diamandis EP. The quest for renal disease proteomic signatures: where should we look? Clin Proteom. 2010;6:45–51.

Kriz W, Kaissling B, Le Hir M. Epithelial-mesenchymal transition (EMT) in kidney fibrosis: fact or fantasy? J Clin Invest. 2011;121(2):468–74.

Kruger T, Benke D, Eitner F, Lang A, Wirtz M, Hamilton-Williams EE, Engel D, Giese B, Muller-Newen G, Floege J, Kurts C. Identification and functional characterization of dendritic cells in the healthy murine kidney and in experimental glomerulonephritis. J Am Soc Nephrol. 2004;15 (3):613–21.

Lattouf R, Younes R, Lutomski D, Naaman N, Godeau G, Senni K, Changotade S. Picrosirius red staining: a useful tool to appraise collagen networks in normal and pathological tissues. J Histochem Cytochem. 2014;62(10):751–8.

LeBleu VS, Taduri G, O'Connell J, Teng Y, Cooke VG, Woda C, Sugimoto H, Kalluri R. Origin and function of myofibroblasts in kidney fibrosis. Nat Med. 2013;19(8):1047–53.

Lian YG, Zhou QG, Zhang YJ, Zheng FL. VEGF ameliorates tubulointerstitial fibrosis in unilateral ureteral obstruction mice via inhibition of epithelial-mesenchymal transition. Acta Pharmacol Sin. 2011;32:1513–21.

Lin SL, Kisseleva T, Brenner DA, Duffield JS. Pericytes and perivascular fibroblasts are the primary source of collagen-producing cells in obstructive fibrosis of the kidney. Am J Pathol. 2008;173 (6):1617–27.

Liu Y. Renal fibrosis: new insights into the pathogenesis and therapeutics. Kidney Int. 2006;69 (2):213–7.

Liu L, Kou P, Zeng Q, Pei G, Li Y, Liang H, Xu G, Chen S. CD4+ T Lymphocytes, especially Th2 cells, contribute to the progress of renal fibrosis. Am J Nephrol. 2012;36 (4):386–96.

Liu M, Liu YZ, Feng Y, Xu YF, Che JP, Wang GC, Zheng JH. Novel evidence demonstrates that epithelial-mesenchymal transition contributes to nephrolithiasis-induced renal fibrosis. J Surg Res. 2013;182(1):146–52.

Mack M, Rosenkranz AR. Basophils and mast cells in renal injury. Kidney Int. 2009;76 (11):1142–7.

Maluf DG, Mas VR, Archer KJ, Yanek K, Gibney EM, King AL, Cotterell A, Fisher RA, Posner MP. Molecular pathways involved in loss of kidney graft function with tubular atrophy and interstitial fibrosis. Mol Med. 2008;14(5–6):276–85.

Mason RM, Wahab NA. Extracellular matrix metabolism in diabetic nephropathy. J Am Soc Nephrol. 2003;14(5):1358–73.

Meas-Yedid V, Servais A, Noel LH, Panterne C, Landais P, Herve N, Brousse N, Kreis H, Legendre C, Thervet E, Olivo-Marin JC, Morelon E. New computerized color image analysis for the quantification of interstitial fibrosis in renal transplantation. Transplantation. 2011;92 (8):890–9.

Mezzano S, Aros C, Droguett A, Burgos ME, Ardiles L, Flores C, Schneider H, Ruiz-Ortega M, Egido J. NF-kappaB activation and overexpression of regulated genes in human diabetic nephropathy. Nephrol Dial Transplant. 2004;19(10):2505–12.

Moreso F, Lopez M, Vallejos A, Giordani C, Riera L, Fulladosa X, Hueso M, Alsina J, Grinyo JM, Seron D. Serial protocol biopsies to quantify the progression of chronic transplant nephropathy in stable renal allografts. Am J Transplant. 2001;1(1):82–8.

Naito T, Ma LJ, Yang H, Zuo Y, Tang Y, Han JY, Kon V, Fogo AB. Angiotensin type 2 receptor actions contribute to angiotensin type 1 receptor blocker effects on kidney fibrosis. Am J Physiol Renal Physiol. 2010;298(3):F683–91.
Niedermeier M, Reich B, Rodriguez Gomez M, Denzel A, Schmidbauer K, Gobel N, Talke Y, Schweda F, Mack M. CD4+ T cells control the differentiation of Gr1+ monocytes into fibrocytes. Proc Natl Acad Sci U S A. 2009;106(42):17892–7.
Paust HJ, Ostmann A, Erhardt A, Turner JE, Velden J, Mittrucker HW, Sparwasser T, Panzer U, Tiegs G. Regulatory T cells control the Th1 immune response in murine crescentic glomerulonephritis. Kidney Int. 2011;80(2):154–64.
Pilling D, Fan T, Huang D, Kaul B, Gomer RH. Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. PLoS One. 2009;4(10), e7475.
Prunotto M, Ghiggeri G, Bruschi M, Gabbiani G, Lescuyer P, Hocher B, Chaykovska L, Berrera M, Moll S. Renal fibrosis and proteomics: current knowledge and still key open questions for proteomic investigation. J Proteomics. 2011;74(10):1855–70.
Reich B, Schmidbauer K, Rodriguez Gomez M, Johannes Hermann F, Gobel N, Bruhl H, Ketelsen I, Talke Y, Mack M. Fibrocytes develop outside the kidney but contribute to renal fibrosis in a mouse model. Kidney Int. 2013;84(1):78–89.
Reinders ME, Bank JR, Dreyer GJ, Roelofs H, Heidt S, Roelen DL, Al Huurman V, Lindeman J, van Kooten C, Claas FH, Fibbe WE, Rabelink TJ, de Fijter JW. Autologous bone marrow derived mesenchymal stromal cell therapy in combination with everolimus to preserve renal structure and function in renal transplant recipients. J Transl Med. 2014;12:331.
Rogers NM, Ferenbach DA, Isenberg JS, Thomson AW, Hughes J. Dendritic cells and macrophages in the kidney: a spectrum of good and evil. Nat Rev Nephrol. 2014;10(11):625–43.
Rybinski B, Franco-Barraza J, Cukierman E. The wound healing, chronic fibrosis, and cancer progression triad. Physiol Genomics. 2014;46(7):223–44.
Satoh M, Kashihara N, Yamasaki Y, Maruyama K, Okamoto K, Maeshima Y, Sugiyama H, Sugaya T, Murakami K, Makino H. Renal interstitial fibrosis is reduced in angiotensin II type 1a receptor-deficient mice. J Am Soc Nephrol. 2001;12(2):317–25.
Schrimpf C, Xin C, Campanholle G, Gill SE, Stallcup W, Lin SL, Davis GE, Gharib SA, Humphreys BD, Duffield JS. Pericyte TIMP3 and ADAMTS1 modulate vascular stability after kidney injury. J Am Soc Nephrol. 2012;23(5):868–83.
Scian MJ, Maluf DG, Archer KJ, Suh JL, Massey D, Fassnacht RC, Whitehill B, Sharma A, King A, Gehr T, Cotterell A, Posner MP, Mas V. Gene expression changes are associated with loss of kidney graft function and interstitial fibrosis and tubular atrophy: diagnosis versus prediction. Transplantation. 2011;91(6):657–65.
Semedo P, Donizetti-Oliveira C, Burgos-Silva M, Cenedeze MA, Avancini Costa Malheiros DM, Pacheco-Silva A, Camara NO. Bone marrow mononuclear cells attenuate fibrosis development after severe acute kidney injury. Lab Invest J Tech Methods Pathol. 2010;90(5):685–95.
Sethi S, Vrana JA, Theis JD, Dogan A. Mass spectrometry based proteomics in the diagnosis of kidney disease. Curr Opin Nephrol Hypertens. 2013;22(3):273–80.
Snelgrove SL, Kausman JY, Lo C, Ooi JD, Coates PT, Hickey MJ, Holdsworth SR, Kurts C, Engel DR, Kitching AR. Renal dendritic cells adopt a pro-inflammatory phenotype in obstructive uropathy to activate T cells, but do not directly contribute to fibrosis. Am J Pathol. 2011;180 (1):91–103
Sorensen I, Susnik N, Inhester T, Degen JL, Melk A, Haller H, Schmitt R. Fibrinogen, acting as a mitogen for tubulointerstitial fibroblasts, promotes renal fibrosis. Kidney Int. 2011;80 (10):1035–44.
Street JM, Souza AC, Alvarez-Prats A, Horino T, Hu X, Yuen PS, Star RA. Automated quantification of renal fibrosis with Sirius Red and polarization contrast microscopy. Physiol Rep. 2014;2(7):e12088:1–9.

Sun S, Ning X, Zhai Y, Du R, Lu Y, He L, Li R, Wu W, Sun W, Wang H. Egr-1 mediates chronic hypoxia-induced renal interstitial fibrosis via the PKC/ERK pathway. Am J Nephrol. 2014;39 (5):436–48.

Sund S, Grimm P, Reisaeter AV, Hovig T. Computerized image analysis vs semiquantitative scoring in evaluation of kidney allograft fibrosis and prognosis. Nephrol Dial Transplant. 2004;19 (11):2838–45.

Tapmeier TT, Fearn A, Brown K, Chowdhury P, Sacks SH, Sheerin NS, Wong W. Pivotal role of CD4+ T cells in renal fibrosis following ureteric obstruction. Kidney Int. 2010;78(4):351–62.

Vilayur E, Harris DC. Emerging therapies for chronic kidney disease: what is their role? Nat Rev Nephrol. 2009;5(7):375–83.

Walker PD, Cavallo T, Bonsib SM, Ad Hoc Committee on Renal Biopsy Guidelines of the Renal Pathology, Society. Practice guidelines for the renal biopsy. Mod Pathol. 2004;17(12):1555–63.

Wang X, Zhou Y, Tan R, Xiong M, He W, Fang L, Wen P, Jiang L, Yang J. Mice lacking the matrix metalloproteinase-9 gene reduce renal interstitial fibrosis in obstructive nephropathy. Am J Physiol Renal Physiol. 2010;299(5):F973–82.

Wang Y, Wang B, Du F, Su X, Sun G, Zhou G, Bian X, Liu N. Epigallocatechin-3-gallate attenuates unilateral ureteral obstruction-induced renal interstitial fibrosis in mice. J Histochem Cytochem. 2015;63(4):270–9.

Wilkinson L, Gilbert T, Sipos A, Toma I, Pennisi DJ, Peti-Peterdi J, Little MH. Loss of renal microvascular integrity in postnatal Crim1 hypomorphic transgenic mice. Kidney Int. 2009;76 (11):1161–71.

Working Group of the International IgA Nephropathy Network and the Renal Pathology Society, Roberts IS, Cook HT, Troyanov S, Alpers CE, Amore A, Barratt J, Berthoux F, Bonsib S, Bruijn JA, Cattran DC, Coppo R, D'Agati V, D'Amico G, Emancipator S, Emma F, Feehally J, Ferrario F, Fervenza FC, Florquin S, Fogo A, Geddes CC, Groene HJ, Haas M, Herzenberg AM, Hill PA, Hogg RJ, Hsu SI, Jennette JC, Joh K, Julian BA, Kawamura T, Lai FM, Li LS, Li PK, Liu ZH, Mackinnon B, Mezzano S, Schena FP, Tomino Y, Walker PD, Wang H, Weening JJ, Yoshikawa N, Zhang H. The Oxford classification of IgA nephropathy: pathology definitions, correlations, and reproducibility. Kidney Int. 2009;76(5):546–56.

Xia Y, Entman ML, Wang Y. CCR2 regulates the uptake of bone marrow-derived fibroblasts in renal fibrosis. PLoS One. 2013;8(10), e77493.

Yang J, Shultz RW, Mars WM, Wegner RE, Li Y, Dai C, Nejak K, Liu Y. Disruption of tissue-type plasminogen activator gene in mice reduces renal interstitial fibrosis in obstructive nephropathy. J Clin Invest. 2002;110(10):1525–38.

Yang L, Besschetnova TY, Brooks CR, Shah JV, Bonventre JV. Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury. Nat Med. 2010;16(5):535–43. 531p following 143.

Zeisberg M, Neilson EG. Mechanisms of tubulointerstitial fibrosis. J Am Soc Nephrol JASN. 2010;21(11):1819–34.

Zununi Vahed S, Samadi N, Ardalan M. Diagnosis of interstitial fibrosis and tubular atrophy in kidney allograft: implementation of microRNAs. Iran J Kidney Dis. 2014;8(1):4–12.

Next-Generation Sequencing (NGS) in Biomarker Discovery and Applications in Nephrology

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Imari Mimura and Masaomi Nangaku

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Abstract

Next-generation sequencing (NGS) has been rapidly evolved in these 10 years. The practical use of high-throughput sequencers makes it possible to identify the localization of epigenetic modifications in detail. Recent technologies including ChIP-seq and RNA-seq allowed demonstration of protein-DNA bindings or splicing variants even with a small number of cells. These technologies have great potential in a wide range of renal diseases because only a few amounts of human renal biopsy samples can be harvested. In addition these techniques have been adapted to a variety of tissues in different model organs. PAT-ChIP (pathology tissue-ChIP)-seq protocol on freshly isolated mouse embryonic kidneys can be used for in vivo analysis of transcriptional factor recruitment on chromatin. RIP-seq can be used to analyze the RNA-binding proteins on genome-wide scale. Chromosome conformation capture (3C) assay makes tremendous progress into Hi-C which can detect genome-wide long interactions on chromosomes which have cell-type specificity. It is important to catch up with the speed of technical development and make use of these tools in order to understand the epigenetic mechanisms systematically.

Keywords

ChIP-seq • RNA-seq • RIP-seq • Chromosome conformation capture assay • Epigenetics

Abbreviations

3C	Chromosome conformation capture
4C-seq	Circular chromosome conformation capture
AGO2	Argonaute 2
AR	Androgen receptor
ChIP-seq	Chromatin immunoprecipitation-sequencing
DCCT/EDIC	Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications
DCR2	Dicer 2
Dpc	Day post-coitum
Ezh2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FFPE	Formalin-fixed paraffin-embedded
GLUT3	Glucose transporter 3
H3K27me3	Lysine 27 trimethylation
H3K4m3	Lysine 4 trimethylation
H3K9me3	Lysine 9 trimethylation
Hi-C	High-resolution circular chromosome conformation capture
HIF-1	Hypoxia-inducible factor-1
HK-2	Human kidney-2
HUVECs	Human umbilical vein endothelial cells
KDM3A	Lysine-specific demethylase 3A
KLF1	Kruppel-like factor

mDCT	Murine distal convoluted tubular epithelial cell line
MM	Metanephric mesenchyme
MR	Mineralocorticoid receptor
PAT-ChIP	Pathology tissue-ChIP
piRNA	PIWI-interacting RNA
PRC2	Polycomb repressive complex 2
pre-miRNAs	Precursor miRNAs
pri-miRNAs	Primary miRNAs
RBP	RNA-binding protein
RISC	RNA-induced silencing complex
SLC2A3	Solute carrier family 2A3
Suv39h1	Suppressor of variegation 3–9 homolog 1
VSMC	Vascular smooth muscle cells

Key Facts of ChIP-seq

- Recently the number of genome-wide analysis such as ChIP-seq has increased because of their technological progress and a reduction in costs.
- ChIP-seq can be used in order to analyze the mechanisms of kidney disease and development of the kidney.
- Long-term effects such as metabolic memory and legacy effect may be mediated by epigenetic changes, and ChIP-seq is a powerful tool to study epigenetic modifications.
- Advanced technology of ChIP-seq called PAT-ChIP makes it possible to analyze the kidney tissue samples for in vivo analysis.
- The improved PAT-ChIP protocol coupled with laser capture microdissection can be used for discovery and validation of novel epigenetic factors in human samples.

Key Facts of RNA-seq

- RNA-seq is used for showing genome-wide gene expressions and gene variants.
- RNA-seq data for 234 renal clear cell carcinoma patients clarified that both gene and isoform expression signatures are useful for distinguishing cancer stages and that they help to identify advanced stage cancers, predict clinical outcome, and present a comprehensive view of cancer development and progression.
- Advanced technique for RNA-seq is a single-cell RNA-seq, which can profile cell-to-cell variability on a genomic scale.
- Making use of this technique, we can identify the difference between tubulointerstitial cells and fibrotic cells or mesangial cell, endothelial cells, and podocytes in the glomerulus.
- RNA-seq using total mouse kidneys at E11.5 and E12.5 as well as the renal vesicles at P4 identified a large number of genes with partially degraded

noncoding RNA. It was also found that single cells at early developmental times often expressed genes related to several developmental pathways, providing powerful evidence that initial organogenesis involves a process of multilineage priming.

Key Facts of RIP-seq

- RIP-seq can analyze the RNA-binding proteins on genome-wide scale.
- RIP-seq using AGO2 antibody demonstrated that AGO2 is strongly enriched in small RNAs that encompass the promoter regions and other regions on both the sense and antisense DNA strands.
- A new method of RIP-seq in combination with northern blotting identified various types of small RNAs associated with the BmAgo2 protein as well as miRNAs and piRNAs.
- It can examine the miRNA-mRNA associations under specific conditions and find novel putative miRNA targets.
- RIPSeeker, a free open-source for de novo RIP peak predictions, demonstrates superior sensitivity and specificity in discriminating high-confidence peaks.

Key Facts of 3C and Hi-C Assay

- Chromosome conformation capture (3C) assay has recently been developed in order to clarify a conformational proximity between promoter and a distal enhancer.
- A study of human genome identified thousands of significant long-range looping interactions between gene promoters and distal loci, revealing that gene promoters engage with distal elements through looping.
- We performed ChIP-seq of HIF-1 (hypoxia-inducible factor-1) and showed that HIF-1 and KDM3A (lysine-specific demethylase 2A3) cooperatively upregulate the downstream target gene, *SLC2A3* (solute carrier family 2A3), via removal of suppressive histone marks and chromosome conformational change under hypoxia.
- In response to glucose, 4C-seq identified the contact in human islets between insulin (INS) promoter and the calcium-activated chloride channel ANO1 gene. The contact was strengthened and the expression of ANO1 increased. It is demonstrated that networks of long-range physical contacts are important to the regulation of insulin metabolism.
- 3C-based techniques have evolved into Hi-C (high-resolution circular chromosome conformation capture assay) at a rapid speed and make it possible to analyze the long-range interactions on multiple chromosomes.

Potential Applications to Prognosis, Other Diseases, or Conditions

We can use NGS to analyze the genome-wide sequencing of patients with diseases and find novel epigenetic factors which are associated with the diseases not only in nephrology but also in other fields. In addition we can analyze and compare the relationships between survival data and genome sequencing. We can predict the prognosis of diseases using the data of NGS coupled with survival data in the future.

Definitions

ChIP-seq Genome-wide sequencing analysis of chromatin immunoprecipitation using high-throughput analyzers.

Hi-C High-resolution circular chromosome conformation capture based on 3C assay.

Legacy Effects The intensive treatment of blood glucose leads to delaying progression of diabetic nephropathy.

Metabolic Memory Early metabolic control affects a beneficial and long-term influence on the clinical outcome of diabetic complications.

PAT-ChIP Pathology tissue-ChIP methods to extract and immunoprecipitate chromatin from paraffin-embedded samples.

Quartz-seq New technique which can detect different cell types and different cell cycle phases of a single-cell type.

RIP-seq Genome-wide sequencing analysis of RNA-binding proteins.

RNA-seq Genome-wide sequencing analysis of total RNA or mRNA.

Introduction

Progression of high-throughput genome sequencers in the early years of the twenty-first century and the determination of human genome in 2003 promotes the genome-wide analysis of many kinds of species. These results clarify that the expressions of genome information are affected intricately not only by the sequences of bases but also by methylation of DNA, modifications of histones which consist of chromatin in the nucleus, and small nucleic acids such as microRNA (Tsukada et al. 2006).

The practical use of high-throughput sequencers recently makes it possible to identify the localization of epigenetic modifications in detail. Increasing number of recent papers has demonstrated the roles of epigenetic mechanisms for embryogenesis, cell differentiation, genome imprinting, inactivation of X chromosome, and aging (Ware et al. 2009). Epigenetic changes are accumulated as a memory in a cell, and epigenetic memory results in the cancer, congenital disorders, and adult-onset diseases (Mimura et al. 2011, 2013). However, molecular mechanisms of transcriptional factors and epigenetic modifications still remain to be solved.

ChIP-seq for Genome-Wide Analysis in Renal Diseases

Applications of ChIP-seq in Kidney Tissue Samples

ChIP-seq has been widely used in fields related to nephrology as shown below. Sun et al. have performed ChIP-seq experiments using RNA polymerase II antibody in five adult mouse tissues including kidney (Gupta et al. 2010; Sun et al. 2011). They clarified that 6,384 promoters are tissue specific among 12,270 novel promoters. ChIP-seq analysis of RNA polymerase II made it possible to identify the novel tissue-specific promoters.

The kidney is composed of various different types of cells, and cell type-specific analysis is desirable. Recent technologies allowed demonstration of protein-DNA bindings using ChIP-seq with a small number of cells (Furey 2012). This technology can be used for a wide range of renal diseases.

Identification of Targets for Nuclear Hormone Receptors

Recently the number of genome-wide analysis such as ChIP-seq has increased because of their rapid progress and prevalence. AR (androgen receptor) binds male sex steroids and other mediators and induces physiological androgen actions. Pihlajamaa et al. performed ChIP-seq analysis of AR-binding sites in murine kidney and epididymis (Pihlajamaa et al. 2014). They found two novel collaborating factors for AR signaling in vivo, Hnf4α (hepatocyte nuclear factor 4α) in the kidney and AP-2α (activating enhancer-binding protein 2α) in the epididymis. They clarified that these factors are constitutively bound to chromatin and guide AR to specific genome loci on hormone exposure. Ueda et al. also demonstrated the genome-wide distribution of MR (mineralocorticoid receptor) using mDCT (murine distal convoluted tubular epithelial cell line) (Ueda et al. 2014). MR is a member of nuclear receptor family proteins and contributes to fluid homeostasis in the kidney. They performed ChIP-seq and microarray analysis using DNA and mRNA samples of mDCT cells overexpressing 3×FLAG-hMR after treatment with aldosterone. Twenty-five genes were indicated as the candidate targets of MR by ChIP-seq and microarrays. Four genes

such as *Sgk1*, *Rasl12*, *Tns1*, and *Tsc22d3* were validated as the direct targets of MR by ChIP-qPCR.

ChIP-seq Analysis in the Developmental Kidney

Li et al. analyzed the genome-wide binding sites of the p53 gene in the embryonic kidney (Li et al. 2014). They found that the p53-regulated transcriptome is largely composed of genes regulating developmental, morphogenesis, and metabolic pathways. They demonstrated that 78 % of p53 peaks in the developing kidney lie within the proximal promoters of annotated genes and that 25 % of the differentially expressed p53-bound genes are present in nephron progenitors and nascent nephrons, including components of Fgf, Wnt, Bmp, and Notch pathways. They showed the comprehensive analysis of the p53 transcriptome and cistrome in a developing mammalian organ for the first time.

The metanephric mesenchyme (MM) is known to give rise to nephrons. The MM is composed of uninduced (*Six2*, high; *Lhx1*, low) and induced (Wnt stimulated, *Six2*, low; *Lhx1*, high) cells. McLaughlin et al. performed ChIP-seq analysis using uninduced (mK3) and induced (mK4) metanephric mesenchyme (McLaughlin et al. 2013). mK3 cells express genes characteristic of early mesenchyme, not epithelial progenitor genes, while mK4 cells resemble induced MM undergoing epithelial conversion. ChIP-seq revealed that H3K4me3 active region peaks are enriched in metabolic genes and that H3K27me3 peaks, histone repressive marks, are enriched mesenchyme and epithelial cell fate commitment genes. They further demonstrated that one of histone methyltransferases, G9a, occupies the promoter region of Six2 in induced cells. Stimulation of Wnt signaling in uninduced cells provokes an active chromatin state (H3K4m3 (lysine 4 trimethylation), high; H3K27me3 (lysine 27 trimethylation), low), recruitment of β-catenin, and loss of prebound Ezh2 (one of histone methyltransferases). As shown above, ChIP-seq analysis revealed that the chromatin signature correlates strongly with their gene expression states.

Renal hypoplasia is a congenital reduction in nephron number. It is a predisposing factor for chronic kidney disease and hypertension. Saifudeen et al. examined whether p53 and Pax2 cooperate in nephrogenesis by using mice with germ line p53 deletion (Saifudeen et al. 2012). Mice or humans with germ line heterozygous mutations in Pax2 exhibit renal hypoplasia. They performed ChIP-seq of p53 and clarified that peaks of p53 occupancy in chromatin regions of the Pax2 promoter and gene in embryonic kidneys. They also demonstrated that p53 binding to Pax2 gene is significantly more enriched in Pax2-expressing than non-expressing MM cells. They suggested that the cross talk between p53 and Pax2 may provide a promotion of nephrogenesis.

ChIP-seq Applications for Metabolic Memory and Legacy Effect

Epidemiological and clinical data support the idea that early metabolic control affects a beneficial and long-term influence on the clinical outcome of diabetic

complications. This phenomenon has recently been called as "metabolic memory." In addition, large clinical studies showed that the intensive treatment of blood glucose leads to delaying progression of diabetic nephropathy (Writing Team for the Diabetes, Complications Trial/Epidemiology of Diabetes et al. 2003; Holman et al. 2008). It is called "legacy effect." Strict control for blood glucose levels in type 1 diabetes patients was effective in inhibition of microalbuminuria and microvascular complications in DCCT/EDIC study (Writing Team for the Diabetes, Complications Trial/Epidemiology of Diabetes et al. 2003). Patients with type 2 diabetes mellitus who receive intensive glucose therapy also had a lower risk of microvascular complications (Holman et al. 2008). In addition, de Boer et al. demonstrated that the albumin excretion rate came out to be controlled and that GFR remained in the normal range with type 1 diabetes mellitus patients during DCCT/EDIC study through 10 years of follow-up (de Boer et al. 2014).

It is natural to presume that this long-term effect such as metabolic memory and legacy effect may be mediated by epigenetic changes and that deep sequencing such as ChIP-seq is a powerful tool to study epigenetic modifications. To support this point of view, ChIP-seq analysis demonstrated hyperglycemia-mediated induction of genes through modulation of acetylated H3K9/K14 (Pirola et al. 2011). They performed ChIP-seq of H3K9/K14 and CpG methylation assays followed by massive parallel sequencing (CpG-seq) using primary cultured vascular cells. They compared the ChIP-seq and CpG-seq and analyzed them and clarified that modulation of acetylated H3K9/K14 is inversely correlated with methyl-CpG content. Villeneuve et al. demonstrated that H3K9me3 (lysine 9 trimethylation) plays an important role in metabolic memory (Villeneuve et al. 2008). H3K9me3 levels were decreased at the promoters of inflammatory genes in cultured *db*/*db* vascular smooth muscle cells (VSMC) relative to control *db*/+ cells. One of the H3K9me3 transmethylferases, Suv39h1 (suppressor of variegation 3–9 homolog 1), was also reduced in *db*/*db* VSMC and overexpression of SUV39H1 in *db*/*db* VSMC reversed diabetic phenotype. These results demonstrate the protective roles for H3K9me3 and Suv39h1 against the pre-activated state of diabetic VSMC. The mechanism of metabolic memory needs further research using deep sequencing.

Technical Evolution of ChIP-seq for In Vivo Analysis

Traditional Method for ChIP-seq

The traditional method for ChIP-seq is indicated in Fig. 1 (Mimura et al. 2014). First, we cross-link cultured cells in the dishes by using paraformaldehyde (Step 1). Next, the cell lysate is homogenized and sonicated into fragments. The antibody is attached with sepharose beads (Step 2). Then we immunoprecipitate the cell lysate and the antibody with beads (Step 3). Then, we reverse cross-linking at 65 °C incubation. And DNA is purified by ethanol precipitation (Step 4). Finally, we use immunoprecipitated DNA for high-throughput sequencing (Step 5).

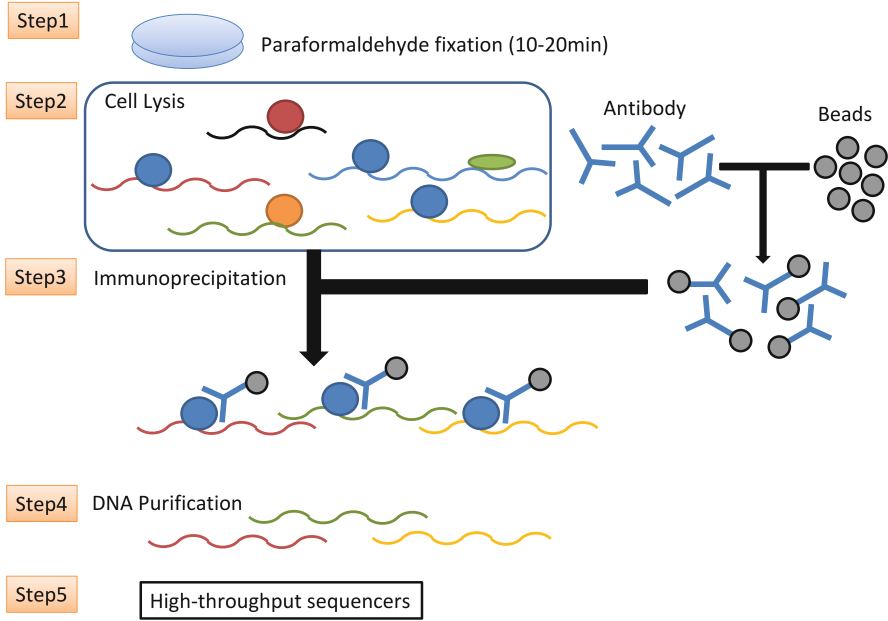

Fig. 1 ChIP-seq methods. Step 1: First we cross-link cultured cells in the dishes by using paraformaldehyde for 10–20 min. Step2: The cell lysis is homogenized and sonicated into fragments. The antibody is attached with sepharose beads. Step3: We immunoprecipitate the cell lysis and antibody with beads for overnight at 4 °C. Step 4: After we reverse cross-linking at 65 °C incubation, DNA is purified by ethanol precipitation. Step5: Immunoprecipitated DNA can be used for high-throughput sequencers

PAT-ChIP-seq

Recently this technique has been adapted to a variety of tissues in different model organs. Heliot et al. demonstrated the ChIP protocol on freshly isolated mouse embryonic kidneys for in vivo analysis of transcription factor recruitment on chromatin (Heliot and Cereghini 2012). Fanelli et al. developed a methodology called PAT-ChIP (pathology tissue-ChIP) to extract and immunoprecipitate chromatin from paraffin-embedded patient samples (Fanelli et al. 2010). The brief protocol of PAT-ChIP is shown in Fig. 2. First, we perform deparaffinization and rehydration of formalin-fixed paraffin-embedded (FFPE) samples (Step 1). After we homogenize the cell lysate, MNase is used for the digestion of cell lysis buffer (Step 2). We sonicate the cell lysate into fragments by using sonicator (Step 3). The antibody is needed to react with sepharose beads. Then, we immunoprecipitate the cell lysate with antibody for overnight (Step 4). Finally, we purify DNA fragment after reversing cross-linking (Step 5). They showed that PAT-ChIP can be coupled with high-throughput sequencing (PAT-ChIP-seq) for the genome-wide analysis of distinct chromatin modifications (Fanelli et al. 2011). They developed the new method for PAT-ChIP-seq coupled with laser capture microdissection (Amatori et al. 2014).

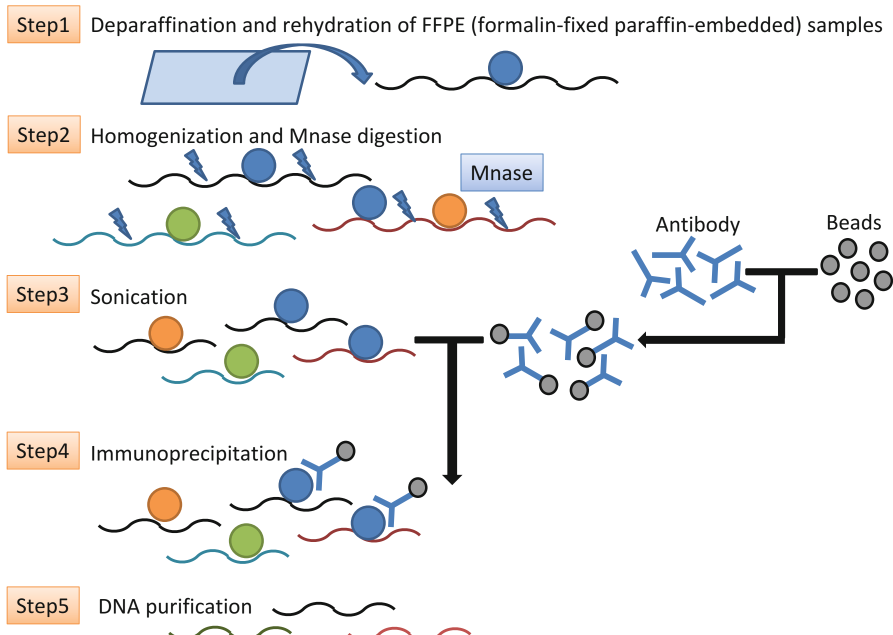

Fig. 2 PAT-ChIP methods. Step 1: We perform deparaffination and rehydration of FFPE (formalin-fixed paraffin-embedded) samples. Step 2: After we homogenize the cell lysis, MNase is used for the digestion of cell lysis buffer. Step 3: We sonicate the cell lysis into fragments by using sonicator. The antibody is needed to react with sepharose beads. Step 4: We immunoprecipitate the cell lysis with antibody for overnight. Step 5: We purify DNA fragment after reversing cross-linking

The improved PAT-ChIP protocol can be used for discovery and validation of novel epigenetic factors in human samples.

RNA-seq and Applications for Kidney Diseases

Technical Method for RNA-seq

As shown in Fig. 3, RNA samples for RNA sequencing are prepared as total RNA or mRNA (Step 1). The cDNA library is constructed using reverse transcription (Step 2). Adaptors are attached to both ends of cDNA library (Step 3). One fragment is sequenced with or without amplification in a high-throughput sequencer to obtain short sequences from one (single end) or both ends (pair end). The resulting reads are aligned to a reference genome or reference transcripts to produce a genome-scale transcription map (Step 4). As shown in step 4, longer reads or pair-end reads reveal connectivity between multiple exons. RNA-seq is useful for revealing the complex

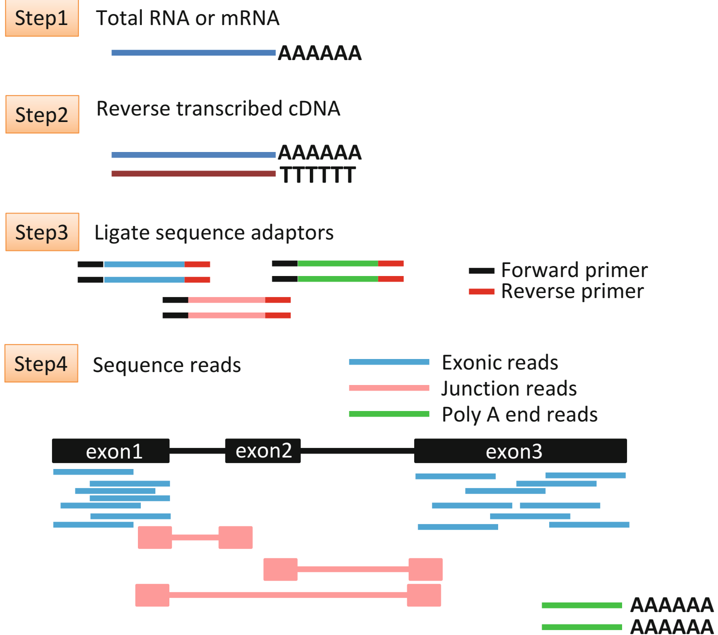

Fig. 3 RNA-seq methods. Step 1: RNA samples for RNA sequences are prepared for total RNA or mRNA. Adaptors need to be added on both sides of them with poly (**A**). Step 2: The cDNA library is constructed using reverse transcription. Step 3: RNA is converted to a library of cDNA fragments with adaptors attached to one or both ends. Step 4: One fragment is sequenced with or without amplification in high-throughput sequencers to obtain short sequences from one (single end) or both ends (pair end). The resulting reads are aligned to a reference genome or reference transcripts to produce a genome-scale transcription map

transcriptomes (Yae et al. 2012). In addition, RNA-seq can also demonstrate sequence variations in the transcribed regions (Mimura et al. 2014).

Applications of RNA-seq for Kidney Diseases

RNA-seq is used for showing genome-wide gene expressions and gene variants. Liu et al. analyzed gene expressions and isoform levels of RNA-seq data for 234 renal clear cell carcinoma patients (Liu et al. 2013). They found that both gene and isoform expression signatures are useful for distinguishing cancer stages and that they help to identify advanced stage cancers, predict clinical outcome, and present a comprehensive view of cancer development and progression. Brunskill and Potter performed RNA-seq using cap mesenchyme progenitors and renal vesicles, identified hundreds of novel splice patterns and new genes, and clarified the RNA processing complexities of the Hox clusters (Brunskill and Steven Potter 2012).

Tallack et al. identified novel target genes of KLF1 (Kruppel-like factor) by RNA-seq from erythroid tissue (Tallack et al. 2012). They clarified two novel long noncoding RNAs that are dynamically expressed during erythroid differentiation. These results suggest that we can find novel functional molecules in the kidney using RNA-seq in vivo. RNA-seq was also helpful in detecting a new fusion of chromosomes from individuals diagnosed with Ewing sarcoma. Pierron discovered a novel fusion between *BCOR* (encoding the BCL6 corepressor) and *CCNB3* (encoding the testis-specific cyclin B3) on the X chromosome (Pierron et al. 2012). Sung found that recurrent HBV integration occurred at the *TERT*, *MLL4*, and *CCNE1* genes, which showed upregulated expression, and that this was observed more frequently by RNA-seq of tumors (Sung et al. 2012). Thiagarajan et al. performed RNA-seq and compared the results with preexisting microarray datasets using 15.5 dpc (day post-coitum) embryonic mouse kidney (Thiagarajan et al. 2011). The resolution of RNA-seq provides the basis for a transition from classical gene-centric models of kidney development.

Quartz-seq for Identifying a Single-Cell RNA-seq Method

A new technique named Quartz-seq has been recently developed (Sasagawa et al. 2013). It is a single-cell RNA-seq method. Sasagawa et al. developed the new technique which can detect different cell types and different cell cycle phases of a single-cell type. Making use of this technique, we can identify the difference between tubulointerstitial cells and fibrotic cells or mesangial cell, endothelial cells, and podocytes in the glomerulus. Brunskill et al. created an atlas of gene expression patterns in the developing kidney by using a single-cell RNA-seq strategy (Brunskill et al. 2014). They performed RNA-seq using total mouse kidneys at E11.5 and E12.5 as well as the renal vesicles at P4. They identify a large number of genes with partially degraded noncoding RNA. They also found that single cells at early developmental times often expressed genes related to several developmental pathways, providing powerful evidence that initial organogenesis involves a process of multilineage priming. As shown in this paper, a single-cell RNA-seq must be a breakthrough in order to analyze the expression of specific types of kidney cells because human samples can be harvested with a very small amount, leading to a limitation of analyzing the data.

New Methods for Revealing RNA-Binding Proteins, RIP-seq

Technical Method for RIP-seq

RIP-seq is firstly developed to capture the polycomb repressive complex 2 (PRC2) transcriptome and identify a genome-wide pool of more than 9,000 PRC2-interacting RNAs in embryonic stem cells in 2010 (Zhao et al. 2010). They demonstrated the direct RNA-protein interaction via Ezh2 (enhancer of zeste 2 polycomb

repressive complex 2 subunit) subunit and identified Gtl2 RNA as a PRC2 cofactor. The method is shown in Fig. 4. To construct RIP-seq libraries, cell nuclei are isolated, and nuclear lysates are prepared with DNAse. Cell lysate is incubated with RBP (RNA-binding protein) antibody or control IgG with agarose beads (Step 1). RNA-protein complexes are immunoprecipitated with beads (Step 2) and RNA are extracted using RNA purification processes (Step 3). Purified RNAs are used for high-throughput sequencers using the same procedures of RNA-seq (Step 4) (Jayaseelan et al. 2014).

Application of RIP-seq in the Research Fields

Small RNAs such as miRNA, siRNA, and piRNA have been known to play roles as a silencer of mRNA or retrotransposons, which is called "RNA silencing." piRNA (PIWI-interacting RNA) comes mainly from retrotransposon and binds to a subset of PIWI proteins. piRNA functions as a guide molecular for targeted RNA which has complementary sequences. A set of Argonaute proteins binds to small RNAs and plays important roles in RNA silencing. Argonaute protein is one of RNA-binding

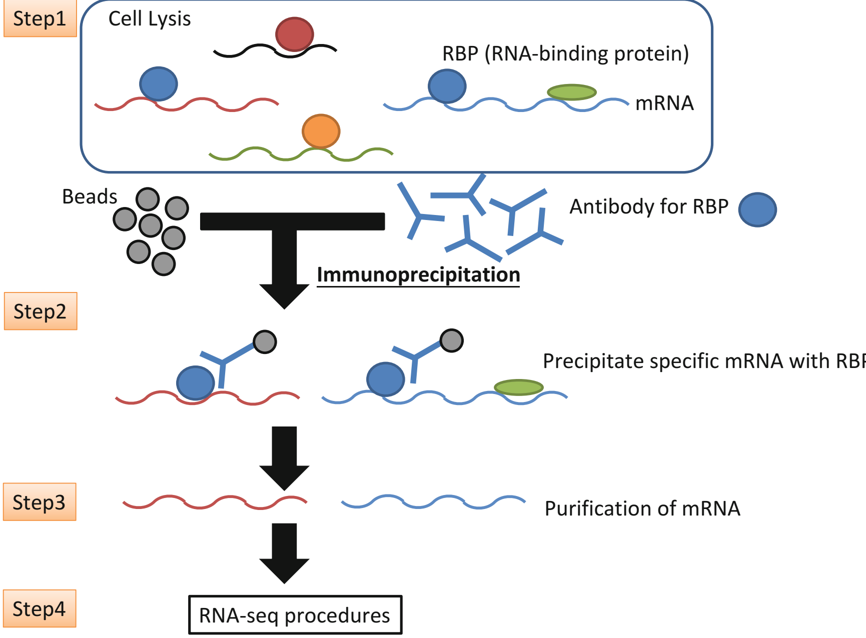

Fig. 4 RIP-seq methods. Step 1: Cell nuclei are isolated, nuclear lysates are prepared with DNAse. Cell lysate is incubated with RBP (RNA-binding protein) antibody or control IgG with agarose beads Step 2: RNA-protein complexes are immunoprecipitated with beads. Step 3: RNA are extracted using RNA purification processes. Step 4: Purified RNA are used for high-throughput sequencers using the same procedures of RNA-seq

proteins, which have PIWI domains. miRNAs are processed as shown in Fig. 5. miRNAs are transcribed by RNA polymerase II (Pol II) into the primary miRNAs (pri-miRNAs). Pri-miRNAs are processed by RNase III Drosha into precursor miRNAs (pre-miRNAs). Pre-miRNAs are exported by the nuclear export factor, Exportin 5, into the cytoplasm. In the cytoplasm the pre-miRNAs are processed by Dicer, another RNase III, into mature miRNAs. The miRNA strand is incorporated into the RISC (RNA-induced silencing complex) and target complementary mRNA. Targeted mRNA receives degradation or translational repression.

Because RIP-seq is a recently developed technique, there are a few papers using RIP-seq. We do not have any report of RIP-seq in the field of the kidney so far. Cernilogar et al. performed RIP-seq using AGO2 antibody and demonstrated that AGO2 is strongly enriched in small RNAs that encompass the promoter regions and other regions of heat shock and other genetic loci on both the sense and antisense DNA strands (Cernilogar et al. 2011). They showed that DCR2 (Dicer 2) and AGO2 are globally associated with transcriptionally active loci and have a pivotal role in shaping the transcriptome by controlling the processivity of RNA polymerase II.

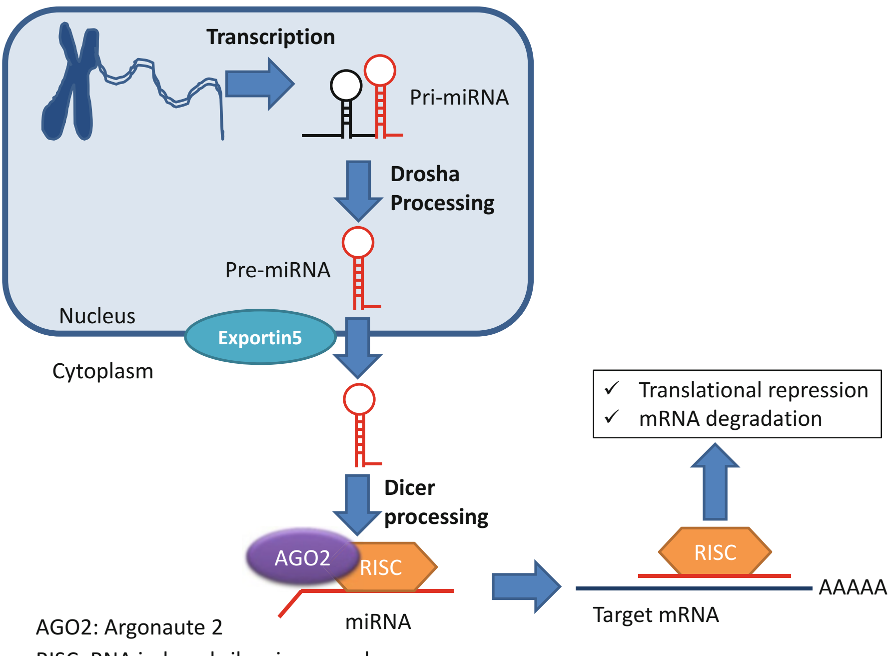

Fig. 5 miRNAs processing in the nucleus and the cytoplasm. miRNAs are transcribed by RNA polymerase II (*Pol II*) into the primary miRNAs (*pri-miRNAs*). Pri-miRNAs are processed by RNase III Drosha into precursor miRNAs (*pre-miRNAs*). Pre-miRNAs are exported by the nuclear export factor, Exportin 5, into the cytoplasm. In the cytoplasm the pre-miRNAs are processed by Dicer, another RNase III, into mature miRNAs. The miRNA strand is incorporated into the RISC (RNA-induced silencing complex) and target complementary mRNA. Targeted mRNA receives degradation or translational repression

Lu et al. developed a multi-targeting RIP-seq strategy to identify Sm-associated RNAs from *Drosophila* ovaries and cultured human cells (Lu et al. 2014). Sm proteins are multimeric RNA-binding factors, and they discovered three major categories of Sm-associated transcripts: small nuclear (sn) RNAs, small Cajal body (sca) RNAs, and mRNAs. They also showed both ubiquitous and tissue-specific interactions. Nie et al. developed a new method of RIP-seq in combination with northern blotting and identified various types of small RNAs associated with the BmAgo2 protein as well as miRNAs and piRNAs (Nie et al. 2013). Kanematsu et al. screened for miRNA-mRNA associations using RIP-seq in colon cancer cell lines (Kanematsu et al. 2014). They also examined the miRNA-mRNA associations under hypoxic condition which included several well-characterized cancer-related genes as novel putative miRNA targets.

Meier et al. performed RIP-seq by using HEK293T cells with stable ectopic expression of miR-155 (Meier et al. 2013). They found 100 AGO2-associated mRNAs in miR-155-expressing cells and indicated that these targets were either regulated by mRNA decay or by translational repression. Li et al. developed RIPSeeker, a free open-source for de novo RIP peak predictions (Li et al. 2013). RIPSeeker demonstrates superior sensitivity and specificity in discriminating high-confidence peaks.

High-Resolution Circular Chromosome Conformation Capture Technology (3C, 4C, 5C, and Hi-C) Methods for Identifying Genome-Wide Chromosome Conformations

Technical Method of 3C, 4C, 5C, and Hi-C

Chromosome conformation capture (3C) assay has recently been developed to clarify a conformational proximity between promoter and a distal enhancer. Dekker developed the 3C assay to detect the frequency of interaction between any two genomic loci (Dekker et al. 2002). As shown in Fig. 6, firstly intact nuclei are isolated, and cells are subjected to formaldehyde fixation, which cross-links proteins to other proteins and to DNA (Step 1). For quantification of cross-linking frequencies, cross-linked DNA is digested with a restriction enzyme (Step 2). Then cross-linked DNA is subjected to ligation at very low concentration (Step 3). Cross-linking is reversed and individual ligation products are detected and quantified by the quantitative PCR (3C) using locus-specific primers or DNA microarray (4C) (Step 4). In Table 1, 3C-based methods were summarized. 3C or 4C generates single interaction profiles for individual loci (Dekker et al. 2013). While 3C typically yields a long-range interaction of a selected gene promoter, 4C generates a genome-wide interaction profile for a single locus. 5C and Hi-C methods are not anchored on a single locus of interest but instead generate matrices of interaction frequencies as two-dimensional heat maps with genomic positions along the two axes. 5C combines 3C with hybrid capture approaches to identify millions of interactions in parallel between two large sets of loci. Hi-C method is genome-wide adaptation of

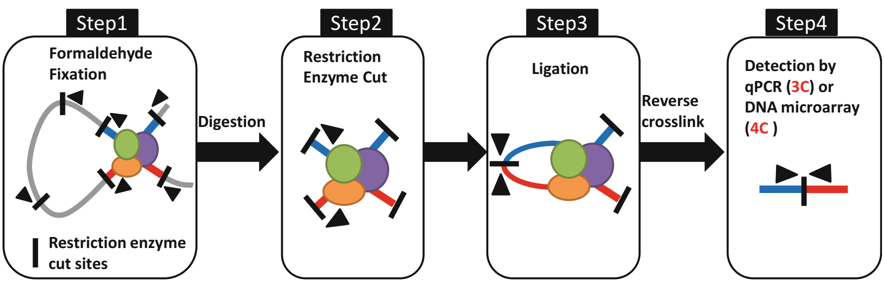

Fig. 6 3C or 4C methods. Step 1: Intact nuclei are isolated and cells are subjected to formaldehyde fixation, which cross-links proteins to other proteins and to DNA. Step 2: For quantification of cross-linking frequencies, cross-linked DNA is digested with a restriction enzyme. Step 3: Cross-linked DNA is subjected to ligation. Step 4: Cross-linking is reversed and individual ligation products are detected and quantified by the quantitative PCR (3C) using locus-specific primers or DNA microarray (4C)

Table 1 Summary of 3C-based methods

	Interactions	Detection methods	Image
3C	One-by-one	PCR or sequencing	Gene Promoter Interaction frequency Distance from prm
4C	One-by-all	Inverse PCR sequencing	anchor Numbers of reads
5C	Many-by-many	Sequencing	5C map Refseq Genes
Hi-C	All-by-all	Sequencing	Hi-C interaction map Chromosome Interaction enrichment

While 3C typically yields a long-range interaction of a selected gene promoter, 4C generates a genome-wide interaction profile for a single locus. 5C and Hi-C methods are not anchored on a single locus of interest but instead generate matrices of interaction frequencies as two-dimensional heat maps with genomic positions along the two axes

3C and includes a unique step in which the DNA ends are filled in with biotinylated nucleotides after restriction digestion. Hi-C could provide all-by-all genome-wide interaction map.

Applications of 3C or Hi-C in Research Fields Including Nephrology

Because chromosome conformation capture assays have recently been developed, there are few papers using these techniques in kidney research fields. In this paragraph, we will check papers of other fields. A study of human genome identified thousands of significant long-range looping interactions between gene promoters and distal loci, revealing that gene promoters engage with distal elements through looping (Sanyal et al. 2012). Many of the looping are cell type-specific interactions between active gene promoters and distal elements. They also demonstrated that relationships between genes and regulatory elements are far from exclusive: genes can interact with multiple distal elements and that elements can interact with multiple genes.

We demonstrated that the chromosome conformation can change under hypoxic condition via HIF-1 (hypoxia-inducible factor-1) and one of histone demethylases, KDM3A (lysine-specific demethylase 3A), using HUVECs (human umbilical vein endothelial cells) by 3C assay (Mimura et al. 2012). We performed ChIP-seq of HIF-1 and clarified that HIF-1 binds to not only promoter regions but also enhancer regions. We showed that HIF-1 and KDM3A cooperatively upregulate the downstream target gene, *SLC2A3* (solute carrier family 2A3; also known as GLUT3, glucose transporter 3), via removal of suppressive histone marks and chromosome conformational change. We also performed 3C assay under normoxic condition with knockdown of HIF-1 by siRNA and clarified that HIF-1 contributes to the loop formation under normoxia on the loci of *SLC2A3*.

Xu et al. performed 4C-seq (circular chromatin conformation capture) to identify a physical contact in human islets between insulin (INS) promoter and the calcium-activated chloride channel ANO1 gene (Xu et al. 2014). In response to glucose, this contact was strengthened and the expression of ANO1 increased. They demonstrated that networks of long-range physical contacts are important to the regulation of insulin metabolism. Bhattacharya et al. demonstrated that chromatin-chromatin interactions exist between upstream regulatory elements and the *Lmo2* (Lim domain only 2) promoter in erythroid cells using 3C experiments (Bhattacharya et al. 2012). *Lmo2* encodes a transcriptional cofactor critical for the development of hematopoietic stem cells. They also showed that the interactions are absent from the kidney where *Lmo2* is transcribed at 12-fold lower levels. Binding of CTCF (CCCTC-binding factor) and cohesin which support chromatin loops was found within the distal regions of *Lmo2* and proximal promoter of *Lmo2*, suggesting that these intergenic transcripts play an important role in regulating *Lmo2*.

Kim et al. performed 3C assays using in vivo kidney samples (Kim et al. 2012). Induction of HO-1 (heme oxygenase-1) is a beneficial response to tissue injury including acute kidney injury. They confirmed that transcription factors such as *USF1/2*, *Jun B*, *Sp1*, and *CTCF* were found to associate with regulatory regions of the human HO-1 in the kidney following rhabdomyolysis by 3C in the formation of chromatin looping in vivo. They also demonstrated hemin-inducible chromatin looping between the intronic enhancer and the promoter region using HK-2 (human kidney-2), a renal epithelial cell line (Deshane et al. 2010). They also showed that Sp1 small interfering RNA and mithramycin A, a Sp1 binding site inhibitor, resulted in loss of the loop formation between the intronic enhancer and the distal *HO-1* promoter. These results also demonstrated molecular interactions that underlie human HO-1 regulation in HK-2.

Cohesin and CTCF are known to be required for long-range interactions in eukaryotic genomes (Lee and Iyer 2012). However, how local chromatin interactions govern higher-order folding of chromatin fibers and the function of cohesion remain poorly understood. Mizuguchi et al. performed Hi-C analysis to explore the high-resolution organization of *Schizosaccharomyces pombe* genome (Mizuguchi et al. 2014). They revealed that heterochromatin mediates chromatin fiber compaction at centromeres and promotes prominent inter-arm interactions within centromere-proximal regions.

Conclusions

Genome-wide analysis using high-throughput sequencing has become more available in various kinds of research fields because of their technological progress and a reduction in costs. ChIP-seq, RNA-seq, RIP-seq, and chromosome conformation capture (3C) techniques are very useful tools for analyzing and clarifying the epigenetic molecular mechanisms. Advanced technology of ChIP-seq makes it possible to analyze the kidney tissue samples for in vivo analysis. Advanced technique for RNA-seq, a single-cell RNA-seq, can profile cell-to-cell variability on a genomic scale. RIP-seq can examine the miRNA-mRNA associations under specific conditions. 3C-based techniques have evolved into Hi-C at a rapid speed and make it possible to analyze the long-range interactions on multiple chromosomes. We make use of these tools to identify new biomarkers and to detect novel epigenetic factors which play important roles in progression of kidney diseases.

Summary Points

- Genome-wide analysis using high-throughput sequencing has become more available in research fields including nephrology.
- ChIP-seq, RNA-seq, RIP-seq, and chromosome conformation capture techniques are very useful tools for analyzing and clarifying the epigenetic molecular mechanisms.

- PAT-ChIP (pathology tissue-ChIP)-seq protocol on freshly isolated mouse embryonic kidneys can be used for in vivo analysis of transcriptional factor recruitment on chromatin.
- Advanced technique for RNA-seq is a single-cell RNA-seq, which can profile cell-to-cell variability on a genomic scale.
- RIP-seq can be used to analyze the RNA-binding proteins on genome-wide scale.
- 3C-based techniques have evolved into Hi-C at a rapid speed and make it possible to analyze the long-range interactions on multiple chromosomes.
- We make use of these tools to identify new biomarkers and to detect novel epigenetic factors which play important roles in progression of kidney diseases.

References

Amatori S, Ballarini M, et al. PAT-ChIP coupled with laser microdissection allows the study of chromatin in selected cell populations from paraffin-embedded patient samples. Epigenetics Chromatin. 2014;7:18.

Bhattacharya A, Chen CY, et al. Upstream distal regulatory elements contact the Lmo2 promoter in mouse erythroid cells. PLoS One. 2012;7(12):e52880.

Brunskill EW, Steven Potter S. RNA-Seq defines novel genes, RNA processing patterns and enhancer maps for the early stages of nephrogenesis: Hox supergenes. Dev Biol. 2012;368 (1):4–17.

Brunskill EW, Park JS, et al. Single cell dissection of early kidney development: multilineage priming. Development. 2014;141(15):3093–101.

Cernilogar FM, Onorati MC, et al. Chromatin-associated RNA interference components contribute to transcriptional regulation in Drosophila. Nature. 2011;480(7377):391–5.

de Boer IH, Afkarian M, et al. Renal outcomes in patients with type 1 diabetes and macroalbuminuria. J Am Soc Nephrol. 2014;25(10):2342–50.

Dekker J, Rippe K, et al. Capturing chromosome conformation. Science. 2002;295(5558):1306–11.

Dekker J, Marti-Renom MA, et al. Exploring the three-dimensional organization of genomes: interpreting chromatin interaction data. Nat Rev Genet. 2013;14(6):390–403.

Deshane J, Kim J, et al. Sp1 regulates chromatin looping between an intronic enhancer and distal promoter of the human heme oxygenase-1 gene in renal cells. J Biol Chem. 2010;285 (22):16476–86.

Fanelli M, Amatori S, et al. Pathology tissue-chromatin immunoprecipitation, coupled with high-throughput sequencing, allows the epigenetic profiling of patient samples. Proc Natl Acad Sci U S A. 2010;107(50):21535–40.

Fanelli M, Amatori S, et al. Chromatin immunoprecipitation and high-throughput sequencing from paraffin-embedded pathology tissue. Nat Protoc. 2011;6(12):1905–19.

Furey TS. ChIP-seq and beyond: new and improved methodologies to detect and characterize protein-DNA interactions. Nat Rev Genet. 2012;13(12):840–52.

Gupta R, Wikramasinghe P, et al. Annotation of gene promoters by integrative data-mining of ChIP-seq Pol-II enrichment data. BMC Bioinforma. 2010;11 Suppl 1:S65.

Heliot C, Cereghini S. Analysis of in vivo transcription factor recruitment by chromatin immunoprecipitation of mouse embryonic kidney. Methods Mol Biol. 2012;886:275–91.

Holman RR, Paul SK, et al. 10-year follow-up of intensive glucose control in type 2 diabetes. N Engl J Med. 2008;359(15):1577–89.

Jayaseelan S, Doyle F, et al. Profiling post-transcriptionally networked mRNA subsets using RIP-Chip and RIP-Seq. Methods. 2014;67(1):13–9.

Kanematsu S, Tanimoto K, et al. Screening for possible miRNA-mRNA associations in a colon cancer cell line. Gene. 2014;533(2):520–31.

Kim J, Zarjou A, et al. In vivo regulation of the heme oxygenase-1 gene in humanized transgenic mice. Kidney Int. 2012;82(3):278–91.

Lee BK, Iyer VR. Genome-wide studies of CCCTC-binding factor (CTCF) and cohesin provide insight into chromatin structure and regulation. J Biol Chem. 2012;287(37):30906–13.

Li Y, Zhao DY, et al. RIPSeeker: a statistical package for identifying protein-associated transcripts from RIP-seq experiments. Nucleic Acids Res. 2013;41(8):e94.

Li Y, Liu J, et al. Genome-wide analysis of the p53 gene regulatory network in the developing mouse kidney. Physiol Genomics. 2014;45(20):948–64.

Liu Q, Zhao S, et al. Gene and isoform expression signatures associated with tumor stage in kidney renal clear cell carcinoma. BMC Syst Biol. 2013;7 Suppl 5:S7.

Lu Z, Guan X, et al. RIP-seq analysis of eukaryotic Sm proteins identifies three major categories of Sm-containing ribonucleoproteins. Genome Biol. 2014;15(1):R7.

McLaughlin N, Yao X, et al. Histone signature of metanephric mesenchyme cell lines. Epigenetics. 2013;8(9):970–8.

Meier J, Hovestadt V, et al. Genome-wide identification of translationally inhibited and degraded miR-155 targets using RNA-interacting protein-IP. RNA Biol. 2013;10(6):1018–29.

Mimura I, Tanaka T, et al. Pathophysiological response to hypoxia – from the molecular mechanisms of malady to drug discovery: epigenetic regulation of the hypoxic response via hypoxia-inducible factor and histone modifying enzymes. J Pharmacol Sci. 2011;115(4):453–8.

Mimura I, Nangaku M, et al. Dynamic change of chromatin conformation in response to hypoxia enhances the expression of GLUT3 (SLC2A3) by cooperative interaction of hypoxia-inducible factor 1 and KDM3A. Mol Cell Biol. 2012;32(15):3018–32.

Mimura I, Tanaka T, et al. Novel therapeutic strategy with hypoxia-inducible factors via reversible epigenetic regulation mechanisms in progressive tubulointerstitial fibrosis. Semin Nephrol. 2013;33(4):375–82.

Mimura I, Kanki Y, et al. Revolution of nephrology research by deep sequencing: ChIP-seq and RNA-seq. Kidney Int. 2014;85(1):31–8.

Mizuguchi T, Fudenberg G, et al. Cohesin-dependent globules and heterochromatin shape 3D genome architecture in S. pombe. Nature. 2014;516:432–5.

Nie Z, Zhou F, et al. RIP-seq of BmAgo2-associated small RNAs reveal various types of small non-coding RNAs in the silkworm, Bombyx mori. BMC Genomics. 2013;14:661.

Pierron G, Tirode F, et al. A new subtype of bone sarcoma defined by BCOR-CCNB3 gene fusion. Nat Genet. 2012;44(4):461–6.

Pihlajamaa P, Sahu B, et al. Tissue-specific pioneer factors associate with androgen receptor cistromes and transcription programs. EMBO J. 2014;33(4):312–26.

Pirola L, Balcerczyk A, et al. Genome-wide analysis distinguishes hyperglycemia regulated epigenetic signatures of primary vascular cells. Genome Res. 2011;21(10):1601–15.

Saifudeen Z, Liu J, et al. A p53-Pax2 pathway in kidney development: implications for nephrogenesis. PLoS One. 2012;7(9):e44869.

Sanyal A, Lajoie BR, et al. The long-range interaction landscape of gene promoters. Nature. 2012;489(7414):109–13.

Sasagawa Y, Nikaido I, et al. Quartz-Seq: a highly reproducible and sensitive single-cell RNA-Seq reveals non-genetic gene expression heterogeneity. Genome Biol. 2013;14(4):R31.

Sun H, Wu J, et al. Genome-wide mapping of RNA Pol-II promoter usage in mouse tissues by ChIP-seq. Nucleic Acids Res. 2011;39(1):190–201.

Sung WK, Zheng H, et al. Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. Nat Genet. 2012;44(7):765–9.

Tallack MR, Magor GW, et al. Novel roles for KLF1 in erythropoiesis revealed by mRNA-seq. Genome Res. 2012;22(12):2385–98.

Thiagarajan RD, Cloonan N, et al. Refining transcriptional programs in kidney development by integration of deep RNA-sequencing and array-based spatial profiling. BMC Genomics. 2011;12:441.

Tsukada Y, Fang J, et al. Histone demethylation by a family of JmjC domain-containing proteins. Nature. 2006;439(7078):811–6.

Ueda K, Fujiki K, et al. Genome-wide analysis of murine renal distal convoluted tubular cells for the target genes of mineralocorticoid receptor. Biochem Biophys Res Commun. 2014;445 (1):132–7.

Villeneuve LM, Reddy MA, et al. Epigenetic histone H3 lysine 9 methylation in metabolic memory and inflammatory phenotype of vascular smooth muscle cells in diabetes. Proc Natl Acad Sci U S A. 2008;105(26):9047–52.

Ware CB, Wang L, et al. Histone deacetylase inhibition elicits an evolutionarily conserved self-renewal program in embryonic stem cells. Cell Stem Cell. 2009;4(4):359–69.

Writing Team for the Diabetes, C., I. Complications Trial/Epidemiology of Diabetes, et al. Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy: the Epidemiology of Diabetes Interventions and Complications (EDIC) study. JAMA. 2003;290(16):2159–67.

Xu Z, Lefevre GM, et al. Mapping of long-range INS promoter interactions reveals a role for calcium-activated chloride channel ANO1 in insulin secretion. Proc Natl Acad Sci U S A. 2014;111(47):16760–5.

Yae T, Tsuchihashi K, et al. Alternative splicing of CD44 mRNA by ESRP1 enhances lung colonization of metastatic cancer cell. Nat Commun. 2012;3:883.

Zhao J, Ohsumi TK, et al. Genome-wide identification of polycomb-associated RNAs by RIP-seq. Mol Cell. 2010;40(6):939–53.

Cell Cycle Arrest Biomarkers in Kidney Disease

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Kianoush Kashani, Erin N. Frazee, and John A. Kellum

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Abstract

Acute kidney injury remains one of the most common and deadly complications of critical illness. Early recognition of this syndrome potentially allows more efficient prophylactic and potentially therapeutic options. The functional biomarkers of kidney injury are very insensitive to the changes of kidney function

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early in the course of AKI and also nonspecific to the etiology of kidney damage. The critical need for novel biomarkers of AKI resulted in a significant number of efforts which concluded discovery and validation of several new AKI biomarkers. The most recent and indeed the most specific biomarkers of kidney stress are recently discovered and validated and currently approved by the Food and Drug Administration (FDA) for identification of AKI high-risk individuals among ICU patients. These biomarkers, i.e., insulin growth factor-binding protein-7 (IGFBP7) and tissue inhibitor metalloproteinases-2 (TIMP-2), are able to identify high-risk patients in ICU, 12 h before the functional biomarkers are able to detect AKI. These proteins are involved in the pathophysiology and natural history of AKI by halting the progression of the cell cycle following injury during the G_1- to S-phase transition. In this review, we will describe the role of the cell cycle during AKI and the relationship between the cell cycle arrest and maladaptive recovery of the kidney following an injury. Then we focus on cell cycle arrest biomarkers and their relationship with AKI, their physiological roles, and finally potential clinical applications.

Keywords

Tissue inhibitor metalloproteinases-2 (TIMP-2) • Insulin-like growth factor binding protein-7 (IGFBP-7) • Cell cycle arrest • Biomarker • Acute kidney injury

Abbreviations

βFGF	β fibroblast growth factor
AKI	Acute kidney injury
CDK	Cyclin-dependent protein kinase
DAMP	Damage-associated molecular pattern
DDR	DNA Damage Response
DNA	Deoxyribonucleic acid
EGF	Epithelial growth factor
FDA	Food and Drug Administration
G_1	Gap 1
G_2	Gap 2
ICU	Intensive care unit
IGFBP7	Insulin-like Growth Factor Binding Protein-7
IL-18	Interleukin-18
ITG α3 β1	Integrin α3/β1
KDIGO	Kidney Disease Improving Global Outcomes
KIM-1	Kidney injury molecule -1
L-FABP	Liver fatty acid binding protein
M	Mitosis
MMP	Metalloproteinases
NGAL	Neutrophil gelatinase-associated lipocalin
NGF	Nerve growth factor
PAMP	Pathogen-associated molecular pattern
PCNA	Proliferating cell nuclear antigen

ROS	Reactive oxygen species
S	Synthesis
SA-β-gal	Senescence-associated galactosidase
TIMP-2	Tissue Inhibitor Metalloproteinases-2
TGF-β	Transforming growth factor- β
VEGF	Vascular endothelial growth factor

Key Facts

- AKI pathophysiology is very complex. Cell cycle arrest is a part of the pathogenesis of AKI.
- Biomarkers of cell cycle arrest can predict moderate to severe AKI at least 12 h before its clinical presentation.
- There are two cutoffs defined for [TIMP-2] [IGFBP7], a high-sensitivity cutoff at 0.3 and a high-specificity cutoff at 2 $(ng/ml)^2/1,000$.

Introduction

Acute kidney injury (AKI) is one of the most common complications of hospital and intensive care unit (ICU) admissions and it is associated with significant clinical consequences. In a large population-based epidemiological study, the incidence of AKI was found to be 1,811 cases per million persons (Ali et al. 2007). Among all hospitalized patients and specifically those in the ICU, the incidences of AKI rise dramatically to 20 % and 67 %, respectively (Uchino et al. 2006; Ostermann and Chang 2007; Hoste et al. 2006). In addition, AKI carries an independent risk of mortality and transition to both chronic kidney disease and end-stage kidney disease (Chawla et al. 1361; Joannidis n.d.; Uchino et al. 2010).

Despite the gravity of AKI as a critical illness complication, very few therapeutic interventions have been tested in humans, the majority of which have been found to be ineffective (Aydin et al. 2007). A plausible explanation for the lack of success with previously tested therapies could relate to an inability to successfully identify patients at high risk for AKI early in their disease course when they most likely benefit from prophylactic or therapeutic strategies. Apart from risk stratification tools to identify high-risk patients, sensitive and early biomarkers of kidney stress or injury could play significant roles in this regard. Early identification and intervention is likely to result in a significant improvement in the outcomes of patients with AKI, a goal that has as yet been difficult to achieve (Himmelfarb et al. 2008).

In the past decade, the number of studies to discover and validate novel biomarkers of AKI has significantly grown. Kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), urinary interleukin-18 (IL-18), and liver fatty acid-binding protein (L-FABP) are among these new discoveries (Ichimura and Mou 2008; Mishra et al. 2005; Siew et al. 1497; Yokoyama et al. 2009). These biomarkers are able to predict AKI development and its

consequences. However, despite the seemingly excellent progress in this field, a recent systematic review concluded that these biomarkers are only effective in early recognition of AKI in a well-defined timed injury in the pediatric population. In adult patients with multiple comorbid conditions and nebulous time course of injury, however, these biomarkers were found to be significantly less robust for early recognition of AKI (Vanmassenhove et al. 2012).

Recently, a new group of biomarkers associated with the cell cycle arrest were found to be predictive of AKI in a heterogenous cohort of critically ill adults and validation studies confirmed these findings (Kashani et al. 2013; Bihorac et al. 2014). In this chapter, we will review the physiology of the cell cycle, its relationship with AKI, and the implications for new biomarkers in clinical practice.

Cell Cycle in Renal Tubular Cells

Under normal physiologic conditions, the majority of renal tubular epithelial cells are in a quiescent [Gap 0 (G0)] state (Shankland et al. 2000). After AKI, as part of the normal repair mechanisms, tubular cells enter the active cell cycle to replace any necrotic or apoptotic cells or any other gaps in the epithelial barrier due to detached cells. The first step of the active cell cycle is interphase, wherein cells prepare for mitosis (M). The interphase includes three stages: gap 1 (G1), synthesis (S), and finally gap 2 (G2). During G1 or the growth phase, cell biosynthetic activities increase significantly. Cells gather supplements required for replication of deoxyribonucleic acid (DNA) content, including proteins and organelles such as ribosomes and mitochondria. In the S phase, all DNA content of the cell is replicated. Cells continue their growth during the G2 phase and then pass into mitosis. Mitosis itself includes four phases (prophase for condensation of chromatin to chromosomes, metaphase for alignment of chromosomes at the equator of the cell, anaphase for splitting sister chromosomes to the opposite pole of the cell, and finally telophase for formation of two daughter cells) (Temple and Raff 1986; Peters 1998; Karp 2005).

During the cell cycle, eukaryotic cells undergo vigorous self-examination to ensure the fidelity of their DNA content. This process happens during at least three well-recognized checkpoints. The first cell cycle checkpoint, also called restriction point at G1/S, happens immediately before cells enter the S phase. Cyclin-dependent protein kinase (CDK) inhibitors, including P21, P16, and P53, halt the progression of the cell cycle from the G1 to S phase by inhibiting the CDK complexes (CyclD-CDK4 and CyclE-CDK2) (Chkhotua et al. 2006; Melk et al. 2005; Price et al. 2004; Tanaka et al. 2005). The second well-known checkpoint is immediately before the beginning of mitosis at the G2 phase. There is evidence that the inability to transition from G2 to M promotes a fibrogenic, "maladaptive" recovery after AKI (Yang et al. 2010). Finally, the third checkpoint is located at the metaphase to assess the tension in bipolar attachments among chromosomes.

If at any of these checkpoints, cells fail to proceed to the next step, they encounter one of these outcomes: (1) transient arrest, repair, and return to cell cycle;

(2) defective repair typically leading to apoptosis or cell senescence; and (3) direct apoptosis when the damage is very severe (Price et al. 2009).

Relationship Between the Cell Cycle and AKI

The pathogenesis of AKI is extremely complex. A combination of injuries to endothelial and epithelial cells in the setting of inflammation leads to the clinical presentation of AKI (Sharfuddin and Molitoris 2011). One of the proposed mechanisms involved in the evolution of AKI is derangements in the cell cycle and its arrest. In response to DNA damage, the DDR (DNA-damage response network) and P53 are activated to determine the ultimate fate of an injured cell. During cell cycle arrest, the cells assess the extent of damage and need for repair.

In a murine model of septic AKI caused by cecal ligation and puncture, G1 cell cycle arrest was found to play an essential role in the development of AKI (Yang et al. 2009). Flow cytometry of the DNA content of kidney cells showed the number of cells in G1 significantly increased within 6 h of insult, while the amount of cells in the S phase decreased in this time frame. The increased G1 to S cell ratio continued for at least 24 h, and it was only after 72 h that the number of S cells increased significantly and clinical improvement of AKI occurred. In this experience, the authors noted a significant upregulation of P53 and P21 during the first 24 h [the cell cycle arrest indicators], CDK [the S phase indicators] after the first 24 h, and finally retinoblastoma protein [a proliferation indicator] 72 h from the original insult. In another murine model of AKI induced by clamping renal arteries, investigators noted changes of injury, G/S transition, and differentiation markers in the S3 segment of proximal tubules happen in series (Witzgall et al. 1994). These investigators found an initial increase in clusterin [injury marker], followed by up-regulation of proliferating cell nuclear antigen (PCNA) [G1 to S transition marker], and finally vimentin [differentiation marker] during the first 120 h of initial insult. In other models of AKI including cisplatin exposure, ischemia reperfusion, and ureteral obstruction, rapid induction of P21 in proximal and distal tubular cells is demonstrated (Megyesi et al. 1996). Knowing that P21 is heavily involved with the G1/S cell cycle arrest would suggest that the cell cycle can be initiated very early in the course of injury, and as an integrated part of the process, G1/S cell cycle arrest happens in the initial steps of the cell cycle if certain conditions are met (e.g., damage, ongoing stress).

Cell Cycle Arrest Urinary Biomarkers

In recent discovery and validation studies, two cell cycle arrest biomarkers of AKI, insulin growth factor-binding protein-7 (IGFBP7), and tissue inhibitor metalloproteinases-2 (TIMP-2) showed high performance in prediction of AKI before its clinical presentation (Kashani et al. 2013; Bihorac et al. 2014). In this

section we describe characteristics of these two proteins and their relationship with the cell cycle arrest during AKI.

IGFBP7

IGFBP7 is a 27-kDA protein from the 16-member IGFBP superfamily and it is expressed in renal epithelial cells (Degeorges et al. 2000; Matsumoto et al. 2010). IGFBP7 is known to weakly bind IGF-1 and IGF-2, and this bond is weaker than other IGFBP family members 1–6. It also binds insulin with over 500-fold greater affinity than other family members (Burger et al. 2005). Its expression is induced by P53, DNA injury induced by retinoic acid, transforming growth factor- β (TGF-β), glucocorticoids, or reactive oxygen species (ROS) (Burger et al. 2005; Pereira et al. 1999; Swisshelm et al. 1995). IGFBP7 regulates tissue availability of insulin-like growth factors, stimulates cell adhesion (Nagakubo et al. 2003), and promotes cell repair. IGFBP7 is induced in renal microvasculature after ischemia (Usui et al. 2002) and is involved in cell senescence (Acosta et al. 2008; Cichowski and Hahn 2008; Vicencio et al. 2008). It also plays a role in the epithelial cell cycle arrest including M12 prostate cells (Sprenger et al. 2002). Given the involvement of IGFBP7 in senescence, proliferation, and the cell cycle arrest, the potential exists that it may also be an early marker of cellular (DNA) damage.

The role of IGFBP7, particularly during AKI, is of interest. In an in vitro experiment with human melanoma cell lines, it was noted that increasing content of recombinant IGFBP7 in the culture environment is associated with a dose-dependent decrease in cell proliferation and also increased apoptosis (Wajapeyee et al. 2008). In another in vitro experiment with MCF-7 breast cancer cells, investigators noted transfecting cells with IGFBP7 or adding it to the culture media induced senescent phenotypes, including decreased cell proliferation, increased G1/S cell cycle arrest cells, altered cell morphology, and increased senescence-associated galactosidase (SA-β-gal) activity. The observed growth stoppage was associated with increased expression of the CDK inhibitor P21 and dephosphorylation of the retinoblastoma protein. This phenomenon was partially reversed by P21 knockdown in MCF-7 cells, while P53 knockdown did not influence the growth inhibition and cellular senescence (Zuo et al. 2012).

TIMP-2

Tissue inhibitor metalloproteinases-2 is a 21-kDA protein from the four-member TIMP family. This protein is expressed in melanoma cells and renal tubular cells (e.g., in polycystic kidney disease). TIMP-2 irreversibly inactivates metalloproteinases (MMP) by binding to their catalytic zinc cofactor. TIMP-2 acts on MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-13,

MMP-14, MMP-15, MMP-16, and MMP-19 and is also required for activation of MMP-2 (Goldberg et al. 1989; Stetler-Stevenson et al. 1989). TIMP-2 expression is influenced by a number of cytokines and chemokines and is upregulated by signals that control proliferation (βFGF and EGF) and differentiation (retinoic acid and NGF) (Jaworski and Pérez-Martínez 2006). Additionally, TIMP-2 has metalloproteinase-independent cell-signaling activities. Studies have shown that TIMP-2 binds to human endothelial cells through integrin α3/β1 (ITG α3 β1), which results in G1 cell cycle arrest and inhibition of proliferation (Seo et al. 2006; Stetler-Stevenson 2008; Chang et al. 2006; Henriet et al. 1999; Perez-Martinez and Jaworski 2005). Similarly, binding of TIMP-2 through ITG α3 β1 mediates suppression of FGF2 or VEGF and induces endothelial cell proliferation in vitro and angiogenesis in vivo (Bourboulia et al. 2011; Seo et al. 2003, 2008, 2011). ITG α3 β1 regulates kidney epithelial cell responses to TGF-β which indicates the ITG-α3-β1-specific mechanisms described for endothelial cells could occur in renal epithelium (Kim et al. 2009). TIMP-2 excretion is induced by ROS, differentiation signals (retinoic acid), and proliferation signals (EGF). During kidney injury TIMP-2 is involved in a variety of events including factors involved in innate immunity such as structural changes influencing leukocyte transmigration from the capillaries to areas of injury in the renal tubule (Opdenakker et al. 2001; Stefanidakis et al. 2006), changes in endothelial permeability (Catania et al. 2007), and modulation of the inflammatory response (Manicone and McGuire 2008; Garton et al. 2006). It is also involved in later events that occur with more serious injury such as apoptosis (cell death) (Ii et al. 2006a; Mannello et al. 2005), loss of cell-cell adhesion, and sloughing of tubular epithelial cells (Catania et al. 2007; Manicone and McGuire 2008; Ii et al. 2006b). Seo et al., in an in vitro investigation of the effect of TIMP-2 on human microvascular endothelial cell proliferation, observed that TIMP-2, via a mechanism independent of MMP inhibition, decreased endothelial cell proliferation (Seo et al. 2003).

Mechanism of Action of Cell Cycle Arrest Biomarkers in AKI

As outlined above, both of the candidate biomarkers are excreted during DNA damage and are involved with the G1/S cell cycle arrest. During early phases of AKI, induced by ROS, pathogen-associated molecular pattern (PAMP), and damage-associated molecular pattern (DAMP) molecules, renal epithelial cells enter the cell cycle and then stop immediately before the S phase. This arrest is highly regulated and several proteins are involved. IGFBP7 and TIMP-2 as part of this machinery are expressed during tubular epithelial cell injury. They appear to act as autocrine signals but also in a paracrine fashion alerting neighboring epithelial cells. P53 and P21 expression is directly induced by IGFBP7 and P27 by TIMP-2. The cell cycle promotion effect of CDK complexes (CyclD-CDK4 and CyclE-CDK2) is directly blocked by P-proteins which results in the initiation of a transient G1/S cell cycle arrest.

Applications of Cell Cycle Arrest Biomarkers for AKI

In recent years the role of cell cycle arrest biomarkers in the early detection of AKI has been validated (Kashani et al. 2013; Bihorac et al. 2014). In the discovery phase, all patients with known AKI at the time of enrollment were excluded. Then, in a protocolized process, more than 340 proteins were measured in multiple samples collected at predefined time points (Kashani et al. 2013; Bihorac et al. 2014). These candidate proteins were selected based on the current knowledge of AKI pathophysiology and its related processes including inflammation, apoptosis, necrosis, endothelial injury, cell-cell and cell-matrix adhesion, cytoprotection, oxidative processes, and the cell cycle. Among all of these potential biomarkers, two molecules related to the cell cycle arrest exhibited superior performance in the early detection of AKI. Hereafter, the clinical validation phase involved a large-scale multicenter study of 728 patients from 35 medical centers in North America and Europe (Kashani et al. 2013). These patients were critically ill adults more than 21 years of age who were admitted to the ICU. Patients with Kidney Disease: Improving Global Outcomes (KDIGO) stage 2 or 3 AKI were excluded during the screening process. This study found that cell cycle arrest biomarkers performed better in the early detection of this syndrome compared to previously described AKI biomarkers such as KIM-1 and NGAL (Kashani et al. 2013). During this study investigators noted that in surgical patients the best individual marker was IGFBP7 while TIMP-2 performed better in patients with sepsis. The product of these two markers ([TIMP-2] [IGFBP7]) was selected as a biomarker panel for AKI risk stratification.

In the follow-up validation study, investigators enrolled 420 patients within the first 24 h of admission to the ICU (Bihorac et al. 2014). Twenty three centers in the USA participated in this study. Investigators excluded patients who had KDIGO stage 2 or 3 AKI at the screening phase. Diagnosis of AKI within 12 h of enrollment adjudicated by a clinical adjudication committee was used as the primary endpoint. Investigators used the highly sensitive threshold of 0.3 $(ng/ml)^2/1,000$ for [TIMP-2] [IGFBP7]. In this study the performance of a clinical model to predict AKI was only fair [the receiving operating characteristic area under curve (AUC) was only 0.70 (95 % CI, 0.63–0.76)]. The performance of the model was significantly enhanced when the urinary [TIMP-2] [IGFBP7] was added to the model (AUC increased to 0.86 (95 % CI, 0.80–0.90)) (Bihorac et al. 2014).

Cutoffs for [TIMP-2] [IGFBP7] for clinical use were validated in another follow-up investigation. In this study 154 patients from six sites in the USA were enrolled (Hoste et al. 2014). Unlike the other two studies that used a central laboratory and ELISA for measurement of [TIMP-2] [IGFBP7], each site used the commercial platform (NephroCheck®) to measure the urinary [TIMP-2] [IGFBP7] at the local level. Eligibility criteria for enrollment were similar to the earlier studies. Two previously determined thresholds from the original validation study (0.3 and 2 $(ng/ml)^2/1,000$) were validated as the sensitivity and specificity cutoffs, respectively. Investigators found a sensitivity of 89 % for the 0.3 $(ng/ml)^2/1,000$ cutoff and a specificity of 90 % for the 2 $(ng/ml)^2/1,000$ as the cutoff. Therefore, authors concluded the lower cutoff could be used for screening and risk stratification

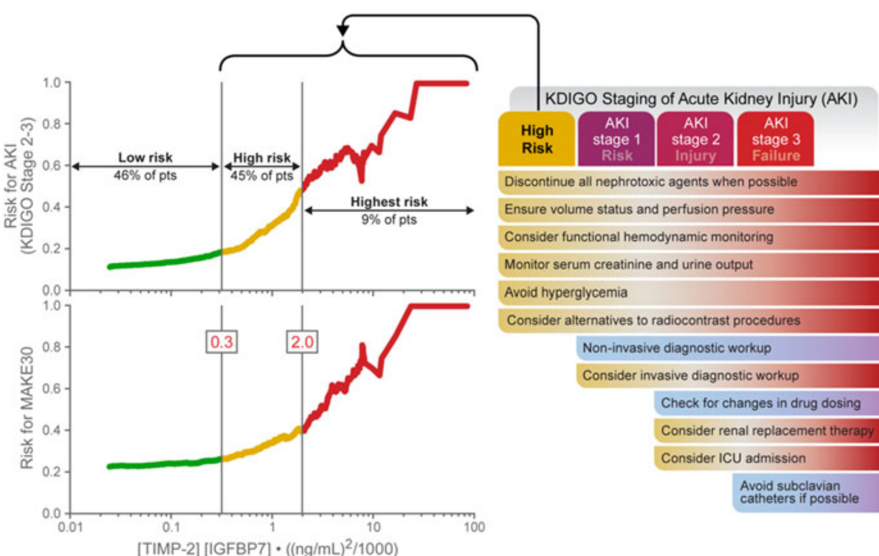

Fig. 1 Clinical application of cell cycle arrest biomarkers. Identifying patients who are at high risk or very high risk for development of AKI is very important. This figure delineates how identification of these patients could be in alignment with the Kidney Disease: Improving Global Outcomes (*KDIGO*) guidelines for AKI (Summary of Recommendation Statements 2012)

processes while the higher cutoff could be used to identify patients at very high likelihood of developing AKI (Hoste et al. 2014) (Fig. 1). A subsequent analysis of data from the Sapphire trial revealed that these cutoffs were able to accurately predict 9-month death or dialysis in ICU patients developing AKI (Koyner et al. 2013).

Further studies have revealed that in patients who underwent cardiac surgery, serial urinary samples of [TIMP-2] [IGFBP7] were predictive of postoperative AKI (Meersch et al. 2014). This cohort included 50 patients, 52 % of whom developed AKI (including stage 1). The authors reported that the maximum urinary [TIMP-2] [IGFBP7] concentration achieved within the first 24 h after cardiopulmonary bypass was predictive of AKI with an AUC of 0.84 – sensitivity of 92 % and specificity of 81 % at a cutoff of 0.5 $(ng/ml)^2/1{,}000$ (Meersch et al. 2014).

In September 2014, the US Food and Drug Administration (FDA) approved the marketing of [TIMP-2] [IGFBP7] to assess a patient's risk for developing AKI (FDA & FDA 2014). This milestone not only allows the clinical use of these biomarkers for early detection of patients with AKI but may also facilitate enrollment in interventional studies designed to prevent or treat this deadly syndrome (Endre et al. 2014). Indeed, this approval announcement is the "beginning of a new era" (Endre et al. 2014).

Importantly, while cell cycle arrest clearly has a "dark side" and is associated with development of AKI and long-term adverse outcomes, there is a "light side" as well. Cell cycle arrest can protect cells from the disastrous consequences of entering cell division with damaged DNA or insufficient bioenergetic resources during injury or

stress. Whether we can use the light side to help prevent AKI remains to be seen, but there is already evidence that cell cycle arrest biomarkers such as IGFBP7 and TIMP-2 are indicators of both sides of this complex physiology.

Summary Points

In conclusion, AKI is a deadly syndrome that affects millions of patients around the world. Damaged renal epithelial cells enter the cell cycle shortly after the injury. There is a cell cycle arrest immediately prior to the S phase, and IGFBP7 and TIMP-2 are among the mediators of this process. These two proteins are sensitive and specific markers for the prediction of AKI and have now been validated in several large-scale investigations. [TIMP-2] [IGFBP7] is currently FDA approved for clinical use to allow accurate assessment for the risk of developing AKI.

References

Acosta JC, et al. Control of senescence by CXCR2 and its ligands. Cell Cycle. 2008;7(19):2956–9.

Ali T, et al. Incidence and outcomes in acute kidney injury: a comprehensive population-based study. J Am Soc Nephrol. 2007;18:1292–8.

Aydin Z, et al. New horizons in prevention and treatment of ischaemic injury to kidney transplants. Nephrol Dial Transplant. 2007;22(2):342–6.

Bihorac A, et al. Validation of cell-cycle arrest biomarkers for acute kidney injury using clinical adjudication. Am J Respir Crit Care Med. 2014;189(8):932–9.

Bourboulia D, et al. Endogenous angiogenesis inhibitor blocks tumor growth via direct and indirect effects on tumor microenvironment. Am J Pathol. 2011;179(5):2589–600.

Burger AM, et al. Essential roles of IGFBP-3 and IGFBP-rP1 in breast cancer. Eur J Cancer. 2005;41(11):1515–27.

Catania JM, Chen G, Parrish AR. Role of matrix metalloproteinases in renal pathophysiologies. Am J Physiol Renal Physiol. 2007;292(3):F905–11.

Chang H, et al. TIMP-2 promotes cell spreading and adhesion via upregulation of Rap1 signaling. Biochem Biophys Res Commun. 2006;345(3):1201–6.

Chawla LS, et al. The severity of acute kidney injury predicts progression to chronic kidney disease. Kidney Int. n.d.;79(12):p. 1361–9.

Chkhotua AB, et al. Up-regulation of cell cycle regulatory genes after renal ischemia/reperfusion: differential expression of p16(INK4a), p21(WAF1/CIP1) and p27(Kip1) cyclin-dependent kinase inhibitor genes depending on reperfusion time. Transpl Int: Off J Eur Soc Organ Transplant. 2006;19(1):72–7.

Cichowski K, Hahn WC. Unexpected pieces to the senescence puzzle. Cell. 2008;133(6):958–61.

Degeorges A, et al. Distribution of IGFBP-rP1 in normal human tissues. J Histochem Cytochem: Off J Histochem Soc. 2000;48(6):747–54.

Endre ZH, Pickering JW. Acute kidney injury: cell cycle arrest biomarkers win race for AKI diagnosis. Nat Rev Nephrol. 2014. Advance online publication.

FDA, FDA allows marketing of the first test to assess risk of developing acute kidney injury. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm412910.htm. 2014.

Garton KJ, Gough PJ, Raines EW. Emerging roles for ectodomain shedding in the regulation of inflammatory responses. J Leukoc Biol. 2006;79(6):1105–16.

Goldberg GI, et al. Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2. Proc Natl Acad Sci U S A. 1989;86 (21):8207–11.
Henriet P, Blavier L, Declerck YA. Tissue inhibitors of metalloproteinases (TIMP) in invasion and proliferation. APMIS: Acta Pathol Microbiol Immunol Scand. 1999;107(1):111–9.
Himmelfarb J, et al. Evaluation and initial management of acute kidney injury. Clin J Am Soc Nephrol. 2008;3(4):962–7.
Hoste EA, et al. RIFLE criteria for acute kidney injury are associated with hospital mortality in critically ill patients: a cohort analysis. Crit Care. 2006;10(3):R73.
Hoste EAJ, et al. Derivation and validation of cutoffs for clinical use of cell cycle arrest biomarkers. Nephrol Dial Transplant. 2014;29(11):2054–61.
Ichimura T, Mou S. Kidney injury molecule-1 in acute kidney injury and renal repair: a review. Zhong Xi Yi Jie He Xue Bao/J Chin Integr Med. 2008;6(5):533–8.
Ii M, et al. Role of matrix metalloproteinase-7 (Matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. Exp Biol Med. 2006;231(1):20–7.
Jaworski DM, Pérez-Martínez L. Tissue inhibitor of metalloproteinase-2 (TIMP-2) expression is regulated by multiple neural differentiation signals. J Neurochem. 2006;98(1):234–47.
Joannidis M, et al. Acute kidney injury in critically ill patients classified by AKIN versus RIFLE using the SAPS 3 database. Intensive Care Med.
Karp G. Cell and molecular biology: concepts and experiments. 4th ed. Hoboken: Wiley; 2005.
Kashani K, et al. Discovery and validation of cell cycle arrest biomarkers in human acute kidney injury. Crit Care. 2013;17(1):R25.
Kim Y, et al. Integrin alpha3beta1-dependent beta-catenin phosphorylation links epithelial Smad signaling to cell contacts. J Cell Biol. 2009;184(2):309–22.
Koyner J, et al. Increased TIMP2•IGFBP7 is associated with increased 9 month mortality in ICU patients at risk for AKI. J Am Soc Nephrol. 2013;24:40A.
Manicone AM, McGuire JK. Matrix metalloproteinases as modulators of inflammation. Semin Cell Dev Biol. 2008;19(1):34–41.
Mannello F, et al. Multiple roles of matrix metalloproteinases during apoptosis. Apoptosis. 2005;10 (1):19–24.
Matsumoto T, et al. Proteomic analysis identifies insulin-like growth factor-binding protein-related protein-1 as a podocyte product. Am J Physiol Renal Physiol. 2010;299(4):F776–84.
Meersch M, et al. Urinary TIMP-2 and IGFBP7 as early biomarkers of acute kidney injury and renal recovery following cardiac surgery. PLoS One. 2014;9(3):e93460.
Megyesi J, et al. The p53-independent activation of transcription of p21 WAF1/CIP1/SDI1 after acute renal failure. Am J Physiol. 1996;271(6 Pt 2):F1211–6.
Melk A, et al. Increased expression of senescence-associated cell cycle inhibitor p16INK4a in deteriorating renal transplants and diseased native kidney. Am J Transplant: Off J Am Soc Transplant Am Soc Transplant Surg. 2005;5(6):1375–82.
Mishra J, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. Lancet. 2005;365(9466):1231–8.
Nagakubo D, et al. A high endothelial venule secretory protein, mac25/angiomodulin, interacts with multiple high endothelial venule-associated molecules including chemokines. J Immunol. 2003;171(2):553–61.
Opdenakker G, et al. Gelatinase B functions as regulator and effector in leukocyte biology. J Leukoc Biol. 2001;69(6):851–9.
Ostermann M, Chang RW. Acute kidney injury in the intensive care unit according to RIFLE. Crit Care Med. 2007;35:1837–43.
Pereira RC, Blanquaert F, Canalis E. Cortisol enhances the expression of mac25/insulin-like growth factor-binding protein-related protein-1 in cultured osteoblasts. Endocrinology. 1999;140 (1):228–32.

Perez-Martinez L, Jaworski DM. Tissue inhibitor of metalloproteinase-2 promotes neuronal differentiation by acting as an anti-mitogenic signal. J Neurosci: Off J Soc Neurosci. 2005;25 (20):4917–29.
Peters J-M. SCF and APC: the Yin and Yang of cell cycle regulated proteolysis. Curr Opin Cell Biol. 1998;10(6):759–68.
Price PM, Megyesi J, Saf Irstein RL. Cell cycle regulation: repair and regeneration in acute renal failure. Kidney Int. 2004;66(2):509–14.
Price PM, Safirstein RL, Megyesi J. The cell cycle and acute kidney injury. Kidney Int. 2009;76 (6):604–13.
Seo DW, et al. TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism. Cell. 2003;114(2):171–80.
Seo D-W, et al. Shp-1 mediates the antiproliferative activity of tissue inhibitor of metalloproteinase-2 in human microvascular endothelial cells. J Biol Chem. 2006;281(6):3711–21.
Seo DW, et al. TIMP-2 disrupts FGF-2-induced downstream signaling pathways. Microvasc Res. 2008;76(3):145–51.
Seo DW, et al. An integrin-binding N-terminal peptide region of TIMP-2 retains potent angio-inhibitory and anti-tumorigenic activity in vivo. Peptides. 2011;32(9):1840–8.
Shankland SJ, Wolf G. Cell cycle regulatory proteins in renal disease: role in hypertrophy, proliferation, and apoptosis. Am J Physiol Renal Physiol. 2000;278:F515–29.
Sharfuddin AA, Molitoris BA. Pathophysiology of ischemic acute kidney injury. Nat Rev Nephrol. 2011;7(4):189–200.
Siew ED, et al. Elevated urinary IL-18 levels at the time of ICU admission predict adverse clinical outcomes. Clin J Am Soc Nephrol. n.d.;5(8):p. 1497–505.
Sprenger CC, et al. Over-expression of insulin-like growth factor binding protein-related protein-1 (IGFBP-rP1/mac25) in the M12 prostate cancer cell line alters tumor growth by a delay in G1 and cyclin A associated apoptosis. Oncogene. 2002;21(1):140–7.
Stefanidakis M, Koivunen E. Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and cancer progression. Blood. 2006;108:1441–50.
Stetler-Stevenson WG. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. 2008;1:re6.
Stetler-Stevenson WG, Krutzsch HC, Liotta LA. Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. J Biol Chem. 1989;264(29):17374–8.
Summary of recommendation statements. Kidney Inter Suppl. 2012;2(1):p. 8–12.
Swisshelm K, et al. Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. Proc Natl Acad Sci U S A. 1995;92(10):4472–6.
Tanaka H, et al. Role of the E2F1-p19-p53 pathway in ischemic acute renal failure. Nephron Physiol. 2005;101(2):27–34.
Temple S, Raff MC. Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions. Cell. 1986;44(5):773–9.
Uchino S, et al. An assessment of the RIFLE criteria for acute renal failure in hospitalized patients. Crit Care Med. 2006;34:1913–7.
Uchino S, et al. Transient azotaemia is associated with a high risk of death in hospitalized patients. Nephrol Dial Transplant: Off Publ Eur Dial Transplant Assoc – Eur Ren Assoc. 2010;25 (6):1833–9.
Usui T, et al. Characterization of mac25/angiomodulin expression by high endothelial venule cells in lymphoid tissues and its identification as an inducible marker for activated endothelial cells. Int Immunol. 2002;14(11):1273–82.
Vanmassenhove J, et al. Urinary and serum biomarkers for the diagnosis of acute kidney injury: an in-depth review of the literature. Nephrol Dial Transplant. 2012.
Vicencio JM, et al. Senescence, apoptosis or autophagy? When a damaged cell must decide its path – a mini-review. Gerontology. 2008;54(2):92–9.

Wajapeyee N, et al. Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. Cell. 2008;132(3):363–74.
Witzgall R, et al. Localization of proliferating cell nuclear antigen, vimentin, c-Fos, and clusterin in the postischemic kidney. Evidence for a heterogenous genetic response among nephron segments, and a large pool of mitotically active and dedifferentiated cells. J Clin Invest. 1994;93 (5):2175–88.
Yang Q-H, et al. Acute renal failure during sepsis: potential role of cell cycle regulation. J Infect. 2009;58(6):459–64.
Yang L, et al. Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury. Nat Med. 2010;16(5):535–43.
Yokoyama T, et al. Urinary excretion of liver type fatty acid binding protein accurately reflects the degree of tubulointerstitial damage. Am J Pathol. 2009;174(6):2096–106.
Zuo S, et al. IGFBP-rP1 induces p21 expression through a p53-independent pathway, leading to cellular senescence of MCF-7 breast cancer cells. J Cancer Res Clin Oncol. 2012;138 (6):1045–55.

Integrin-Linked Kinase (ILK) Expression as a Biomarker in Cancer of the Kidney

44

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Abstract

Integrin-linked kinase (ILK) is a serine/threonine protein kinase implicated in cell-cycle control via integration of integrins with the extracellular matrix. Integrin-linked kinase overexpression promotes anchorage-independent growth and may induce tumorigenesis and invasion. Integrin-linked kinase suppresses anoikis, suggesting that it has a role in oncogenic transformation, particularly in the process of metastasis. Due to its effects on the cell cycle, apoptosis, cell adherence, and in the breakdown of collagen and cellular mobility, integrin-linked kinase has been the subject of numerous studies, particularly in the field of oncology. ILK expression and activity are increased in several human cancers, including prostate, colon, stomach, ovary, malignant melanomas, Ewing's sarcoma, primitive

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neuroectodermal tumor, non-small-cell lung cancer, bladder cell carcinoma, basal cell carcinoma, squamous cell/adenosquamous carcinomas, adenocarcinoma of the gallbladder, malignant pleural mesothelioma, chondrosarcoma, and pancreatic cancer. Renal cell carcinoma is a tumor derived from epithelial cells of the renal tubules and represents 80–85 % of all primary malignant tumors of the kidney and 2–3 % of all cancers in adults. Among renal cell carcinomas, clear renal cell carcinomas are the most frequent, accounting for 70–80 % of cases. These neoplasms may be family associated or, in the majority of cases (95 %), sporadic. Both types are related to loss of function of the VHL gene, which acts as a tumor suppressor. Clear renal cell carcinomas at identical stages and pathological grades may exhibit distinct biological behavior, and consequently prognosis markers are urgently needed. In clear renal cell carcinoma, integrin-linked kinase immunoexpression is related to loss of intercellular adhesion and degree of differentiation, and it is positively correlated with the proliferation index, renal capsule and renal vein invasion, tumor size, and Robson stage. Integrin-linked kinase may be essential for invasion and metastasis in renal cell carcinoma. Integrin-linked kinase may also act through the phosphoinositide 3-kinase (PI3K)-Akt pathway to promote cell survival in human renal cell carcinoma. Integrin-linked kinase may act as a phosphoinositide-dependent kinase (PDK)-2 by facilitating Akt phosphorylation at S473. Altogether, results reviewed here indicate that the PI3K/ILK/Akt axis is a promising target for therapeutic intervention in renal cell carcinoma and that integrin-linked kinase expression might be used in clinical practice as an important predictive and prognostic tumor marker for treatment customization.

Keywords

Immunohistochemistry • Biomarker • Carcinoma • Renal cell • Integrin-linked kinase • Human

Abbreviations

ADRP	Adipose differentiation related protein
AKT/PKB	Protein kinase B
AP1	Activator protein 1
BCC	Basal cell carcinoma
CCRC	Clear cell renal carcinoma
COX-2	Cyclooxygenase-2
CRC	Renal cell carcinoma
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
FA	Focal adhesion
FAK	Focal adhesion kinase
FFA	Free fatty acid
FKHR	Forkhead transcription factor
GPR40	G protein-coupled receptor 40
GPR40	G protein-coupled receptors
GSK-3	Glycogen synthase kinase

HFI 1α	Hypoxia-inducible factor-1alpha
HFI 2α	Hypoxia-inducible factor-1alpha
IGF-1	Growth factor 1 insulin
IHC	Immunostaining
ILK	Integrin-linked kinase
ILKAP	ILK phosphatase associated
MMP9	Metalloproteinase-9
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MUC 1	Mucin1
NF-κB	Nuclear factor kappa B
NSCLC	Non-small cell lung cancer
PanIN	Pancreatic intraepithelial neoplasm
PAS	Periodic acid-Schiff
PCR	Polymerase chain reaction
PDGF	PLATELET derived growth factor
PDK-1	PI 3-kinase-dependent kinase
PI3K	3 kinase fosfatilinositol
PTEN	Phosphatase (fosfatilinositol of 3, 4, 5-triphosphate) and tensin homolog
Pthrp	Parathyroid hormone-related protein
RNA	Ribonucleic acid
TGF-α	Transforming growth factor α
TMA	Tissue microarray
VEGF	Vascular endothelial growth factor
VLH	Von Hippel-Lindau
α-NAC	Alpha-chain of the nascent polypeptide-associated complex
α-Pix	PAK (p21-activated kinase)-interacting exchange factor
α-SMA	α-smooth muscle actin

Key Facts: Activation of the Phosphoinositide 3-Kinase (PI3K)-Akt-Signaling Pathway and Integrin-Linked Kinase in Clear-Cell Renal Carcinoma

- The immunohistochemical expression of integrin-linked kinase in clear-cell renal carcinomas correlates with loss of intercellular adhesion, degree of differentiation, cell proliferation index, invasion of the renal capsule and renal vein, tumor size, and Robson stage. ILK may be essential for invasion and metastasis in RCC.
- The phosphoinositide 3-kinase (PI3K)-Akt-signaling pathway is involved in many cellular processes including proliferation, death, migration, and angiogenesis.
- Once recruited to the plasma membrane through the activity of PI3K, Akt is activated by phosphorylation at two sites: T308 in the kinase domain by PDK-1 and S473 in the regulatory tail by PDK-2.

- Integrin-linked kinase phosphorylates PKB/Akt at the Ser473 residue, leading to evasion of apoptosis by inhibition of caspase-3 activation or activation of nuclear factor κ B (NF-κB). Activation of this factor may also stimulate cyclooxygenase-2 (COX-2), which is implicated in tumor progression by stimulating angiogenesis and invasion.
- Integrin-linked kinase promotes cell survival in human renal cell carcinomas. PTHrP activates integrin-linked kinase, which acts as a phosphoinositide-dependent kinase (PDK)-2 in facilitating Akt phosphorylation at S473.
- Free-fatty acids are associated with the development of renal cell carcinomas, through the activation of GPR40/ILK/Akt, revealing a new mechanism for the correlation between metabolic disorders and renal carcinoma.

Definitions

Anoikis From the ancient Greek, which means homelessness. It is the apoptosis of normal epithelial cells in response to detachment from their extracellular matrix.

Epithelial–mesenchymal transition (EMT) Mechanism by which cancer cells acquire the invasive and stem-like traits necessary for distant metastasis.

GPR40, also known as free-fatty-acid receptor 1 (FFA1) A class A G-protein-coupled receptor for medium- and long-chain free-fatty acids that may be involved in the metabolic regulation of insulin secretion.

Integrin Proteic transmembrane receptors which interact and connect cell to cell and their extracellular matrix.

Oncogenes Proto-oncogenes are genes that normally help cells grow. When a proto-oncogene mutates or there are too many copies of it, it becomes a gene that can become permanently turned on or activated when it is not supposed to be. When this happens, the cell grows out of control, which can lead to cancer and this gene is called an oncogene.

Parathyroid hormone-related protein (PTHrP) A cytokine-like polyprotein expressed in fetal and adult tissues and a key regulator of cellular calcium transport and smooth muscle cell contractility, as well as a control factor in cell proliferation, development, and differentiation.

Predictive marker A biomarker that predicts the likelihood of benefit from a specific clinical intervention or the differential outcomes of two or more interventions or treatment.

Prognostic marker A biomarker that assesses a patient's overall outcome, such as the probability of cancer recurrence after standard treatment.

Tumor suppressor genes Normal genes that slow down cell division, repair DNA mistakes, or tell cells when to die (apoptosis or programmed cell death). When tumor suppressor genes don't work properly, cells can grow out of control, which can lead to cancer.

Von Hippel–Lindau (VHL) disease Autosomal dominantly inherited tumor syndrome, caused by mutations of the von Hippel–Lindau tumor suppressor (VHL) gene. It is characterized by frequent development of specific types of tumors in selective organs such as: retinal hemangioblastomas, cerebellar and spinal hemangioblastomas, renal clear-cell carcinomas and cysts, epididymal and broad ligament cystadenomas, pancreatic cysts, microcystic serous adenomas, neuroendocrine tumors, and pheochromocytomas.

Introduction

Cell interaction with the extracellular matrix (ECM) regulates fundamental processes such as cell shape, motility, growth, survival, differentiation, and gene expression through integrin-mediated signal transduction. Integrin-linked kinase (ILK) was isolated approximately 20 years ago in a yeast two-hybrid screen using the cytoplasmic tail of integrin β as bait (Hannigan et al. 1996). This serine/threonine kinase interacts with the β-integrin cytoplasmic domain in focal adhesions, where it functions as a scaffolding protein in the formation of multiprotein complexes connecting integrins to the actin cytoskeleton and modulating intracellular signaling pathways originated by those connections (McDonald et al. 2008).

ILK comprises three structurally distinct domains. The N-terminus consists of five ankyrin repeats that mediate the interaction with PINCH, a family of LIM-domain-only proteins that consists of two members, PINCH-1 and PINCH-2. Both PINCH proteins contain five LIM domains, the first of which binds ILK (Tu et al. 1999; Chiswell et al. 2008). The pleckstrin homology (PH)-like domain of ILK has been shown to bind phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) (Delcommenne et al. 1998; Pasquali et al. 2007). The C-terminal kinase-like domain binds several adaptor proteins including α-parvin, also known as actopaxin or CH-ILKBP, β-parvin, also known as affixin, and γ-parvin (Nikolopoulos and Turner 2000; Olski et al. 2001; Tu et al. 2001; Yamaji et al. 2001; Chu et al. 2006) (Fig. 1).

The ILK/PINCH/parvin (IPP) complex represents a central constituent of adhesion sites that contain β1 and β3 integrin, from where it regulates multiple cellular processes (Wickström et al. 2010). ILK mediates the phosphorylation of a variety of intracellular substrates, including protein kinase B (PKB/Akt) and glycogen synthase kinase-3 (GSK-3). However, ILK lacks key catalytic residues, and thus

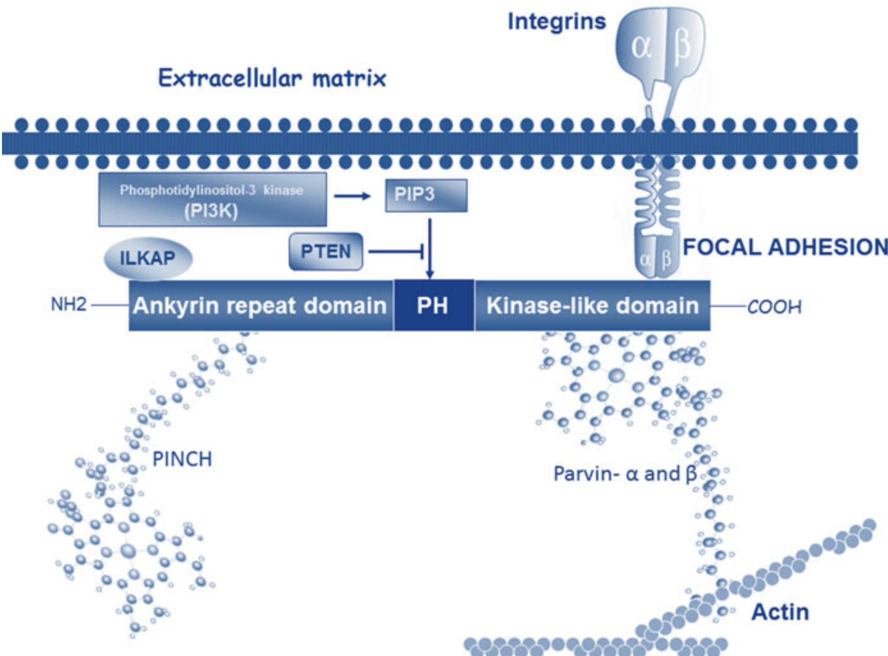

Fig. 1 ILK functional domains and interactions. ILK is localized in focal adhesions, where it forms multiprotein complexes with several proteins that are involved in cytoskeletal dynamics and cell-signaling cascades. The N-terminal ankyrin repeats of ILK interact directly with several key proteins, including PINCH and ILKAP. The central PH-like domain of ILK binds to PtdIns(3,4,5) P3 and is required for PI3K-dependent activation of ILK. The C-terminal kinase-like domain of ILK interacts with integrins, as well as with several actin-binding adaptor proteins, including α-parvin, β-parvin, and paxillin

its ability to function as a "true" kinase was questioned (Qin and Wu 2012). Gain- and loss-of-function strategies have shown that overexpression and/or constitutive activation of ILK results in oncogenic transformation and progression to invasive and metastatic phenotypes in human tumors (Persad and Dedhar 2003).

Usually, ILK is overexpressed in human malignancies, and it correlates with tumor stage and grade (Tables 1 and 2). Moreover, ILK overexpression predicts poor patient survival in several types of cancers. Because of ILK roles in the cell cycle, apoptosis, cellular adhesion, collagen degradation, angiogenesis, and cell motility, numerous studies have documented it as a potentially tumorigenic molecule (Fig. 2).

Among RCCs, clear renal cell carcinomas (CRCC) are the most frequent, accounting for 70–80 % of cases. These neoplasms may be family associated or, in the majority of cases (95 %), sporadic. Both cases are related to a loss of function of the VHL gene, which acts as a tumor suppressor. CRCCs at identical stages and pathological grades may exhibit distinct biological behavior. Therefore, prognosis markers are urgently needed.

Table 1 ILK expression in human malignancies

Malignancy	Reference	ILK expression
Medulloblastoma	Chung et al. 1998	ILK expression in 100 % off cells
Primitive neuroectodermal tumor	Chung et al. 1998	ILK expression in 100 % off cells
Ewing's sarcoma	Chung et al. 1998	ILK expression in 100 % off cells
Prostate carcinoma	Graff et al. 2001	ILK expression increased with tumor progression and correlated with poor prognosis
Ovarian tumor	Ahmed et al. 2003	ILK expression increased with tumor progression. Normal epithelium negative
Colon carcinoma	Marotta et al. 2003	ILK overexpression in malignant acini compared with normal crypts
Gastric carcinoma	Ito et al. 2003	ILK expression associated with tumor thickness and nodal metastasis
Melanoma	Dai et al. 2003	ILK expression associated with tumor thickness and correlated with poor prognosis
Anaplastic thyroid carcinoma	Younes et al. 2005	ILK overexpression in tumor tissue, but not in normal thyroid tissue
Lung carcinoma, non-small cell	Takanami 2005	ILK expression correlated with tumor invasion, grade, stage, and poor prognosis
Colon carcinoma	Bravou et al. 2006	ILK expression correlated with tumor invasion, grade, and stage. Overexpression in metastasis
Lung carcinoma, non-small cell	Okamura et al. 2007	ILK overexpression in a subset of tumor associated with poor prognosis
Hepatocellular carcinomas	Peroukides et al. 2008	ILK overexpression in liver oncogenesis and hepatic cirrhosis correlates with activation of Akt
Squamous laryngeal carcinomas	Goulioumis et al. 2008	ILK expression nuclear associated with p-Akt expression
Lung carcinoma, non-small cell	Watzka et al. 2010	ILK overexpression and strong pAkt staining that are mutually associated with poor prognosis
Basal cell carcinoma	Papanikolaou et al. 2010	ILK overexpression in 100 % of cases and correlated with tumor invasion
Pancreatic carcinoma	Schaeffer et al. 2010	ILK expression elevated in tumors but minimal in pancreatic intraepithelial neoplasm lesions
Bladder cell carcinoma	Matsui et al. 2011	ILK expression correlates to the invasiveness of cancer
Clear-cell renal carcinoma	Engelman et al. 2013	ILK expression correlated with tumor invasion, grade, increase in proliferation index, loss of intercellular adhesion, and stage
Renal cell carcinoma	Han et al. 2015	ILK expression regulates vimentin and E-cadherin expression by regulating the EMT-related transcription factors Snail and Zeb1

ILK is overexpressed in several human malignancies
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Table 2 ILK expression in human tumor progression

ILK overexpression		
Association	Tumor	Reference
Histological grade	Prostatic carcinoma	Graff et al. 2001
	Ovarian tumor	Ahmed et al. 2003
	Colonic carcinoma	Bravou et al. 2006
	Small-cell pulmonary carcinoma	Takanami 2005
	Clear-cell renal carcinoma	Engelman et al. 2013
Increase in proliferation	Prostatic carcinoma	Graff et al. 2001
	Clear-cell renal carcinoma	Engelman et al. 2013
Apoptosis evasion	Anaplastic thyroid cancer	Younes et al. 2005
	Pancreatic adenocarcinoma	Duxbury et al. 2005
	Clear-cell renal carcinoma	Sourbier 2007
Apoptosis evasion	Clear-cell renal carcinoma	Engelman et al., 2013
Invasion and stage	Colon cancer	Bravou et al.2006
	Melanoma	Dai et al. 2003
	Non-small-cell lung cancer	Takanami 2005
	Gastric carcinoma	Ito et al. 2003
	Basal cell carcinoma	Papanikolaou et al. 2010
	Pancreatic carcinoma	Schaeffer et al. 2010
	Bladder cell carcinoma	Matsui et al. 2011
	Clear-cell renal carcinoma	Engelman et al. 2013
	Renal cell carcinoma	Han et al. 2015

ILK is overexpressed in several human malignancies, and it correlates with histological grade, increase in proliferation, apoptosis evasion, invasion, and tumor stage

In this chapter we discuss the potential of ILK as a biomarker for these kidney tumors.

ILK and Tumorigenesis

Overexpression of ILK promotes anchorage-independent growth and can induce tumorigenesis and invasiveness. In normal epithelial cells, loss of integrin–ECM interaction induces a form of apoptosis called anoikis. Work by Attwell et al. (2000) has shown that ILK suppresses anoikis and might therefore play an important role in oncogenic transformation, particularly in metastasis (Attwell et al. 2000). ILK is overexpressed in Ewing’s sarcoma, primitive neuroectodermal tumors, and medulloblastomas (Chung et al. 1998). ILK also takes part in chemically induced tumors in mice, probably via alteration of the PKB/Akt pathway (Segrelles et al. 2002).

Numerous studies have related the development and progression of different malignancies to ILK overexpression and ILK-mediated activation of important signaling pathways involved in neoplastic transformation such as PI3K, PKB/Akt, and GSK-3β. In colon cancer, β-catenin activation, decreased immunoexpression of

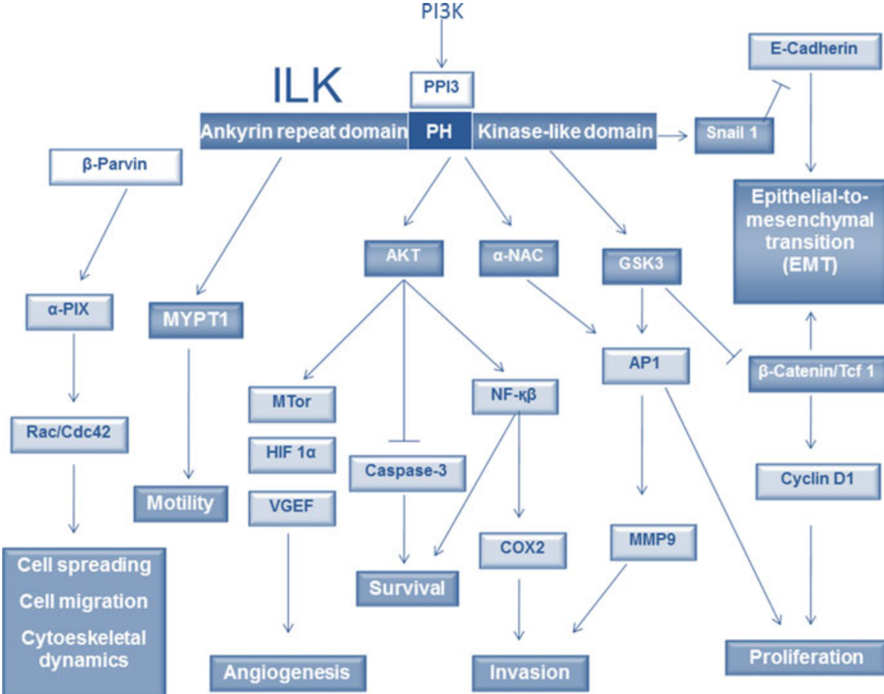

Fig. 2 Overview of the intracellular signaling pathways regulated by ILK. ILK activation promotes processes such as motility and contractility, survival, EMT, invasion, proliferation, and angiogenesis that are crucial for the progression of malignant PIP3, PtdIns(3,4,5)P3; NAC, nascent polypeptide-associated complex and coactivator; and *HIF1* hypoxia-inducible factor 1 (Republished with permission of Company of Biologists Ltd., from Journal of Cell Science, Paul C. McDonald, Andrew B. Fielding, Shoukat Dedharauthor, 121, 2008; permission conveyed through Copyright Clearance Center, Inc.)

E-cadherin, and activation of Akt correlate with increased expression of ILK and tumor progression (Bravou et al. 2006). Specifically in colon adenocarcinomas, ILK has been associated with overexpression of phosphorylated GSK-3β and nuclear translocation of β-catenin (Marotta et al. 2003). In gliomas, the growth factor Cyr61 activates ILK, thus triggering the phosphorylation of GSK-3β and downstream activation of the β-catenin signaling pathway, β-TCF/Lef-1, and activation of Akt phosphorylation with Pik3 and antiapoptotic protein Bad (Xie et al. 2004). The evaluation of 118 samples of non-small-cell lung cancers showed strong ILK cytoplasmic expression, strong integrin β1 membranous staining, and strong phosphorylated Akt (pAkt) cytoplasmic staining and provided evidence that ILK, integrin β1, and pAkt are mutually associated with poor prognosis (Okamura et al. 2007).

In liver oncogenesis and hepatic cirrhosis, ILK overexpression correlates with Akt activation but not with other conventional ILK targets. The expression levels of ILK, β-catenin, E-cadherin, and pAkt were evaluated by immunohistochemistry in

69 human hepatocellular carcinomas (HCC) and adjacent normal and cirrhotic liver parenchyma. ILK and pAkt immunostaining was observed in 100 % and 79.7 % of HCCs, respectively, and their protein levels correlated significantly with each other. Activation of β-catenin and downregulation of E-cadherin were frequently observed in HCC, but they were not related to ILK expression (Peroukides et al. 2008).

A study of 97 invasive laryngeal squamous cell carcinomas showed that ILK overexpression correlates with activation of Akt but not with downregulation of E-cadherin or activation of β-catenin. Activated Akt seems to characterize well-differentiated tumors, while loss of E-cadherin and activation of β-catenin correlated with high-grade carcinomas (Goulioumis et al. 2008).

Moreover, tumors with an inactive PTEN gene, such as prostate carcinomas (Persad et al. 2000) and glioblastomas (Obara et al. 2004), had upregulated ILK. Other work has shown that ILKAP, a protein phosphatase that inhibits both ILK and PTEN, also mediates the inhibition of GSK-3β and has an important role in suppressing carcinogenesis (Kumar et al. 2004).

Patients with familial adenomatous polyposis (FAP), a condition that precedes colon cancer, showed impaired regulation of ILK (Marotta et al. 2001). More recently, work by the same group suggested that disturbances in ILK signaling represent an early event in the development of colon cancer. ILK was overexpressed in the crypts of both primary and metastatic lesions. In functional tests, high ILK activity coincided with changes in the target molecule GSK3 β. Finally, the authors showed that in colon carcinomas, activation of β-catenin, downregulation of E-cadherin, and activation of the Akt-FKHR pathway were all significantly correlated with ILK expression and tumor progression (Marotta et al. 2003).

ILK expression also inversely correlates with the degree of histological differentiation in several human malignancies. High-grade prostate carcinomas display higher levels of ILK immunoexpression compared to low-grade carcinomas (Graff et al. 2001). In 53 cases of ovarian tumors, intensity of immunohistochemical staining for ILK directly correlates with tumor grade. An ovarian tumor cell line expressed high ILK levels, as observed by Western blotting, whereas immortalized cells derived from normal ovarian tissue displayed low basal expression of ILK (Ahmed et al. 2003).

ILK expression is also significantly correlated with histological grade in non-small-cell lung carcinomas (Takanami 2005; Okamura et al. 2007; Watzka et al. 2010). Bravo et al. (2006), studying 125 primary colon carcinomas, found ILK expression in 98.4 % of tumors and reported strong correlation between immunoprotein levels and degree of histological differentiation.

Elevated ILK immunoexpression in pancreatic carcinomas correlates with the expression of Snail, which suppresses E-cadherin. In 23 of 25 carcinoma cases, ILK displayed extensive positivity (>50 %). On the other hand, pancreatic intraepithelial neoplastic (PanIN) lesions stained minimally for Snail and ILK (Schaeffer et al. 2010).

Many studies encompassing various types of cancers suggest the involvement of ILK in metastatic spread. For example, 4 out of 5 gastric carcinoma cell lines and 22 out of 35 (63 %) microdissected tumor samples of primary gastric carcinomas

expressed ILK mRNA. In this study, strong expression of ILK protein significantly correlated with deep invasion of tumor cells into the gastric wall and presence of nodal metastasis (Ito et al. 2003).

Immunohistochemical ILK expression significantly correlates with human melanoma thickness. Biopsies of melanomas overexpressed ILK protein in 0 %, 22 %, 33 %, and 63 % of the individual samples when tumors measured $\leq$0.75, 0.76–1.50, 1.51–3.0, and >3.0 mm in thickness, respectively. Similarly, 83 % of tumors with lymph node invasion strongly expressed ILK in comparison to only 18 % of tumors without lymph node invasion (Dai et al. 2003).

Increased ILK immunoexpression was also significantly associated with lymph node metastasis and tumor stage in 134 cases of non-small-cell lung carcinoma. The study suggests that increased ILK expression is associated with poor prognosis in patients with non-small-cell lung carcinoma (Okamura et al. 2007).

Bravo et al. (2006) also showed a correlation of immunohistochemical ILK expression with local invasion and metastasis in colon carcinomas. All metastatic tumors showed ILK immunohistochemical expression. Moreover, the intensity of immunoreaction was higher in metastatic tumors. However, there was no direct correlation between protein overexpression and depth of wall invasion or stage of the disease.

In human basal cell carcinomas (BCC), ILK expression also correlates with epithelial–mesenchymal transition markers and tumor invasion. Histological sections of 100 human BCCs were evaluated by immunohistochemistry for the expression of ILK, E-cadherin, Snail, β-catenin, and alpha-smooth muscle actin (alpha-SMA). ILK overexpression was observed in 100 % of the sections and expression levels strongly correlated with tumor invasion and infiltrative BCCs. There was a significant correlation between ILK expression and all ECM markers investigated (Papanikolaou et al. 2010).

Results from a tissue microarray (TMA) performed with human bladder cell carcinomas showed that ILK expression correlated with invasiveness. This study suggests that invasive bladder cancers overexpress ILK, which plays an important role in the epithelial–mesenchymal transition (EMT) of bladder cancer via control of E-cadherin and MMP-9 expression (Matsui et al. 2011).

Another study indicated that ILK expression in 45 human clear-cell renal carcinomas (CRCC) correlated with the loss of intercellular adhesion, loss of differentiation and increased cell proliferation, renal capsule and renal vein invasion, tumor size, and Robson stage (Engelman et al. 2013). ILK immunoexpression directly correlated with CRCC, paving the way for the potential development of new molecular therapies targeting specific pharmacologic inhibitors of the ILK pathway.

The phosphoinositide 3-kinase (PI3K)-Akt-signaling pathway is constitutively activated in human CRCC independently of VHL expression, and it plays an essential role in CRCC progression through inhibition of tumor cell apoptosis (Sourbier et al. 2006). PI3K constitutes a family of enzymes involved in monitoring of cell growth, proliferation, motility, adhesion, survival, and angiogenesis. Binding of a number of growth factors to membrane receptor tyrosine kinases (EGFR – epidermal growth factor receptor, c-kit, and INS-1 – insulin receptor 1) initiates

signaling via the PI3K pathway. Growth factors include insulin-like growth factor, epidermal growth factor, fibroblast growth factor, interleukin 3 and 6, and vascular endothelial growth factor (Clark et al. 2002; Meier et al. 1997). The ligand–receptor interaction determines the conversion of phosphatidylinositol 4,5-2P (PIP2) into phosphatidylinositol 3,4,5-3P (PIP3), which relays growth and survival signals by recruiting Akt, also known as protein kinase B, and phosphoinositide-dependent kinase (PDK). Cytoplasmic Akt is activated in the cell membrane by phosphorylation at two independent positions with the involvement of PDK1 and mTOR. Once activated, the mTOR complex (mTORC) acts through its downstream effectors to stimulate protein synthesis and entrance into G1 phase as well as to control proteins that regulate apoptosis (Hay 2005).

At the renal level, the PI3K phosphorylation product PIP3 recruits cytoplasmic Akt to the membrane. Once activated, Akt inhibits apoptosis by phosphorylation and inactivation of the proapoptotic proteins procaspase-9, apoptosis signal-regulating kinase-1 (ASK1), and BAD, a member of the bcl-2 family. PI3K inhibits GSK-3β, which normally phosphorylates and induces degradation of cell-cycle control protein cyclin D1 and of transcription factors that promote proliferation such as c-myc, β-catenin, c-Jun, and Notch (Cojocaru et al. 2015). The PI3K–Akt pathway is constitutively activated in various human cancers where it plays a critical role in tumor progression and in tumor resistance to therapies (Hanada et al. 2004).

Parathyroid hormone-related protein (PTHrP) is a cytokine-like polyprotein that is normally expressed throughout the body where it plays a variety of roles, including regulation of cellular growth, differentiation, and death. PTHrP was initially identified as the factor responsible for the paraneoplastic syndrome humoral hypercalcemia of malignancy (Martin et al. 1997; Philbrick et al. 1996). PTHrP represents an essential growth factor for CRCC and a target for the von Hippel–Lindau (VHL) tumor suppressor gene. In fact, pVHL suppresses PTHrP expression at both the mRNA and protein levels (Massfelder et al. 2004). PTHrP-mediated inhibition of tumor cell apoptosis is crucial for human RCC growth (Sourbier et al. 2006).

The PI3K/ILK/Akt/NF-κB axis provides a promising target for therapeutic intervention in CRCC. PTHrP is one of the main factors involved in the constitutive activation of the PI3K-Akt-signaling pathway in human RCC, regardless of VHL expression. PTHrP induces phosphorylation of Akt at S473 but not at T308. Transfections with ILK constructs and RNA interference provide evidence that ILK is involved in human RCC cell survival. PTHrP activates ILK, which then acts as a phosphoinositide-dependent kinase (PDK)-2 in facilitating phosphorylation of Akt at S473. NF-κB is the downstream Akt target regulated by PTHrP (Agouni et al. 2007).

Induced overexpression of ILK also leads to RCC progression via free-fatty-acid (FFA)-mediated activation of the GPR40/ILK/Akt pathway, revealing a novel mechanism for the correlation between metabolic disturbances and renal carcinomas (Liu et al. 2013). Oleic acid is an n-9 monounsaturated fatty acid that activates G-protein-coupled receptors, which, in turn, lead to ERK1/2 phosphorylation and cancer cell proliferation in breast cancer. High concentration levels of oleic acid have been used

to imitate the effects of abnormal levels of FFAs on tumor growth in human RCC 786-O cells (Liu et al. 2013). Results indicated that oleic acid stimulates 786-O cell viability and delayed apoptosis, both in a concentration-dependent manner. Western blot analysis revealed that oleic acid treatment upregulated ILK expression in a concentration-dependent manner. Overexpression of ILK was found to increase the phosphorylation of Akt on Ser-473, and, in addition, when siRNA was used to target and knock down ILK, the effects of oleic acid on 786-O cell growth were weakened and the expression of ILK was suppressed.

Recent investigation regarding the role of ILK in cancer progression and metastasis in RCC showed that ILK may be essential for invasion and metastasis and that it regulates vimentin and E-cadherin expression by regulating the EMT-related transcription factors Snail and Zeb1 (Han et al. 2015). ILK is expressed at a low level in normal cells and low-stage RCC cells and is highly expressed in advanced and metastatic cells. In Caki-1, a metastatic RCC cell, both ILK and its downstream EMT-related effectors are highly expressed. However, ILK knockdown suppressed the formation of stress fibers and focal adhesions and impeded phenotypic EMT markers, including cell migration and invasion. In vivo knockdown of ILK suppressed the progression, invasion, and metastasis of primary RCC in nude mice by downregulation of EMT markers. Overexpression of ILK increased tumor cell migration and invasion.

Tumor growth requires the conjunction of several factors, including increased replication potential, anchor-independent growth capacity, resistance to apoptosis, angiogenesis, adjacent tissue invasion, and metastasis. When disturbed or overexpressed, ILK promotes all of these factors. Currently, ILK is considered a potential prognosis marker for the following human malignancies: melanoma (Lu et al. 2013), non-small-cell lung cancer (Posch et al. 2014; Watzka et al. 2011; Okamura et al. 2007), colorectal cancer (Li et al. 2013b), squamous cell/ adenosquamous carcinomas and adenocarcinoma of the gallbladder (Li et al. 2013a), malignant pleural mesothelioma (Watzka et al. 2010; Schramm et al. 2010), chondrosarcoma (Papachristou et al. 2008), and pancreatic cancer (Sawai et al. 2006) (Table 3).

Potential Applications to Prognosis and Other Diseases or Conditions

RCC represents almost 3 % of malignant tumors in adult humans and most of the neoplasms arising from the kidney. RCC is characterized by a lack of early warning signs as well as by diverse and variable clinical manifestations, thus more than 30 % of patients have advanced-stage RCC at diagnosis (Koul et al. 2011). Only 10 % of individuals with RCC present with the classic triad of hematuria, pain, and a flank mass. RCC has the highest mortality rate of the genitourinary cancers, as more than a third of patients with RCC will die from the disease. Novel and well-established approaches for the early detection and management of renal cancer are therefore extremely important.

Table 3 ILK as a prognostic marker in human cancer

Type of cancer	Clinical significance	Detection	Reference
Melanoma	Concomitant JWA and ILK expression is closely correlated with survival	IHC	Lu et al. 2013
Non-small-cell lung cancer	Increased sILK is associated with adverse survival	Serum ILK quantified by ELISA	Posch et al. 2014
Non-small-cell lung cancer	ILK is an adverse prognostic factor	IHC	Watzka et al. 2010
Non-small-cell lung cancer	ILK and Akt are mutually associated with poor prognosis and are independent prognostic factors	IHC	Okamura et al. 2007
Colorectal cancer	ILK overexpression is associated with tumor progression and poor prognosis	IHC, PCR, and Western blotting	Li et al. 2013b
Carcinomas of the gallbladder	Positive ILK and PRDX1 expressions are closely related to the progression and poor prognosis	IHC	Li et al. 2013a
Chondrosarcoma	ILK might serve as biological marker that could accurately predict a high-grade tumor	IHC	Papachristou et al. 2008
Pancreatic cancer	Association between strong expression of ILK and poor prognosis	IHC	Sawai et al. 2006

ILK has prognostic significance in several types of human tumors

The incidence of RCC directly correlates with the existence of genetic factors such as von Hippel–Lindau disease, hereditary papillary renal cancer, and tuberous sclerosis (Delahunt 2009). Other suggested risk factors include cigarette smoking, obesity, diuretic use, high-protein diets, hypertension, kidney transplantation, and HIV infection as well as exposure to petroleum-derived products, chlorinated solvents, cadmium, lead, asbestos, and ionizing radiation (Cairns 2011).

RCC comprises a heterogeneous group of epithelial tumors with variable clinical outcomes. Genetic hallmarks identify the various histological subtypes: clear-cell RCC also called conventional RCC, papillary RCC, chromophobe RCC, collecting duct RCC, and unclassified forms. Clear-cell renal cell carcinoma (CRCC) represents the most common histological type (80 %) and originates in renal proximal tubular epithelium (Cairns 2011).

In 95 % of the cases, CRCC occurs without an obvious cause, but it may also result from genetic factors such as von Hippel–Lindau disease. In both cases, biallelic inactivating mutations of the VHL tumor suppressor gene occur (Kaelin 2007; Arjumand and Sultana 2012). The presence of germ-line mutations of the VHL gene has been ascertained in 100 % of the cases of hereditary CRCC and in two-thirds of sporadic CRCC cases. The VHL gene is inactivated by point mutations, deletions, or hypermethylation of gene promoters (Li et al. 2007; Lianjie et al. 2007).

Historically, CRCC prognosis has relied on clinicopathologic variables, such as pathological stage, histological grade (Fuhrman nuclear grade), presence of sarcomatoid differentiation, histological type, vascular invasion, and presence of necrosis. However, carcinomas at identical stages and pathological grades may have different biological behaviors.

At present, no markers segregate between aggressive and indolent behaviors. Prognostic information regarding long-term CRCC outcome for an individual patient is based primarily upon pathologic data obtained from definitive surgical resection. Markers that can be detected through noninvasive methods and still indicate the malignancy of small renal masses or predict growth rates are urgently needed (Cairns 2011).

In recent years, many studies have focused on the molecular basis of RCC. The discovery of reliable biomarkers in RCC could have an important impact on diagnosis, prognosis, and prediction of therapeutic benefit. To date, most biomarker research has centered on by-products of the VHL pathway including VHL mutations, vascular endothelial growth factor ligands and receptors, HIF, and carbonic anhydrase IX. Elucidation of the VHL/HIF pathway has led to the successful evaluation and regulatory approval of agents targeting the VEGF and mTOR axes.

Multiple research reports provide solid evidence for the implication of PI3K/Akt/mTOR in CRCC carcinogenesis (Cojocaru et al. 2015). Other prognostic factors have little consistency, thus they are not routinely used. The most commonly studied include DNA ploidy, which strongly correlates with the Fuhrman grade, cell proliferation by flow cytometry, Ki-67 or PCNA, CD44 expression, mucin1 (MUC 1), and insulin growth factor 1 (IGF-1). The p53 mutation seems to correlate with poor survival and metastatic disease in patients with early-stage disease.

Several potential RCC biomarkers have been identified, although none has progressed beyond the discovery phase. Some of the most promising RCC markers are proteins such as the B7-H1 factor, insulin-like growth factor II, and mRNA-binding protein 3, which display strong independent prognosis but have not been validated. Several studies have shown that C-reactive protein, an easily measured routine marker, is produced by RCC cells and has significant prognostic value for metastasis and mortality (Saito and Kihara 2011), but it was not further evaluated.

The best-known genetic abnormalities associated with CRCC include loss of chromosome 3p (70–80 %) and gain of chromosome 5q (50–60 %). It is believed that loss-of-function mutations in the remaining allele and VHL represent early events in CRCC carcinogenesis, albeit not sufficient to result in tumor evolution. Antiangiogenic therapies targeting vascular endothelial growth factor (VEGF) have benefited patients with advanced RCC (Singer et al. 2011), but results are generally thought to be cytostatic and do not cure patients. These therapies effectively inhibit tumor progression through deprivation of oxygen and nutrition from the tumor microenvironment but cannot block metastasis of CRCC cells.

However, these treatments do not target tumor cells directly, leaving room for disease progression (Virtanen and Lehto 2004). A recent large-scale analysis suggested that CRCCs have substantial genetic heterogeneity that involves genes implicated in the regulation of methylation in 15 % of cases, confirming the

Table 4 ILK expression in RCC

Reference	ILK expression
Sourbier (2007)	ILK overexpression correlates with activation of Akt and induces cell survival
Engelman et al. (2013)	ILK expression correlated with tumor invasion, increase in proliferation index, grade, loss of intercellular adhesion, and stage
Liu et al. (2013)	ILK expression associated with p-Akt expression
Han et al. (2015)	ILK expression regulates vimentin and E-cadherin expression by regulating the EMT-related transcription factors Snail and Zeb1

ILK expression is correlated with tumor progression and activation of Akt in RCC

importance of epigenetic modification and truncating mutations of PRMB1 in 41 % of cases (Audenet et al. 2012).

The implication of multiple genes suggests the existence of genetically distinct CRCC subgroups, similarly to what has been shown for breast cancer. In fact, CRCC studies established that these tumors may also be segregated based on gene expression profiles, and this effort provides important independent information. Work focused on the genetic profile of tumors separated CRCC into two distinct subtypes, named CCA and CCB. CCA tumors overexpress genes associated with hypoxia, angiogenesis, and fatty acid metabolism and have a favorable prognosis compared to CCB tumors. The latter group overexpresses a more aggressive panel of genes associated with epithelial–mesenchymal transition, cell cycle, and wound healing (Brannon et al. 2012). Interestingly, VHL profiles are similar in the two groups.

ILK represents a potential prognostic marker for CRCC as a consequence of its tumorigenic properties as well as recent evidence of its immunoexpression in various forms of human malignancies (Table 4). Increased ILK immunoreactivity in the primary tumor is an adverse prognostic factor in a variety of preclinical and clinical models of human cancer. Despite the small number of studies focusing on the role of ILK in renal carcinogenesis, existing results provide relevant data and may have important prognostic and therapeutic implications.

In fact, ILK immunoreactivity correlates with tumor severity in CRCC (Engelman et al. 2013), and ILK is essential for invasion and metastasis of RCC both in vitro and in vivo (Han et al. 2015). In the latter study, the RCC cell line from a metastatic lesion showed the highest expression of ILK and displayed higher expression of EMT-related transcriptional factors Snail and Zeb1. This finding suggests that ILK immunoreactivity might be related to the metastatic potential of RCC cells, which would point to ILK as a potential prognostic marker for CRCC.

Two recent studies have provided clear descriptions of the route of CRCC tumor progression. There is strong evidence for the participation of ILK in CRCC carcinogenesis, involving the signaling pathway PI3K-Akt (Agouni et al. 2007; Liu et al. 2013). ILK phosphorylates PKB/Akt at the Ser473 residue, leading to evasion of apoptosis by inhibition of caspase-3 activation or activation of nuclear factor κ B (NF-B κ). Activation of this factor may also stimulate cyclooxygenase-2 (COX-2), which is implicated in tumor progression by stimulating angiogenesis and invasion. Moreover, the PI3K-Akt-signaling pathway intersects with the signaling cascade

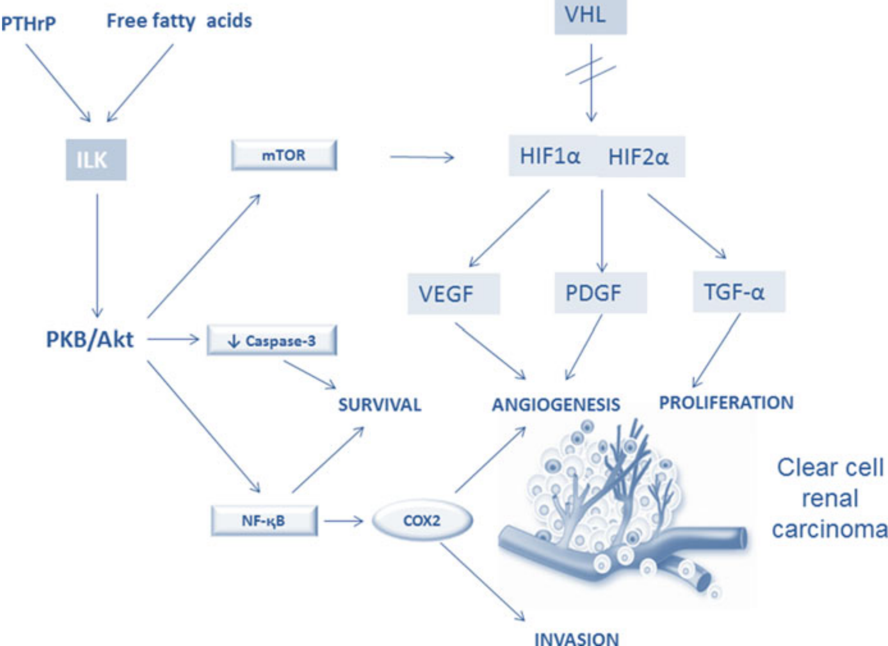

Fig. 3 Schematic representation of activation of the Akt/PKB pathway in human CRCC. The VHL gene is mutated in a majority of sporadic CRCCs. As a result of mutation, the VHL protein cannot target and degrade hypoxia-inducible factors (HIF) 1α and 2α. Excess HIF causes increased transcription of downstream genes, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor alpha (TGF-α). The PI3K-Akt-signaling pathway intersects with the signaling cascade triggered by VHL inactivation via AKT-mediated induction of HIF-1α through mTOR. PTHrP or free-fatty acids activate NF-κB through stimulation of ILK, one of the kinases with PDK-2 activity in human CRCC

triggered by VHL inactivation, as shown in Fig. 3, provided that Akt can upregulate HIF-1α expression by increasing mTOR-mediated protein translation (Chou et al. 2015). The same authors describe a novel regulatory feedback loop in which hypoxia induces ILK expression through an HIF-1α-dependent mechanism and ILK, in turn, stimulates HIF-1α expression through cell-type- and cell-context-dependent pathways. The ILK-HIF-1α regulatory loop could underlie the maintenance of high HIF-1α expression levels and the promotion of EMT under hypoxic conditions. The small-molecule ILK inhibitor T315 can disrupt this regulatory loop in vivo and suppress xenograft tumor growth, thereby providing proof of concept that targeting ILK represents an effective strategy to block HIF-1α expression and aggressive phenotypes in cancer cells.

Both VHL inactivation and ILK-mediated activation of the Akt pathway result in HIF-1α overexpression, which promotes hyperangiogenesis in CRCC. Antiangiogenic therapies do not prevent CRCC metastases, and ILK is essential for RCC invasiveness and metastases (Han et al. 2015). Thus, the PI3K/ILK/Akt axis provides a promising

target, and ILK could be a predictive marker indicating the activity in this pathway in a group of patients and guiding therapy toward ILK inhibition.

Notably, ILK expression can be measured at the protein level by immunohistochemistry of histological CRCC samples, a low-cost and routinely available technique. Alternatively, serum ILK (sILK) could be measured in CRCC patients. This possibility is supported by the fact that ILK has been detected in the serum of non-small-cell lung cancer (NSCLC) patients. Moreover, sILK was preoperatively quantified by ELISA in 50 newly diagnosed NSCLC patients. After surgery, patients received follow-up examinations for a median interval of 2.5 years. Mean sILK was 2.3 times more elevated in the 16 patients who died as compared to the 34 patients who survived (Posch et al. 2014).

Recently published data on urinary bladder cancer show that methylation markers can predict the progression of early lesions to muscle-invasive bladder tumors with a high degree of accuracy, suggesting that these markers can be used to diagnose bladder cancer (Costa et al. 2011; Kandimalla et al. 2012). Similarly, tumor markers such as urinary ILK may, in the near future, be used in CRCC allowing early detection of disease.

Although the causes for ILK overexpression in various tumors have not been fully unveiled, data reviewed here suggest that ILK may be involved in various pathogenic mechanisms of human malignancies. It has become increasingly clear that a complex network of protein–protein interactions controls the ILK-mediated loss of cell adhesion, migration, growth, cell-cycle progression, and survival. On the other hand, much like oncoproteins that stimulate cell proliferation during embryonic development, ILK plays essential roles during embryogenesis. Could a new super-oncogene code for ILK?

Summary Points

- Integrin-linked kinase is a serine/threonine kinase implicated in cell-cycle control via integration of integrins with the extracellular matrix.
- Integrin-linked kinase overexpression promotes anchorage-independent growth and may induce tumorigenesis and invasion. Integrin-linked kinase suppresses anoikis, suggesting an important role for it in oncogenic transformation, particularly in the process of metastasis.
- Integrin-linked kinase expression and activity increase in several human cancers, such as prostate, colon, stomach, ovary, malignant melanomas, Ewing's sarcoma, primitive neuroectodermal tumor, non-small-cell lung cancer, bladder cell carcinoma, basal cell carcinoma, squamous cell/adenosquamous carcinomas, adenocarcinoma of the gallbladder, malignant pleural mesothelioma, chondrosarcoma, and pancreatic cancer.
- Renal cell carcinomas are tumors derived from epithelial cells of the renal tubules and represent 80–85 % of all primary malignant tumors of the kidney and 2–3 % of all cancers in adults. The most common renal cell carcinoma forms are clear-cell renal carcinoma, papillary, and chromophobe.

- Clear-cell renal carcinomas represent the most frequent form of renal cell cancer, accounting for 70–80 % of cases. These neoplasms may be family associated or, in a majority of cases (95 %), sporadic. In both cases, tumors are related to loss of function of the VHL gene, a tumor suppressor gene.
- Clear-cell renal carcinomas at identical stages and similar pathological grades may exhibit distinct biological behavior. Therefore, other markers are needed for prognostic evaluation.
- In 2013, Integrin-linked kinase immunoexpression was evaluated in a tissue microarray of 45 human clear-cell renal carcinomas. Results suggested that integrin-linked kinase immunoexpression is related to the loss of intercellular adhesion and with the degree of differentiation. It also is positively correlated with the proliferation index, invasion of the renal capsule and renal vein, tumor size, and Robson stage.
- Recent investigation has shown that integrin-linked kinase may be essential for renal cell carcinoma progression and metastasis, and that it regulates vimentin and E-cadherin expression by regulating the extracellular matrix-related transcription factors Snail and Zeb1.
- Integrin-linked kinase affects cell survival in human renal cell carcinomas through the PI3K-Akt-signaling pathway. PTHrP activates ILK, which acts as a PDK-2 in facilitating Akt phosphorylation at S473. These results indicate that the PI3K/ILK/Akt/NF-κB axis may provide a target for therapeutic intervention in clear-cell renal carcinomas.
- In 2013, free-fatty acids, through the activation of GPR40/ILK/Akt, were associated with renal cell carcinoma. This finding revealed a new mechanism for the correlation between metabolic disorders and renal carcinoma.

References

Agouni A, Sourbier C, Danilin S, et al. Parathyroid hormone-related protein induces cell survival in human renal cell carcinoma through the PI3K Akt pathway: evidence for a critical role for integrin-linked kinase and nuclear factor kappa B. Carcinogenesis. 2007;28:1893–901.

Ahmed N, Riley C, Oliva K, et al. Integrin-linked kinase expression increases with ovarian tumour grade and is sustained by peritoneal tumour fluid. J Pathol. 2003;201:229–37.

Arjumand W, Sultana S. Role of VHL gene mutation in human renal cell carcinoma. Tumour Biol. 2012;33:9–16. doi:10.1007/s13277-011-0257-3. Epub 2011 Nov 29.

Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase suppresses anoikis. Oncogene. 2000;33:811–5.

Audenet F, Yates DR, Cancel-Tassin G, et al. Genetic pathways involved in carcinogenesis of clear cell renal cell carcinoma: genomics towards personalized medicine. BJU Int. 2012;109:1864–70. doi:10.1111/j.1464-410X.2011.10661.x. Epub 2011 Oct 28.

Brannon AR, Haake SM, Hacker KE, et al. Meta-analysis of clear cell renal cell carcinoma gene expression defines the variant subgroup Identifies and gender influences on tumor biology. Eur Urol. 2012;61:258–68. doi:10.1016/j.eururo.2011.10.007. Epub 2011 Oct 18.

Bravou V, Klironomos G, Papadaki E, et al. ILK over-expression in human colon cancer progression correlates with activation of beta-catenin, down-regulation of E-cadherin and activation of the Akt-FKHR pathway. J Pathol. 2006;208:1–9.

Cairns P. Renal cell carcinoma. Cancer Biomark. 2011;9:461–73. doi:10.3233/CBM-2011-0176.

Chiswell BP, Zhang R, Murphy JW, Boggon TJ, Calderwood DA. The structural basis of integrin-linked kinase-PINCH interactions. Proc Natl Acad Sci USA. 2008;105(52):20677–82.

Chou CC, Chuang HC, Salunke SB, Chen CS. A novel HIF-1α-integrin-linked kinase regulatory loop that facilitates hypoxia-induced HIF-1α expression and epithelial-mesenchymal transition in cancer cells. Oncotarget. 2015;6(10):8271–85.

Chu H, Thievessen I, Sixt M, Lämmermann T, Waisman A, Braun A, Noegel AA, Fässler R. Gamma-parvin is dispensable for hematopoiesis, leukocyte trafficking, and T-cell-dependent antibody response. Mol Cell Biol. 2006;26:1817–25.

Chung DH, Lee JI, Kook MC, et al. ILK (β1-integrin-linked protein kinase): a novel immunohistochemical marker for Ewing's sarcoma and primitive neuroectodermal tumour. Virchows Arch. 1998;433:113–7.

Clark EA, Shatill SJ, Brugge JS. Regulation of protein tyrosine kinases in platelets. Trends Biochem Sci. 2002;19:464–9.

Cojocaru E, Lozneanu L, Giuşcă SE, Căruntu ID, Danciu M. Renal carcinogenesis – insights into signaling pathways. Rom J Morphol Embryol. 2015;56:15–9.

Costa VL, Henrique R, Danielsen SA, Eknaes M, Patricio P, Morais A, Oliveira J, Lothe RA, Teixeira MR, Lind GE, Jerónimo C. TCF21 and PCDH17 methylation: an innovative panel of biomarkers for a simultaneous detection of urological cancers. Epigenetics. 2011;6:1120–30.

Dai DL, Makretsov N, Campos EI, Huang C, Zhou Y, Huntsman D, Martinka M, Li G. Increased expression of integrin-linked kinase is correlated with melanoma progression and poor patient survival. Clin Cancer Res. 2003;9:4409–14.

Delahunt B. Advances and controversies in grading and staging of renal cell carcinoma. Mod Pathol. 2009;22 Suppl 2:S24–36. doi:10.1038/modpathol.2008.183.

Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc Natl Acad Sci U S A. 1998;95:11211–6.

Duxbury MS, Matros E, Clancy T, Bailey G, Doff M, Zinner M.J, Ashley SW, Maitra A, Redston M, Whang EE. CEACAM6 is a novel biomarker in pancreatic adenocarcinoma and PanIN lesions. Ann Surg. 2005;241(3):491–6.

Engelman Mde F, Grande RM, Naves MA, de Franco MF, de Paulo Castro Teixeira V. Integrin-linked kinase (ILK) expression correlates with tumor severity in clear cell renal carcinoma. Pathol Oncol Res. 2013;19:27–33. doi:10.1007/s12253-012-9554-4. Epub 2012 Jul 20.

Goulioumis AK, Varakis J, Goumas P, Papadaki H. Differential beta-catenin expression between glottic and supraglottic laryngeal carcinoma. Eur Arch Otorhinolaryngol. 2010;267 (10):1573–8.

Graff JR, Deddens JA, Konicek BW, Colligan BM, Hurst BM, Carter HW, Carter JH. Integrin-linked kinase expression increases with prostate tumor grade. Clin Cancer Res. 2001;7:1987–91.

Han KS, Li N, Raven PA, Fazli L, Ettinger S, Hong JJ, Gleave ME, So AI. Targeting integrin-linked kinase suppresses invasion and metastasis through downregulation of epithelial-to-mesenchymal transition in renal cell carcinoma. Mol Cancer Ther. 2015;14:1–11.

Hanada M, Feng J, Hemmings BA. Structure, regulation and function of PKB/AKT–a major therapeutic target. Biochim Biophys Acta. 1697;2004:3–16.

Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, et al. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature. 1996;379:91–6.

Hay N. The Akt-mTOR tango and its relevance to cancer. Cancer Cell. 2005;8:179–83.

Ito R, Oue N, Zhu X, Yoshida K, Nakayama H, Yokozaki H, Yasui W. Expression of integrin-linked kinase is closely correlated with invasion and metastasis of gastric carcinoma. Virchows Arch. 2003;442:118–23.

Kaelin Jr WG. The von Hippel-Lindau suppressor protein and clear cell renal carcinoma. Clin Cancer Res. 2007;13:680–2.

Kandimalla R, van Tilborg AA, Kompier LC, Stumpel DJ, Stam RW, Bangma CH, Zwarthoff EC. Genome-wide analysis of CpG island methylation in bladder cancer identified TBX2, TBX3, GATA2, and ZIC4 as pTa-specific prognostic markers. Eur Urol. 2012;61:1245–56.

Koul H, Huh JS, Rove KO, et al. Molecular aspects of renal cell carcinoma: a review. Am J Cancer Res. 2011;1:240–54.

Kumar AS, Naruszewicz I, Wang P, Leung-Hagesteijn C, Hannigan GE. ILKAP regulates ILK signaling and inhibits anchorage-independent growth. Oncogene. 2004;23:3454–61.

Li L, Zhang L, Zhang X, Yan Q, Minamishima YA, Olumi AF, Mao M, Bartz S, Kaelin Jr WG. Hypoxia-inducible factor linked to differential kidney cancer risk seen with type 2A and type 2B VHL mutations. Cell Biol. 2007;27:5381–92.

Li J, Yang ZL, Ren X, et al. ILK and PRDX1 are prognostic markers in squamous cell/adenosquamous carcinomas and adenocarcinoma of gallbladder. Tumour Biol. 2013a;34:359–68. doi:10.1007/s13277-012-0557-2.

Li R, Liu B, Yin H, Sun W, Yin J, Su Q. Overexpression of integrin-linked kinase (ILK) is associated with tumor progression and an unfavorable prognosis in patients with colorectal cancer. J Mol Histol. 2013b;44:183–9. doi:10.1007/s10735-012-9463-6.

Lianjie L, Liang Z, Xiaoping Z et al. Hypoxia-inducible factor linked to differential kidney cancer risk seen with type 2A and type 2B VHL mutations. Mol Cell Biol, 2007;27(15):5381–92.

Liu ZH, Xiao Y, Yuan Y, et al. Effects of oleic acid on cell proliferation through an integrin-linked kinase signaling pathway in 786-O renal cell carcinoma cells. Oncol Lett. 2013;5(4):1395–1399.

Lu J, Tang Y, Farshidpour M, et al. JWA inhibits melanoma angiogenesis by suppressing ILK signaling and is an independent prognostic biomarker for melanoma. Carcinogenesis. 2013;34:2778–88. doi:10.1093/carcin/bgt318.

Marotta A, Tan C, Gray V, Malik S, Gallinger S, Sanghera J, Dupuis B, Owen D, Dedhar S, Salh B. Dysregulation of integrin-linked kinase (ILK) signaling in colonic polyposis. Oncogene. 2001;20:6250–67.

Marotta A, Parhar K, Owen D, Dedhar S, Salh B. Characterization of integrin-linked kinase signaling in sporadic human colon cancer. Br J Cancer. 2003;88:1755–62.

Martin TJ, Moseley JM, Williams ED. Parathyroid hormone-related protein: hormone and cytokine. J Endocrinol. 1997;154:S23–37.

Massfelder T, Lang H, Schordan E, Lindner V, Rothhut S, Welsch S, Simon-Assmann P, Barthelmebs M, Jacqmin D, Helwig JJ. Parathyroid hormone-related protein is an essential growth factor for human clear cell renal carcinoma and a target for the von Hippel-Lindau tumor suppressor gene. Cancer Res. 2004;64:180–8.

Matsui Y, Assi K, Ogawa O, Raven PA, Dedhar S, Gleave ME, Salh B, So AI. The importance of integrin-linked kinase in the regulation of bladder cancer invasion. Int J Cancer. 2011;130:521–31. doi:10.1002/ijc.26008.

McDonald PC, Fielding AB, Dedhar S. Integrin-linked kinase – essential roles in physiology and cancer biology. J Cell Sci. 2008;1:3121–32. doi:10.1242/jcs.017996.

Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA. Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta. J Biol Chem. 1997;272:30491–7.

Nikolopoulos SN, Turner CE. Actopaxin, a new focal adhesion protein that binds paxillin LD motifs and actin and regulates cell adhesion. J Cell Biol. 2000;151:1435–48.

Obara S, Nakata M, Takeshima H, Katagiri H, Asano T, Oka Y, Maruyama I, Kuratsu J. Integrin-linked kinase (ILK) regulation of the cell viability in PTEN mutant glioblastoma and in vitro inhibition by the specific COX-2 inhibitor NS-398. Cancer Lett. 2004;208:115–22.

Okamura M, Yamaji S, Nagashima Y, et al. Prognostic value of integrin beta1-ILK-pAkt signaling pathway in non-small cell lung cancer. Hum Pathol. 2007;38:1081–91.

Olsk TM, Noegel AA, Korenbaum E. Parvin, a 42 kDa focal adhesion protein, related to the alpha-actinin superfamily. J Cell Sci. 2001;114:525–38.

Papachristou DJ, Gkretsi V, Rao UN, et al. Expression of integrin-linked kinase and its binding partners in chondrosarcoma: association with prognostic significance. Eur J Cancer. 2008;44:2518–25. doi:10.1016/j.ejca.2008.07.021.

Papanikolaou S, Bravou V, Gyftopoulos K, Nakas D, Repanti M, Papadaki LK. Expression in human basal cell carcinoma correlates with epithelial-mesenchymal transition markers and tumour invasion. Histopathology. 2010;56:799–809.

Pasquali C, Bertschy-Meier D, Chabert C, Curchod ML, Arod C, Booth R, Mechtler K, Vilbois F, Xenarios I, Fergurson CG, et al. A chemical proteomics approach to phosphatidylinositol 3-kinase signaling in macrophages. Mol Cel Proteonomics. 2007;6:1829–41.

Peroukides S, Bravou V, Varakis J, Alexopoulos A, Kalofonos H, Papadaki H. ILK overexpression in human hepatocellular carcinoma and liver cirrhosis correlates with activation of Akt. Oncol Rep. 2008;20(6):1337–44.

Persad S, Dedhar S. The role of integrin-linked kinase (ILK) in cancer progression. Cancer Metastasis Rev. 2003;22(4):375–84.

Persad S, Attwell S, Gray V. Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. Proc Natl Acad Sci U S A. 2000;97:3207–12.

Philbrick WM, Wysolmerski JJ, Galbraith S, Holt E, Orloff JJ, Yang KH, Vasavada RC, Weir EC, Broadus AE, Stewart AF. Defining the roles of parathyroid hormone-related protein in normal physiology. Physiol Rev. 1996;76:127–73.

Posch F, Setinek U, Flores RM, et al. Serum integrin-linked kinase (sILK) concentration and survival in non-small cell lung cancer: a pilot study. Clin Transl Oncol. 2014;16:455–62. doi:10.1007/s12094-013-1101-6.

Qin J, Wu C. ILK: a pseudokinase in the center stage of cell-matrix adhesion and signaling. Curr Opin Cell Biol. 2012;24(5):607–13. doi:10.1016/j.ceb.2012.06.003. Epub 2012 Jul 2.

Saito K, Kihara K. Role of C-reactive protein as a biomarker for renal cell carcinoma. Expert Rev Anticancer Ther. 2011;10:1979–89.

Sawai H, Okada Y, Funahashi H, et al. Integrin-linked kinase activity is associated with interleukin-1 alpha-induced progressive behavior of pancreatic cancer and poor patient survival. Oncogene. 2006;25:3237–46.

Schaeffer DF, Assi K, Chan K, Buczkowski AK, Chung SW, Scudamore CH, Weiss A, Salh B, Owen DA. Tumor expression of integrin-linked kinase (ILK) correlates with the expression of the E-cadherin repressor snail: an immunohistochemical study in ductal pancreatic adenocarcinoma. Virchows Arch. 2010;456:261–8.

Schramm A, Opitz I, Thies S, et al. Prognostic significance of epithelial-mesenchymal transition in malignant pleural mesothelioma. Eur J Cardiothorac Surg. 2010;37:566–72. doi:10.1016/j.ejcts.2009.08.027.

Segrelles C, Ruiz S, Perez P et al. Functional roles of Akt signaling in mouse skin tumorigenesis. Oncogene. 2002;21:53–64.

Singer EA, Gupta GN, Srinivasan R. Update on targeted therapies for clear cell renal carcinoma. Curr Opin Oncol. 2011;23:283–9.

Sourbier C, Lindner V, Lang H, et al. The phosphoinositide 3-kinase/Akt pathway: a new target in human renal cell carcinoma therapy. Cancer Res. 2006;66:5130–42.

Takanami I. Increased expression of integrin-linked kinase is associated with shorter survival in non-small cell lung cancer. BMC Cancer. 2005;5:1. doi:10.1186/1471-2407-5-1.

Tu Y, Li F, Goicoechea S, Wu C. The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. Mol Cell Biol. 1999;19:2425–34.

Tu Y, Huang Y, Zhang Z, Hua Y, Wu C. A new focal adhesion protein that interacts with integrin-linked kinase and regulates cell adhesion and spreading. J Cell Biol. 2001;153:585–98.

Virtanen I, Lehto VP. Progression of malignancy in clear cell renal cell carcinomas. Scand J Surg. 2004;93:112–7.

Watzka SB, Rauscher-Pötsch I, Stubenberger E, Getman V, Setinek U, Tötsch M, Pötschger U, Müller MR. Immunoreactivity of integrin-linked kinase in primary non-small-cell lung cancer and survival after curative resection. Eur J Cardiothorac Surg. 2010;38:254–9.

Watzka SB, Setinek U, Stubenberger EB et al. Integrin-linked kinase, phosphorylated AKT and the prognosis of malignant pleural mesothelioma. Eur J Cardiothorac Surg. 2011;39(2):180-4. doi:10.1016/j.ejcts.2010.05.007.

Wickström SA, Lange A, Montanez E, Fässler R. The ILK/PINCH/parvin complex: the kinase is dead, long live the pseudokinase! EMBO J. 2010;29(2):281–91. doi:10.1038/emboj. 2009; 376. Epub 2009 Dec 24.

Xie D, Yin D, Tong X, O'Kelly J, Mori A, Miller C, Black K, Gui D, Said JW, Koeffler HP. Cyr61 is overexpressed in gliomas and involved in integrin-linked kinase-mediated Akt and beta-catenin-TCF/Lef signaling pathways. Cancer Res. 2004;64:1987–96.

Yamagi S, Suzuki A, Koide Y, Kamamori H, Mohri H, Ohno S, Yshigatsubo Y. A novel integrin-linked kinasebinding protein, affixin, is involved in the early stage of cell–substrate interaction. J Cell Biol. 2001;153:1251–64.

Younes MN, Kim S, Yigitbasi OG, Mandal M, Jasser SA, Dakak Yazici Y, Schiff BA, El-Naggar A, Bekele BN, Mills GB, Myers JN. Integrin-linked kinase is a potential therapeutic target for anaplastic thyroid cancer. Mol Cancer Ther. 2005;4:1146–56.

Molecular Biomarkers and Treatments for Renal Cell Carcinoma

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Abstract

Recent advances in the biological understanding of renal cell carcinoma (RCC) have led to the identification of several molecular biomarkers with prognostic and therapeutic value. Clinical trials have proven the effectiveness of targeted therapies for advanced RCC and a growing body of work is underway to provide molecular biomarkers for response of these new therapies which act primarily against the vascular endothelial growth factor or mammalian target of rapamycin pathways. Finding optimal biomarkers will help tailor targeted therapies according to tumor and patient innate molecular factors and provide the best individualized treatments that can prolong survival while minimizing adverse side effects.

Keywords

Renal cell carcinoma • Molecular biomarkers • Prognostic markers • Vascular endothelial growth factor • Tyrosine kinase inhibitor • Mammalian target of rapamycin inhibitor

Abbreviations

ccRCC	Clear cell renal cell carcinoma
CECs	Circulating endothelial cells
CEPCs	Circulating endothelial progenitor cells
CRP	C-reactive protein
CTCs	Circulating tumor cells
ECOG	Eastern Cooperative Oncology Group
FDA	Food and Drug Administration
HIF	Hypoxia-inducible factor
IFN	Interferon
ILN	Interleukin
LDH	Lactate dehydrogenase level
LLN	Lower limit of normal
Metastatic RCC	Metastatic renal cell carcinoma
miRNA	MicroRNA
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NGAL	Neutrophil gelatinase-associated lipocalin
OS	Overall survival
PDGF	Platelet-derived growth factor
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinase
RCC	Renal cell carcinoma
RECIST	Response evaluation criteria in solid tumors
SAA	Serum amyloid A
SEER	Surveillance, Epidemiology, and End Results
ULN	Upper limit of normal

VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor(s)
VHL	Von Hippel-Lindau

Key Facts

- Renal cell carcinoma (RCC) is one of the major genitourinary cancers. RCCs are often found incidentally and surgically curable if still localized. Once RCC spreads (metastasizes), it becomes a deadly disease. There are different types of RCC, which have different invasive and biological behaviors. In order to effectively treat these types, it is important to understand their differences, which are linked to tumor-related or tumor-produced by-products, so-called biomarkers. Biomarkers are used to help clinicians to choose the best treatment for patients and different tumor types. They can be useful to improve diagnostics or to monitor the effect of treatment. This chapter takes a closer look and gives an overview over many different biomarkers important for treatment and diagnostics.

Definitions

Prognostic marker A biomarker that predicts disease progression, treatment response, or mortality (disease specific or overall).

Renal cell carcinoma Renal cell carcinoma (RCC) is an adenocarcinoma of the renal parenchyma.

Von Hippel-Lindau (VHL) Tumor suppressor gene frequently deleted in kidney cancer

Introduction

In 2014, an estimated 63,920 new kidney and renal pelvis cancers will be diagnosed in the USA, and approximately 13,860 will die of this disease (Siegel et al. 2014). Renal cell carcinoma (RCC) is an adenocarcinoma of the renal parenchyma and accounts for more than 80 % of kidney cancer in adults (King et al. 2014). Associated risk factors for RCC are cigarette smoking, obesity, and hypertension, and its highest incidence and death rates are found among American Indians and Alaska Natives, which may be due in part to high rates of obesity and smoking in these populations.

There are several subtypes of RCC based on histopathological and genetic characteristics. The most common histology is clear cell followed by papillary, chromophobe, oncocytic, and rare types such as collecting duct and medullary

Type	Incidence	Mutations
Clear Cell	65 - 70%	VHL gene
Papillary 1	5 - 7.5%	C-Met oncogene
Papillary 2	5 - 7.5%	Fumarate hydratase

Fig. 1 Incidence of RCC and genetic associations

carcinomas. Both hereditary and sporadic types have been associated with mutation in the short arm of chromosome 3, with implication in tumor suppressor genes (i.e., VHL) or oncogenes (i.e., c-Met). Many of the hereditary types are associated with familial syndromes such as Birt-Hogg-Dubé, hereditary papillary renal carcinoma, and von Hippel-Lindau disease (see Fig. 1).

Surgical extirpation has been the mainstay of treatment for patients who present with stage I–III RCC. For those who present late with advanced and metastatic disease (stage IV), the overall clinical course of RCC varies; approximately 50 % of patients survive less than 1 year and 10 % survive for more than 5 years (Yuen 2009). Chemotherapy has been shown to be ineffective in the treatment of this disease, and until recently, the only effective treatment for metastatic RCC (mRCC) was cytokine-based immunotherapy with interferon (IFN)-α or interleukin (IL)-2.

Besides anatomic and histologic characteristics, others have evaluated various clinical factors for prognostic purposes. Motzer and associates evaluated the relationship between pretreatment clinical features and survival in 670 patients enrolled in phase II and phase III clinical trials of chemotherapy or immunotherapy at the Memorial Sloan Kettering Cancer Center (MSKCC) in order to create a multivariate model that can predict survival. Prognostic factors associated with shorter survival were low Karnofsky performance status (KPS) (<80 %), lactate dehydrogenase level (LDH) $>1.5 \times$ the upper limit of normal (ULN), hemoglobin level $<$ the lower limit of normal (LLN), high corrected serum calcium level (>10 mg/dL), and absence of nephrectomy. The median time to death in patients with 0 risk factors (favorable risk), 1 or 2 risk factors (intermediate risk), and ≥ 3 risk factors (poor risk) were 20 months, 10 months, and 4 months, respectively (Motzer et al. 2004).

The MSKCC model was modified in 2002 and was restricted to 400 patients who received IFN-α. Five variables were used as risk factors for short survival: low KPS, high LDH, low serum hemoglobin, high corrected serum calcium, and time from initial RCC diagnosis to start of IFN-α therapy of less than 1 year. The median time to death for patients deemed favorable, intermediate, and poor risk was 30 months, 14 months, and 5 month, respectively (Motzer et al. 2002). Fortunately, advances in the understanding of molecular and genetic factors of RCC have led to the emergence of newer targeted therapeutic agents for its treatment. According to the Surveillance, Epidemiology, and End Results (SEER) cancer registry, advanced

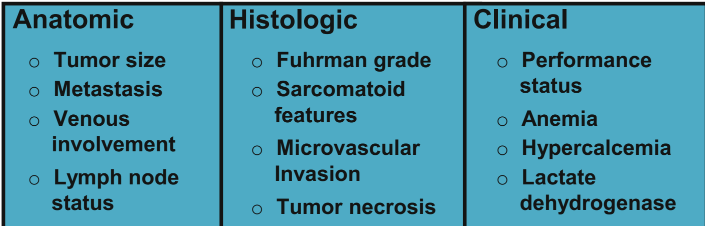

Fig. 2 Anatomic, histologic, and clinical prognostic factors in RCC

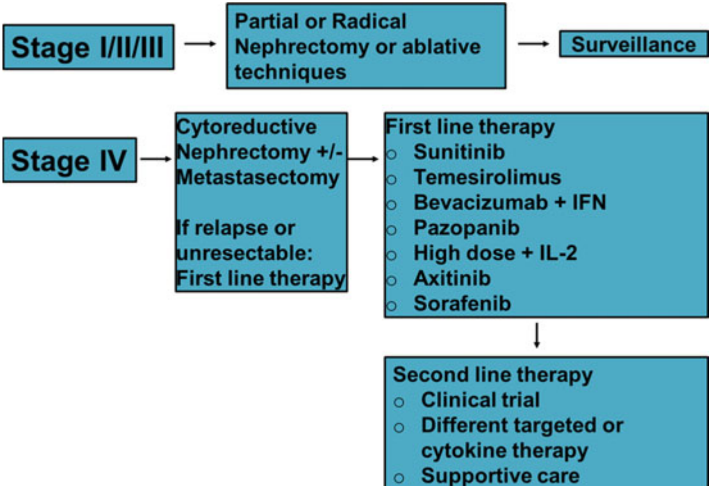

Fig. 3 Treatments for RCC

RCC cases (regional and distant disease) in the targeted therapy era had a statistically significant improvement in overall survival (OS) than the pre-targeted therapy group ($P < 0.001$) (Vaishampayan et al. 2014) (see Fig. 2).

The treatment of advanced RCC has evolved significantly following the identification of the von Hippel-Lindau (VHL) gene and the subsequent development of antiangiogenic therapies. There are currently eight US Food and Drug Administration (FDA)-approved agents available for the treatment of mRCC. Five of these agents target either the vascular endothelial growth factor (VEGF) or its receptors (VEGFR), two inhibit activity of the mammalian target of rapamycin (mTOR), and one is a recombinant form of the endogenous cytokine IL-2. Each of these agents provides clinical benefit to a subset of patients, and the overall outlook for patients with mRCC is better today than it was 10 years ago (see Fig. 3).

There are still many unmet needs when it comes to treating mRCC. More specifically, there are no validated systems that predict those patients at risk for early metastatic disease that may benefit from some type of adjuvant therapy. In

addition, there is no clear mechanism to identify nonresponders as well as those patients more susceptible to certain drug toxicities. For years, molecular biomarkers from serum, tissue, or urine have been acknowledged as potential prognostic and predictive adjuncts in the treatment of mRCC. In this review, we provide an update on current investigations of the most well-known molecular biomarkers associated with RCC.

Systemic Therapy for Renal Cell Carcinoma

The immune system has long been believed to have an important role in the development of renal cancer. Reports of complete remissions have been described in the literature, although it is estimated that the true incidence of spontaneous regression is less than 1 %. Clear cell RCC (ccRCC) is the most common subtype, occurring in approximately 80 % of patients. It is characterized by the inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene located on chromosome 3p25 and encodes for proteins in charge of proteolysis of hypoxia-inducible factors (HIFs). Disruption of VHL results in production of defective VHL protein and accumulation of HIF, which leads to the transcription of several hypoxia-inducible genes such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor receptor (EGFR), erythropoietin, and others (Choueiri 2008; Bardos and Ashcroft 2004).

Overproduction of these angiogenic factors promotes tumor growth and progression after binding with its corresponding receptors. VEGF functions by promoting the proliferation and migration of endothelial cells, stimulating vessel formation, and inhibiting apoptosis. The crucial role of VEGF in RCC growth and metastasis has led to the development of FDA-approved small molecule VEGF inhibitors such as sunitinib, sorafenib, and axitinib, that target the VEGF and PDGF receptors, and also of a recombinant human monoclonal anti-VEGF antibody, bevacizumab. All of these agents have been shown to improve clinical outcomes in patients with clear cell mRCC, although sunitinib is superior to sorafenib (Larkin et al. 2015).

The targeting of the mTOR pathway has also been proven effective in the treatment of mRCC, although its mechanism of action is not completely understood. Along with phosphatidylinositol 3-kinase (PI3K) and Akt, its signaling regulates cell growth, metabolism, proliferation, and motility (Hudes 2009). The relevance of PI3K/Akt/mTOR pathway led to the development of the new therapies such as temsirolimus which was shown to prolong OS and progression-free survival (PFS) compared to IFN alone in poor-risk patients (Hudes et al. 2007). Another mTOR inhibitor, everolimus, was approved after its phase III trial was halted early once interim analysis showed significant improvement in PFS for patients who had failed previous VEGF-targeted therapy (Motzer et al. 2008). Table 1 summarizes current therapies that target the VHL-VEGF and PI3k/Akt/mTOR pathways.

Table 1 FDA-approved targeted agents for the treatment of mRCC

Name	Class	Pivotal study	Primary endpoint	HR (95 % CI)	Common side effects
Sunitinib	TKI	Motzer et al. (2007)	PFS: 11 vs 5 months (INF-α)	0.42 (0.32–0.54) $p < 0.001$	Diarrhea, N/V, HTN, hand-foot syndrome
Sorafenib	TKI	Escudier et al. (2009)	OS: 17.8 vs 14.3 months (placebo)	0.78 (0.62–0.97) $p < 0.05$[a]	Diarrhea, HTN, hand-foot syndrome, rash/desquamation
Pazopanib	TKI	Sternberg et al. (2010)	PFS: 9.2 vs 4.2 months (placebo)	0.46 (0.34–0.62) $p < 0.0001$	Diarrhea, HTN, hair color changes, N/V
Axitinib	TKI	Rini et al. (2011)	PFS: 6.7 vs 4.7 months (sorafenib)	0.665 (0.544–0.812) $p < 0.0001$	Diarrhea, HTN, fatigue
Bevacizumab	VEGF monoclonal antibody	Escudier et al. (2010)	OS: 23.3 (bevacizumab + IFN) vs 21.3 months (IFN + placebo)	0.91 (0.76–1.10) $p = 0.336$	Fatigue, asthenia, proteinuria, HTN
Temsirolimus	mTOR inh	Hudes et al. (2007)	OS: 10.9 (alone) vs 7.3 (IFN) vs 8.4 months (both)	0.73 (0.58–0.92) $p = 0.008$	Rash, edema, hyperglycemia, hyperlipidemia
Everolimus	mTOR inh	Motzer et al. (2008)	PFS: 4 vs 1.9 months (placebo)	0.30 (0.22–0.40) $p < 0.0001$	Stomatitis, rash, fatigue, pneumonitis

HTN hypertension, *mo* months, *mTOR inh* mammalian target of rapamycin inhibitor, *N/V* nausea or vomiting, *OS* overall survival, *PFS* progression-free survival, *TKI* tyrosine kinase inhibitor, *VEGF* vascular endothelial growth factor

[a]Analysis after crossover from placebo

Markers in Targeted Therapy

Efficacy of markers is challenging due to the molecular heterogeneity in advanced RCC. These molecular differences can account for the mixed results and treatment responses of targeted therapies. Several groups have confirmed that molecular markers may predict tumor behavior, in addition to response to treatment. Molecular markers could become helpful adjuncts in deciding particular therapies and dosage for optimal treatment of patients. Table 2 summarizes selected prognostic markers in advanced RCC.

Carbonic Anhydrase IX (CAIX)

Tissue-based biomarkers have been used to predict progression and survival outcomes. CAIX is a well-known immunohistochemical marker in the diagnosis of RCC. It is a cell surface enzyme that is overexpressed in upward of 90 % of ccRCC cases, and its expression is regulated by HIF-1α and the inactivation of VHL (Sun et al. 2011). A retrospective study by Bui et al. showed that decreased CAIX levels are associated with poor survival in advanced RCC. Low CAIX (≤85 %) staining was an independent poor prognostic factor for survival in mRCC ($p < 0.001$), and overall expression of CAIX decreased with development of metastasis (Bui et al. 2003). In a recent meta-analysis of 2611 patients, low CAIX expression was associated with poor disease-specific survival (DSS) (HR = 1.89, 95 %CI: 1.20–2.98), unfavorable PFS (HR = 2.62, 95 %CI: 1.14–6.05), and worse OS (HR = 2.03, 95 %CI: 1.28–3.21). Furthermore, low CAIX expression was significantly associated with lymph node metastases (OR = 0.31, 95 %CI: 0.15–0.62) and higher tumor grade (OR = 0.41, 95 % CI: 0.31–0.5; (Zhao et al. 2014)). In general, a high expression of CAIX appears to be a favorable prognostic marker, and it may even contribute to improved response rates for IL-2 therapy (Atkins et al. 2005).

VHL

ccRCC is characterized by VHL inactivation through biallelic gene loss due to a dual hit in most patients (Garcia-Donas et al. 2013). It has been proposed that VHL-inactivated tumors are more susceptible to VEGF-targeted therapies (Choueiri 2008). It is inactivated in almost all patients with VHL syndrome and in approximately 70 % of sporadic ccRCC (Yao et al. 2002) VHL mutations (intragenic or hypermethylation) were strongly associated with better cancer-free survival and cancer-specific survival for 134 patients with stage I–III ccRCC ($p = 0.024$ and 0.023, respectively; (Yao et al. 2002)).

Table 2 Selected prognostic markers in advanced RCC

Author	n	Specimen	Study	Marker	Therapy	Endpoint
Bui et al. (2003)	321	Tissue	RT	CAIX	n/a	DSS
Choueiri et al. (2008)	123	Tissue	RT	VHL (LOF mutation)	Sunitinib, sorafenib, axitinib, bevacizumab	RR
Rini et al. (2006)	43	Tissue	RT	VHL (mutation or methylation)	Bevacizumab + IFN-α	TTP
Escudier et al. (2009)	900	Serum	RCT	VEGF	Sorafenib	OS, RR
Harmon et al. (2014)	30	Serum	RCT	VEGF-A, VEGF-C, sVEGFR-3, IL-8	Sunitinib or IFN-α	PFS, OS
Deprimo et al. (2007)	63	Serum	PT	VEGF, sVEGFR-2, PIGF, sVEGFR-3	Sunitinib	RR
Porta et al. (2010)	85	Serum	RT	NGAL, VEGF	Sunitinib	PFS
Steffens et al. (2012)	1,161	Serum	RT	CRP	n/a	CSS
Armstrong et al. (2012)	404	Serum	RT	LDH	Temsirolimus or IFN-α	OS
Garcia-Donas et al. (2011)	101	Serum, saliva	PT	VEGFR3 polymorphisms (rs307826, rs307821), CYP3A5*1	Sunitinib	PFS, toxicity
Xu et al. (2011)	397	Serum	PT	Polymorphisms in IL-8 (2767TT/-251TT), HIF1A (1790G), NR1I2 (-25385TT), VEGF-A (-1498CC)	Pazopanib	PFS, RR
Lambrechts et al. (2012)	110	Serum	RCT	Polymorphism in VEGFR1 (rs799341/ rs9554316, rs9513070)	IFNα-2a + bevacizumab or placebo	PFS, OS

CAIX carbonic anhydrase IX, *CSS* cancer-specific survival, *DSS* disease-specific survival, *IL-8* interleukin-8, *IFN-α* interferon alpha, *LOF* loss of function, *OS* overall survival, *PIGF* placenta growth factor, *PFS* progression-free survival, *PT* prospective, *RCT* randomized control trial, *RR* response rate, *RT* retrospective, *sVEGFR* soluble vascular endothelial growth factor receptor, *TTP* time to tumor progression, *VHL* von Hippel-Lindau protein

A retrospective analysis of temsirolimus-treated patients observed no difference in outcome for tumors harboring VHL mutations. CAIX was also studied but found no correlation with objective response or clinical benefit (Cho et al. 2007). Another study of patients treated with sunitinib, sorafenib, axitinib, or bevacizumab at the Cleveland Clinic and University of California, San Francisco, showed VHL inactivation had a Response Evaluation Criteria in Solid Tumors (RECIST)-defined response rate of 41 % vs 31 % for patients with wild-type VHL ($p = 0.34$). However, PFS and OS did not correlate with VHL status (Choueiri et al. 2008).

VEGF

VEGF is a glycoprotein that plays a critical role in the induction of endothelial cell division and migration by interacting with the transmembrane tyrosine kinase receptors VEGFR-1 and VEGFR-2 selectively expressed on vascular endothelial cells (Rini et al. 2005). Another VEGF-receptor family member is VEGFR-3, which is mainly expressed in lymphatic, endothelial cells (Iljin et al. 2001). Porta et al. reported VEGF baseline values were significant predictors of PFS for patients treated with sunitinib on multivariate analysis (RR = 1.96, 95 %CI: 1.47–2.45; (Porta et al. 2010)). The use of VEGF as a prognostic biomarker was also evaluated in the TARGET study of 900 patients randomly assigned to sorafenib versus placebo. Baseline VEGF levels correlated with Eastern Cooperative Oncology Group (ECOG) PS, MSKCC score, PFS, and OS in univariate and multivariate analyses. Results suggested that patients in the 75th percentile of VEGF level may experience greater benefit from sorafenib (HR = 0.27, 95 % CI: 0.15–0.460) than those with low VEGF (HR = 0.58, 95 %CI: 0.43–0.78; (Escudier et al. 2009)). An update demonstrated elevated VEGF levels correlated with shorter PFS in placebo-treated patients on univariate analysis (HR = 1.645, 95 % CI: 1.19–2.28) but failed to show as an independent prognostic factor on multivariate analysis (Pena et al. 2010). No links have been found so far regarding the relation of VEGFR-3 and RCC. A study by Virman et al. could only confirm that a low expression of CD31 was significantly associated with poorer survival but failed to show any association toward VEGFR-3 (Virman et al. 2015).

Circulating Endothelial Cells (CECs)

A growing body of evidence shows that CECs and circulating endothelial progenitor cells (CEPCs) contribute to tumor angiogenesis. RCC shows a high expression of VEGF, which plays a role in the mobilization, recruitment, homing, and incorporation of endothelial progenitor cells for tumor vasculogenesis during tumor progression (Ding et al. 2008). An analysis of 53 patients showed the preoperative CEPC level correlated with serum VEGF level in patients with RCC ($r = 0.710$, $p < 0.001$). The mean CEPC level in patients with RCC was significantly higher than in patients with benign renal tumors and healthy controls (0.281 % vs 0.073 %

and 0.076 %, respectively; (Yang et al. 2012)). Recently, Gu and associates were able to isolate and culture CEPCs from patients with RCC and found CEPC levels significantly higher than control group (0.276 % vs 0.086 %, $p < 0.001$). Furthermore, earlier emergence of CEPC colonies (6.72 vs 14.67 days), higher number of colonies (10.06 vs 1.83), and higher cell culture success rate (87.8 % vs 40 %; each $p < 0.001$) were also noted in RCC patients (Gu et al. 2015). Given that CECs decrease after nephrectomy in patients with localized RCC, CECs may be a potential marker of progression and recurrence, although the difficulty in quantifying them reliably has decreased the enthusiasm for their use a biomarker.

Circulating Tumor Cells (CTCs)

Although there has been great interest in CTCs as biomarkers in RCC, the detection of RCC cells in the blood has been hindered by the absence of markers that are specific enough for RCC cells against the background of hematological cells (Hernandez-Yanez et al. 2012). The only commercially available FDA-approved platform for the enumeration of CTCs is the CellSearch™ assay which relies on the expression of epithelial cell adhesion molecule (EpCAM) and cytokeratins for cellular detection (Mego et al. 2011). Although this system has been useful for detection of CTCs in metastatic breast, colorectal, and prostate cancer patients, its use has been limited in RCC due to the lack of EpCAM positivity of RCC cells. Other filtration-based technologies have been developed to allow for antigen-independent isolation of CTCs based on size and cytomorphological features in comparison to hematological cells (Vona et al. 2000). Using such methods, El-Heliebi et al. aimed to identify CTCs in the blood of 30 RCC patients; nonetheless, they were unable to distinguish epithelial from endothelial non-hematologic cells based on morphological criteria alone (El-Heliebi et al. 2013).

Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Matrix Metalloproteinase-9 (MMP-9)

NGAL was identified as 25 kDa protein covalently linked to MMP-9 in neutrophils. NGAL protects MMP-9 from degradation, thereby preserving the activity of MMP-9 which has been shown to play a critical role in tumor cell invasion and metastasis in many cancers (Yan et al. 2001). NGAL, along with VEGF, showed significant prediction of PFS in 85 patients treated with sunitinib. NGAL level above threshold resulted in increased risk of progression (RR = 1.86, 95 % CI: 1.42–3.019) and decreased PFS compared to those below the NGAL threshold level (3.4 vs 8.2 months, $p = 0.03$; (Porta et al. 2010)). The role of MMP-9 and other metalloproteinases has been investigated in RCC progression. Kugler et al. found that MMP-2 and MMP-9 are elevated in RCC tumor tissue with more expression seen in advanced tumors (Kugler et al. 1998). One study of paraffin-embedded tissue samples from 232 patients revealed MMP-9 had a high correlation with systemic

symptoms (OR = 1.02, 95 %CI: 0.36–2.62; (Kawata et al. 2006)). Although there is tissue evidence, other studies have failed to find any correlation of MMP-2 and MMP-9 activity in serum and urine (Di Carlo 2012).

MicroRNA (miRNA)

miRNAs are endogenous ~23 nt RNAs that have gene-regulatory roles by pairing to the mRNAs of protein-coding genes to direct their posttranscriptional repression. Due to having crucial impact on the regulation of cell growth, evasion of apoptosis, angiogenesis, tissue invasion, and metastasis, miRNAs have become a focus of intensive investigation throughout recent years (Bartel 2009). Expression analyses have shown that a variety of miRNAs are either up- or downregulated in RCC when compared to normal kidney tissue. Patients with RCC have higher levels of circulating miRNA-221 and miRNA-222 when compared to healthy individuals. Furthermore, even higher levels of these miRNAs were observed in mRCC patients when compared to nonmetastatic patients (Teixeria et. al. 2014). Signatures of certain combinations of miRNAs, e.g., miRNA-451, miRNA-221, miRNA-30a, miRNA-10b, and miRNA-29a, may also be useful in differentiating between metastatic and nonmetastatic RCC (Heinzelmann et. al. 2011). However, it remains to be determined whether changes in the expression of specific miRNAs that were reported in different studies are either the reason or the result of RCC (Li et. al. 2015).

mTOR

As described above, the targeting of mTOR provides one of the best approaches for clinicians to effectively treat mRCC. Predictors for success of this treatment option are still under investigation. In 2015 Bodnar et al. showed that for histological grade 1 and 2 tumors, increased LDH level before treatment and the PIK3CA gene variant rs6443624 were predictors of treatment-related effects for everolimus (Bodner et. al. 2015).

Other RNA-Related Markers

Due to the advances in genomic profiling and easy to use laboratory techniques, such as RT-(q)-PCR, it has become easier to measure the expression of genes and, thereby, to identify up- and downregulated genes in RCC patients. For example, CD1d is a member of the CD1 family of glycoproteins that is expressed on the surface of various human antigen-presenting cells and is involved in the presentation of lipid antigens to T cells. CD1d expression was found to significantly correlate with stage, grade, higher relapse rates, poor cancer specific, and OS (Chong et. al. 2015).

Inflammatory Markers

Studies have investigated the role of inflammatory proteins and cytokines in RCC. Serum amyloid A (SAA) and C-reactive protein (CRP) have been identified as potential biomarkers for RCC. Fischer et al. analyzed blood samples of 115 patients with localized or advanced RCC. Preoperative values of IL-6, CRP, and SAA were significantly higher in advanced versus localized RCC cases. SAA had the best diagnostic accuracy with sensitivity of 78 % and specificity of 82 % compared to CRP (69 % and 82 %) and IL-6 (44 % and 94 %; (Fischer et al. 2012)). In another study of 422 RCC patients, SAA showed prognostic significance with higher levels in advanced stage disease (i.e., T3-M1; (Mittal et al. 2012)). Large studies have correlated high serum preoperative CRP levels as a significant prognostic factor for unfavorable clinical outcome (Steffens et al. 2012; de Martino et al. 2013; Johnson et al. 2010). A preoperative CRP level of >10 mg/l indicates a 2.5-fold increased risk of death from RCC when compared to a preoperative CRP level of ≤4 mg/l (Steffens et al. 2012).

LDH has long been recognized as a prognostic biomarker as part of the MSKCC risk classification model for mRCC (Motzer et al. 2004). LDH is an enzyme involved in anaerobic glycolysis and is regulated by the PI3K/Akt/mTOR pathway (Armstrong et al. 2012). A phase III trial evaluated pre-and posttreatment LDH levels in 404 poor-risk patients treated with temsirolimus or IFN-α. Patients with LDH >1 × ULN indicated a hazard ratio for death of 2.8 with a median survival time of 6.9 months for patients treated with temsirolimus versus 4.2 months median survival for those treated with IFN-α (Armstrong et al. 2012). This study validated that a higher baseline LDH is a predictor of OS in patients treated with mTOR inhibitors.

Polymorphisms

Genetic factors have been associated with deficiencies of certain therapies according to their targeted pathway. Various VEGF polymorphisms have been found as risk factors for renal cancer (Bruyere et al. 2010). The association between VEGF single nucleotide polymorphisms (SNPs) and sunitinib has been the most widely studied among the anti-VEGF therapies. In an observational, prospective study of 101 patients, sunitinib tolerability and RECIST response were assessed in correlation with 16 polymorphisms of nine genes involved in sunitinib pharmacokinetics. Two VEGFR3 polymorphisms (rs307826 and rs307821) and CYP3A5 (rs776746) were associated with reduced PFS (HR = 3.57 and HR = 3.31) and increased toxicity (HR = 3.75), respectively. Three additional polymorphisms in ABCB1, ABCG2, and VEGFR2 (rs1128503, rs2231142, and rs1870377) showed a tendency for worse response although not significant (Garcia-Donas et al. 2011). Other studies that investigated tolerability of sunitinib in RCC patients found an increased risk of leukopenia associated with CYP1A1 (OR = 6.24), FLT3 (OR = 2.8), the NR1|3

haplotype (OR = 1.74), mucosal inflammation with CYP1A1 (OR = 4.03), and hand-foot syndrome with ABCB1 haplotype (OR = 2.56; (van Erp et al. 2009)).

Using data from the recent phase III COMPARZ trial of pazopanib- vs sunitinib-treated patients, two IL8 SNPs (rs1126647 and rs4073) showed significant association with poor OS outcomes ($p \leq 0.05$); rs1126647 also showed association with OS in another independent dataset of sunitinib-treated patients ($p \leq 0.05$) (Xu et al. 2015). In the AVOREN bevacizumab trial, VEGFR1 SNP rs7993418, as well as rs9554316 (full linkage) and rs9513070, correlated with PFS (HR = 1.81 and 1.68, respectively), but only rs7933418 remained significant after covariate adjustment (Lambrechts et al. 2012).

Potential Applications to Prognosis, Other Diseases, or Conditions

Greater understanding of the biological heterogeneity of RCC has led to the discovery of a number of biomarkers with prognostic and therapeutic value. These prognostic biomarkers show an opportunity to individualize treatment therapies based on patient and tumor molecular background and require further external validation before they can be incorporated in routine clinical practice. Eventually, markers can help the health-care community guide available targeted therapies toward improving prognostication, risk stratification, and survival outcomes for patients with advanced RCC and even cut down on costs for the health-care system.

Summary Points

- Clinical course and subtypes of kidney cancer
- Treatment strategies for renal cell carcinoma
- Biomarkers for metastasis
- Biomarkers for treatment
- Biomarkers for clinical decision making

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References

Armstrong AJ, George DJ, Halabi S. Serum lactate dehydrogenase predicts for overall survival benefit in patients with metastatic renal cell carcinoma treated with inhibition of mammalian target of rapamycin. J Clin Oncol. 2012;30:3402–7.

Atkins M, Regan M, McDermott D, Mier J, Stanbridge E, Youmans A, Febbo P, Upton M, Lechpammer M, Signoretti S. Carbonic anhydrase IX expression predicts outcome of interleukin 2 therapy for renal cancer. Clin Cancer Res. 2005;11:3714–21.

Bardos JI, Ashcroft M. Hypoxia-inducible factor-1 and oncogenic signalling. Bioessays. 2004;26:262–9.

Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136:215–33.

Bodner L, Stec R, Cierniak S, Synowiec A, Wcisło G, Jesiotr M, Koktysz R, Kozłowski W, Szczylik C. Clinical usefulness of PI3K/Akt/mTOR genotyping in companion with other clinical variables in metastatic renal cell carcinoma patients treated with everolimus in the second and subsequent lines. Ann Oncol. 2015;26(7):1385–9.

Bruyere F, Hovens CM, Marson MN, D'Arcier BF, Costello AJ, Watier H, Linassier C, Ohresser M. VEGF polymorphisms are associated with an increasing risk of developing renal cell carcinoma. J Urol. 2010;184:1273–8.

Bui MH, Seligson D, Han KR, Pantuck AJ, Dorey FJ, Huang Y, Horvath S, Leibovich BC, Chopra S, Liao SY, Stanbridge E, Lerman MI, Palotie A, Figlin RA, Belldegrun AS. Carbonic anhydrase IX is an independent predictor of survival in advanced renal clear cell carcinoma: implications for prognosis and therapy. Clin Cancer Res. 2003;9:802–11.

Cho D, Signoretti S, Dabora S, Regan M, Seeley A, Mariotti M, Youmans A, Polivy A, Mandato L, McDermott D, Stanbridge E, Atkins M. Potential histologic and molecular predictors of response to temsirolimus in patients with advanced renal cell carcinoma. Clin Genitourin Cancer. 2007;5:379–85.

Chong TW, Goh FY, Sim MY, Huang HH, Thike DAA, Lim WK, Teh BT, Tan PH. CD1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival. J Clin Pathol. 2015;68:200–5.

Choueiri TK. Factors associated with outcome in patients with advanced renal cell carcinoma in the era of antiangiogenic agents. Clin Genitourin Cancer. 2008;6:15–20.

Choueiri TK, Vaziri SA, Jaeger E, Elson P, Wood L, Bhalla IP, Small EJ, Weinberg V, Sein N, Simko J, Golshayan AR, Sercia L, Zhou M, Waldman FM, Rini BI, Bukowski RM, Ganapathi R. von Hippel-Lindau gene status and response to vascular endothelial growth factor targeted therapy for metastatic clear cell renal cell carcinoma. J Urol. 2008;180:860–5. discussion 865–6.

DE Martino M, Klatte T, Seemann C, Waldert M, Haitel A, Schatzl G, Remzi M, Weibl P. Validation of serum C-reactive protein (CRP) as an independent prognostic factor for disease-free survival in patients with localised renal cell carcinoma (RCC). BJU Int. 2013;111:E348–53.

Deprimo SE, Bello CL, Smeraglia J, Baum CM, Spinella D, Rini BI, Michaelson MD, Motzer RJ. Circulating protein biomarkers of pharmacodynamic activity of sunitinib in patients with metastatic renal cell carcinoma: modulation of VEGF and VEGF-related proteins. J Transl Med. 2007;5:32.

di Carlo A. Matrix metalloproteinase-2 and -9 in the sera and in the urine of human oncocytoma and renal cell carcinoma. Oncol Rep. 2012;28:1051–6.

Ding YT, Kumar S, Yu DC. The role of endothelial progenitor cells in tumour vasculogenesis. Pathobiology. 2008;75:265–73.

El-Heliebi A, Kroneis T, Zohrer E, Haybaeck J, Fischereder K, Kampel-Kettner K, Zigeuner R, Pock H, Riedl R, Stauber R, Geigl JB, Huppertz B, Sedlmayr P, Lackner C. Are morphological criteria sufficient for the identification of circulating tumor cells in renal cancer? J Transl Med. 2013;11:214.

Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Staehler M, Negrier S, Chevreau C, Desai AA, Rolland F, Demkow T, Hutson TE, Gore M, Anderson S, Hofilena G, Shan M, Pena C, Lathia C, Bukowski RM. Sorafenib for treatment of renal cell carcinoma: Final efficacy and safety results of the phase III treatment approaches in renal cancer global evaluation trial. J Clin Oncol. 2009;27:3312–8.

Escudier B, Bellmunt J, Negrier S, Bajetta E, Melichar B, Bracarda S, Ravaud A, Golding S, Jethwa S, Sneller V. Phase III trial of bevacizumab plus interferon alfa-2a in patients with metastatic renal cell carcinoma (AVOREN): final analysis of overall survival. J Clin Oncol. 2010;28:2144–50.

Fischer K, Theil G, Hoda R, Fornara P. Serum amyloid A: a biomarker for renal cancer. Anticancer Res. 2012;32:1801–4.

Garcia-Donas J, Esteban E, Leandro-Garcia LJ, Castellano DE, DEL Alba AG, Climent MA, Arranz JA, Gallardo E, Puente J, Bellmunt J, Mellado B, Martinez E, Moreno F, Font A, Robledo M, Rodriguez-Antona C. Single nucleotide polymorphism associations with response and toxic effects in patients with advanced renal-cell carcinoma treated with first-line sunitinib: a multicentre, observational, prospective study. Lancet Oncol. 2011;12:1143–50.

Garcia-Donas J, Rodriguez-Antona C, Jonasch E. Molecular markers to predict response to therapy. Semin Oncol. 2013;40:444–58.

Gu W, Sun W, Guo C, Yan Y, Liu M, Yao X, Yang B, Zheng J. Culture and characterization of circulating endothelial progenitor cells in patients with renal cell carcinoma. J Urol. 2015;194:214–22.

Harmon CS, Deprimo SE, Figlin RA, Hudes GR, Hutson TE, Michaelson MD, Negrier S, Kim ST, Huang X, Williams JA, Eisen T, Motzer RJ. Circulating proteins as potential biomarkers of sunitinib and interferon-alpha efficacy in treatment-naive patients with metastatic renal cell carcinoma. Cancer Chemother Pharmacol. 2014;73:151–61.

Heinzelmann J, Henning B, Sanjmyatav J, Posorski N, Steiner T, Wunderlich H, Mieczyslaw RG, Junker G. Specific miRNA signatures are associated with metastasis and poor prognosis in clear cell renal cell carcinoma. World J Urol. 2011;29:367–73.

Hernandez-Yanez M, Heymach JV, Zurita AJ. Circulating biomarkers in advanced renal cell carcinoma: clinical applications. Curr Oncol Rep. 2012;14:221–9.

Hudes GR. Targeting mTOR in renal cell carcinoma. Cancer. 2009;115:2313–20.

Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, Kapoor A, Staroslawska E, Sosman J, McDermott D, Bodrogi I, Kovacevic Z, Lesovoy V, Schmidt-Wolf IG, Barbarash O, Gokmen E, O'Toole T, Lustgarten S, Moore L, Motzer RJ, Global AT. Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. N Engl J Med. 2007;356:2271–81.

Iljin K, Karkkainen MJ, Lawrence EC, Kimak MA, Uutela M, Taipale J, Pajusola K, Alhonen L, Halmekyto M, Finegold NF, Ferrell RE, Alitalo K. VEGFR3 gene structure, regulatory region, and sequence polymorphisms. FASEB J. 2001;6:1028–36.

Johnson TV, Abbasi A, Owen-Smith A, Young A, Ogan K, Pattaras J, Nieh P, Marshall FF, Master VA. Absolute preoperative C-reactive protein predicts metastasis and mortality in the first year following potentially curative nephrectomy for clear cell renal cell carcinoma. J Urol. 2010;183:480–5.

Kawata N, Nagane Y, Igarashi T, Hirakata H, Ichinose T, Hachiya T, Takimoto Y, Takahashi S. Strong significant correlation between MMP-9 and systemic symptoms in patients with localized renal cell carcinoma. Urology. 2006;68:523–7.

King SC, Pollack LA, Li J, King JB, Master VA. Continued increase in incidence of renal cell carcinoma, especially in young patients and high grade disease: United States 2001 to 2010. J Urol. 2014;191:1665–70.

Kugler A, Hemmerlein B, Thelen P, Kallerhoff M, Radzun HJ, Ringert RH. Expression of metalloproteinase 2 and 9 and their inhibitors in renal cell carcinoma. J Urol. 1998;160:1914–8.

Lambrechts D, Claes B, Delmar P, Reumers J, Mazzone M, Yesilyurt BT, Devlieger R, Verslype C, Tejpar S, Wildiers H, DE Haas S, Carmeliet P, Scherer SJ, VAN Cutsem E. VEGF pathway genetic variants as biomarkers of treatment outcome with bevacizumab: an analysis of data from the AViTA and AVOREN randomised trials. Lancet Oncol. 2012;13:724–33.

Larkin J, Paine A, Foley G, Mitchell S, Chen C. First-Line treatment in the management of advanced renal cell carcinoma: systematic review and network meta-analysis. Expert Opin. Pharmacother. 2015;16:1915–27.

Li M, Wang Y, Song Y, Bu R, Yin B, Fei X, Guo Q, Wu B. MicroRNAs in renal cell carcinoma: a systematic review of clinical implications. Oncol Rep. 2015;33:1571–8.

Mego M, DE Giorgi U, Dawood S, Wang X, Valero V, Andreopoulou E, Handy B, Ueno NT, Reuben JM, Cristofanilli M. Characterization of metastatic breast cancer patients with nondetectable circulating tumor cells. Int J Cancer. 2011;129:417–23.

Mittal A, Poudel B, Pandeya DR, Gupta SP, Sathian B, Yadav SK. Serum amyloid a as an independent prognostic factor for renal cell carcinoma – a hospital based study from the Western region of Nepal. Asian Pac J Cancer Prev. 2012;13:2253–5.

Motzer RJ, Bacik J, Murphy BA, Russo P, Mazumdar M. Interferon-alfa as a comparative treatment for clinical trials of new therapies against advanced renal cell carcinoma. J Clin Oncol. 2002;20:289–96.

Motzer RJ, Bacik J, Mazumdar M. Prognostic factors for survival of patients with stage IV renal cell carcinoma: memorial sloan-kettering cancer center experience. Clin Cancer Res. 2004;10:6302S–3.

Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, Oudard S, Negrier S, Szczylik C, Kim ST, Chen I, Bycott PW, Baum CM, Figlin RA. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med. 2007;356:115–24.

Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grunwald V, Thompson JA, Figlin RA, Hollaender N, Urbanowitz G, Berg WJ, Kay A, Lebwohl D, Ravaud A, GROUP, R.-S. Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. Lancet. 2008;372:449–56.

Pena C, Lathia C, Shan M, Escudier B, Bukowski RM. Biomarkers predicting outcome in patients with advanced renal cell carcinoma: results from sorafenib phase III Treatment Approaches in Renal Cancer Global Evaluation Trial. Clin Cancer Res. 2010;16:4853–63.

Porta C, Paglino C, DE Amici M, Quaglini S, Sacchi L, Imarisio I, Canipari C. Predictive value of baseline serum vascular endothelial growth factor and neutrophil gelatinase-associated lipocalin in advanced kidney cancer patients receiving sunitinib. Kidney Int. 2010;77:809–15.

Rini BI, Sosman JA, Motzer RJ. Therapy targeted at vascular endothelial growth factor in metastatic renal cell carcinoma: biology, clinical results and future development. BJU Int. 2005;96:286–90.

Rini BI, Jaeger E, Weinberg V, Sein N, Chew K, Fong K, Simko J, Small EJ, Waldman FM. Clinical response to therapy targeted at vascular endothelial growth factor in metastatic renal cell carcinoma: impact of patient characteristics and Von Hippel-Lindau gene status. BJU Int. 2006;98:756–62.

Rini BI, Escudier B, Tomczak P, Kaprin A, Szczylik C, Hutson TE, Michaelson MD, Gorbunova VA, Gore ME, Rusakov IG, Negrier S, Ou YC, Castellano D, Lim HY, Uemura H, Tarazi J, Cella D, Chen C, Rosbrook B, Kim S, Motzer RJ. Comparative effectiveness of axitinib versus sorafenib in advanced renal cell carcinoma (AXIS): a randomised phase 3 trial. Lancet. 2011;378:1931–9.

Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics. CA Cancer J Clin. 2014;64:9–29.

Steffens S, Kohler A, Rudolph R, Eggers H, Seidel C, Janssen M, Wegener G, Schrader M, Kuczyk MA, Schrader AJ. Validation of CRP as prognostic marker for renal cell carcinoma in a large series of patients. BMC Cancer. 2012;12:399.

Sternberg CN, Davis ID, Mardiak J, Szczylik C, Lee E, Wagstaff J, Barrios CH, Salman P, Gladkov OA, Kavina A, Zarba JJ, Chen M, McCann L, Pandite L, Roychowdhury DF, Hawkins RE. Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. J Clin Oncol. 2010;28:1061–8.

Sun M, Shariat SF, Cheng C, Ficarra V, Murai M, Oudard S, Pantuck AJ, Zigeuner R, Karakiewicz PI. Prognostic factors and predictive models in renal cell carcinoma: a contemporary review. Eur Urol. 2011;60:644–61.

Teixeira AL, Ferreira M, Silva J, Gomes M, Dias F, Santos JI, Maurício J, Lobo F, Medeiros R. Higher circulating expression levels of miR-221 associated with poor overall survival in renal cell carcinoma patients. Tumor Biol. 2014;35:4057–66.

Vaishampayan U, Vankayala H, Vigneau FD, Quarshie W, Dickow B, Chalasani S, Schwartz K. The Effect of Targeted Therapy on Overall Survival in Advanced Renal Cancer: A Study of the National Surveillance Epidemiology and End Results Registry Database. Clin Genitourin Cancer. 2014;12:124–9.

van Erp NP, Eechoute K, van der Veldt AA, Haanen JB, Reyners AK, Mathijssen RH, Boven E, van der Straaten T, Baak-Pablo RF, Wessels JA, Guchelaar HJ, Gelderblom H. Pharmacogenetic pathway analysis for determination of sunitinib-induced toxicity. J Clin Oncol. 2009;27:4406–12.

Virman J, Bono P, Luukkaala T, Sunela K, Kujala P, Kellokumpu-Lehtinen PL. VEGFR3 and CD31 as prognostic factors in renal cell cancer. Anticancer Res. 2015;35:921–7.

Vona G, Sabile A, Louha M, Sitruk V, Romana S, Schutze K, Capron F, Franco D, Pazzagli M, Vekemans M, Lacour B, Brechot C, Paterlini-Brechot P. Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulating tumor cells. Am J Pathol. 2000;156:57–63.

Xu CF, Bing NX, Ball HA, Rajagopalan D, Sternberg CN, Hutson TE, De Souza P, Xue ZG, McCann L, King KS, Ragone LJ, Whittaker JC, Spraggs CF, Cardon LR, Mooser VE, Pandite LN. Pazopanib efficacy in renal cell carcinoma: evidence for predictive genetic markers in angiogenesis-related and exposure-related genes. J Clin Oncol. 2011;29:2557–64.

Xu CF, Johnson T, Garcia-Donas J, Choueiri TK, Sternberg CN, Davis ID, Bing N, Deen KC, Xue Z, McCann L, Esteban E, Whittaker JC, Spraggs CF, Rodriguez-Antona C, Pandite LN, Motzer RJ. IL8 polymorphisms and overall survival in pazopanib- or sunitinib-treated patients with renal cell carcinoma. Br J Cancer. 2015;112(Suppl):1190–8.

Yan L, Borregaard N, Kjeldsen L, Moses MA. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. J Biol Chem. 2001;276:37258–65.

Yang B, Gu W, Peng B, Xu Y, Liu M, Che J, Geng J, Zheng J. High level of circulating endothelial progenitor cells positively correlates with serum vascular endothelial growth factor in patients with renal cell carcinoma. J Urol. 2012;188:2055–61.

Yao M, Yoshida M, Kishida T, Nakaigawa N, Baba M, Kobayashi K, Miura T, Moriyama M, Nagashima Y, Nakatani Y, Kubota Y, Kondo K. VHL tumor suppressor gene alterations associated with good prognosis in sporadic clear-cell renal carcinoma. J Natl Cancer Inst. 2002;94:1569–75.

Yuen JS. Molecular targeted therapy in advanced renal cell carcinoma: a review of its recent past and a glimpse into the near future. Indian J Urol. 2009;25:427–36.

Zhao Z, Liao G, Li Y, Zhou S, Zou H, Fernando S. Prognostic value of carbonic anhydrase IX immunohistochemical expression in renal cell carcinoma: a meta-analysis of the literature. PLoS One. 2014;9:e114096.

M-Type Phospholipase A_2 Receptor as a Biomarker in Kidney Disease

46

Elion Hoxha and Rolf A.K. Stahl

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Abstract

The identification of the M-type phospholipase A_2 receptor as a major antigen in membranous nephropathy is leading to a paradigm shift in our understanding of the pathomechanisms involved in this disease as well as the clinical management of these patients. On the one side, the autoimmune nature of the disease, in which circulating autoantibodies bind to podocytic antigens, is confirmed, and on the other side, a whole new area of research on this condition has opened. Genome-wide association studies show an association of membranous nephropathy with PLA_2R and HLA loci, and major antibody-binding epitope regions have been identified on the PLA_2R. These findings might enable us in the future to better

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characterize the processes leading to the development of these antibodies, thus leading to new treatment options. At the same time, PLA_2R antibody findings are playing an increasing role in the differential diagnosis of primary from secondary membranous nephropathy and treatment management helping to better adapt therapy to the risk profile and disease activity of an individual patient and are a perfect example of how experimental research can lead to individualized medicine.

Keywords
Membranous nephropathy • PLA_2R antibodies • Differential diagnosis • Treatment • Prognosis

Abbreviations

ACTH	Adrenocorticotropic hormone
PLA_2R	M-type phospholipase A_2 receptor
THSD7A	Thrombospondin type-1 domain-containing 7A

Key Facts of Membranous Nephropathy

Primary membranous nephropathy is an autoimmune disease, caused by binding of circulating autoantibodies on antigen(s) on the podocytes.

The clinical course of primary membranous nephropathy is very variable ranging from spontaneous remission of disease in approximately one-third of patients to development of end-stage renal disease in another third of patients.

Considering the high rate of spontaneous remissions, the severe potential adverse effects of an immunosuppressive therapy, and the lack of prospective biomarkers to characterize the risk for disease progression, the decision which patients should be treated with immunosuppressants is challenging.

PLA_2R and, less frequently, THSD7A are target antigens in primary membranous nephropathy.

There is increasing evidence that PLA_2R antibody levels are reliable biomarkers in primary membranous nephropathy.

Definitions

Immune deposits Immune deposits can be detected by electron microscopy in the kidney tissue of patients with membranous nephropathy. They are localized in the subepithelial space and consist of antibodies against a target epitope, which is localized on the podocyte, antigen, and molecules of the complement system.

In situ immune-complex formation In situ immune-complex formation is presumably a key step leading to the development of membranous nephropathy. In this regard, in situ formation describes the binding of circulating antibodies to an

endogenous podocytic antigen; thus, the antibody-antigen complexes are formed directly at the subepithelial space, where they can be detected in renal biopsies.

Nephrotic syndrome Nephrotic syndrome is a kidney disorder characterized by loss of large amounts of protein (>3.5 g/1.73 m^2 body surface area per day) in the urine, low levels of serum albumin, high levels of cholesterol in the blood and edema. A nephrotic syndrome is caused by a number of diseases such as diabetic nephropathy, amyloidosis, or kidney-specific diseases such as membranous nephropathy, focal segmental glomerulosclerosis, minimal change disease, etc.

Spontaneous remission Spontaneous remission of proteinuria in patients with membranous nephropathy is a frequent event. A remission of proteinuria is usually defined as a decrease of proteinuria by at least 50 % to a level of <3.5 g per day (partial remission) or <0.3 g per day (complete remission). The remission of proteinuria is considered spontaneous when the patient did not receive any immunosuppressive medication, but was treated with supportive therapy only.

Subepithelial space Subepithelial space is the area between the glomerular basement membrane and the podocytes in the kidney.

Introduction

Membranous nephropathy is a common cause of nephrotic syndrome in adults (Ponticelli and Glassock 2014). At presentation, patients usually show a normal or only slightly impaired renal function. The clinical hallmark of the disease is the large proteinuria and the nephrotic syndrome (Ponticelli and Glassock 2014). Often patients also show an arterial hypertension. The diagnosis is made by renal biopsy (Fig. 1), which typically shows (a) no pronounced glomerular infiltration of inflammatory cells, while the glomerular capillary wall might appear thickened by light microscopy, (b) a granular positivity for immunoglobulins along the glomerular basement membrane by immunohistochemistry or immunofluorescence, and

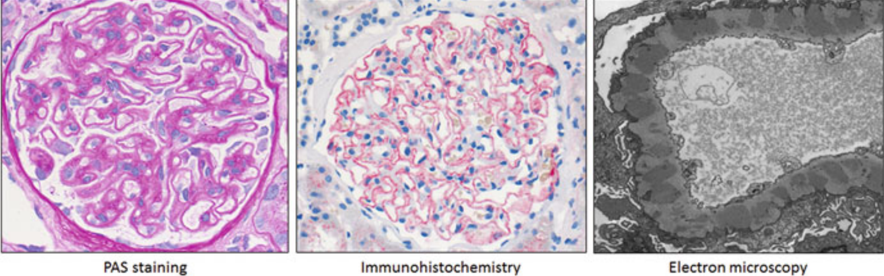

Fig. 1 PAS staining, immunohistochemical staining for IgG, and transmission electron microscopy of a renal biopsy from a patient with membranous nephropathy with kind permission of Prof. T. Wiech

(c) electron-dense immune deposits strictly on the epithelial side of the glomerular basement membrane. In most cases membranous nephropathy is an autoimmune disease restricted to the kidney, where autoantibodies against podocytic antigens lead to the formation of the subepithelial immune deposits (Glassock 2012). For the last 50 years, great efforts have been made to identify the podocytic antigen(s) and the corresponding autoantibodies responsible for the development of this disease, leading to the discovery of the M-type phospholipase A_2 receptor (PLA_2R) as a major antigen in membranous nephropathy (Beck et al. 2009).

Pathogenesis of Primary and Secondary Membranous Nephropathy

In some cases membranous nephropathy is clinically presented in the context of a systemic disease, e.g., systemic lupus erythematosus, malignant diseases, and chronic active viral infection (e.g., hepatitis B), or use of certain medications (e.g., nonsteroidal anti-inflammatory drugs, gold, penicillamine) (Glassock 2013). These cases make approximately 20 % of all cases of membranous nephropathy. In the absence of detailed knowledge on the pathophysiological processes leading to membranous nephropathy, all cases, in which no secondary cause of disease could be identified, were considered to be primary or idiopathic. For a long time, it has been postulated that immune processes play a central role in the development of primary membranous nephropathy. Most of the evidence derives from experimental work on the Heymann nephritis, a rat model of primary membranous nephropathy (Heymann et al. 1959). It was shown that circulating autoantibodies to antigens on the podocytes lead to an in-situ immune-complex formation, complement activation, and proteinuria (Beck and Salant 2014). While these findings were very important for more in-depth investigation and better understanding of the development of membranous nephropathy, the responsible podocytic antigen in Heymann nephritis, megalin, is not found in humans (Ronco and Debiec 2011). Thus, it was postulated that primary membranous nephropathy is an autoimmune disease, caused by binding of circulating autoantibodies on antigen(s) on the podocytes; however, the responsible antigen remained elusive for years to come.

First Antigens Discovered in Human Membranous Nephropathy

The first direct evidence that in situ immune-complex formation is responsible for development of primary membranous nephropathy in humans came in 2002 (Debiec et al. 2002). A newborn from a woman genetically deficient for neutral endopeptidase developed membranous nephropathy due to the fetomaternal alloimmunization of the mother to neutral endopeptidase in a previous pregnancy. Transplacental passage of the autoantibodies to neutral endopeptidase leads to the development of membranous nephropathy in the newborn. These cases of membranous nephropathy are very rare, and neutral endopeptidase was not found to be the target antigen in membranous nephropathy in adults. However, these findings were important,

showing that binding of circulating autoantibodies to a podocytic antigen leads to the development of membranous nephropathy (Debiec et al. 2002). Over the course of years, other mechanisms were attributed to the formation of immune deposits and development of membranous nephropathy. In children it was shown that circulating autoantibodies against bovine serum albumin bind to the antigen, which is natively not expressed on the podocytes, but rather "planted" in the first months or years of life (Debiec et al. 2011). These children develop membranous nephropathy, which usually resolves without need of specific treatment. This form of early-childhood membranous nephropathy has not been described in adults, and serum bovine albumin was not the target antigen in membranous nephropathy in adults. Some evidence exists that circulating immune complexes can lead to development of immune deposits; however, one would expect immune deposits deriving from circulating immune complexes to also be present in the mesangium and the endothelial side of the glomerular basement membrane, which is not the case in membranous nephropathy (Fujigaki et al. 1993). Thus, it is not known which role (if any) such processes might play in human membranous nephropathy.

Clinical Course and Treatment of Membranous Nephropathy

The clinical course of primary membranous nephropathy ranges from spontaneous remission of disease in approximately one-third of patients to development of end-stage renal disease in another third of patients (Schieppati et al. 1993). The remaining patients have a persisting proteinuria but a stable renal function. Patients with spontaneous remission have an excellent prognosis and don't need specific treatment. On the other side, patients who don't have a remission of disease have a very unfavorable prognosis (Polanco et al. 2010). Different immunosuppressants have been shown to be effective in patients with primary membranous nephropathy (Waldman and Austin III 2012). However, considering the high rate of spontaneous remissions and the severe potential adverse effects of an immunosuppressive therapy, the decision which patients should be treated is challenging. Different models have been proposed in order to define the patient's risk for disease progression (Branten et al. 2005; Cattran et al. 1997; van den Brand et al. 2012). Proteinuria and serum creatinine play a central role in these models; however, without knowledge on the pathophysiological and immunological processes leading to disease progress, no conclusive decision could be made which patients should receive immunosuppression. This is due to a number of factors: (i) spontaneous remission may take more than 1 year to happen, (ii) patients with very high proteinuria can still have a spontaneous remission, (iii) patients with low proteinuria may develop nephrotic proteinuria, or other factors, and there were no markers which would specifically differentiate these patients prospectively (Polanco et al. 2010; Hladunewich et al. 2009). Therefore, clinical management of patients with membranous nephropathy relied on short-term and midterm clinical observation, reacting to eventual disease progress (decrease of renal function), failure of disease recovery (persistence

of proteinuria), or severe uncontrollable symptoms (severe edema, not reacting to supportive treatment).

Treatment guidelines recommend a supportive treatment of all patients with primary membranous nephropathy for at least 6 months, consisting of inhibitors of the renin-angiotensin axis, diuretics, lipid-lowering drugs, and, depending on the individual thromboembolic risk of a patient, anticoagulants. Patients with no remission of proteinuria and decrease in renal function and who are at a high risk for disease progression should then be treated with immunosuppressive drugs (KDIGO 2012). Alkylating agents have been shown to be effective in inducing remission of proteinuria and preserve renal function (Hofstra and Wetzels 2010). For these agents the most long-term data on treatment of membranous nephropathy are available (Ponticelli et al. 1992, 1998; Jha et al. 2007). Cyclophosphamide seems to be as efficacious as chlorambucil, however, with fewer side effects (Ponticelli et al. 1998). Calcineurin inhibitors are also effective in inducing remission of proteinuria in patients with membranous nephropathy; however, high relapse rates upon cessation of treatment have been reported (Cattran et al. 2001, 1995; Praga et al. 2007; Caro et al. 2014). At the same time, use of alkylating agents might be associated with a better preserved renal function, especially in patients with impaired renal function (Howman et al. 2013). Rituximab has been shown to be efficacious in inducing remission of proteinuria; however, long-term data on renal function are still lacking (Ruggenenti et al. 2012; Fervenza et al. 2010). Most importantly, up to now, there are no randomized, controlled studies comparing the effectiveness and safety of rituximab to the other treatment options. The use of glucocorticoids alone is not recommended in patients with membranous nephropathy (Ponticelli et al. 1992). Other immunosuppressants such as mycophenolate mofetil, ACTH, etc., are also used for treatment of membranous nephropathy; however, there are not sufficient data on their long-term efficacy (Chan et al. 2007; Dussol et al. 2008; Ponticelli et al. 2006; Bomback et al. 2012).

The Discovery of PLA_2R Antibodies in Membranous Nephropathy

In 2009, a pivotal work from Beck et al. identified the PLA_2R as a major target antigen in patients with primary membranous nephropathy (Beck et al. 2009). Autoantibodies against PLA_2R are found in the blood of patients with primary membranous nephropathy (Fig. 2), but not in healthy controls, in patients with secondary membranous nephropathy or other glomerular diseases (Hoxha et al. 2011). PLA_2R antibodies were found to be detectable in patients with active disease or with a relapse; however, they were negative in patients with stable remission of disease (Hofstra et al. 2011). The fact that the PLA_2R antibodies co-localize with IgG4 in, and were eluted from the immune deposits of a kidney with membranous nephropathy, makes a direct pathogenetic role of these antibodies more plausible (Beck et al. 2009). This is further highlighted by the finding that a patient who was on dialysis because of membranous nephropathy had a very rapid relapse of membranous nephropathy after renal transplantation and was positive for PLA_2R antibodies in the serum (Stahl et al. 2010). The

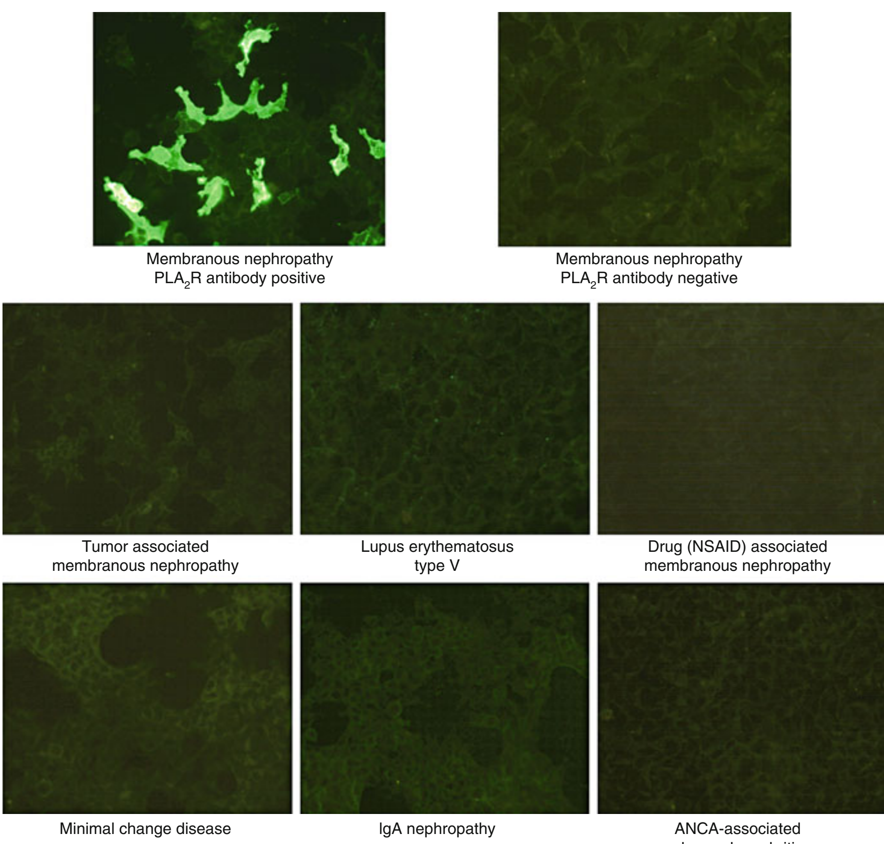

Fig. 2 Detection of circulating PLA_2R antibodies by an immunofluorescence test. Serum samples were diluted 1:10 prior to measurement. No PLA_2R antibodies can be detected in the serum of patients with secondary forms of membranous nephropathy or other glomerular diseases

formal pathogenesis of membranous nephropathy, positive for PLA_2R antibodies, however, still has to be verified in animal models. A major still unanswered question is how and why PLA_2R antibodies develop. Genome-wide association studies have identified several SNP mutations in the PLA_2R and HLA genes which were associated with the incidence of membranous nephropathy, PLA_2R antibody positivity, and enhanced PLA_2R staining of renal biopsy (Stanescu et al. 2011; Lv et al. 2013). Whether these genetic factors play a role or are associated with clinical parameters, disease progression or outcome remains to be seen. The description of major antigenic epitope sites on the PLA_2R from several groups might further help understand the pathogenesis of disease and perhaps lead to new prognostic markers and therapeutic targets (Fresquet et al. 2015; Kao et al. 2015).

Since 2009 different methods have been established to easily and reliably measure PLA_2R antibodies in the serum, thus allowing a number of studies to establish

the role of PLA_2R antibodies as biomarkers for diagnosis, disease activity, treatment success, and prognosis in membranous nephropathy (Hoxha et al. 2011; Dähnrich et al. 2013; Hofstra et al. 2012; Behnert et al. 2014).

The Role of PLA_2R Antibodies Differentiating Primary from Secondary Membranous Nephropathy

Many studies have reported on the role of PLA_2R antibodies to differentiate primary form secondary membranous nephropathy (Qin et al. 2011; Hoxha et al. 2011, 2012; Dähnrich et al. 2013). Since the pathogenesis, treatment, and prognosis of primary membranous nephropathy are different from that of secondary membranous nephropathy, differentiating the two forms of disease is very important. In primary membranous nephropathy, the dominant immunoglobulin in the immune deposits is usually IgG4, while in the secondary forms of disease, the other immunoglobulin subtypes are more dominant (Ohtani et al. 2004). However, neither the histological patterns nor the clinical findings can reliably differentiate primary from secondary membranous nephropathy, since the coexistence of membranous nephropathy and a potential secondary cause does not prove the secondary pathogenesis of membranous nephropathy, but might be coincidental. PLA_2R antibodies can be detected in the serum of approximately 70 % of patients with primary membranous nephropathy (Beck et al. 2009). Since PLA_2R antibodies can disappear from the blood (spontaneously or under immunosuppressive therapy), a negative PLA_2R antibody test does not rule out the possibility that the patient has PLA_2R-associated disease (Beck et al. 2011; Hoxha et al. 2014c). In these cases, histological finding on the PLA_2R expression in renal biopsy can be helpful. Patients with PLA_2R antibodies in the serum show a markedly enhanced glomerular PLA_2R expression (Fig. 3), which is not seen in renal tissue of patients with other glomerular diseases or with secondary

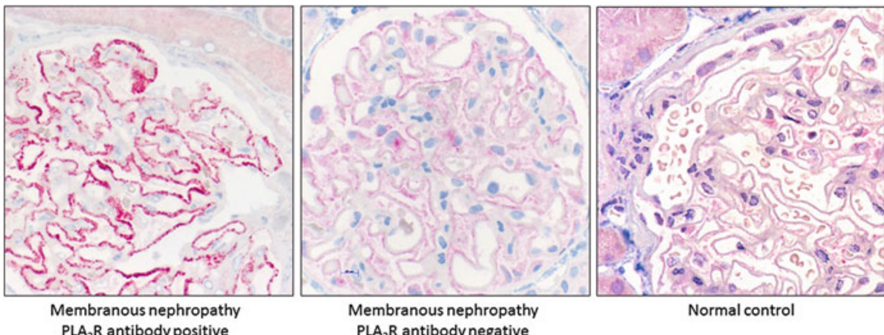

Fig. 3 Immunohistochemical staining of PLA_2R in renal biopsies from a patient with primary membranous nephropathy and detectable PLA_2R antibodies in the serum, a patient with secondary membranous nephropathy (PLA_2R antibody negative), and a normal kidney with kind permission of Prof. T. Wiech

membranous nephropathy (Hoxha et al. 2012; Svoboda et al. 2013). The enhanced staining for PLA_2R does not seem to be caused by an increase in the glomerular PLA_2R production (Hoxha et al. 2012). Future studies will show if these findings are due to conformational changes on the PLA_2R or increased PLA_2R deposition. Until then, one can only speculate on the mechanisms leading to these findings. An eventual conformational change on the PLA_2R could be induced by PLA_2R antibody binding, but it could also be a preexisting condition, leading to the development of PLA_2R antibodies. An increased PLA_2R deposition may be a result of podocytic antigen shedding and/or antigen-antibody captured in the subepithelial space.

Patients who are negative for PLA_2R antibodies in the serum, but have an enhanced PLA_2R staining in renal biopsy, might have an immunological remission of disease, or PLA_2R antibody levels in these patients are so low that the antibodies are captured in the kidney and elude detection in the blood (Debiec and Ronco 2011). Thus, the combination of PLA_2R antibody measurement in blood and PLA_2R staining of renal tissue can be used to differentiate primary from secondary membranous nephropathy.

It is important to note that there have been reports of patients with PLA_2R antibody-positive membranous nephropathy, in whom hepatitis B, lupus erythematosus, sarcoidosis, or malignancies have been diagnosed (Qin et al. 2011; Larsen et al. 2013; Knehtl et al. 2011). In these cases the two diseases might have coincidentally developed independently of each other, or by a yet unknown mechanism, the secondary cause might have led to the development of PLA_2R antibodies. While the former is more likely to be true, further research is needed to fully understand the development of membranous nephropathy in these cases. Of particular importance is the question on the incidence of malignancies in PLA_2R antibody-positive patients, whether it is lower than in PLA_2R antibody-negative patients or perhaps same as in the general population. This is of utmost importance, since membranous nephropathy characteristically develops in patients older than 50 years, who have a higher risk for developing malignancies.

THSD7A Is Another Target Antigen in Membranous Nephropathy

In a considerable number of patients who are negative for PLA_2R antibodies in the serum and show no enhanced staining for PLA_2R in renal biopsy, no secondary cause of membranous nephropathy can be found (Hoxha et al. 2012). There are a number of factors which may play a role in this finding: (i) these patients might have a secondary cause of membranous nephropathy, which could not be detected; (ii) antibodies against podocytic antigens other than the PLA_2R might lead to the development of membranous nephropathy. This was shown to be the case for thrombospondin type-1 domain-containing 7A (THSD7A), a podocytic membrane protein, against which antibodies are found in the blood of patients with membranous nephropathy (Tomas et al. 2014). THSD7A antibodies co-localize with IgG4 in glomeruli and were isolated from immune deposits of a kidney from a THSD7A antibody-positive patient with membranous nephropathy. THSD7A antibodies and

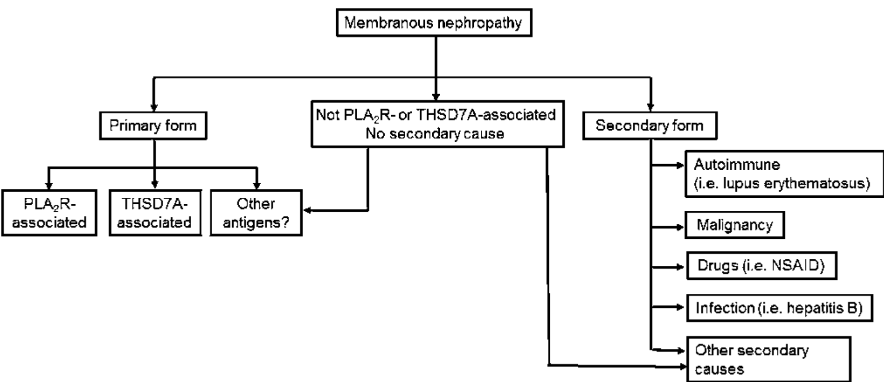

Fig. 4 In primary membranous nephropathy, PLA_2R and THSD7A have been identified as potential antigens. However, there are patients who are negative for PLA_2R antibodies and THSD7A antibodies in the serum, don't show an enhanced PLA_2R or THSD7A staining in the renal biopsy, and also don't have any known secondary cause of membranous nephropathy. In these patients, the search for further antigens or clinical causes that might lead to the development of membranous nephropathy continues

PLA_2R antibodies seem to be exclusive to each other, since until now no cases of THSD7A antibody-positive patients are reported, who are also positive for PLA_2R antibodies (Tomas et al. 2014). More work is needed to clinically characterize THSD7A antibody-positive patients and analyze if there are differences in the clinical characteristics of THSD7A antibody-positive from PLA_2R antibody-positive membranous nephropathy. Of particular importance for future research work is the understanding of the pathophysiological mechanisms leading to the development of membranous nephropathy in patients who are negative for PLA_2R and THSD7A but don't show any known secondary cause of disease (Fig. 4).

There have been other antibodies found in the serum of patients with membranous nephropathy; however, the characterization of the role of these antibodies for the development of membranous nephropathy is still lacking (Murtas et al. 2012). Further studies are needed to analyze whether these antibodies play a direct role in the pathogenesis of membranous nephropathy or they develop as a consequence of the podocytic damage upon formation of immune deposits by binding of PLA_2R antibodies on the podocytes and activation of the complement system.

The Role of PLA_2R Antibodies in the Clinical Management of Patients with Membranous Nephropathy

PLA_2R antibody levels in the serum are associated with the clinical activity of disease (Hofstra et al. 2011). This was shown for patients who had a spontaneous remission of disease, as well as for patients treated with immunosuppressants, where a decrease of PLA_2R antibody levels preceded a decrease in proteinuria

(Hoxha et al. 2014c). There have been conflicting data whether PLA_2R antibody levels and proteinuria correlate at a defined time point, with retrospective studies showing a correlation, which was not confirmed in prospective analyses (Hofstra et al. 2012; Hoxha et al. 2012, 2014c). The fact that changes in PLA_2R antibody levels precede changes in proteinuria by months is an explanation why no direct correlation of PLA_2R antibody levels and proteinuria at a defined time point was found (Hoxha et al. 2014c; Beck et al. 2011). At the same time, proteinuria depends not only on the immunological disease activity but also on other factors such as glomerular sclerosis, concomitant diseases (e.g., arterial hypertension, diabetes), etc. The precise relationship between PLA_2R antibody levels and proteinuria at a defined time remains to be verified in animal models of PLA_2R antibody-positive membranous nephropathy.

Upon initiation of an immunosuppressive treatment, PLA_2R antibody levels decrease more rapidly than proteinuria, perhaps because of the time glomerular reparatory processes need to effect proteinuria (Beck and Salant 2010; Hoxha et al. 2014c). Experimental work on animal models will further elucidate the mechanisms which may play a role in these processes. At the same time, patients with higher PLA_2R antibody levels at the time of diagnosis were less prone and needed longer time to achieve a remission of proteinuria (Hofstra et al. 2012, 2014c). Preliminary data indicate that the effect of different immunosuppressive drugs on the PLA_2R antibody levels is not different, suggesting that PLA_2R antibody levels may serve as a target for treatments and the efficacy of any immunosuppressive agent may depend on its capability to lower PLA_2R antibody levels (Hoxha et al. 2014c). However, randomized, controlled clinical trials are needed to prove which immunosuppressive drugs have the best efficacy and safety profile in PLA_2R antibody-positive membranous nephropathy. At the same time, experimental work on animal models will help us better understand how immunosuppressive drugs influence disease activity and if there is a direct podocytic effect, in addition to the immunomodulatory effect, of drugs such as cyclosporine A and rituximab in PLA_2R antibody-positive membranous nephropathy.

In patients with low proteinuria, high PLA_2R antibody levels are associated with the risk for developing a nephrotic syndrome (Hoxha et al. 2014b). If it can be confirmed that relapse of proteinuria in patients with membranous nephropathy is preceded by an increase in PLA_2R antibody levels or is associated with persistence of PLA_2R antibodies as suggested by a study (Bech et al. 2014), treatment of patients might be adapted accordingly. If the high relapse rate of proteinuria upon cessation of an immunosuppressive treatment is due to persisting PLA_2R antibodies in the blood, than another immunosuppressant, a higher treatment dosage or a longer treatment time might be recommended. In other patients, if PLA_2R antibodies disappear very quickly, some immunosuppressive therapy might be safely saved, thus resulting in less adverse events.

One of the most important findings was that patients with high PLA_2R antibody levels are at higher risk for progression of renal function impairment (Kanigicherla et al. 2013; Hoxha et al. 2014a). This finding might lead to further changes in the way treatment decisions are made in patients with membranous nephropathy. Future

clinical studies will shed more light into these findings. If they are confirmed, the therapeutic strategy of membranous nephropathy might profoundly change, and PLA_2R antibody levels might take a central role when deciding which patient should receive what treatment and when, leading to a more personalized treatment approach in this disease.

The discovery of the PLA_2R antibodies has led to a major burst in the clinical and experimental research of membranous nephropathy and has already substantially improved the diagnosis and the clinical management of patients with membranous nephropathy.

Potential Applications to Prognosis, Other Diseases, or Conditions

PLA_2R antibody levels are associated with disease activity in membranous nephropathy and may predict the prognosis of these patients. This is shown by the fact that higher PLA_2R antibody levels are associated with a lower chance to reach a remission of proteinuria and, perhaps, more importantly with the risk of renal function deterioration. Therefore, PLA_2R antibody levels could be helpful when making treatment decisions. Experimental animal models of PLA_2R-associated membranous nephropathy are needed to further analyze whether PLA_2R antibody levels lead to a worse prognosis in membranous nephropathy because of the prolonged high proteinuria in these patients or if higher PLA_2R antibody levels have a direct glomerular effect. When we better understand how reduction or disappearance of PLA_2R antibodies leads to remission of proteinuria and maintenance of renal function, treatment of membranous nephropathy might focus on these antibodies.

Summary Points

- The PLA_2R is the major target antigen in primary membranous nephropathy.
- The combination of PLA_2R antibody measurement in blood and PLA_2R staining of renal tissue can be used to differentiate primary from secondary membranous nephropathy.
- PLA_2R antibody levels in the serum are associated with the clinical activity of disease, and a decrease of PLA_2R antibody levels precedes a decrease in proteinuria.
- Patients with higher PLA_2R antibody levels at the time of diagnosis are less prone to have a remission of proteinuria and need longer time to achieve a remission of proteinuria.
- In patients with low proteinuria, high PLA_2R antibody levels are associated with the risk for developing a nephrotic syndrome.
- Patients with high PLA_2R antibody levels are at higher risk for progression of renal function impairment.

- PLA_2R antibody levels may serve as a target for treatments, and the efficacy of any immunosuppressive agent may depend on its capability to lower PLA_2R antibody levels.

References

Bech AP, Hofstra JM, Brenchley PE, Wetzels JFM. Association of anti-PLA2R antibodies with outcomes after immunosuppressive therapy in idiopathic membranous nephropathy. Clin J Am Soc Nephrol. 2014;9:1386–92.

Beck LH, Salant DJ. Membranous nephropathy: recent travels and new roads ahead. Kidney Int. 2010;77(9):765–70.

Beck LH, Salant DJ. Membranous nephropathy: from models to man. J Clin Invest. 2014;124 (6):2307–14.

Beck LH, Bonegio RG, Lambeau G, Beck DM, Powell DW, Cummins TD, Klein JB, Salant DJ. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med. 2009;361(1):11–21.

Beck LH, Fervenza FC, Beck DM, Bonegio RG, Malik FA, Erickson SB, Cosio FG, Cattran DC, Salant DJ. Rituximab-induced depletion of anti-PLA2R autoantibodies predicts response in membranous nephropathy. J Am Soc Nephrol. 2011;22(8):1543–50.

Behnert A, Schiffer M, Müller-Deile J, Beck LH, Mahler M, Fritzler MJ. Antiphospholipase A2 receptor autoantibodies: a comparison of three different immunoassays for the diagnosis of idiopathic membranous nephropathy. J Immunol Res. 2014. doi:10.1155/2014/143274.

Bomback AS, Canetta PA, Beck LH, Ayalon R, Radhakrishnan J, Appel GB. Treatment of resistant glomerular diseases with adrenocorticotropic hormone gel: a prospective trial. Am J Nephrol. 2012;36(1):58–67.

Branten AJ, du Buf-Vereijken PW, Klasen IS, Bosch FH, Feith GW, Hollander DA, Wetzels JF. Urinary excretion of beta2-microglobulin and IgG predict prognosis in idiopathic membranous nephropathy: a validation study. J Am Soc Nephrol. 2005;16(1):169–74.

Caro J, Gutiérrez-Solís E, Rojas-Rivera J, Agraz I, Ramos N, Rabasco C, Espinosa M, Valera A, Martín M, Frutos MA, Perea L, Juárez GF, Ocaña J, Arroyo D, Goicoechea M, Fernández L, Oliet A, Hernández Y, Romera A, Segarra A, Praga M, for the Grupo de Estudio de las Enfermedades Glomerulares de la Sociedad Española de Nefrología (GLOSEN). Predictors of response and relapse in patients with idiopathic membranous nephropathy treated with tacrolimus. Nephrol Dial Transplant. 2014;30(3):467–74.

Cattran DC, Greenwood C, Ritchie S, Bernstein K, Churchill DN, Clark WF, Morrin PA, Lavoie S. A controlled trial of cyclosporine in patients with progressive membranous nephropathy. Canadian Glomerulonephritis Study Group. Kidney Int. 1995;47(4):1130–5.

Cattran DC, Pei Y, Greenwood CM, Ponticelli C, Passerini P, Honkanen E. Validation of a predictive model of idiopathic membranous nephropathy: its clinical and research implications. Kidney Int. 1997;51(3):901–7.

Cattran DC, Appel GB, Hebert LA, Hunsicker LG, Pohl MA, Hoy WE, Maxwell DR, Kunis CL. Cyclosporine in patients with steroid-resistant membranous nephropathy: a randomized trial. Kidney Int. 2001;59(4):1484–90.

Chan TM, Lin AW, Tang SC, Qian JQ, Lam MF, Ho YW, Tse KC, Chan KW, Lai KN, Tang CS. Prospective controlled study on mycophenolate mofetil and prednisolone in the treatment of membranous nephropathy with nephrotic syndrome. Nephrology. 2007;12 (6):576–81.

Dähnrich C, Komorowski L, Probst C, Seitz-Polski B, Esnault V, Wetzels JF, Hofstra JM, Hoxha E, Stahl RA, Lambeau G, Stöcker W, Schlumberger W. Development of a standardized ELISA for the determination of autoantibodies against human M-type phospholipase A2 receptor in primary membranous nephropathy. Clin Chim Acta. 2013;421:213–8.

Debiec H, Ronco P. PLA2R autoantibodies and PLA2R glomerular deposits in membranous nephropathy. N Engl J Med. 2011;364(7):689–90.

Debiec H, Guigonis V, Mougenot B, Decobert F, Haymann JP, Bensman A, Deschênes G, Ronco PM. Antenatal membranous glomerulonephritis due to anti-neutral endopeptidase antibodies. N Engl J Med. 2002;346(26):2053–60.

Debiec H, Lefeu F, Kemper MJ, Niaudet P, Deschênes G, Remuzzi G, Ulinski T, Ronco P. Early-childhood membranous nephropathy due to cationic bovine serum albumin. N Engl J Med. 2011;364(22):2101–10.

Dussol B, Morange S, Burtey S, Indreies M, Cassuto E, Mourad G, Villar E, Pouteil-Noble C, Karaaslan H, Sichez H, Lasseur C, Delmas Y, Nogier MB, Fathallah M, Loundou A, Mayor V, Berland Y. Mycophenolate mofetil monotherapy in membranous nephropathy: a 1-year randomized controlled trial. Am J Kidney Dis. 2008;52(4):699–705.

Fervenza FC, Abraham RS, Erickson SB, Irazabal MV, Eirin A, Specks U, Nachman PH, Bergstralh EJ, Leung N, Cosio FG, Hogan MC, Dillon JJ, Hickson LJ, Li X, Cattran DC, Mayo Nephrology Collaborative Group. Rituximab therapy in idiopathic membranous nephropathy: a 2-year study. Clin J Am Soc Nephrol. 2010;5(12):2188–98.

Fresquet M, Jowitt TA, Gummadova J, Collins R, O'Cualain R, McKenzie EA, Lennon R, Brenchley PE. Identification of a major epitope recognized by PLA2R autoantibodies in primary membranous nephropathy. J Am Soc Nephrol. 2015;26(2):302–13.

Fujigaki Y, Nagase M, Honda N. Intraglomerular basement membrane translocation of immune complex (IC) in the development of passive in situ IC nephritis of rats. Am J Pathol. 1993;142 (3):831–42.

Glassock RJ. The pathogenesis of membranous nephropathy: evolution and revolution. Curr Opin Nephrol Hypertens. 2012;21(3):235–42.

Glassock RJ. Pathogenesis of membranous nephropathy: a new paradigm in evolution. Contrib Nephrol. 2013;181:131–42.

Heymann W, Hackel DB, Harwood S, Wilson SG, Hunter JL. Production of nephrotic syndrome in rats by Freund's adjuvants and rat kidney suspensions. Proc Soc Exp Biol Med. 1959;100:660–4.

Hladunewich MA, Troyanov S, Calafati J, Cattran DC, Metropolitan Toronto Glomerulonephritis Registry. The natural history of the non-nephrotic membranous nephropathy patient. Clin J Am Soc Nephrol. 2009;4(9):1417–22.

Hofstra JM, Wetzels JF. Alkylating agents in membranous nephropathy: efficacy proven beyond doubt. Nephrol Dial Transplant. 2010;25(6):1760–6.

Hofstra JM, Beck LH, Beck DM, Wetzels JF, Salant DJ. Anti-phospholipase A2 receptor antibodies correlate with clinical status in idiopathic membranous nephropathy. Clin J Am Soc Nephrol. 2011;6(6):1286–91.

Hofstra JM, Debiec H, Short CD, Pellé T, Kleta R, Mathieson PW, Ronco P, Brenchley PE, Wetzels JF. Antiphospholipase A2 receptor antibody titer and subclass in idiopathic membranous nephropathy. J Am Soc Nephrol. 2012;23(10):1735–43.

Howman A, Chapman TL, Langdon MM, Ferguson C, Adu D, Feehally J, Gaskin GJ, Jayne DR, O'Donoghue D, Boulton-Jones M, Mathieson PW. Immunosuppression for progressive membranous nephropathy: a UK randomised controlled trial. Lancet. 2013;381 (9868):744–51.

Hoxha E, Harendza S, Zahner G, Panzer U, Steinmetz O, Fechner K, Helmchen U, Stahl RA. An immunofluorescence test for phospholipase-A2-receptor antibodies and its clinical usefulness in patients with membranous glomerulonephritis. Nephrol Dial Transplant. 2011;26 (8):2526–32.

Hoxha E, Kneißler U, Stege G, Zahner G, Thiele I, Panzer U, Harendza S, Helmchen UM, Stahl RA. Enhanced expression of the M-type phospholipase A2 receptor in glomeruli correlates with serum receptor antibodies in primary membranous nephropathy. Kidney Int. 2012;82 (7):797–804.

Hoxha E, Harendza S, Pinnschmidt H, Panzer U, Stahl RA. M-type phospholipase A2 receptor autoantibodies and renal function in patients with primary membranous nephropathy. Clin J Am Soc Nephrol. 2014a;9(11):1883–90.

Hoxha E, Harendza S, Pinnschmidt H, Panzer U, Stahl RA. PLA2R antibody levels and clinical outcome in patients with membranous nephropathy and non-nephrotic range proteinuria under treatment with inhibitors of the renin-angiotensin system. PLoS One. 2014b;9(10):e110681. doi:10.1371/journal.pone.0110681.

Hoxha E, Thiele I, Zahner G, Panzer U, Harendza S, Stahl RA. Phospholipase A2 receptor autoantibodies and clinical outcome in patients with primary membranous nephropathy. J Am Soc Nephrol. 2014c;25(6):1357–66.

Jha V, Ganguli A, Saha TK, Kohli HS, Sud K, Gupta KL, Joshi K, Sakhuja V. A randomized, controlled trial of steroids and cyclophosphamide in adults with nephrotic syndrome caused by idiopathic membranous nephropathy. J Am Soc Nephrol. 2007;18(6):1899–904.

Kanigicherla D, Gummadova J, McKenzie EA, Roberts SA, Harris S, Nikam M, Poulton K, McWilliam L, Short CD, Venning M, Brenchley PE. Anti-PLA2R antibodies measured by ELISA predict long-term outcome in a prevalent population of patients with idiopathic membranous nephropathy. Kidney Int. 2013;83(5):940–8.

Kao L, Lam V, Waldman M, Glassock RJ, Zhu Q. Identification of the immunodominant epitope region in phospholipase A2 receptor-mediating autoantibody binding in idiopathic membranous nephropathy. J Am Soc Nephrol. 2015;26(2):291–301.

KDIGO (Kidney Disease: Improving Global Outcomes). Clinical practice guideline for glomerulonephritis. Kidney Int Suppl. 2012;2(2):186–98.

Knehtl M, Debiec H, Kamgang P, Callard P, Cadranel J, Ronco P, Boffa JJ. A case of phospholipase A2 receptor-positive membranous nephropathy preceding sarcoid-associated granulomatous tubulointerstitial nephritis. Am J Kidney Dis. 2011;57(1):140–3.

Larsen CP, Messias NC, Silva FG, Messias E, Walker PD. Determination of primary versus secondary membranous glomerulopathy utilizing phospholipase A2 receptor staining in renal biopsies. Mod Pathol. 2013;26(5):709–15.

Lv J, Hou W, Zhou X, Liu G, Zhou F, Zhao N, Hou P, Zhao M, Zhang H. Interaction between PLA2R1 and HLA-DQA1 variants associates with anti-PLA2R antibodies and membranous nephropathy. J Am Soc Nephrol. 2013;24(8):1323–9.

Murtas C, Bruschi M, Candiano G, Moroni G, Magistroni R, Magnano A, Bruno F, Radice A, Furci L, Argentiero L, Carnevali ML, Messa P, Scolari F, Sinico RA, Gesualdo L, Fervenza FC, Allegri L, Ravani P, Ghiggeri GM. Coexistence of different circulating anti-podocyte antibodies in membranous nephropathy. Clin J Am Soc Nephrol. 2012;7(9):1394–400.

Ohtani H, Wakui H, Komatsuda A, Okuyama S, Masai R, Maki N, Kigawa A, Sawada K, Imai H. Distribution of glomerular IgG subclass deposits in malignancy-associated membranous nephropathy. Nephrol Dial Transplant. 2004;19(3):574–9.

Polanco N, Gutiérrez E, Covarsí A, Ariza F, Carreño A, Vigil A, Baltar J, Fernández-Fresnedo G, Martín C, Pons S, Lorenzo D, Bernis C, Arrizabalaga P, Fernández-Juárez G, Barrio V, Sierra M, Castellanos I, Espinosa M, Rivera F, Oliet A, Fernández-Vega F, Praga M, Grupo de Estudio de las Enfermedades Glomerulares de la Sociedad Española de Nefrología. Spontaneous remission of nephrotic syndrome in idiopathic membranous nephropathy. J Am Soc Nephrol. 2010;21 (4):697–704.

Ponticelli C, Glassock RJ. Glomerular diseases: membranous nephropathy – a modern view. Clin J Am Soc Nephrol. 2014;9(3):609–16.

Ponticelli C, Zucchelli P, Passerini P, Cesana B. Methylprednisolone plus chlorambucil as compared with methylprednisolone alone for the treatment of idiopathic membranous nephropathy. N Engl J Med. 1992;327(9):599–603.

Ponticelli C, Altieri P, Scolari F, Passerini P, Roccatello D, Cesana B, Melis P, Valzorio B, Sasdelli M, Pasquali S, Pozzi C, Piccoli G, Lupo A, Segagni S, Antonucci F, Dugo M, Minari M, Scalia A, Pedrini L, Pisano G, Grassi C, Farina M, Bellazzi R. A randomized

study comparing methylprednisolone plus chlorambucil versus methylprednisolone plus cyclophosphamide in idiopathic membranous nephropathy. J Am Soc Nephrol. 1998;9(3):444–50.
Ponticelli C, Passerini P, Salvadori M, Manno C, Viola BF, Pasquali S, Mandolfo S, Messa P. A randomized pilot trial comparing methylprednisolone plus a cytotoxic agent versus synthetic adrenocorticotropic hormone in idiopathic membranous nephropathy. Am J Kidney Dis. 2006;47(2):233–40.
Praga M, Barrio V, Juárez GF, Luño J. Tacrolimus monotherapy in membranous nephropathy: a randomized controlled trial. Kidney Int. 2007;71(9):924–30.
Qin W, Beck LH, Zeng C, Chen Z, Li S, Zuo K, Salant DJ, Liu Z. Anti-phospholipase A2 receptor antibody in membranous nephropathy. J Am Soc Nephrol. 2011;22(6):1137–43.
Ronco P, Debiec H. Advances in membranous nephropathy: success stories of a long journey. Clin Exp Pharmacol Physiol. 2011;38(7):460–6.
Ruggenenti P, Cravedi P, Chianca A, Perna A, Ruggiero B, Gaspari F, Rambaldi A, Marasà M, Remuzzi G. Rituximab in idiopathic membranous nephropathy. J Am Soc Nephrol. 2012;23 (8):1416–25.
Schieppati A, Mosconi L, Perna A, Mecca G, Bertani T, Garattini S, Remuzzi G. Prognosis of untreated patients with idiopathic membranous nephropathy. N Engl J Med. 1993;329(2):85–9.
Stahl RA, Hoxha E, Fechner K. PLA2R autoantibodies and recurrent membranous nephropathy after transplantation. N Engl J Med. 2010;363(5):496–8.
Stanescu HC, Arcos-Burgos M, Medlar A, Bockenhauer D, Kottgen A, Dragomirescu L, Voinescu C, Patel N, Pearce K, Hubank M, Stephens HA, Laundy V, Padmanabhan S, Zawadzka A, Hofstra JM, Coenen MJ, den Heijer M, Kiemeney LA, Bacq-Daian D, Stengel B, Powis SH, Brenchley P, Feehally J, Rees AJ, Debiec H, Wetzels JF, Ronco P, Mathieson PW, Kleta R. Risk HLA-DQA1 and PLA(2)R1 alleles in idiopathic membranous nephropathy. N Engl J Med. 2011;364(7):616–26.
Svobodova B, Honsova E, Ronco P, Tesar V, Debiec H. Kidney biopsy is a sensitive tool for retrospective diagnosis of PLA2R-related membranous nephropathy. Nephrol Dial Transplant. 2013;28(7):1839–44.
Tomas NM, Beck LH, Meyer-Schwesinger C, Seitz-Polski B, Ma H, Zahner G, Dolla G, Hoxha E, Helmchen U, Dabert-Gay AS, Debayle D, Merchant M, Klein J, Salant DJ, Stahl RA, Lambeau G. Thrombospondin type-1 domain-containing 7A in idiopathic membranous nephropathy. N Engl J Med. 2014;371(24):2277–87.
van den Brand JA, Hofstra JM, Wetzels JF. Prognostic value of risk score and urinary markers in idiopathic membranous nephropathy. Clin J Am Soc Nephrol. 2012;7(8):1242–8.
Waldman M, Austin III HA. Treatment of idiopathic membranous nephropathy. J Am Soc Nephrol. 2012;23(10):1617–30.

Part V

Functional and Structural Variables

Ultrasound Elastography in Kidney Disease 47

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Abstract

Ultrasound elastography is a relatively new imaging modality that can measure tissue elasticity quantitatively by using different techniques. With the development of technology, kidney elasticity measurements can be performed accurately and quickly using ultrasound. Nowadays, kidney stiffness is used not only in fibrosis but also in the characterization of focal lesions, staging of metabolic kidney damage including diabetes mellitus, gout disease, etc.., and evaluating renal damage in urinary tract abnormality**.** However, kidney is a unique tissue and has a complex internal architecture. Therefore intrinsic or extrinsic factors such as perfusion, urinary pressure, anisotropy, depth of kidney, and hydronephrosis may affect the viscoelasticity of the kidney.

Elastography is a useful, quick, noninvasive complementary method in the diagnosis of kidney diseases but needs specific training and optimization techniques as well as acknowledging technical and pathological factors which may influence it.

Keywords

Elastography • Renal-acoustic radiation force imaging • Real-time elastography • Transient elastography-supersonic shear wave imaging • Renal transplant

Abbreviations

1D	One-dimensional
2D	Two-dimensional
3D	Three-dimensional
ARFI	Acoustic radiation force imaging
CDUS	Color Doppler ultrasonography
CKD	Chronic kidney disease
CT	Computed tomography
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
IF/TA	Interstitial fibrosis and tubular atrophy
kPa	Kilopascal
L-NAME	Nω-nitro-L-arginine methyl ester hydrochloride
MRI	Magnetic resonance imaging
ROI	Region-of-interest
RTE	Real-time elastography
SDUV	Shear wave dispersion ultrasound vibrometry
SSI	Supersonic shear wave imaging
SWV	Shear wave velocity
Tc-99m DMSA	Technetium-99m dimercaptosuccinic acid
TE	Transient elastography
US	Ultrasonography
VTI	Virtual touch imaging
VTQ	Virtual touch quantification
VUR	Vesicoureteral reflux
β2-MG	*β2*-microglobulin

Key Facts

Key Facts of Quasi-static Elastography

- The principle of compression-based elastography (quasi-static) is based on the relationship between differences in tissue deformability and its elastic properties.
- Mechanical pressure (transducer pressure exerted by operator, carotid pulses, breathing movements, muscular contractions) on a tissue produces a minor axial deformability in the hard tissue (incompressible) and high deformability in the soft areas (compressible).
- Quasi-static elastography software evaluates in real time this tissue deformability by evaluating the sound wave frequency alteration data before and after the application of a compressive force.
- The different stiffness of tissues is measured and represented as a color-scale image. Hard areas are represented as blue, soft areas as red, and firm areas as an intermediate consistency in green.
- Strain ratio, semiquantitative estimation of elasticity, compares a lesion's elasticity to elasticity of surrounding normal tissue, providing a numeric outcome by using elastograms.

Key Facts of Dynamic Elastography

- The methods are classified according to applied force.
- In ARFI imaging, short-duration acoustic "push pulses" generate shear waves perpendicular to the main ultrasound beam; detection pulses are generated simultaneously.
- The shear wave speed can be expressed as numeric values in m/s by VTQ in ARFI imaging.
- TE evaluates shear wave speed no forming an image because it uses a surface mechanical force rather than acoustic radiation force.
- In SSI, multiple focal spots are created in depth along a same longitudinal axis, generating a Mach cone acting as a shear wave source. The measurement of the velocity of this plane in each point of the image in real time provide a 2D quantitative elasticity map by using the ultrafast imaging technique.

Definitions

Elasticity Elasticity is the feature of objects to retrieve their original shape and size after the stress is stopped.

Strain Strain (ε) is the outcome of subjecting stress to an elastic object. It describes alteration in shape size of an elastic object as a result of stress. Longitudinal strain,

like compression, gives rise to change in length of an object. Shear strain gives rise to changes in angles of an object. Strain has no units.

Stress Stress (σ) is defined as power producing deformation (elongation, compression, torsion). Unit of stress is pascal or pounds per square inch (psi). (pascal = newton/m^2). Although both stress and pressure have same units, stress mostly can be a vector and is applied in a specified direction, whereas pressure effects equally in all directions.

Viscoelasticity Biological tissues exhibit both viscous and elastic properties. This means that, at low strain rates, because molecular structural "flow" can occur, there is a time delay between application of force and displacement. However, this does not have time to become at high strain rates. Therefore, for shear wave methods, the use of higher vibration frequencies will give rise to higher values of wave speed.

Viscosity Viscosity is the quantity of resistance of a fluid when it subjects shear stress or tensile (compression or stretching) stress.

Introduction

To date, renal imaging has primarily been based on morphologic evaluation of the kidney parenchyma, excretory system, and vascularity using ultrasonography, color Doppler ultrasonography (CDUS), computed tomography (CT), and magnetic resonance imaging (MRI). Moreover, functional parameters could easily be studied with MRI such as perfusion, filtration, and diffusion measurements. Since radiological evaluation of structural differences between normal parenchyma and pathological tissues remains a challenge, elastography has been accepted as an attractive technique firstly having been established in the liver (Bavu et al. 2011; Castera et al. 2008; Palmeri et al. 2011).

Elastography is a noninvasive imaging technique of stiffness or elasticity of the tissue via measuring movement or distortion of the tissue in response to a slight pressure (Singla et al. 2013). Although clinical practice of ultrasonography (US) elastography in liver tissue alters related to fibrosis or steatosis, yet little validation has been done to estimate its potential role in renal tissue changes (Grenier et al. 2013).

Progressive changes of extracellular matrix causing progression of fibrosis are associated with decreased functional alteration in kidneys, as in the liver. This process, called chronic kidney disease (CKD), is mostly encountered in developed countries, particularly depending on diabetes and hypertension-related nephropathies (El Nahas 2005). It leads to end-stage renal failure, with high morbidity and mortality and raised health costs. Most types of kidney diseases may progress to CKD, with progressive fibrotic process affecting first either glomeruli (glomerulosclerosis) or the interstitial space (interstitial fibrosis), depending on the early-stage nephropathy (Chatziantoniou et al. 2004; Ricardo et al. 2008). Likewise, the development of interstitial fibrosis and tubular atrophy (IF/TA), formerly named chronic

allograft nephropathy (Nankivell et al. 2003; Stegall et al. 2011), is the main reason of renal allograft failure in renal transplantation.

Renal biopsy is a valuable procedure for the diagnosis of renal disease, and it can help to guide treatment and state prognosis, especially with the evaluation of the degree of fibrosis or inflammation in the assessment of allograft rejection (Urban et al. 2013). However, the biopsy procedure can be complicated with small and large hematomas, gross hematuria, arteriovenous fistula, and even death (Maya and Allon 2009; Urban et al. 2013). Moreover, the biopsy is inherently prone to sampling errors due to the small volume of tissue extracted (Urban et al. 2013). Therefore, more efficient diagnostic strategies are emerging to evaluate the renal pathologies in vivo and noninvasively. These new approaches are expected to require defining and confirming adequate imaging biomarkers, with their intrinsic variability and interoperator reproducibility before clinical practice. Recently, diffusion-weighted MRI has been suggested to assess the parenchymal fibrosis via evaluating the restriction of water molecule motion (Togao et al. 2010; Warner et al. 2011) but it has no specificity for the fibrosis. Besides, US elastography is shown to be a promising technique in the evaluation of stiffness with first encouraging results of hepatic evaluation (Grenier et al. 2013). However, kidneys have a complex intrarenal architecture compared with liver, having two anatomical compartments, cortex and medulla, a high vascularity, and a function of urinary excretion.

The purpose of this chapter is to represent the main results, advantages, and limitations of US elastography technique in the evaluation of several kidney diseases and to present the new horizons of US elastography application in renal imaging.

US Elastography Techniques

The elastography techniques can be classified into two main categories: the quasi-static technique and the dynamic technique. These two categories do not obtain the same knowledge on mechanical parameters, so the difference between them is quite important. Although dynamic elastography yields a quantitative image or only one quantitative value of elasticity, quasi-static elastography yields a strain image of the investigated tissue and demonstrates qualitative knowledge in proportional to the elasticity (Grenier et al. 2013).

Quasi-Static Elastography

This is also called by manufacturers as “real-time elastography” (RTE) and is the first elastography technique, developed by Ophir et al. in the beginning of the 1990s (Ophir et al. 1991). It is an easily applicable and widespread technique (Grenier et al. 2013) based on a quasi-static distortion of a tissue due to a stress (σ). A stress (σ) induces a strain (ε), depending on the degree of tissue elasticity. The Young modulus E is identified by the ratio between the stress and the strain (Eq. 1):

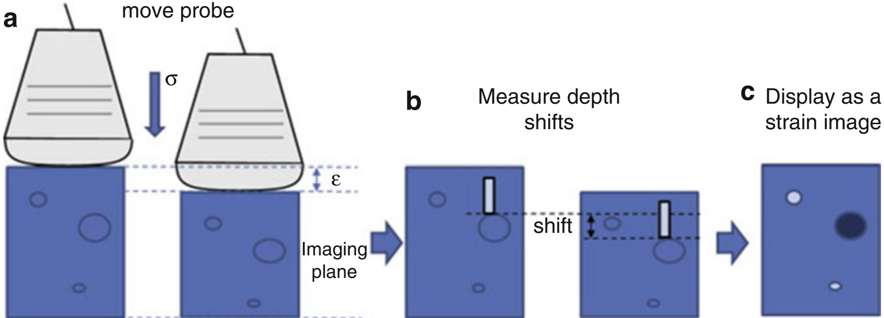

Fig. 1 Principle of quasi-static elastography. The probe is placed at the surface of the investigated organ and small compression is applied. Images are acquired before and after compression (**a**). By taking small windows within the image of raw US signals, a displacement shift in depth is recovered (**b**). Then, by calculating the derivative of the displacement, the final strain image is obtained and displayed (**c**) (From Grenier et al. 2013, with permission)

$$E = \sigma/\varepsilon \tag{1}$$

In this technique, a stress is applied to the tissue (through the ultrasonic probe itself) and observed by ultrasound imaging. The strain induced by the propagation of the stress is evaluated by comparing data before and after stress, and we estimate the displacement (Fig. 1). Though the stress (S) is not measurable, the resulting map does not display the Young modulus. This system provides parametric maps that differentiate rigid and soft tissues, and these maps may be in color and/or gray scale depending on the constructor.

In this technique, the tissue strain is reliant on the amount of applied compression. Therefore, this makes the technique operator-dependent. Although this technique is known as a qualitative imaging, nowadays semiquantitative measurement can be made with evaluation of strain ratio, which is the ratio of strain between the lesion and the adjacent normal tissue.

This technique seems useful for superficial organs, mainly in the evaluation of breast or thyroid, due to the limitation of operator-dependent strain (Grenier et al. 2013). Though it is accepted as non-applicable for renal pathologies because of the deep location of kidneys, a recent study has reported a quantitative evaluation of tissue elasticity from deep soft tissue which could be achievable with modified form of this technique (Baghani et al. 2012).

Dynamic Elastography

This technique is based on the propagation of mechanical or elastic waves in the body (Grenier et al. 2013). In a quasi-incompressible medium such as the human body consisted with 75 % of water, which is an incompressible material, the Young modulus is directly related to the speed (V_S) of a kind of elastic waves, the shear

waves (Grenier et al. 2013), and the tissue density (ρ) is assumed to be constant and equal to 1,000 kg m^{-3} (Eq. 2):

$$E = 3\rho V^2_S \tag{2}$$

Dynamic elastography can state a quantitative value of the Young modulus by estimating the speed of the shear waves by both generating a shear wave within the tissues using an external source (mechanical vibration and impulse or acoustic radiation force) and using one-dimensional (1D) or two-dimensional (2D) US to follow their propagation (Grenier et al. 2013).

Fibroscan Transient Elastography

It was originally called the "shear elasticity probe" and was the first quantitative elastography method available on the market (Grenier et al. 2013). It was manufactured at the Langevin Institute at the end of the 1990s (Sandrin et al. 2002, 2003). This system is based on a mechanical pulse made by a mechanical impulse or vibrating "punch" which generates a transient shear wave (pulse) that propagates longitudinally through the skin. The velocity and amplitude of the shear wave are calculated in a region-of-interest. The velocity (V) is converted into kilopascal (kPa), and it reflects the tissue stiffness. This is not an imaging-guided system and the probe is positioned randomly in the skin surface. The measurements are obtained at a depth between 20 and 60 mm (Grenier et al. 2013).

The Fibroscan transient elastography (TE) system is also inappropriate for the kidneys: (1) first, there is no B-mode control, and as the sample volume is fixed at a certain depth and size, it is extremely difficult and hazardous to position the sample volume on the renal cortical parenchyma, which is located at a variable depth (Grenier et al. 2013). A manual adjustment could be considered in the transplanted kidney, due to its superficial localization. Nevertheless it is hazardous without real-time US control, and this technique would require a variable amount of pressure applied on the probe, which would change the values of elasticity measurements (Grenier et al. 2013); (2) second, the mechanical wave should be applied on a rigid surface, such as the rib cage, to avoid compression effects of the probe, which is not able to occur for the kidney. Despite these limitations, Fibroscan transient elastography was used to evaluate the elasticity in three recent series of transplanted kidneys (Arndt et al. 2010; Sommerer et al. 2013; Lukenda et al. 2014). Sommerer et al. have reported that body mass index, skin-transplant distance, and perirenal or intrarenal fluid collection were important parameters of successful measurement of kidney stiffness by this technique. The same authors revealed that heterogeneous renal morphology and factors mentioned above may affect measurability of elasticity negatively. More technical modifications were needed to better assess the kidney.

ARFI = Acoustic Radiation Force Imaging

In this technique which was initially developed by Siemens (ARFI Virtual Touch™), estimation of stiffness of deep tissues can be ensured with short-term acoustic forces

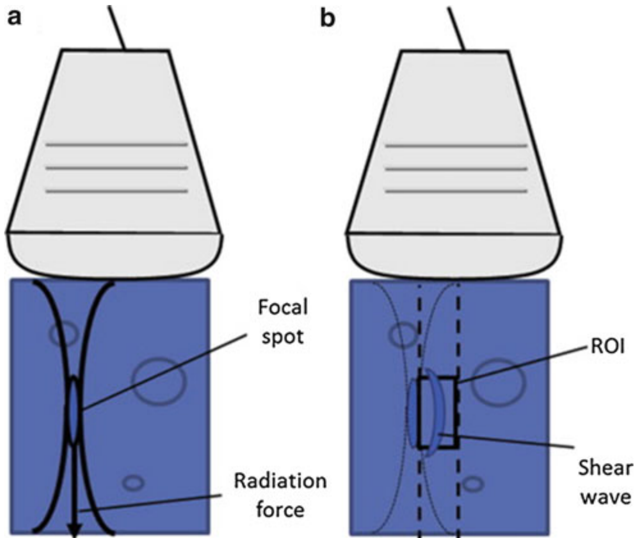

Fig. 2 Principle of acoustic radiation force imaging (*ARFI*). A radiation force is created from a focal spot within the organ by focusing US, creating a local shear wave source that is propagated perpendicularly to the US axis (**a**). On a few transducers close to the focal spot, the displacements of the shear wave are recovered, allowing a 1D measurement of shear elasticity in the region-of-interest (*ROI*) (**b**) (From Grenier et al. 2013, with permission)

named as pushing pulses. When USs are focused for a long time (hundreds of μs vs. 1–10 μs for classical US B-mode), a force, known as US radiation pressure, is generated at the focal spot, and this acoustic force is used to deform the tissue (Grenier et al. 2013). Tissue comes back to the original position after the force application and meanwhile generates shear waves which spread away from the focal excitation point as a local vibration (Singla et al. 2013). The velocity of the shear waves depends on the tissue stiffness and is quantitatively measured by software in an evaluated area and expressed as m/s (Virtual Touch Quantification, Siemens Healthcare, Mountain View, CA). The Virtual Touch Quantification (VTQ) is a noninvasive and painless examination and can obtain multiple measurements on a small box located at the chosen depth within organs. Though US devices use 1D technique for the elasticity measurement, 2D US image (B-mode) is preferred to position the estimation window at the needed location within the organ (Grenier et al. 2013) (Fig. 2).

The inability of this technique results from underestimation of tissues at a depth of more than 8–10 cm due to weakening of the radiation forces at greater depths (Singla et al. 2013).

Supersonic Shear Wave Imaging

Acoustic radiation force (US radiation pressure) generating a Mach cone, and US ultrafast imaging are the two basic models of Supersonic shear wave imaging (SSI)

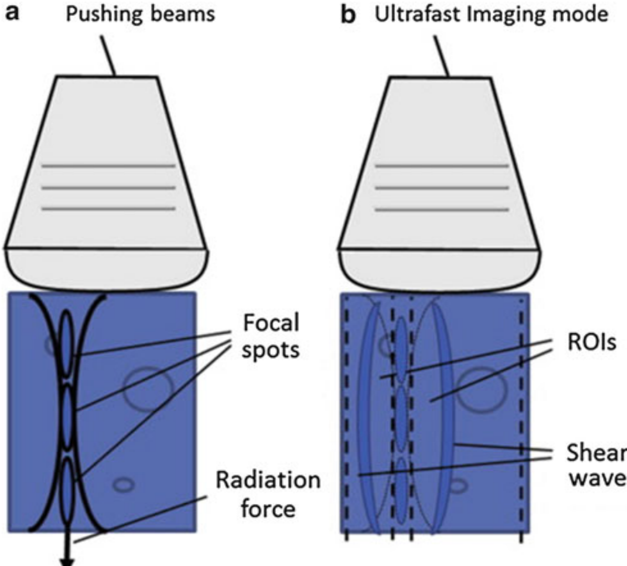

Fig. 3 Principle of supersonic shear wave imaging (*SSI*). Multiple focal spots are created in depth along the same longitudinal axis, generating a Mach cone acting as a shear wave source (**a**). By using an ultrafast imaging mode, the propagating shear wave is caught in the entire field of imaging, allowing the reconstruction of a 2D elasticity map (**b**) (From Grenier et al. 2013, with permission)

technique (Bercoff et al. 2004; Grenier et al. 2013). Various repeated focalized US spots at different depths (typically four to five spots) are used to evaluate a wide area at the same time (Grenier et al. 2013). Multiply located shear waves form a conical shear wave front like a Mach cone of an aircraft with supersonic speed (Singla et al. 2013) (Fig. 3). An ultrafast scanner with 5,000 frames per second capability is needed for the imaging of this shear wave propagation (Singla et al. 2013). This ultrafast scanner allows the formation of real-time images by achieving the entire shear wave field in less than 30 ms, and fast average processing of acquired images to increase robustness without stroboscoping the acquisition several times to obtain a whole shear wave field (Grenier et al. 2013). In conclusion, a real-time dimensional quantitative mapping is obtained, and low-frequency curved arrays are used for native and deep transplanted kidneys and high-frequency linear arrays for superficial transplanted kidneys for examination with SSI technique (Grenier et al. 2013).

The main limitation of this technique is certain depth of tissue is required for shear wave penetration. Therefore evaluation of superficial structures can be difficult.

Shear Wave Dispersion Ultrasound Vibrometry

Shear wave dispersion ultrasound vibrometry (SDUV) has a focused ultrasound beam to form harmonic shear waves or impulse multi-frequency shear waves that spread out from the central vibration point (Amador et al. 2011). Dispersion of shear

wave is a feature that means the measured speed of shear waves vary depending on the frequency in a viscoelastic medium and may specify viscoelastic properties of the tissue quantitatively.

Viscoelasticity of the Kidney

In recent years, several previous studies have reported on the usefulness of elastographic methods focusing on the elastic properties of the tissue, not the viscosity (Scola et al. 2012). However, it has been claimed that if viscosity is not regarded, measurements of stiffness can be biased, and the results may be higher than their real values (Amador et al. 2011).

The kidney can be modeled as a viscoelastic material due to its both elastic and viscous physical characteristics (Amador et al. 2011). For an accurate diagnosis, single evaluation of shear elasticity is not enough for a correct diagnosis despite the correlation of stiffness with pathology (Deffieux et al. 2009). Therefore it is essential to measure some mechanical parameters such as shear viscosity for accurate characterization of the kidney. SSI with specialized hardware can form maps of stiffness and viscosity as distinct from TE and MR elastography that only evaluate the elastic part, not the viscous part of the tissue response (Amador et al. 2011).

SDUV uses an intermittent pulse sequence suitable with ultrasound scanners on the market and can measure the tissue viscosity and elasticity quantitatively (Amador et al. 2011).

Influence of Renal Characteristics on Elasticity Values

Tissue stiffness is characterized by its acquired intrinsic mechanical characteristics (e.g., fibrosis) and hemodynamic factors of turgor (Korsmo et al. 2013).

The sensitivity of the elastography to mechanical parameters causes the measurement of renal shear wave velocities to be made with caution (Grenier et al. 2013). There are some intrinsic factors of kidneys such as anisotropy of renal architecture, compressibility of renal transplants, high degree of renal vascularity, and possible increase of urinary pressure. It is important to assess the variation of these parameters to increase the reproducibility of the technique and to decrease the intrinsic variability of the measurements (Grenier et al. 2013).

A manual compression by the US probe may be possible during scanning of renal transplants which are more superficially located in the iliac fossa whereas native kidneys are bedded quite deeply and hardly compressible. The same phenomenon is valid for elastographic evaluation of the liver parenchyma with higher values on the left lobe obtained using a compliant epigastric window, than on the right lobe, using a rigid intercostal window. Syversveen et al. (2012) evaluated the effect of probe compression on elasticity values of transplanted kidneys, and they calibrated the compression forces

with ARFI on the renal cortex of 31 kidney transplants by a mechanical device. They claimed that there were highly significant differences in mean shear wave velocity between five different compression levels (Syversveen et al. 2012).

In the kidney Henle's loops and vasa recta within medulla and the collecting ducts within cortex and medulla are parallel and directed from the capsule to the papilla within each renal segment. Therefore, the intrinsic architecture of the renal parenchyma is accepted as anisotropic, and (Grenier et al. 2013) the degree of anisotropy has been assessed to be around 15 % in the cortex and 30 % in the medulla with MR evaluation (Ries et al. 2001). It has been claimed that when emission of the US beam is sent parallel to renal microstructures, the shear wave propagates perpendicular to these, and this causes multiple vascular and tubular interfaces, decreases the speed of shear wave propagation, and results in lower elasticity values (Grenier et al. 2013). Conversely, when emission of the US beam is sent perpendicular to these structures, higher elasticity values are obtained (Grenier et al. 2013) (Fig. 4). It has been also claimed that the mean variation of the Young modulus was 10.5 % in the outer cortex, 29.7 % in the inner cortex, and 31.8 % in the medulla due to anisotropy in normal conditions (Gennisson et al. 2012). Therefore, a clear identification of sampled renal segments and their orientation according to the US beam are mandatory when performing renal US elastography, and it is important to position the main axis of US probe as parallel as possible to the main axis of pyramids to reduce the effect of anisotropy in order to quantify the shear wave velocity.

The kidney is a highly vascularized organ, receiving approximately 20 % of cardiac output, and this vascularity may significantly affect in vivo elasticity measurements (Warner et al. 2011; Asano et al. 2014). It has been reported that reduction of blood flow may affect shear wave velocity values more than tissue fibrosis in patients with CKD (Asano et al. 2014). It has been shown with MR elastography that cortical and medullary stiffness gradually decreased ~30 % and ~20 %, respectively, during reduction in renal blood flow (Warner et al. 2011). Similarly, a significant decrease in elasticity affecting mainly cortex was described after ligation of the renal artery (Gennisson et al. 2012). Medullary stiffness is less affected from the alterations in renal perfusion since medullary blood flow has less dependence on hydrostatic pressure and renal vascular resistance, contributing around 90 % of RBF, exists generally in cortical microvessels (Warner et al. 2011). Conversely, a giant increase in renal elasticity was observed after ligation of the renal vein in an animal study (Gennisson et al. 2012).

Urinary obstruction, especially acute and complete forms, may increase the intrarenal pressure. Gennisson et al.'s pig study defined that elasticity values were linearly affected with the severity of urinary obstruction (Gennisson et al. 2012). Consequently, before remarking increased elasticity measurements, urinary obstruction will have to be ruled out, and especially in renal transplant patients, it should be noted to the degree of bladder filling due to the shortness of the ureter and its denervation; eventually a filled bladder may affect the pyelocaliceal system (Grenier et al. 2013).

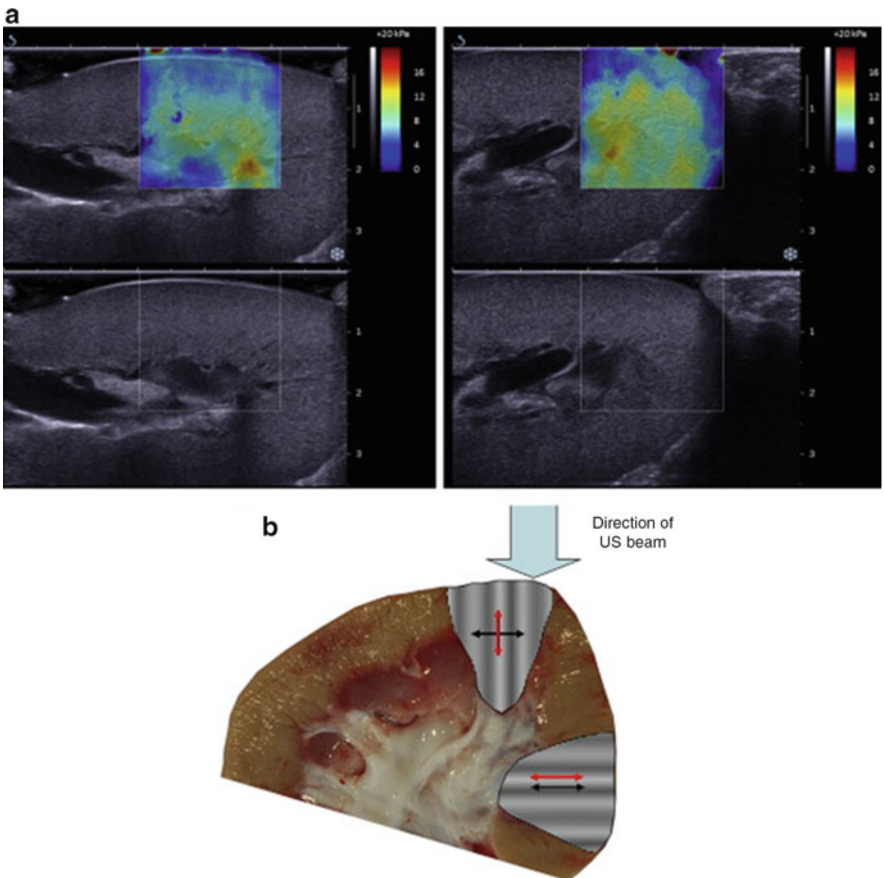

Fig. 4 Effect of anisotropy. Elasticity map of a pig kidney showing higher values in the lower pole than on the anterior aspect (**a**). Macroscopic image of a lower half of a pig kidney showing the corticomedullary differentiation and, superimposed, schematic representation of 2 renal segments: one shows a predominant vertical anisotropy in the direction of US beam and the other a horizontal anisotropy perpendicular to the direction of US beam (*red arrows* illustrate the direction of anisotropy). Axis of propagation of the shear wave (*black arrows*) is perpendicular to the oriented renal structures in the first case and parallel in the second (**b**) (From Grenier et al. 2013, with permission)

Normal Renal Elasticity Values and Reproducibility

It is important to know "normal" kidney elasticity before interpretation of the results obtained from patients with kidney pathology, and there are many factors that influence the renal shear wave speed: age, gender, overweight/obesity, and measurement depth (Bota et al. 2014). In the first study with SSI technique (Arda et al. 2011), normal cortical elasticity values were defined as 5.2 ± 2.9 (1–13) kPa and 4.9 ± 2.9

(1–26) kPa in men and women, respectively. They stated that there were no significant difference in elasticity measurements between sexes and no significant positive or negative correlation between age and elasticity of the renal cortex.

In a study by Guo et al. (2013), a negative correlation between shear wave velocity (SWV) of renal parenchyma evaluated by ARFI and age and significantly different SWV values between men and women (2.0660.48 m/s vs. 2.260.52 m/s, respectively) was declared in contrast to Arda et al.'s study (Grenier et al. 2013).

Bota et al. also reported that kidney shear wave speed values measured by ARFI elastography were decreasing with age, and values were significantly lower in men than in women in patients between 31–50 and 51–65 years of age (Bota et al. 2014).

It was previously reported that mean SWVs in normal adult kidneys were 2.24–2.37 m/s, with no significant difference between the right kidney and the left kidney (Goertz et al. 2011; Sohn et al. 2014).

In a study evaluating age-related changes in kidney stiffness in healthy children by ARFI technique with an average age of 8.1 ± 4.7 years (Lee et al. 2013), it was stated that the mean SWVs were 2.19 m/s for the right kidney and 2.33 m/s for the left kidney. It has been declared that SWV of kidneys changed with aging in pediatric population with the most notable increase less than 5 years of age (Lee et al. 2013).

Interobserver variability has been accepted as a major limitation of elastography. Though some authors claimed that elastography was an operator-dependent exam and higher intra- and interobserver variabilities were stated as inevitable (Yoon et al. 2011; Ozkan et al. 2013), many other studies reported higher interobserver agreement especially in RTE (Merino et al. 2011; Orlacchio et al. 2014). These contradictory results may be due to the different elastography techniques and softwares preferred.

Different results were reported in interobserver agreement of transient elastography, ARFI, and SSI techniques. Although previous studies reported low reproducibility of all these techniques, recent studies reported good reproducibility especially in RTE and ARFI techniques by using extra dedicated software or gained experience.

Measurement of Renal Fibrosis

Increased extracellular matrix synthesis, with excessive fibrillary collagens in the glomerular, interstitial, and vascular compartments, may progressively cause the end-stage renal failure (El Nahas 2005; Grenier et al. 2013). These pathophysiological pathways have been well investigated and numerous therapeutic interventions should be made to prevent or favor regression of fibrosis in experimental models (Chatziantoniou et al. 2004; Boffa et al. 2003). Therefore, improvement of new noninvasive methods for definition and quantification of fibrosis is emerging.

Preclinical Studies

The first study evaluating US elastography in an experimental model reported the influence of renal anisotropy and vascular and urinary pressure on elasticity values in six pig kidneys by ultrasonic supersonic shear wave elastography (Gennisson et al. 2012). Second study used SSI to detect kidney cortex elasticity changes and tried to predict histopathological development of fibrosis in a rat model of glomerulosclerosis induced by Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) administration (Derieppe et al. 2012). It was shown that increased cortical elasticity values were correlated with the degree of renal dysfunction, but no correlation was found between the global histological score and the severity of cortical stiffness (Derieppe et al. 2012).

Evaluation of Renal Transplants

The natural history of IF/TA in transplanted kidneys has been well studied through the protocol biopsies. The early phase, which generally occurs during the first years of post-transplantation, is characterized by fibrogenesis and the occurrence of tubulointerstitial damage due to immunologic phenomena; the late phase is characterized by the worsening of parenchymal lesions (irreversible IF, TA, arteriolar hyalinosis) and glomerular sclerosis leading to graft lost (Nankivell et al. 2003; Stegall et al. 2011). Protocol biopsies are the only reliable tool for diagnosis of IF/TA due to lacking of noninvasive markers of abovementioned pathological changes. However, the role of renal biopsy protocol is controversial in renal transplantation due to its invasive nature and highly cost (Orlacchio et al. 2014). Recently, ultrasonographic elasticity imaging has been emerging to evaluate the viscoelastic properties of renal tissue in transplantation patients (Urban et al. 2013; Amador et al. 2012) (Fig. 5). There are several studies about elasticity properties of renal transplants reporting controversial information using different elastographic systems (Table 1).

Arndt et al. (2010) found a high positive correlation between renal stiffness and the degree of internal fibrosis in renal transplants by using transient elastography (Fibroscan). Sommerer et al. have measured significant higher renal stiffness in renal transplants with histologically verified advanced fibrosis on a large cohort of transplanted patients by transient elastography (Sommerer et al. 2013). They have declared that the sensitivity and specificity of transient elastography detecting renal allograft fibrosis were 54 % and 73 %, respectively. Stock et al. (2011) stated that an average increase of shear wave velocities of ARFI technique was seen in 15 % of transplants with pathologically proven acute rejection. However, Syversveen et al. (2011) reported that there was no correlation between shear wave velocities and the presence and/or severity of fibrosis by TE technique. Both TE and ARFI techniques were used to detect the tissue fibrosis, but they were not directly comparable because of their dissimilar technologies (although ARFI used short duration, high-intensity acoustic pulses to produce shear waves, TE used vibrations)

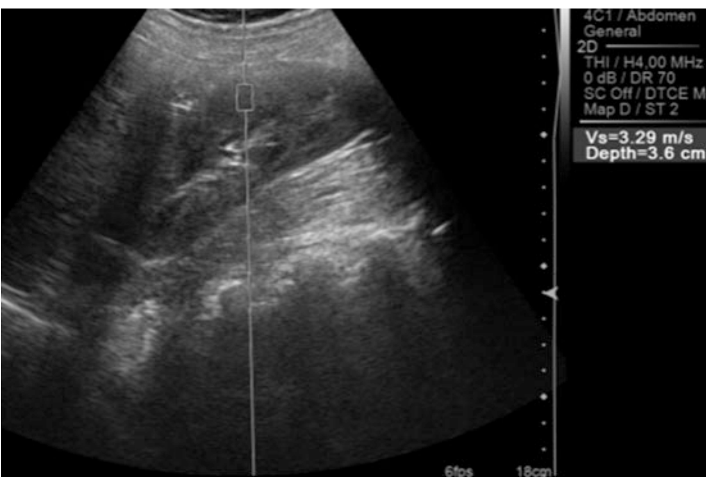

Fig. 5 Virtual Touch Quantification (*VTQ*) measurement of renal allograft rejection. Acoustic radiation force impulse quantification measurement in the 47-year-old man transplanted kidney is performed. Shear wave velocity = 3.29 m/s shows renal allograft rejection

Table 1 Several studies performed on renal transplants with different elastography techniques

Authors	Technique	Results
Arndt et al. (2010)	TE	Correlation between stiffness and fibrosis (Pearson r=0.67; p=0.002; R^2=0.45)
		Stiffness difference according to Banff score
Syversveen et al. (2011)	ARFI	No significant difference in stiffness according to Banff score
Stock et al. (2011)	ARFI	Moderate correlation between stiffness and fibrosis (r = 0.468, p = 0.026)
Grenier et al. (2012)	SSI	No correlation (excepted with total Banff score)
Orlacchio et al. (2014)	RTE	High correlation between stiffness and fibrosis according to Banff score ($r = -0.52$, $p < 0.05$)
Gao et al. (2013)	RTE	Strong correlation between stiffness and fibrosis according to Banff score ($p < 0.05$)
Lukenda et al. (2014)	TE	High correlation between stiffness and fibrosis according to Banff score ($r = 0.727$; $p = 0.0001$)
Cui et al. (2014)	ARFI	Significant difference between the stiffness values of the nonfibrosis group and mild-moderate fibrosis group ($p < 0.01$)

(Orlacchio et al. 2014). TE is a quasi-static elastography method, and its measurements are qualitative or semiquantitative.

Gao et al. have introduced a novel technique for assessing the severity of cortical fibrosis in renal transplants by applying the corticomedullary strain ratio as a quantitative

marker (Gao et al. 2013). In this technique, two-dimensional speckle-tracking software was used to make offline analysis of cortical and medullary strain produced by external compression by the ultrasound probe (Gao et al. 2013). Then, corticomedullary strain ratio (cortical normalized strain/medullary normalized strain; normalized strain = developed strain/applied strain [deformation from the abdominal wall to the pelvic muscles]) was calculated (Gao et al. 2013). They concluded that measuring the renal strain by a strong speckle-tracking technique, analyzing the internal tissue deformation stimulated by external compression, might have advantages over other methods based on shear wave velocity (Gao et al. 2013).

Grenier et al. evaluated 49 consecutive kidney transplant recipients by using SSI scheduled for renal biopsy (Grenier et al. 2012), and none of each individual score of the semiquantitative Banff classification was correlated with the measurement of cortical stiffness. Moreover, cortical stiffness was correlated with neither the level of IF measured by quantitative image analysis nor the scoring and grading of IF/TA. These results may be due to impact of other parameters on parenchymal stiffness besides the IF. Nevertheless, in a study measuring viscoelastic properties of renal transplants with varied biopsy scores by using some post-processing software applications (Urban et al. 2013), the measurements of shear modulus in transplanted kidneys were found to be positively correlated with increased Banff scores. Though the measurement of viscoelasticity in renal transplants may be feasible, it is needed to be studied in large cohorts to evaluate its prognostic and diagnostic value (Urban et al. 2013).

Evaluation of Renal Mass

There are only a few studies investigating the renal masses. The first study about this topic reported a series of 15 cases (2 pseudotumors, 2 hemorrhagic cysts, 8 renal cell carcinomas, 1 chromophobe cell carcinoma, 2 tubulo-papillary carcinomas), by ARFI technique, and the elastographic values of renal masses were between 1.61 and 3.97 m/s, without distinction among different types (Clevert et al. 2009). The second study found a difference between the ratios of angiomyolipomas to adjacent parenchyma and renal cell carcinoma to adjacent parenchyma by using strain imaging (Tan et al. 2013). The third study evaluated shear wave velocities of the tumors by ARFI quantification, and the authors claimed that there were differences in the shear wave velocities between the SWV values of benign renal lesions including hematomas and malignant renal lesions (Goya et al. 2014a) (Figs. 6, 7, 8, and 9). More experience is necessary to evaluate the potential role of elastography in separating benign and malignant tumors.

Elastographic Properties of End-Stage Renal Disease

CKD is an important health problem in developed countries, particularly in the context of diabetes and hypertension-related nephropathies. It is important to diagnose the disease in early phase, because the earlier treatment is efficient in slowing

Fig. 6 Virtual Touch Quantification (*VTQ*) measurement of renal angiomyolipoma**.** VTQ measurement of a renal angiomyolipoma in anterior upper pole of the left kidney of a 47-year-old male patient

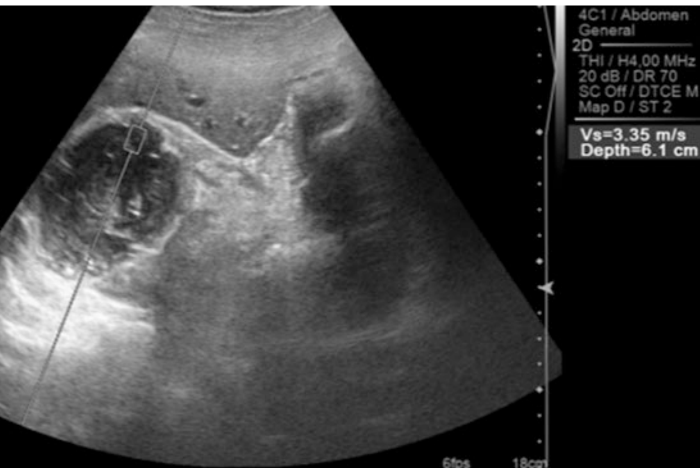

Fig. 7 Virtual Touch Quantification (*VTQ*) measurement of papillary renal cell carcinoma. VTQ measurement of a histopathologically confirmed papillary renal cell carcinoma in the upper pole of the right kidney of a 51-year-old female patient

the progression to kidney failure irrespective of the reason (K/DOQI clinical practice guidelines 2002). However, conventional signs of CKD, such as serum creatinine, proteinuria, and urea nitrogen, are insensible and may cause loss of time to diagnose (Guo et al. 2013; Levey et al. 2009). Guo et al. have used ARFI elastography to determine changes in elasticity in chronic renal disease, and they found that the

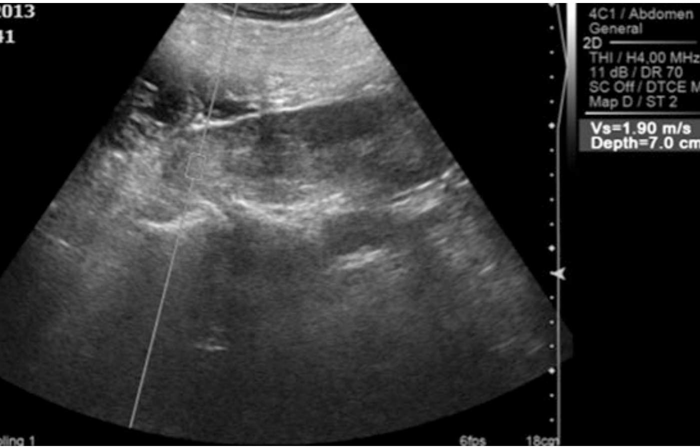

Fig. 8 Virtual Touch Quantification (*VTQ*) measurement of hydatid cyst. VTQ measurement of a histopathologically confirmed hydatid cyst in the upper pole of the right kidney of a 32-year-old male patient

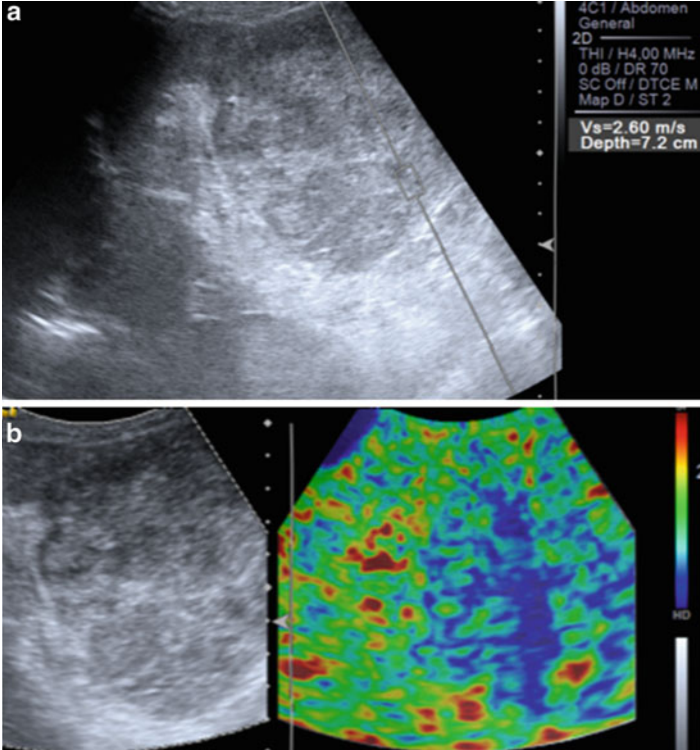

Fig. 9 Wilms' tumor. VTQ measurement (**a**) and eSie Touch Elasticity Imaging (**b**) of a Wilms' tumor in a 5-year-old male patient

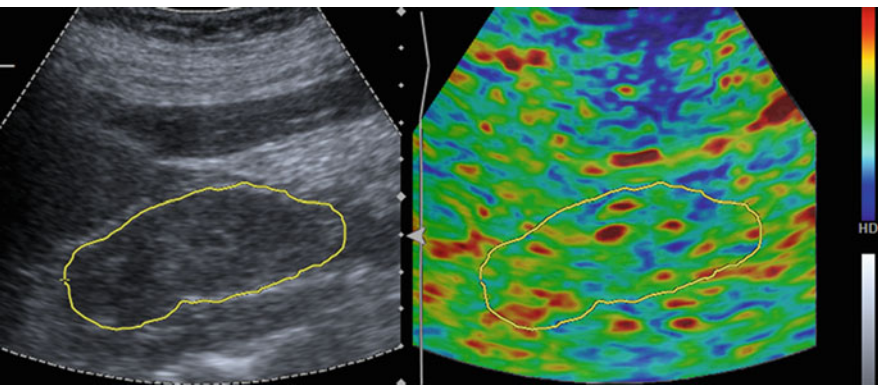

Fig. 10 eSie Touch Elasticity Imaging of the chronic kidney disease. Acoustic radiation force impulse (*ARFI*) imaging in a 45-year-old man with chronic kidney disease due to hypertension shows decreased stiffness (*yellow-green-red* areas) in atrophic kidney

SWV was significantly higher in control group than each stage of CKD patients (Fig. 10).

However, Wang et al. stated that SWV did not correlate with any pathological marks of fibrosis, and ARFI could not predict the dissimilar stages of CKD in a study evaluating ARFI technique in CKD with common etiology of IgA nephropathy (Wang et al. 2014).

The contradictory results of these studies may arise from complex intrarenal architecture that affects the viscoelasticity of the kidney. Further studies are warranted to elucidate the sources of the variability.

Metabolic Diseases of the Kidney

Primary gout is a metabolic disease characterized with increased blood uric acid and tissue injury depending on a long-term purine metabolism disorder (Tian et al. 2014). Renal involvement may be seen as acute and chronic renal insufficiency due to chronic interstitial nephritis and uric acid stones (Tian et al. 2014). In a study evaluating the value of VTQ technology combined with urinary β2-microglobulin (β2-MG) measurement, a small-molecule immunoglobulin increases in urine in early phase of renal damage (Fassett et al. 2011), in the early diagnosis of gouty kidney (Tian et al. 2014). In this study, patients with kidney damage had significantly increased renal parenchyma and sinus shear wave velocities, and urinary β2-MG level was positively linearly correlated with the parenchymal shear wave velocities.

Diabetes mellitus is the leading cause of end-stage renal disease (ESRD), and diabetic nephropathy is one of the most significant complications that is directly related to progression of the disease (Yu et al. 2014). Laboratory examinations such as albumin-to-creatinine ratio, blood β2-MG, and urine β2-MG are indicators that appear before renal diabetic involvement (Yu et al. 2014). It has been stated that there

was significant difference in SWV of the renal cortex between the patients with microalbuminuria and macroalbuminuria groups and normal population (Yu et al. 2014), and the renal cortical SWVs were positively correlated with the albumin-to-creatinine ratio.

Goya et al. found that ARFI imaging was able to distinguish diabetic nephropathy stages (except for stage 5 in the right and except for stages 4–5 in the left kidneys) by VTQ technique, and they stated a correlation between the SWV and estimated glomerular filtration rate (eGFR), serum urea nitrogen, urinary protein, and creatinine (Goya et al. 2015).

Evaluation of Renal Damage in Urinary Tract Abnormality

There are only a few studies evaluating renal pathologies in children with urinary tract abnormalities. Bruno et al. reported that the kidneys in pediatric patients with vesicoureteral reflux (≥grade III) had significantly higher SWV values compared with healthy kidneys by ARFI technique (Bruno et al. 2013). The authors claimed that ARFI might ensure reliable information about the severity of renal damage and might be useful in the diagnostic workup in children with chronic reflux disease (Bruno et al. 2013). In a study evaluating hydronephrotic kidney stiffness in 30 children (range, 0–23 months), a significant difference in the median SWVs between normal kidneys (1.75 m/s) and high-grade hydronephrotic kidneys (2.02 m/s) was also stated by using ARFI technique (Sohn et al. 2014). These results were compatible with decreased elasticity and increased stiffness in high-grade hydronephrotic kidneys (Sohn et al. 2014). In a comprehensive study about detection of the renal damage with ARFI technique as a complementary tool to technetium-99m dimercaptosuccinic acid (Tc-99m DMSA) scintigraphy, high-grade (grade V–IV) refluxing kidneys had the lowest SWV values, while non-refluxing kidneys had the highest values, and severely damaged kidneys had the lowest SWV values (Goya et al. 2014b) (Fig. 11). Goya et al. considered that other parameters such as vascularization, anisotropy, external pressure, and hydronephrosis might be more effective in renal elasticity than fibrosis (Goya et al. 2014b). It has been also claimed that ARFI measurements could not differentiate the reason of the stiffness such as tissue fibrosis and edema (Sohn et al. 2014). Therefore, further researches with larger patient population and pathologic correlation are needed.

In summary, ultrasound elastography is a more favorable imaging modality than others including magnetic resonance elastography. Firstly, ultrasound is less expensive and easier to use and especially capable of ensuring quantitative measurements of tissue both viscosity and elasticity with new techniques. Therefore it will likely become the most widely used modality for clinical elasticity estimation and imaging. However, kidney has unique biomechanical properties including viscosity and anisotropy due to complex intrarenal architecture. This means that changeability of measurements in the renal parenchyma is an actual concern. Further studies have to be made to obtain more experience with preclinical models

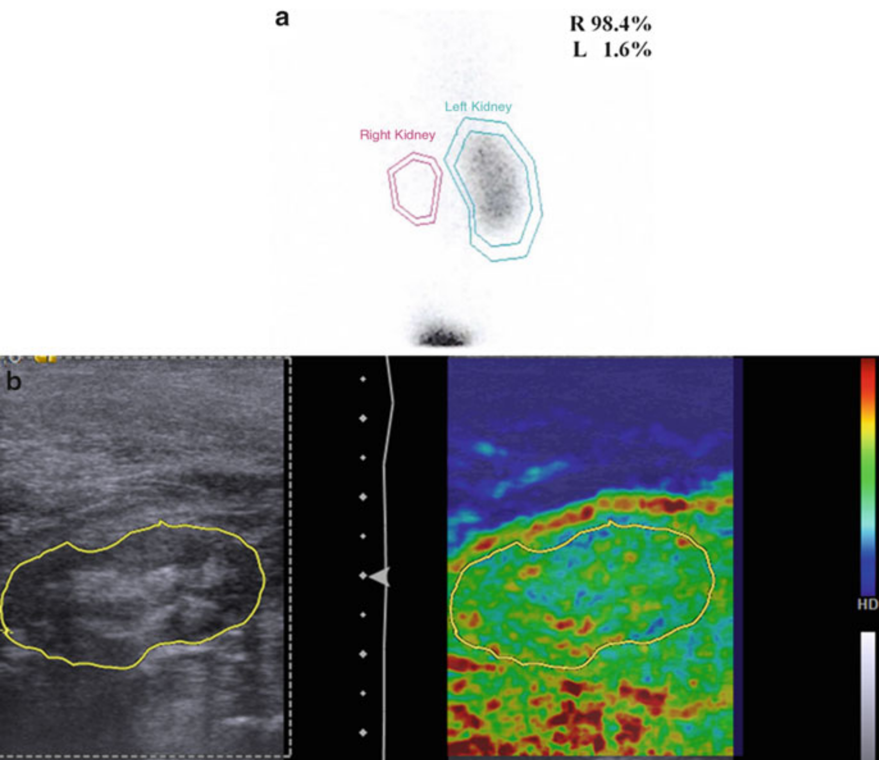

Fig. 11 eSie Touch Elasticity Imaging of the grade V vesicoureteral reflux (*VUR*). Technetium-99m dimercaptosuccinic acid (*Tc-99m DMSA*) summed image shows right atrophic kidney in a 5-year-old boy with grade V VUR (**a**). Acoustic radiation force impulse (*ARFI*) imaging shows a small kidney with no corticomedullary differentiation and entirely decreased stiffness as yellow-green-red areas (**b**) (Images taken by a high-frequency probe)

and to clarify better the known/unknown sources of variability and the histopathological causes of elasticity changes in patient cohorts, with pathological correlation.

Potential Applications to Prognosis, Other Diseases or Conditions

The innovation of elastography is giving rise to new avenues for prognosis, diagnosis, and therapy. This is especially welcome in the renal disease because of an increasing incidence of chronic kidney disease worldwide with its high morbidity and mortality. Technological advances in ultrasound probe, for example, three-dimensional (3D) and combining with new elastographic techniques may offer advantages in diagnosis and treatment of diseases (Treece et al. 2008). 3D evaluation is easy and feasible and provides post-processing evaluation in different planes. Combining these techniques may allow not only volumetric evaluation which could

be important in order to plan invasive treatment but also better visualization of features of the organ/lesion (type of vascularization).

Ultrafast imaging technique which is used to track the wave in SSI leads to many groundbreaking applications of ultrasound (Tanter and Fink 2014). This technique may detect slow flow in very small vessels, which allows functional imaging (fUltrasound). Moreover, it may pave the way to molecular imaging owing to higher contrast and non-disruption of microbubbles.

The unique combination of these properties with elastography may provide a multiparametric biomarker which will not necessitate histopathological techniques for prognosis, diagnosis, and therapy in kidney diseases.

Summary Points

- This chapter mainly focuses on recent developments in the field of renal ultrasound elastography, especially its use in kidney diseases.
- Ultrasound elastography is a new imaging modality that can measure stiffness of the tissue qualitative/quantitatively.
- The most common elastography techniques for clinical use are quasi-static (compression-based) and dynamic elastography.
- Kidney is a unique tissue and has complex internal architecture. Therefore intrinsic or extrinsic factors such as perfusion, urinary pressure, anisotropy, depth of kidney, and hydronephrosis may affect viscoelasticity of the kidney.
- Elastography can be considered as a potential diagnostic biomarker in kidney diseases; however, it needs specific training and optimization techniques as well as acknowledging technical and pathological factors which may influence it.

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References

Amador C, Urban MW, Chen SG, et al. Shearwave dispersion ultrasound vibrometry (SDUV) on swine kidney. IEEE Trans Ultrason Ferroelectr. 2011a;58:2608–19.

Amador C, Urban MW, Chen SG, et al. In vivo assessment of renal tissue viscoelasticity during acute and gradual renal ischemia. 2011 I.E. International Ultrasonics Symposium (Ius). 2012;2102–5. doi:10.1109/Ultsym.2011.0521.

Arda K, Ciledag N, Aktas E, et al. Quantitative assessment of normal soft-tissue elasticity using shear-wave ultrasound elastography. AJR. 2011;197:532–6.

Arndt R, Schmidt S, Loddenkemper C, et al. Noninvasive evaluation of renal allograft fibrosis by transient elastography – a pilot study. Transpl Int. 2010;23:871–7.

Asano K, Ogata A, Tanaka K, et al. Acoustic radiation force impulse elastography of the kidneys: is shear wave velocity affected by tissue fibrosis or renal blood flow? J Ultrasound Med. 2014;33:793–801.

Baghani A, Eskandari H, Wang W, et al. Real-time quantitative elasticity imaging of deep tissue using free-hand conventional ultrasound. Med Image Comput Comput Assist Interv. 2012;15:617–24.
Bavu E, Gennisson JL, Couade M, et al. Noninvasive in vivo liver fibrosis evaluation using supersonic shear imaging: a clinical study on 113 hepatitis C virus patients. Ultrasound Med Biol. 2011;37:1361–73.
Bercoff J, Tanter M, Fink M. Supersonic shear imaging: a new technique for soft tissue elasticity mapping. IEEE Trans Ultrason Ferroelectr Freq Control. 2004;51:396–409.
Boffa JJ, Lu Y, Placier S, Stefanski A, et al. Regression of renal vascular and glomerular fibrosis: role of angiotensin II receptor antagonism and matrix metalloproteinases. J Am Soc Nephrol. 2003;14:1132–44.
Bota S, Bob F, Sporea I, et al. Factors that influence kidney shear wave speed assessed by acoustic radiation force impulse elastography in patients without kidney pathology. Ultrasound Med Biol. 2014. doi:10.1016/j.ultrasmedbio.2014.07.023.
Bruno C, Caliari G, Zaffanello M, et al. Acoustic radiation force impulse (ARFI) in the evaluation of the renal parenchymal stiffness in paediatric patients with vesicoureteral reflux: preliminary results. Eur Radiol. 2013;23:3477–84.
Castera L, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. J Hepatol. 2008;48:835–47.
Chatziantoniou C, Boffa JJ, Tharaux PL, et al. Progression and regression in renal vascular and glomerular fibrosis. Int J Exp Pathol. 2004;85:1–11.
Clevert DA, Stock K, Klein B, et al. Evaluation of Acoustic Radiation Force Impulse (ARFI) imaging and contrast-enhanced ultrasound in renal tumors of unknown etiology in comparison to histological findings. Clin Hemorheol Microcirc. 2009;43:95–107.
Cui G, Yang Z, Zhang W, et al. Evaluation of acoustic radiation force impulse imaging for the clinicopathological typing of renal fibrosis. Exp Ther Med. 2014; 7:233–235.
Deffieux T, Montaldo G, Tanter M, et al. Shear wave spectroscopy for in vivo quantification of human soft tissues visco-elasticity. IEEE Trans Med Imaging. 2009;28:313–22.
Derieppe M, Delmas Y, Gennisson JL, et al. Detection of intrarenal microstructural changes with supersonic shear wave elastography in rats. Eur Radiol. 2012;22:243–50.
El Nahas M. The global challenge of chronic kidney disease. Kidney Int. 2005;68:2918–29.
Fassett RG, Venuthurupalli SK, Gobe GC, et al. Biomarkers in chronic kidney disease: a review. Kidney Int. 2011;80:806–21.
Gao J, Min R, Hamilton J, et al. Corticomedullary strain ratio: a quantitative marker for assessment of renal allograft cortical fibrosis. J Ultrasound Med. 2013;32:1769–75.
Gennisson JL, Grenier N, Combe C, et al. Supersonic shear wave elastography of in vivo pig kidney: influence of blood pressure, urinary pressure and tissue anisotropy. Ultrasound Med Biol. 2012;38:1559–67.
Goertz RS, Amann K, Heide R, et al. An abdominal and thyroid status with acoustic radiation force impulse elastometry: a feasibility study: acoustic radiation force impulse elastometry of human organs. Eur J Radiol. 2011;80:e226–e230.
Goya C, Daggulli M, Hamidi C, et al. The role of quantitative measurement by acoustic radiation force impulse imaging in differentiating benign renal lesions from malignant renal tumours. Radiol Med. 2014a. doi:10.1007/s11547-014-0443-7.
Goya C, Hamidi C, Ece A, et al. Acoustic radiation force impulse (ARFI) elastography for detection of renal damage in children. Pediatr Radiol. 2014b. doi:10.1007/s00247-014-3072-3.
Goya C, Kilinc F, Hamidi C, et al. Acoustic radiation force impulse imaging for evaluation of renal parenchyma elasticity in diabetic nephropathy. AJR. 2015;204:324–9.
Grenier N, Poulain S, Lepreux S, et al. Quantitative elastography of renal transplants using supersonic shear imaging: a pilot study. Eur Radiol. 2012;22:2138–46.
Grenier N, Gennisson J-L, Cornelis F, et al. Ultrasound elastography of the kidney. Ultrasound Clin. 2013;8:551–64.

Guo LH, Xu HX, Fu HJ, et al. Acoustic radiation force impulse imaging for noninvasive evaluation of renal parenchyma elasticity: preliminary findings. PLoS One. 2013;8:e68925. doi:10.1371/journal.pone.0068925.

Korsmo MJ, Ebrahimi B, Eirin A, et al. Magnetic resonance elastography noninvasively detects in vivo renal medullary fibrosis secondary to swine renal artery stenosis. Invest Radiol. 2013;48:61–8.

Lee MJ, Kim MJ, Han KH, et al. Age-related changes in liver, kidney, and spleen stiffness in healthy children measured with acoustic radiation force impulse imaging. Eur Radiol. 2013;82:290–4.

Levey AS, Cattran D, Friedman A, et al. Proteinuria as a surrogate outcome in CKD: report of a scientific workshop sponsored by the National Kidney Foundation and the US Food and Drug Administration. Am J Kidney Dis. 2009;54:205–26.

Lukenda V, Mikolasevic I, Racki S, et al. Transient elastography: a new noninvasive diagnostic tool for assessment of chronic allograft nephropathy. Int Urol Nephrol. 2014;46:1435–40.

Maya ID, Allon M. Percutaneous renal biopsy: outpatient observation without hospitalization is safe. Semin Dial. 2009;22:458–61.

Merino S, Arrazola J, Cardenas A, et al. Utility and interobserver agreement of ultrasound elastography in the detection of malignant thyroid nodules in clinical care. AJNR. 2011;32:2142–8.

Nankivell BJ, Borrows RJ, Fung CL, et al. The natural history of chronic allograft nephropathy. N Engl J Med. 2003;349:2326–33.

National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis. 2002; 39(2 Suppl 1):S1–266.

Ophir J, Cespedes I, Ponnekanti H, et al. Elastography: a quantitative method for imaging the elasticity of biological tissues. Ultrason Imaging. 1991;13:111–34.

Orlacchio A, Chegai F, Del Giudice C, et al. Kidney transplant: usefulness of real-time elastography (RTE) in the diagnosis of graft interstitial fibrosis. Ultrasound Med Biol. 2014. doi:10.1016/j.ultrasmedbio.2014.06.002.

Ozkan F, Yavuz YC, Inci MF, et al. Interobserver variability of ultrasound elastography in transplant kidneys: correlations with clinical-Doppler parameters. Ultrasound Med Biol. 2013;39:4–9.

Palmeri ML, Wang MH, Rouze NC, et al. Noninvasive evaluation of hepatic fibrosis using acoustic radiation force-based shear stiffness in patients with nonalcoholic fatty liver disease. J Hepatol. 2011;55:666–72.

Ricardo SD, van Goor H, Eddy AA. Macrophage diversity in renal injury and repair. J Clin Invest. 2008;118:3522–30.

Ries M, Jones RA, Basseau F, et al. Diffusion tensor MRI of the human kidney. J Magn Reson Imaging. 2001;14:42–9.

Sandrin L, Tanter M, Gennisson JL, et al. Shear elasticity probe for soft tissues with 1-D transient elastography. IEEE Trans Ultrason Ferroelectr Freq Control. 2002;49:436–46.

Sandrin L, Fourquet B, Hasquenoph JM, et al. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. Ultrasound Med Biol. 2003;29:1705–13.

Scola MR, Baggesen LM, Gallippi CM. Multi-push (MP) acoustic radiation force (ARF) ultrasound for assessing tissue viscoelasticity, in vivo. Conf Proc IEEE Eng Med Biol Soc. 2012;2012:2323–6.

Singla V, Singh T, Sinha A. Ultrasound elastography: principles and application. In: Gupta AK, Chowdhury V, Khandelwal N, editors. Diagnostic radiology: recent advances and applied physics in imaging. New Delhi: Jaypee; 2013. p. 37–57.

Sohn B, Kim MJ, Han SW, et al. Shear wave velocity measurements using acoustic radiation force impulse in young children with normal kidneys versus hydronephrotic kidneys. Ultrasonography. 2014;33:116–21.

Sommerer C, Scharf M, Seitz C, et al. Assessment of renal allograft fibrosis by transient elastography. Transpl Int. 2013;26:545–51.

Stegall MD, Park WD, Larson TS, et al. The histology of solitary renal allografts at 1 and 5 years after transplantation. Am J Transplant. 2011;11:698–707.
Stock KF, Klein BS, Cong MT, et al. ARFI-based tissue elasticity quantification and kidney graft dysfunction: first clinical experiences. Clin Hemorheol Microcirc. 2011;49:527–35.
Syversveen T, Brabrand K, Midtvedt K, et al. Assessment of renal allograft fibrosis by acoustic radiation force impulse quantification – a pilot study. Transpl Int. 2011;24:100–5.
Syversveen T, Midtvedt K, Berstad AE, et al. Tissue elasticity estimated by acoustic radiation force impulse quantification depends on the applied transducer force: an experimental study in kidney transplant patients. Eur Radiol. 2012;22:2130–7.
Tan S, Ozcan MF, Tezcan F, et al. Real-time elastography for distinguishing angiomyolipoma from renal cell carcinoma: preliminary observations. AJR. 2013;200:W369–75.
Tanter M, Fink M. Ultrafast imaging in biomedical ultrasound. IEEE Trans Ultrason Ferroelectr Freq Control. 2014;61:102–19.
Tian F, Wang ZB, Meng DM, et al. Preliminary study on the role of virtual touch tissue quantification combined with a urinary beta2-microglobulin test on the early diagnosis of gouty kidney damage. Ultrasound Med Biol. 2014;40:1394–9.
Togao O, Doi S, Kuro-o M, et al. Assessment of renal fibrosis with diffusion-weighted MR imaging: study with murine model of unilateral ureteral obstruction. Radiology. 2010;255:772–80.
Treece GM, Lindop JE, Gee AH, et al. Freehand ultrasound elastography with a 3-D probe. Ultrasound Med Biol. 2008;34:463–74.
Urban MW, Amador C, Greenleaf JF. In vivo measurement of renal transplant viscoelasticity. IEEE Int Ultra Sym 2013;1517–20. doi:10.1109/Ultsym.2013.0136.
Wang L, Xia P, Lv K, et al. Assessment of renal tissue elasticity by acoustic radiation force impulse quantification with histopathological correlation: preliminary experience in chronic kidney disease. Eur Radiol. 2014;24:1694–9.
Warner L, Yin M, Glaser KJ, et al. Noninvasive In vivo assessment of renal tissue elasticity during graded renal ischemia using MR elastography. Invest Radiol. 2011;46:509–14.
Yoon JH, Kim MH, Kim EK, et al. Interobserver variability of ultrasound elastography: how it affects the diagnosis of breast lesions. AJR. 2011;196:730–6.
Yu N, Zhang Y, Xu Y. Value of virtual touch tissue quantification in stages of diabetic kidney disease. J Ultrasound Med. 2014;33:787–92.

Aortic Pulse Wave Velocity as a Biomarker in Chronic Dialysis Patients

48

Petar Avramovski and Aleksandar Sikole

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Abstract

Cardiovascular mortality is considered the main cause of death in patients receiving dialysis and is 10–20 times higher in such patients than in the general population. A high percentage of all cardiovascular mortality diseases are

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associated with stiffening of the arteries, a direct consequence of atherosclerosis. Increased central arterial stiffening is a hallmark of the aging process and consequence of many disease states, such as diabetes, atherosclerosis, and chronic renal compromise. Accelerated arteriosclerosis is a major risk to long-term survivors on maintenance hemodialysis.

Measuring of the pulse wave velocity provides useful information regarding the mechanical properties of the arterial tree and can be used to assess the stiffness and endothelial function. From all the different methods to assess arterial stiffness, carotid to femoral pulse wave velocity has emerged as the gold standard method. Two Doppler waves are recorded transcutaneously at the base of the neck for the right common carotid artery and over the right common femoral artery. After that, the Doppler waves are identified and their time delay diversity is measured simultaneously with electrocardiography. Time delay (transition time, ΔT) is the time from the R wave to the foot of the carotid or femoral Doppler waveform.

There is a high prevalence of increased pulse wave velocity in a relatively young hemodialysis patient population. Vascular stiffening likely begins much earlier and progresses more rapidly in hemodialysis patients. Accelerated arteriosclerosis is a major risk to long-term survivors on maintenance hemodialysis. The pulse wave velocity measured at baseline was markedly higher in chronic hemodialysis patients than in general population patients, with a greater than twofold higher annual increase. In the general population group, only factors associated with the progression of arterial stiffness in the elderly were evident (traditional risk factors), but in chronic kidney disease patients, arterial stiffness (i.e. pulse wave velocity) is accelerated due to synergism between age and traditional risk factors plus factors related to renal comorbidity (nontraditional risk factors). Patients with end stage renal disease face a particularly high risk of cardiovascular disease and total mortality. It is now known that pulse wave velocity, C-reactive protein and serum albumin are strongly and independently predictive of outcome in chronic hemodialysis patients. Whether enhanced arterial stiffness is a risk factor contributing to the development of cardiovascular disease or a marker of established cardiovascular disease is a matter of debate. The pulse wave velocity is a strong independent predictor of over-all and cardiovascular mortality with high-level performance values, assessed by simple, indirect, reproducible and noninvasive evaluation of regional arterial stiffness.

Keywords

Pulse wave velocity • Stiffness • Clinical biomarker • Chronic dialysis • Doppler • Cardiovascular mortality • Traditional risks factors • Nontraditional risk factors

Abbreviations

C	Incisura
CCA	Common carotid artery
CFA	Common femoral artery
CHP	Chronic dialysis patients

CKD	Chronic kidney disease
CRP	C-reactive protein
CV	Cardiovascular
D	Dicrotic wave
DD	Dialysis duration
ECG	Electrocardiogram
ESRD	End-stage renal disease
GPP	General population patients
P	Percussion wave
PWV	Pulse wave velocity
S	Starting point
T	Tidal wave
TT	Transit time
ΔD	Relative change in vascular diameter
ΔS	Vascular cross sectional surface area
ΔT	Time delay, time diversity

Key Facts of Arteriosclerosis

- Increased arterial stiffness is the result of atherosclerosis, vascular calcification and changes in collagen/elastin ratio content in the vessel wall.
- Stiffening and thickening of the arterial walls are two important components of atherosclerosis.
- Arterial stiffness is a cause of premature return of reflected waves in late systole, increasing central pulse pressure and the load on the ventricle.
- Accelerated atherosclerosis in renal disease is also driven by diffuse calcifications in the arterial media without much inflammation, producing a histological picture quite different from calcifications in complex atherosclerotic plaque.
- Accelerated arteriosclerosis is a major risk to long-term survivors on maintenance hemodialysis and the most frequent cause of cardiovascular morbidity in patients with end stage renal disease.

Key Facts of Doppler Effect

- The Doppler ultrasound, measuring the changes in ultrasound waves, can actually measure how fast or slow blood is moving, which can indicate a circulatory problem.
- Doppler ultrasound imaging can also be used to identify atherosclerotic plaque buildup in the blood vessels, narrowed or blocked arteries.
- The most widely used method of evaluating arterial stiffness is Doppler ultrasound that measures aortic pulse wave velocity in the area running from the common carotid artery to the common femoral artery.

Key Facts of Aortic Pulse Wave Velocity

- Pulse wave velocity is a measure of arterial stiffness, or the rate at which pressure wave (not blood) moves down the vessel.
- In aging and arteriosclerosis, central elastic arteries become stiffer, diastolic pressure decreases while systolic and pulse pressures are augmented due to increased pulse wave velocity.
- By measuring the pulse wave velocity it is possible to noninvasively assess stiffness and age of the arteries and thus the risk of cardiovascular events with fatal ending.

Key Facts of Hemodialysis

- Hemodialysis is the default therapy for patients in end stage renal disease.
- Hemodialysis is a method that is used to achieve the extracorporeal removal of waste products such as urea, creatinine and free water from the blood when the kidneys are in end stage renal disease.
- In chronic hemodialysis patients, increased arterial stiffness has recently become intensively investigated as a major novel cardiovascular risk factor.
- Dialysis patients have rigid blood vessels in which stiffening starts earlier and are more pronounced by accelerated aging of blood vessels compared with patients from the general population.

Definitions

Arterial stiffness Arterial stiffness is a term that describes rigidity of the arterial walls and the reduced capability of arteries to expand and contract according pressure changes.

Arteriosclerosis It is a broad term that describes hardening and elasticity loss of the inner and middle layers of the artery wall.

Atherosclerosis This is a condition where the arteries become stiffer and narrowed due to progressive thickening and hardening of their walls from waxy plaques on the inner lining.

Chronic Renal Insufficiency It is a stage based on reduced filtering capacity of the kidneys so that they are no longer capable to remove fluids and wastes from the body or of maintaining the adequate level of certain kidney-regulated chemical in the bloodstream.

Doppler ultrasound This is a noninvasive ultrasound test that is used to estimate blood flow through blood vessels based of series of generated pulses that is transmitted and then reflected from blood cells to detect movement of blood.

Foot of the wave This is the terminal point of diastolic spectral waveform that occurs at the beginning of the first ascent after diastole.

Hemodialysis Hemodialysis is a renal replacement therapy method in renal failure disease that is used to achieve the extracorporeal removal of waste products and free water from the blood.

Pulse Wave Velocity Measures the rate of pressure wave propagation across the vessel. Pressure wave is generated during blood flows through the vessels of circulatory system.

Introduction

The pulse wave is a physiological phenomenon that propagates through the arteries due to the reciprocal transformation between the kinetic energy of a segment of the expelled blood volume and the potential energy of a stretched segment of the resilient vascular wall. The pulse wave analysis provides useful information regarding the mechanical properties of the arterial tree and can be used to assess the stiffness and endothelial function. Arterial stiffness is a general term that collectively describes distensiblility, compliance, and elastic modulus of the arterial vascular system. These properties are not homogeneous along the arterial tree, muscular and elastic vessels differ. The elastic properties of arteries vary along the arterial tree, with more elastic proximal arteries and stiffer distal arteries. The velocity of propagation of the pulse wave increases with decreased arterial distensibility. Moreover, wave reflections, which amplify the pressure wave, are generated at the level of peripheral arterial bifurcations and smaller muscular arteries (Laurent et al. 2006).

In recent years, great emphasis has been placed on the role of arterial stiffness in the development of CV diseases. Indeed, the assessment of arterial stiffness is increasingly used in the clinical assessment of patients. Increased central arterial stiffening is a hallmark of the aging process and the consequence of many disease states, such as diabetes, atherosclerosis, and chronic renal compromise. The most consistent and well-reported changes are luminal enlargement with wall thickening (remodeling) and reduction of elastic properties (stiffening) at the level of large elastic arteries, namely arteriosclerosis (Izzo and Shykoff 2001). Arteriosclerosis refers to reduced arterial compliance due to increased fibrosis, loss of elasticity, and vessel wall calcification affecting the media of large and middle-sized arteries. In dialysis patients, both atherosclerosis (affecting mainly the intima of the arteries) and arteriosclerosis (affecting predominantly the media of large and middle-sized arteries diffusely) is prominent (Kanbay 2010).

Arterial stiffness describes the reduced capability of an artery to expand and contract in response to pressure changes. Parameters that describe vessel stiffness include compliance and distensibility. The consequence of reduced compliance/distensibility is an increased propagation velocity of the pressure pulse along the arterial tree, called pulse wave velocity (PWV). PWV inversely correlates with arterial distensibility and relative arterial compliance (Cecelja and Chowienczyk 2012).

Mechanical behaviour of large arteries is extremely complex and provides serious difficulties, both on the theoretical and technical aspects. Indeed, arteries have marked anisotropy, exhibit non-linear visco-elastic properties, and have powerful adaptive mechanisms (Nichols and O'Rourke 2005). An understanding of the basic principles of haemodynamics and generating pulse wave, require integrated knowledge of physicists, physiologists, biologists and medical doctors. Earlier physicists such as Young (1808), Poiseuille (1840), and Korteweg (1878) established hydraulic and elastic theory. Important contributions to the analysis of the pressure wave and PWV were made by physiologist Marey (1860) and Mackenzie (1902) by developing and experimenting with various types of sphygmographs. It is impossible to extrapolate segmental arterial stiffness to the whole arterial tree, because no single arterial segment has identical viscoelastic properties. Despite these obstacles, simple parameters derived either from the Windkessel model or based on the arterial wave propagation that have been developed. Otto Frank (1899) originally used the principle of conservation of mass to quantify the Windkessel model of the arterial system. According to this description, the large conductance arteries distend to accommodate blood ejected from the heart during systole and recoil to propel blood through the small resistance vessels in diastole (Sagawa K et al. 1990).

Total arterial compliance (C_{tot}), the sum of all arterial compliances in the system, determines the ability of the arterial system to store blood, whereas the total peripheral resistance (R_{tot}), the average input pressure divided by the average flow, determines the ability of the arterial system to resist blood flow. According to this description, C_{tot} is higher in young subjects, allowing the arterial system to accommodate an entire stroke volume without generating much pulse pressure (i.e., difference between systolic and diastolic pressures). However, with an increase in arterial stiffness with age, C_{tot} decreases and ejection creates a larger pulse pressure (Quick et al. 2006).

Arterial stiffening increases in patients with chronic renal insufficiency, and aortic PWV, a marker of stiffening, is a strong independent predictor of mortality in this population.

In uremic patients, elasticity and digestibility of collagen and other extracellular matrix proteins are reduced because of reactions with methylglyoxal and other reactive carbonyl compounds, which are increased. Intima-medial thickening occurs in response to increased wall stress from hypertension. Arterial stiffening in renal disease is also driven by diffuse calcifications in the arterial media without much inflammation, producing a histological picture quite different from calcifications in complex atherosclerotic plaque (Goldsmith et al. 2004).

Document Map

While we mention three ways for PWV measuring, attention is kept on the stiffness measuring using Doppler ultrasound. Foot-to-foot Doppler estimating method is explained by time delay of the signal acquired by the carotid and femoral arteries with synchronous ECG monitoring. It is a method of PWV calculating based on the carotid to femoral signals time delay using a standard equation of speed. In the following text, an associative correlation between hemodialysis duration and arterial stiffness expressed by PWV through coefficient of determination and scatter diagram is presented. We emphasize the associated interaction of traditional and dialysis-specific factors in the impact of increasing stiffness of the arteries in patients on dialysis.

Estimation of PWV progression have concluded that dialysis patients have rigid blood vessels with more pronounced stiffening by accelerated aging of blood vessels compared with GPPs. To distinguish the patients who survived and who did not survived, we used discrimination ability of a model, by assessing cut-off point of PWV.

Both groups (survived or not survived) formed by the PWV cut-off point value present statistically significant difference regarding survival. A plot of the Kaplan-Meier estimate of the survival function indicates significantly higher CV mortality observed in patients with PWV $\geq$11.8 m/s. PWV as an independent predictor for CV outcome is assessed by Cox-regression model analysis.

Pulse Wave Velocity Estimation

Arterial stiffness, estimated by aortic PWV, is an independent predictor of CV mortality and morbidity. However, the clinical applicability of these measurements and the elaboration of PWV reference values are difficult due to differences between the various devices used Salvi et al. (2008)). Arterial stiffness is assessed noninvasively by PWV measurement, that is, the velocity of the pulse wave to travel a given distance between two sites of the arterial system.

There are three different non-invasive techniques to measure arterial stiffness:

1. Measuring of PWV expressed through time delay ΔT [ms].
2. Measuring of the relative change in vascular diameter $\Delta D = D_1/D_2$, or relative change in vascular cross sectional surface area $\Delta S = S_1/S_2$ during PWV pressure propagation.
3. Registration and analysis of different segments from arterial pressure waveforms (S, P, T, C and D) and calculating indexes: ejection elastic index, dicrotic dilatation index and dicrotic elastic index (Fig. 1).

Using the first technique, PWV is estimated from foot-to-foot transit time in the aorta and path length measurement. This technique offers a simple, reproducible, cheap, and noninvasive evaluation of segmental aortic stiffness.

Transit times are assessed as the time difference between two characteristic points on carotid and femoral waveforms. The characteristic points chosen are dependent

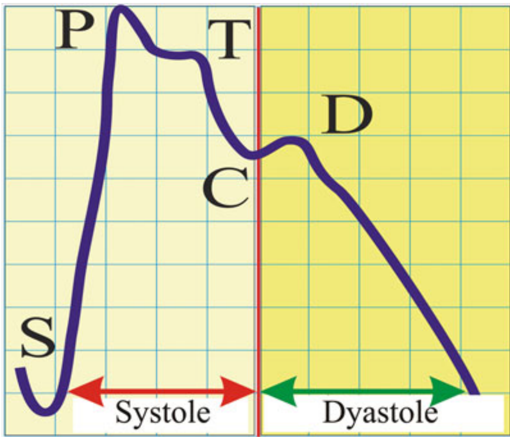

Fig. 1 Arterial pressure waveforms in systole and diastole. Graphic variation of pressure changes throughout one full heart cycle. This diagram can be used for classification of the arterial elasticity. There are several specific points on the pressure diagram that reflect appropriate hemodynamic events in the specific phases of the cardiac cycle: S (starting point) – blood discharging after aortic valve is open; P (percussion wave) – linearly increasing of arterial wall by left ventricular (*LV*) ejection; T (tidal wave) – reflected wave from the distal small arteries; C (Incisura) – end-point of systolic phase, aortic valve is closed now and D (dicrotic wave) – reflective blood pressure of aorta that crash into aortic valve

on the type of waveform (flow, pressure, or diameter distension) and the algorithm used for its detection. The two most popular algorithms are: (I) the intersecting tangent algorithm (Sphygmocor® system and for manual identification) and (II) the point of maximal upstroke during systole (as used in the Complior® system). Different algorithms applied on the same waveforms can lead to differences in measured PWV values of 5–15 % (Millasseau et al. 2005).

PWV Measured by Doppler

Carotid and femoral waves are analyzed by a General Electric Logiq pro 5 Doppler ultrasound machine. Although it is not possible to analyze the carotid and femoral waves simultaneously, they can be normalized separately with the electrocardiogram (ECG) (gatting).

Three parameters needed for foot-to-foot PWV calculating are obtained by Doppler ultrasound with a linear array (10 MHZ) probe synchronized with ECG during 2-s minimum sliding window: T_1 – time delay from “R – wave” of ECG to foot of the Common Carotid Artery (CCA) wave, T_2 – time delay from “R – wave” of ECG to foot of the Common Femoral Artery (CFA) wave and distance D measured from sternal notch (CCA) to the groin (CFA). The foot of the wave is defined at the end of diastole, when the steep rise of the systolic waveform begins. Path length (distance D) was defined by direct anthropometric measurement of the distance between suprasternal

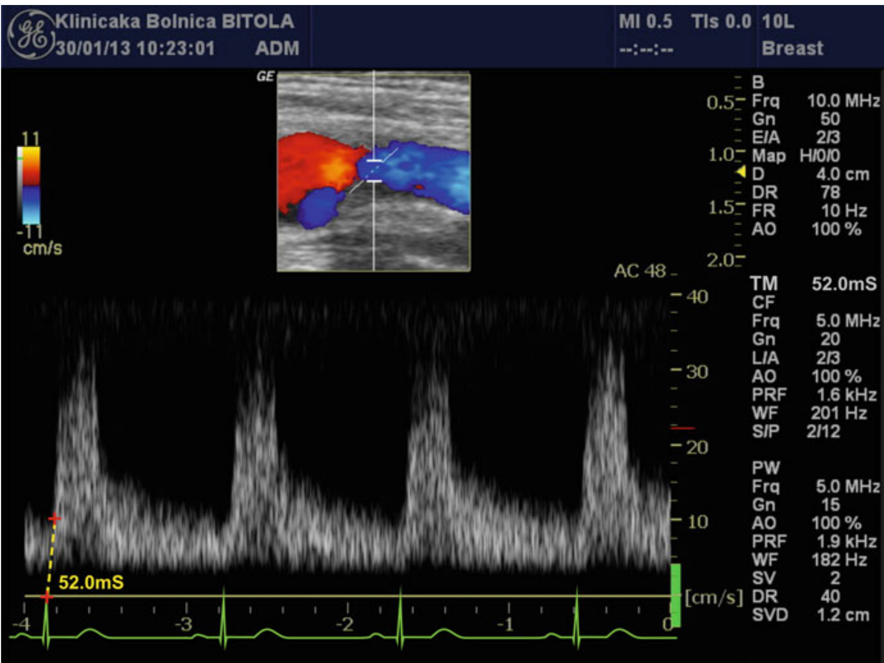

Fig. 2 Doppler of carotid artery – time delay measurement by CCA Doppler synchronized with ECG. Picture shows the basic principle of time delay estimation from heart beat to the emergence of carotid flow wave. Doppler of right common carotid artery obtains grey spectral wave and the *green curve* below it is obtained by synchronous recording of electrocardiography. The distance determined by two calipers mark, presented by two "red cross" is calculated as 50.0 ms time delay (TM). This time delay period is needed for the pulse wave to arrive from the heart to the carotid artery

notch (fossa jugularis sternalis) and groin. Each of the three consequent recordings involved two or three cardiac cycles. To find the transit time (TT) we measured the time from the R wave of ECG to the foot of the waveform using digital calipers (Fig. 2).

It is now known that the measurement of carotid-femoral PWV is calculated by dividing the distance D by the ΔT, so-called TT (Transit Time). Time diversity ΔT is calculated by the time differences T_1 and T_2 yielding the time delay: $\Delta T = T_2 - T_1$. The speed of pulse wave (V) is calculated by standard equation for the speed: V (m/s) = S (m)/ΔT (s). Hence, PWV = D/Δt (m/s).

Arterial Stiffness and Hemodialysis Duration

While traditional risk factors predominated in the general population, in chronic dialysis patients (CHP), nontraditional risk factors play an increasingly important role, being perhaps dominant in end-stage renal disease (ESRD) patients. Recently, many studies have focused on newly discovered nontraditional risk factors, such as

vitamin D deficiency, CRP, fibrinogen, hyperhomocysteinemia, high plasma norepinefrin, accumulation of the endogenous inhibitor of the nitric oxide synthase asymmetric dimethylarginine, extracellular volume overload, hyperphosphatemia, and oxidant stress as a link between traditional and other nontraditional risk factors in CHPs. Nontraditional risk factors are more prevalent in ESRD patients compared to the general population (Zoccali et al. 2005). These include specific factors like uremia, infection, biocompatibility of dialysis membranes, hyperhomocysteinemia, acidosis, and hyperphosphatemia. Parallel testing and comparing the two groups (the hemodialysis and the general population) are providing an important data for the influence of traditional risk factors for atherosclerosis in the general population and the combined impact of traditional and dialysis-specific risk factors in patients undergoing dialysis (Avramovski et al. 2013).

Coefficient of determination R^2 (0.3723) is showing that 37.23 % from the total variability is explained with the linear relation between PWV and dialysis duration (DD) or that 37.23 % from PWV is dependent on the DD. Only 37.23 % from the changes in PWV are a result of the DD value changes and the rest 62.77 % from the total variability between them are not explained (62.77 % of aortic PWV are dependent on other factors, which are not covered with the regression model). This model is used as criterion for best regression equation choice, so the greater its value is, the better the model of approximation will be (Table 1).

The regression parameter $b_o = 9.7797$ is showing the expected theoretical value of aortic PWV in case if DD would have a value equal to zero. This parameter also shows the point of the y-axis (dependent variable axis, aortic PWV) through which the regression line passes across. The regression parameter $b_1 = 0.2914$ signifies that at each increase of one unit (year) in DD, aortic PWV score increases for 0.2914 m/s. The equation of simple linear regression $\mathbf{y=9.7797+0.2914 \cdot X}$ shows the average coordination of aortic PWV and DD variations. With this equation, we get the evaluated (theoretical) aortic PWV values in opposition to its empirical values.

A Fig. 3 shows a scatter plot of aortic PWV and DD. There is a positive association between these variables. The data from each one of 80 examined patients is displayed as a collection of colored points (blue circles) determining the cross-sectional point of “x” axis – DD value, with “y” axis – PWV value. Each point has the value of one variable determining the position on the horizontal axis and the value of the other variable determining the position on the vertical axis. Linear regression lines computed by data acquired from different aortic PWV patient’s status dependent on aortic stiffness are plotted and shown by different colors and line styles (light blue solid line, red dashed line and dark blue solid line). Linear regression line plotted with dark blue solid line shows a positive correlation between aortic PWV and DD. The 95 % confidence interval is presented by red dashed line and prediction interval is presented by light blue solid line.

The high CV mortality rate in dialysis population has become a major issue and it’s not simply related to the increased acceptance of elderly subjects. A recent cross-sectional haemodialysis study found that while traditional coronary risk factors may apply to this population, other factors including the uraemic milieu and the haemodialysis procedure itself were probably contributory (Cheung et al. 2000).

Table 1 Linear regression analysis of aortic PWV and dialysis duration. This table shows the results of relationship between a scalar dependent variable (*PWV*) and explanatory variable (*DD*) obtained by linear regression. The high value of the coefficient of determination ($R^2 = 0.3723$) and the low value of the coefficient of statistical significance ($p < 0.0001$) shows that there is a strong positive correlation between PWV and DD. This means that 37.23 % from the total variability is explained with the linear relation between PWV and DD or that 37.23 % from PWV is dependent of the DD

Regression						
Dependent Y			Pulse wave velocity, m/s			
Independent X			Dialysis duration, years			
Sample size			80			
Coefficient of determination R^2			0.3723			
Residual standard deviation			1.9666			
Regression equation						
$\mathbf{y = 9.7797 + 0.2914 \cdot X}$						
Parameter		**Coefficient**	**Std. error**	**95 % CI**	**t**	**P**
Intercept	$\mathbf{b_0}$	**9.7797**	0.3216	9.1395–10.4199	30.4118	**<0.0001**
Slope	$\mathbf{b_1}$	**0.2914**	0.04285	0.2061–0.3767	6.901	**<0.0001**

PWV pulse wave velocity, *DD* dialysis duration, *Std. Error* standard error, *CI* confidence interval

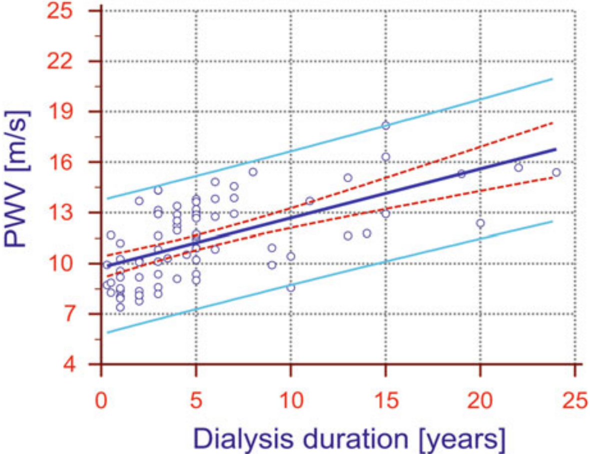

Fig. 3 Linear regression scatter plot of aortic PWV and dialysis duration. A Fig. 3 shows the results from linear regression analysis between pulse wave velocity (*PWV*) and dialysis duration (*DD*) presented as scatter plot, a graph of plotted points that shows the relationship between two sets of data. Linear regression line plotted with *dark blue solid line* shows a positive correlation between aortic PWV and DD, the 95 % confidence interval is presented by *red dashed line* and prediction interval is presented by *light blue solid line*. Abbreviations: *PWV* pulse wave velocity, *DD* dialysis duration

Haemodialysis causes numerous changes including abnormal complement activation with disordered leukocyte-endothelial interactions, the release of plasma factors including tumour necrosis factor-alpha and reactive oxygen species (Himmelfarb et al. 2002; Schroder et al. 2001). These processes cause vascular oxidative stress and consecutive elevation of PWV in latter period as result of the vascular stiffness increase. Because of this, the impact of nontraditional risk factors and the abovementioned factors, nontraditional and specific dialysis factors are conditioning progressive yearly increase of artery stiffness in dialysis patients or PWV increase of average 0,291 m/s in every single year spent in dialysis.

Progression of Arterial Stiffness

Aortic stiffness is associated with increased CV mortality in patients with chronic kidney disease (CKD). Traditional CV risk factors may play some role in the progression of aortic stiffness before development of advanced CKD, and that the enhanced rates of progression of aortic stiffness in CKD patients on dialysis are probably determined by more specific CKD-related risk factors such as advanced-glycation end products (AGEs) (Utescu et al. 2013). Modification of vascular extracellular matrix by advanced AGEs may result in progression of vascular stiffness. Because of higher exposure to glucose and uremic toxins, patients on dialysis have higher tissue levels of AGEs, increased vascular stiffness, and

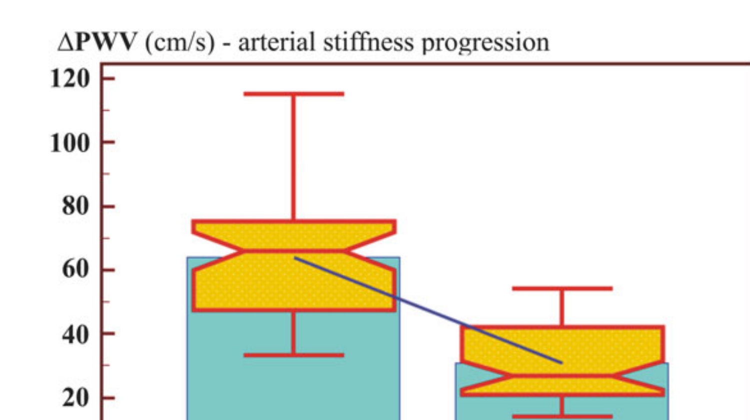

Fig. 4 Pronounced progression of arterial stiffness in chronic hemodialysis patient (*CHP*) compared to the general population patient (*GPP*) group. This *box whisker* diagram shows comparative results from PWV progression in 36-month follow-up period among CHPs and GPPs. ΔPWV in both groups is presented as a difference between PWV in the baseline and after 36 months. Its mean value and SD is 63.95 ± 18.373 cm/s for CHP and 27.28 ± 28.519 cm/s for GPP. It is obvious that there is a faster and pronounced progression of PWV in CHP than in the GPP group. The results of mean, range, 75th percentiles, median and 25th percentiles, test statistics, difference and two-tailed probability of P are presented in this figure, too (Figure courtesy of Korean Journal of Internal Medicine (KJIM 2013; 28: Fig. 4, p 469). The figure is published with permission from the KJIM and copyrights are reserved). Abbreviations: *PWV* pulse wave velocity, *CHP* chronic hemodialysis patients, *GPP* general population patients

enhanced central augmentation pressure compared to general population patients (GPP). However, the rate of progression of arterial stiffness and the role of CV risk factors in the progression of arterial stiffness are not enough established in longitudinal prospective studies. Although age is one of the most important determinants of CV risk, large artery stiffness is also a key independent predictor of CV mortality (London et al. 2001).

It is now known that progression of arterial stiffness in CHPs compared to the GPPs is pronounced. It was evaluated in longitudinal prospective study in 3 years period (Avramovski et al. 2013). The progression of PWV in CHP during the 36-month period (mean difference 0.6395 ± 0.18373 m/s, $p < 0.001$) compared to the progression of PWV in GPP during the same period (mean difference 0.2728 ± 0.28519 m/s, $p < 0.001$) is pronounced (Fig. 4).

Estimated and compared patients from the control group did not include a young healthy population. The control group consisted of participants from the general population who were not spared from the normal process of atherosclerosis, aging, and osteoporosis. The patients in this group had functioning kidneys, to exclude the influence of renal comorbidity. There is a high prevalence of increased PWV in a relatively young hemodialysis patient population. Vascular stiffening likely begins much earlier and progresses more rapidly in hemodialysis patients ($p < 0.001$). The PWV value measured at baseline was markedly higher (24 %) in CHP than in GPP, with a greater than twofold higher annual increase. In the GPP group, only factors associated with the progression of arterial stiffness in the elderly were evident (traditional risk factors), but in CKD patients, arterial stiffness (i.e., PWV) is accelerated due to synergism between age and traditional risk factors plus factors related to renal comorbidity (nontraditional risk factors).

The marked increase in aortic stiffness with aging and little change in peripheral arterial stiffness results in a reversal of the gradient of arterial stiffness from the youthful pattern of a compliant proximal aorta, which was evident in individuals aged <50 years, to a pattern of greater aortic stiffness in older participants (Fantin et al. 2007). The progression of blood vessel aging is significantly greater in dialysis patients. In this population, the chronological age is greater than biological age, expressed through the increased arterial stiffness (Fig. 5).

It is now known that progression of PWV over a 36-month period, and the significant difference between the CHP and GPP groups, suggest that arterial stiffening has progressed further in dialysis patients compared to the general population, which suggests a significant distinction in the aging and stiffness of their arteries, and so thus the biological age of both populations (despite almost identical chronological age: 59.3 ± 11.8 vs. 59.7 ± 11.9 years).

GPPs have an increased vascular stiffness; this is associated with traditional risk factors and urea, hemoglobin, albumin, CRP, and glucose levels. Nontraditional risk factors, or uremia-related specific factorssuch as anemia (hemoglobin), inflammation (CRP), hypoalbuminemia, and abnormal lipoproteins-might play a role in the accelerated progression of arterial stiffness only in CHPs (Avramovski et al. 2013). Fortunately, arterial stiffening can be monitored by a simple noninvasive method, measuring the PWV, which enables evaluation of the risk of CVr events.

Arterial Stiffness and Cardiovascular Mortality

A high percentage of all CV diseases are associated with stiffening of the arteries, a direct consequence of atherosclerosis. Increased arterial stiffness is the result of many contributing factors, such as atherosclerosis, vascular calcification and changes in collagen/elastin ratio content in the vessel wall. The increase in artery wall stiffness is noticeable from the beginning of the arteriosclerosis process, before anatomical changes and clinical manifestations are observed. Atherosclerosis is the most frequent cause of CV morbidity in patients with ESRD. Patients with ESRD face a particularly high risk of CV disease and total mortality (Zaccali et al. 2003).

PWV (m/s)
20
16
12
8
4
0
11.18 ± 2.29
11.82 ± 2.34
CHP (baseline)
CHP (36 mon)
Mean
Range
75th percentile
Median
25th percentile
PWV, Pulse Wave Velocity; CHP, Chronic Hemodialysis Patients;

Fig. 5 Values of 3-year PWV follow-up period in CHP (baseline and after 36 months). The results from the PWV progression in CHPs during 36-months follow-up period are presented in Fig. 5. The mean value of 11.18 ± 2.29 m/s is compared with the mean value of 11.82 ± 2.34 m/s after 3-years. *Box plots*, *notched box plots and lines* are presenting results of mean, range, 75th percentiles, median and 25th percentiles of PWV in CHPs. Abbreviations: *PWV* pulse wave velocity, *CHP* chronic hemodialysis patients, *36 mon* 36-months follow-up period

Accelerated arteriosclerosis is a major risk to long-term survivors on maintenance hemodialysis (Safar et al. 2002). Myocardial infarction and cerebrovascular events occupy an important place in the mortality of these patients. The CV mortality rate of CHPs is approximately 20 times higher than that of the general population, and the cerebrovascular death rate is nearly 10 times higher.

The arterial system in ESRD patients undergoes structural remodeling very similar to changes with aging, and is characterized by diffuse dilation, hypertrophy and stiffening of the aorta and major arteries. In comparison with nonuremic patients, the intima-media thickness of major central arteries is increased in ESRD patients (London et al. 1997). There are large-scale cohort studies, which provide evidence that high PWV as a noninvasive marker for arterial stiffness is a useful predictive marker for CV events in subjects with CKD, hypertension and diabetes. Since epidemiological and clinical studies have shown that damage of large arteries is a major contributory factor to the high CV morbidity and mortality of patients with ESRD, such a population is particularly appropriate to analyze the impact of arterial stiffness on mortality (Blacher et al. 1999, 2003). PWV is a strong independent predictor of overall and CV mortality in a population of ESRD patients undergoing

hemodialysis but (Guérin et al . 2001) have recently showed that arterial stiffness is not only a risk factor contributing to the development of CV disease but is also a marker of established more advanced, less reversible arterial changes.

Avramovski et al. (2014) in 36-month follow-up period, comparing the PWV results in survived (11.26 ± 2.37 m/s) and nonsurvived ESRD patients (13.13 ± 1.70 m/s) got significantly ($p < 0.001$) higher PWV in deceased patients. PWV in deceased patients from CV disease is more pronounced, it is equal to 13.7 ± 1.24 m/s ($p < 0.001$). At first sight, it is not very big difference, only about two and a half meters. But, if we know the fact, that an increase of aortic PWV by 1 m/s corresponds to an age, sex and risk factor adjusted, risk increases for 14 %, 15 % and 15 % in total CV events, CV mortality and all-cause mortality, respectively, the above mentioned fact is not for underestimation. An increase in aortic PWV by 1 SD (standard deviation) was associated with respective increases of 47 %, 47 % and 42 % (Vlachopoulos et al. 2010b).

Estimation of Cut-Off Point

The most relevant way of structuring the comparison groups of ESRD, in order to obtain the statistical significance between them is grouping by cut-off PWV value. The PWV cut-off point value for ESRD patients where the sensitivity and specificity are highest (94.1 % and 61.4 %, respectively) is 11.8 m/s. Avramovski et al. found that the PWV cut-off point of 11.8 m/s is predictive of increased mortality in ESRD patients, especially for CV mortality. However, different studies have determined different cut-off points of PWV that is predictive of increased overall and CV mortality. The cut-off point of 12 m/s or greater was chosen based upon a study demonstrating this to be the level associated with clinically significant negative prognosis in patients with ESRD (Covic et al. 2005). Based on receiver operating characteristics (ROC) curve analysis mean PWV levels in CHPs show an optimal cut-off point at 12.0 m/s, while mean PWV levels in GPPs show an optimal cut-off point at 9.6 m/s/ (Boutouyrie 2010). The role of age in presenting normal, reference and cut-off point values needs careful consideration. As for blood pressure, it is not immediately clear whether normality should be defined according to age. It is now known that considering the PWV of 11.8 m/s as a relevant cut-off point speed, for all-cause and especially for CV mortality prediction generates two different subgroups of ESRD patients. There are statistically significant differences between those subgroups ($p < 0.001$) according to PWV value (13.65 ± 1.32 vs. 9.76 ± 1.29 for each one).

Predictors of Cardiovascular Survival

Vertical drop in a plot of the Kaplan-Meier indicates an event as series of horizontal steps of declining magnitude approaching the true survival function in CHPs (Fig. 6). Considering the fact that the mean value of PWV is 8.3 m/s (mean age

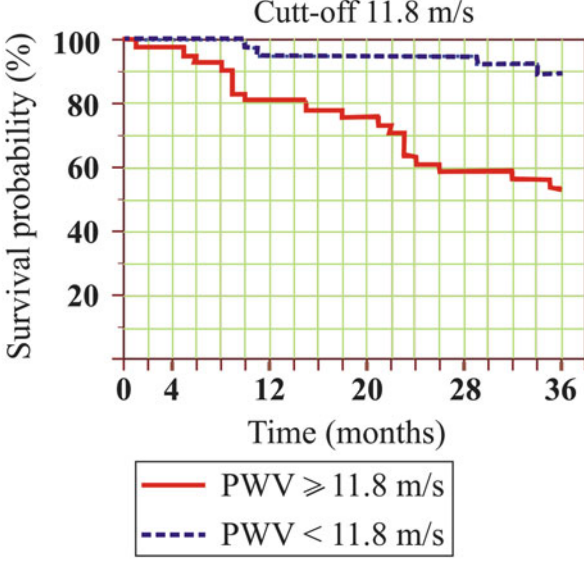

Fig. 6 Kaplan-Meier survival time according different cut-off value of PWV and CV events. This figure presents survival probability in CHPs according different PWV (above or below the cut-off velocity): PWV ≥11.8 m/s (*red staircase line*) and PWV <11.8 m/s (*blue staircase line*). Every vertical drop in a plot of the Kaplan-Meier indicates a CV event as series of horizontal steps of declining magnitude approaching the true survival function in CHPs during 36-months follow-up period. Abbreviations: *PWV* pulse wave velocity, *CV* cardiovascular, *CHP* chronic hemodialysis patients

61.0 years) in the general population (Inoue et al. 2009) the majority of patients with ESRD could be considered to have increased arterial stiffness and elevated PWV equal to 12.50 m/s (mean age 59.3) as results of accelerated atherosclerosis (Avramovski et al. 2014). It is now known that there is a more than fourfold increased relative risk for lethal outcomes (all-causes mortality) in subgroups with more stiffened arteries (PWV ≥11.8 m/s, $P = 0.0037$). Relative risk for exposed groups according to CV lethal outcomes is a about 14-fold increased risk in subgroups with more stiffened arteries (PWV ≥11.8 m/s, $P < 0.0080$).

Avramovski hypothesized that there is no differences in survival in both subgroups of patients on dialysis just below and above the cut-off point (11.8 m/s). Comparative results of the two curves [(logrank), $x^2 = 13{,}1001$; degree of freedom (DF) = 1; significance (P) = 0,0003; relative risk = 0.1744; 95 % CI = (0.0767–0.3965)] indicate significantly higher CV mortality in patients with PWV above cut-off point (PWV ≥11.8 m/s). With threshold of 0.95 or security risk error of 0.05, Avramovski rejected the null hypothesis and concluded that there is a statistically high significant difference (significance is very large) in both subgroups of dialysis patients with different PWV, regarding survival. Survival time according to different cut-off value of PWV dependent on CV events is presented in Fig. 6.

Are the traditional risks factors for atherosclerosis sufficient alone to describe high prevalence of CV disease in this condition? The traditional risk factors for

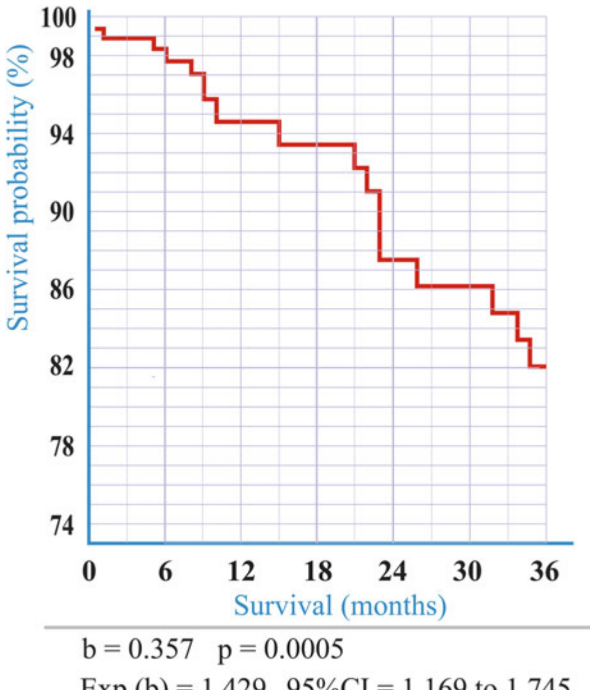

Fig. 7 Cox-regression survival analysis (predictors of CV outcome). The results from Cox-regression analysis of CV survival according to PWV as an independent predictor for the CV outcome in CHPs: regression coefficient $b = 0.357$, p value $= 0.0005$, hazard ratio coefficient Exp (b) = 1.429 and 95 % CI of Exp (b) = 1.169–1.745 are presented in Fig. 7. *Red staircase line* indicates CV survival probability in CHPs in 36-months follow-up period. Every vertical drop in a plot means one fatal CV event. Abbreviations: *CV* cardiovascular, *CHPs* chronic hemodialysis patients, *PWV* pulse wave velocity, *CI* confidence interval

atherosclerosis (age, elevated blood pressure, smoking status, low levels of HDL cholesterol, high levels of LDL cholesterol and triglycerides, obesities and diabetes) interacting to initiate atherosclerosis and promote the development of CV disease have enhanced our ability to assess risk in individual patients. In addition, understanding of new, so-called novel risk factors (CRP, homocysteine, plasma fibrinogen, interleukin-10, impaired glucose tolerance and metabolic syndrome) and when these are included along with the classic risk factors in assessing the global risk profile, may improve ability to predict future risk precisely. In uremic patients, traditional risk factors are added to specific, disease-related (inflammation and malnutrition) and treatment-related risk factors (incompatibility of dialysis membrane and dialysis adequacy) (Fruchart et al. 2004).

Using Mantel – Cox- regression analysis (proportional hazards regression) of CV survival in hemodialysis patients, the potential predictors of events ending with death we're analyzed. Assessments (regression coefficient [b], hazard ratio coefficient Exp [b], p value, and 95 % CI [confidence interval] of Exp [b]) of independent predictors for CV outcome after Cox-regression model analysis are presented in Fig. 7.

According to the Cox-regression analysis, the significant covariates retained by the model (backward stepwise) are only PWV, CRP and albumin. Covariates with positive regression coefficients (b), PWV (0.357) and CRP (0.083) are predictors of the CV events. They indicate decreased hazard and increased survival time. Albumin, as covariate with negative regression coefficient (b) (−0.1881), indicates

decreased hazard and increased survival time. The predictor PWV has an Exp (b) hazard ratio coefficient of 1.429. The HR increases by 1.429 (42.9 %) with each unit increase in PWV. Foremost biomarker in predicting CV risk is PWV with more expressed statistical significance ($p < 0.0001$) than statistical significance of other covariates (CRP, $p < 0.001$; albumin, $p < 0.003$).

Aortic PWV may represent a surrogate end point, which may in fact indicate in which patients the traditional CV risk factors translate into real risk. Summary comparative results from meta-analysis of the predictive value of aortic stiffness (carotid-femoral PWV) for all-cause and CV events are presented by Vlachopoulos et al.: HR: 1.63 for CV and 1.61 for all-cause mortality; HR: 1.44 for CV and 1.35 for all-cause mortality; HR: 1.20 for CV and 1.14 for all-cause mortality. Considering earlier before mentioned arguments, it remains to explain whether PWV as the main determinant of arterial stiffness, has some independent predictive value for the overall and CV – mortality. Several pathophysiological mechanisms may explain the association between increasing PWV and CV-mortality. Increased stiffness of the arteries is the cause of premature return of reflected waves in late systole, resulting in increased central pulse pressure and further ventricular overload. It reduces ejection fraction and increases the myocardial oxygenation demand.

Potential Applications to Prognosis, Other Diseases or Conditions

The estimation of PWV as an indicator of artery stiffness has never been ascertained as a CV risk marker. Recently, many studies have confirmed its importance that aortic PWV is strongly associated with the presence and extent of atherosclerosis and constitutes a forceful marker and predictor of CV risk in *hypertensive patients*, *diabetes*, *chronic kidney disease*, *rheumatoid arthritis*, *degenerative disease* and many other diseases. PWV as a biomarker of disease is a predictor of coronary heart disease and stroke in a population-based study among apparently healthy subjects (Mattace-Raso et al. 2006), and provides additional predictive value above CV risk factors, measures of atherosclerosis, stiffness and pulse pressure. Viscoelastic properties of large arteries play an essential role in CV hemodynamics, especially in systolic blood pressure determination.

Large artery damage is a major contributing factor to the elevated CV morbidity and mortality observed in CV risk factors such as **hypertension**. Rich qualitative and quantitative information about the large arteries (stiffness, distensibility, pulsatility, compliance) is easily obtained by Doppler determination of PWV. Reduced arterial distensibility contributes to a disproportionate increase in systolic pressure and an increase in arterial pulsatility that is associated with an increase in CV morbidity and mortality. A number of longitudinal studies among hypertensive patients report the effects of elevated PWV as an independent predictor of cerebrovascular diseases and all-cause mortality. The relative risk of stroke mortality is 1.7 for PWV elevation of 4 m/s and that of all-cause mortality is 2.1 for PWV elevation of 5 m/s (Laurent et al. 2003).

Patients with **type 2 diabetes** have increased stiffness of central elastic arteries. However, whether peripheral muscular artery stiffness is equally affected by the

disease remains sparsely examined. Diabetes is a predictor of central artery stiffness, and glucose is a determinant of peripheral artery stiffness (Zhang et al. 2011). Hyperhomocystinaemia is associated with macro and microangiopathic diabetic complications. Vitamin B_{12} is significantly associated with homocystein concentrations and is identified as a marginally independent correlate of PWV in diabetic patients in the absence of folate deficiency. Elevated homocystein and reduced vitamin B_{12} have a key role in the development of atherogenesis in diabetic patients (Shargorodsky et al. 2009). Mortality risk doubled in subjects with diabetes (hazard ratio 2.34, 95 % CI 1.5–3.74) and in those with glucose intolerance (2.12, 95 % CI 1.11–4.0) compared with controls (Cruickshank et al. 2002).

Rheumatoid arthritis (**RA**) is a chronic, systemic, inflammatory disease, which is associated with increased CV risk that is not explained by traditional CV risk factors but may be due in part to increased aortic stiffness, an independent predictor of CV mortality (Mäki-Petäjä et al. 2006). The involvement of the CV system in the course of inflammatory lesion and connective tissue diseases may result in serious morbidity and mortality. PWV as an indicator of arterial distensibility predicts CV risk in RA patients. Decreased dilatation capacity leads to a reduction in arterial blood pressure and flow dynamics and impairment in coronary perfusion. Aortic PWV increases by only +6 % per decade in healthy individuals, which suggests that subjects with RA have arteries 20 years older than chronological control subjects. Contrary to findings by Klocke et al. (2003), there is not a significant difference in PWV between the RA and control groups.

Systemic inflammation may contribute to the increased incidence of CV disease in RA, because inflammation is known to play a pivotal role in the pathogenesis of CV disease (Libby 2002). All these changes in the CV system are in line with increased CV morbidity and mortality in RA patients. The CV mortality risk in patients with RA is approximately equal to the CV risk in patients with diabetes. In a prospective study, the 3-year incidence rate of fatal and nonfatal CV events was 9.0 % in RA patients and 4.3 % in the general population. Compared with the non-diabetic population, non-diabetic patients with RA and those with type 2 diabetes had comparable hazard ratios, 2.16 and 2.04 respectively (Peters et al. 2009). It is very likely that the use of common risk calculators (e.g., Framingham, SCORE) will underestimate the CV risk in RA patients.

Effective control of inflammation reduces the CV risk in patients with RA as it improves arterial stiffness and endothelial function. The measurement of PWV gives data values about the current state of blood vessels inflammation and assesses to the risk of CV disease in RA patients. This diagnostic method for assessment of arterial stiffness is useful not only to assess vascular features of blood vessels (rigidity, elastance, compliance, distensibility) but can assess the effectiveness of certain drugs in the course of therapy in certain diseases.

Conventional and noninvasive tools for evaluating atherosclerosis have been recently developed and are currently in use. The use of PWV has received increasing attention as a non-invasive method to measure vascular injury (Khoshdel et al. 2007). The first use of PWV was reported in 1922 in a study examining an association between age and arterial stiffness (Bramwell and Hill 1922). During the

1960s, as new techniques were developed to assess pulse wave and pressure, reports on PWV increased. At the time, PWV measurement procedures were complicated and not suitable for diagnostic purposes. The development of ultrasound Doppler technique made a big step forward, so now measuring the PWV has become a relatively simple method for fast, inexpensive, accurate and routine assessment of arterial stiffness. Following its commercialization in 1999, many reports on PWV have been published.

Here, we have indicated the potential value of using PWV, which is a convenient, inexpensive, and noninvasive test to identify vascular injury and predict vascular disease. The European Society of Hypertension (ESH) and the European Society of Cardiology (ESC) have added PWV measurement as an early index of large artery stiffening in the "2007 Guideline for the Management of Arterial Hypertension" (Mancia et al. 2007).

Summary Points

- This chapter focuses on arterial stiffness, which describes the reduced capability of an artery to expand and contract in response to pressure changes.
- Increased central arterial stiffening is a hallmark of the aging process and the consequence of many disease states, such diabetes, atherosclerosis, and chronic renal compromise.
- The pulse wave velocity is a physiological phenomenon that is used to assess the stiffness of large vessels, measuring the speed of pressure wave propagation, not the displacement of the blood.
- While traditional risk factors predominated in the general population, nontraditional risk factors (uremia, infection, biocompatibility of dialysis membranes, acidosis, etc. . .) play an increasingly important role, being perhaps dominant in end-stage renal disease patients.
- The larger stiffness of the blood vessels in patients on hemodialysis, which occurs earlier and progresses rapidly, increases the speed of the pulse wave and the number of cardiovascular events.
- Aortic pulse wave velocity may represent a surrogate end point, which may in fact indicate in which patients the traditional cardiovascular risk factors translate into real risk.
- The salient finding of this chapter is that the pulse wave velocity was a strong independent predictor of cardiovascular mortality with high-level performance values, assessed by simple, indirect, reproducible, and noninvasive evaluation of regional arterial stiffness in chronic dialysis patients.

References

Avramovski P, Janakievska P, Sotiroski K, Sikole A. Accelerated progression of arterial stiffness in dialysis patients compared with the general population. Korean J Intern Med. 2013;28:464–74.

Avramovski P, Janakievska P, Sotiroski K, Zafirova-Ivanovska B, Sikole A. Aortic pulse wave velocity is a strong predictor of all-cause and cardiovascular mortality in chronic dialysis patients. Ren Fail. 2014;36:176–86.

Blacher J, Guerin AP, Pannier B, Marchais SJ, Safar ME, London GM. Impact of aortic stiffness on survival in end-stage renal disease. Circulation. 1999;99:2434–9.

Blacher JB, Safar ME, Guérin AP, Pannier B, Marchais JS, London MG. Aortic pulse wave velocity index and mortality in end-stage renal disease. Kidney Int. 2003;63(5):1852–60.

Boutouyrie P. Determinants of pulse wave velocity in healthy people and in the presence of cardiovascular risk factors: "establishing normal and reference values". Eur Heart J. 2010;31:2338–50.

Bramwell JC, Hill AV. Velocity of transmission of the pulse-wave and elasticity of arteries. Lancet. 1922;199:891–2.

Cecelja M, Chowienczyk P. Role of arterial stiffness in cardiovascular disease. JRSM Cardiovasc Dis. 2012;1:11.

Cheung AK, Sarnak MJ, Yan G, et al. Atherosclerotic cardiovascular disease risks in chronic hemodialysis patients. Kidney Int. 2000;58:353–62.

Covic A, Haydar AA, Bhamra-Ariza P, Gusbeth-Tatomir P, Goldsmith DJ. Aortic pulse wave velocity and arterial wave reflections predict the extent and severity of coronary artery disease in chronic kidney disease patients. J Nephrol. 2005;18:388–96.

Cruickshank K, Riste L, Anderson SG, Wright JS, Dunn G, Gosling RG. Aortic pulse-wave velocity and its relationship to mortality in diabetes and glucose intolerance: an integrated index of vascular function? Circulation. 2002;106:2085–90.

Fantin F, Mattocks A, Bulpitt CJ, Banya W, Rajkumar C. Is augmentation index a good measure of vascular stiffness in the elderly? Age Ageing. 2007;36:43–8.

Fruchart JC, Nierman MC, Stroes EG, Kastelein JP, Duriez P. Atherosclerosis: evolving vascular biology and clinical implications. Circulation. 2004; New risk factors for atherosclerosis and patient risk assessment 109(Suppl III):15–9.

Goldsmith D, Ritz E, Covic A. Vascular calcification: a stiff challenge for the nephrologist: does preventing bone disease cause arterial disease? Kidney Int. 2004;66:1315–33.

Guérin A, Blacher J, Pannier B, Marchais ME, Safar EM, London MG. Impact of aortic stiffness attenuation on survival of patients in end-stage renal disease. Circulation. 2001;103:987–92.

Himmelfarb J, Stenvinkel P, Jkizler TA, Hakim RM. The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. Kidney Int. 2002;62:1524–38.

Inoue N, Maeda R, Kawakami H, Shokawa T, Yamamoto H, Ito C, et al. Aortic pulse wave velocity predicts cardiovascular mortality in middle-aged and elderly Japanese men. Circ J. 2009;73:549–53.

Izzo Jr JL, Shykoff BE. Arterial stiffness: clinical relevance, measurement, and treatment. Rev Cardiovasc Med. 2001;2:29–34.

Kanbay M, Afsar B, Gusbeth-Tatomir P, Covic A. Arterial stiffness in dialysis patients: where are we now? Int Urol Nephrol. 2010;42:741–52.

Khoshdel AR, Carney SL, Nair BR, Gillies A. Better management of cardiovascular diseases by pulse wave velocity: combining clinical practice with clinical research using evidence-based medicine. Clin Med Res. 2007;5:45–52.

Klocke R, Cockcroft JR, Taylor GJ, Hall IR, Blake DR. Arterial stiffness and central blood pressure, as determined by pulse wave analysis, in rheumatoid arthritis. Ann Rheum Dis. 2003;62:414–8.

Laurent S, Katsahian S, Fassot C, et al. Aortic stiffness is an independent predictor of fatal stroke in essential hypertension. Stroke. 2003;34:1203–12.

Laurent S, Cockcroft J, Van Bortel L, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. Eur Heart J. 2006;27:2588–605.

Libby P. Inflammation in atherosclerosis. Nature. 2002;420:868–74.

London GM, Marchais SJ, Metivier F, Pannier B. Vascular and cardiac remodeling in end-stage renal disease. Nefrologia. 1997;17 Suppl 1:17–22.

London GM, Blacher J, Pannier B, Guerin AP, Marchais SJ, Safar ME. Arterial wave reflections and survival in end-stage renal failure. Hypertension. 2001;38:434–8.

Mäki-Petäjä KM, Hall FC, Booth AD, et al. Rheumatoid arthritis is associated with increased aortic pulse-wave velocity, which is reduced by anti-tumor necrosis factor-alpha therapy. Circulation. 2006;114:1185–92.

Mancia G, De Backer G, Dominiczak A, et al. 2007 guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). J Hypertens. 2007;25:1105–87.

Mattace-Raso FU, van der Cammen TJ, Hofman A, et al. Arterial stiffness and risk of coronary heart disease and stroke: the Roterdam study. Circulation 2006;113(5):657–63.

Millasseau SC, Stewart AD, Patel SJ, Redwood SR, Chowienczyk PJ. Evaluation of carotid-femoral pulse wave velocity: influence of timing algorithm and heart rate. Hypertension. 2005;45:222–6.

Nichols WW, O'Rourke MF. McDonald's blood flow in arteries: theoretical, experimental and clinical principles. 5th ed. UK: Taylor & Francis; 2005. p. 624.

Otto F. Die Grundform des arterielen pulses. Zeitschrift für Biologie. 1899;37:483–526.

Peters MJ, van Halm VP, Voskuyl AE, et al. Does rheumatoid arthritis equal diabetes mellitus as an independent risk factor for cardiovascular disease? A prospective study. Arthritis Rheum. 2009;61:1571–9.

Quick CM, Berger DS, Stewart RH, Laine GA, Hartley CJ, Noordergraaf A. Resolving the hemodynamic inverse problem. IEEE Trans Biomed Eng. 2006;53:361–8.

Safar ME, Henry O, Meaume S. Aortic pulse wave velocity: an independent marker of cardiovascular risk. Am J Geriatr Cardiol. 2002;11:295–304.

Sagawa K, Lie RK, Schaefer J. Translation of Otto Frank's paper "Die Grundform des Arteriellen Pulses" Zeitschrift für Biologie. 1899;37:483–526. J Mol Cell Cardiol. 1990;22:253–44.

Salvi P, Magnani E, Valbusa F, et al. Comparative study of methodologies for pulse wave velocity estimation. J Hum Hypertens. 2008;22:669–77.

Schroder M, Riedel E, Beck W, Deppisch M, Pommer W. Increased reduction of dimethylarginines and lowered interdialytic blood pressure by the use of biocompatible membranes. Kidney Int Suppl. 2001;78:19–24.

Shargorodsky M, Boaz M, Pasternak S, et al. Serum homocysteine, folate, vitamin B_{12} levels and arterial stiffness in diabetic patients: which of them is really important in atherogenesis? Diabetes Metab Res Rev. 2009;25:70–5.

Utescu MS, Couture V, Mac-Way F, et al. Determinants of progression of aortic stiffness in hemodialysis patients: a prospective longitudinal study. Hypertension. 2013;62:154–60.

Vlachopoulos C, Alexopoulos N, Stefanadis C. Aortic stiffness: prime time for integration into clinical practice? Hellenic J Cardiol. 2010a;51:385–90.

Vlachopoulos C, Aznaouridus K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. J Am Coll Cardiol. 2010b;55:1318–27.

Zaccali C, Mallamaci F, Tripepi G. Traditional and emerging cardiovascular risk factors in end-stage renal disease. Kidney Int Suppl. 2003;85:105–10.

Zhang M, Bai Y, Ye P, Luo L, et al. Type 2 diabetes is associated with increased pulse wave velocity measured at different sites of the arterial system but not augmentation index in a Chinese population. Clin Cardiol. 2011;34:622–7.

Zoccali C, Enia G, Tripepi G, Panuccio V, Mallamaci F. Clinical epidemiology of major nontraditional risk factors in peritoneal dialysis patients. Perit Dial Int. 2005;25 Suppl 3:84–7.

Renal Arterial Resistance Index 49

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Abstract

The kidney is a profusely vascularized organ which, unlike other organs, does not regulate renal blood flow (RBF) mainly by oxygen demand. Reflex (myogenic effect and tubular-glomerular feedback) and neurohormonal mechanisms modulate RBF and

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renal resistances by regulating the tone of afferent and efferent arterioles as well as that of the major resistance vessels in the kidneys. Arterial renal resistances are also influenced by a number of other pathophysiological factors, such as increased arterial stiffness, arterial atherosclerosis, renal parenchymal abnormalities, and intra-abdominal and central venous pressure. Finally, renal resistances can be permanently increased if a microvascular disease and vascular rarefaction occur as a result of vasoconstriction-related ischemia, endothelial dysfunction, and inflammatory cytokine activity.

In this setting, a parameter reflecting renal resistances could offer a useful tool to better characterize renal disease and the risk of progression. This chapter focuses on renal resistance index (RRI), a parameter obtained by pulsed Doppler which reflects renal arterial resistances. The possible clinical usefulness of RRI has been demonstrated in studies which show the pathophysiological correlates and prognostic role in predicting a greater risk of chronic kidney disease progression and cardiovascular events.

Keywords
Renal resistance index • Renal circulation • Chronic kidney disease • Cardiovascular diseases • Heart failure • Worsening of renal failure

Abbreviations

AKI	Acute kidney injury
CHF	Chronic heart failure
CKD	Chronic kidney disease
CVP	Central venous pressure
GFR	Glomerular filtration rate
HF	Heart failure
HFPEF	Heart failure with preserve ejection fraction
HFREF	Heart failure with reduced ejection fraction
NO	Nitric oxide
RAAS	Renin-angiotensin-aldosterone system
RAS	Renal artery stenosis
RBF	Renal blood flow
RI	Resistance index
ROS	Reactive oxygen species
RRI	Renal resistance index
WRF	Worsening of renal function

Key Facts

Key Facts of Cardiorenal Syndrome (CRS)

- Kidney and heart diseases share many pathophysiological mechanisms such as tissue hypoperfusion, venous congestion, inflammatory status, and neurohormonal activation.

- Acute or chronic dysfunction of one of these two organs can lead to functional worsening of the other. Cardiorenal syndrome is the condition in which the heart and kidney can negatively affect each other.
- The Consensus Conference of the Acute Dialysis Quality Group divided this syndrome into five subtypes.
- Types 1 and 2 (cardiorenal syndrome): acute heart failure causing acute kidney injury (AKI) and chronic heart failure leading to a progressive renal dysfunction, respectively.
- Types 3 and 4 (renocardial syndrome): acute kidney dysfunction causing acute heart failure and chronic kidney disease leading to a worsening of cardiac function, respectively.
- Type 5: systemic diseases involving both the kidney and heart.
- Therefore, in patients with heart failure, an accurate evaluation of renal function is important, and vice versa; therefore, close collaboration between nephrologists and cardiologists is necessary in order to improve patient prognosis.

Key Facts of Doppler Ultrasonography

- Doppler ultrasonography is a noninvasive technique that uses high-frequency sound waves (ultrasound) to estimate blood flow through blood vessels.
- During examination the operator uses a transducer that sends and receives ultrasounds. The sound waves are reflected by blood cells, and their frequency is modified (increase or decrease) by the movement of red cells (Doppler effect).
- The Doppler shift is the difference in frequency between the reflected ultrasound and the initial ultrasound and is correlated with the velocity of the blood cells.
- A computer receives and processes this information and gives a spectrum or an image that represents the blood flow.
- Three main Doppler techniques are used: continuous wave, pulsed wave, and color Doppler.
- Continuous wave Doppler: the transducer continuously emits trains of ultrasounds along a line and continuously receives reflected signals. Continuous wave Doppler ultrasound is unable to determine the specific location of velocities within the beam.
- Pulsed wave Doppler: the transducer emits a train of ultrasound to a certain depth and then captures the reflected ultrasounds. The computer then calculates the flow velocity at that point.
- Color Doppler: the principle is the same as for pulsed wave Doppler, but analyzes an area of small sample volumes. Based on the velocities calculated in that area, the computer builds a color image that represents the blood flow.

Key Facts of Heart Failure

- Heart failure represents one of the main causes of morbidity and mortality in industrialized countries. The prevalence ranges from 0.4 % to 2.0 % in the European population. The incidence increases with age.
- It is a condition in which the heart is not able to pump an adequate supply of blood to satisfy the body's needs (oxygen and nutrients).
- Heart dysfunction can involve the left or right ventricle or both. However, the left ventricle is usually affected first.
- There are two different mechanisms underlying left heart dysfunction: the heart is not able to contract with enough force (systolic dysfunction), or the ventricle becomes stiff and develops resistance to filling.
- Heart failure is defined as acute if signs and symptoms arise suddenly and require immediate medical treatment. It is defined as chronic if the patient is in a stable clinical condition but has, or has had, signs and symptoms of heart failure for some time.
- Causes of heart failure are coronary artery disease; myocardial infarction; valvular, pericardium, endocardium, or heart muscle disease; congenital heart disease; severe lung disease; diabetes; abnormalities of heart rhythm; etc.
- Possible symptoms are shortness of breath (dyspnea) during activity or at rest, tiredness, and palpitations. Possible signs are high jugular venous pressure, pulmonary crepitations and displaced apical impulse, and excess fluid in body tissues (edema) with swelling of feet, ankles, legs, or abdomen.
- Heart failure treatment includes lifestyle changes, drugs (angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, beta-blockers, diuretics, vasodilators, antiplatelet agents, and anticoagulants), implantable defibrillators to prevent arrhythmic complications, left ventricular assist devices (a mechanical device that helps the heart to pump blood), and, ultimately, heart transplantation.

Definitions

Arteriolosclerosis This is a disease of the small blood vessels characterized by a thickening and hardening of the arteriole wall. It can be due to concentric, smooth muscle wall hypertrophy or the buildup of hyaline material. It reduces blood flow and determines tissue ischemia.

Central venous pressure (CVP) This is the pressure in the thoracic vena cava near the heart; therefore, it is an estimate of pressure in the right atrium. It can be measured invasively using a central venous catheter, but noninvasive evaluation is also possible. The most common method used is to evaluate the diameter of the vena cava and its changes during inspiration.

Ejection fraction This is the percentage of blood ejected by the heart with each beat. It is an indicator of heart contractility. Mathematically, it is the difference

between the end-diastolic volume and the end-systolic volume divided by the end-diastolic volume. Normal values are 55–70 %.

Glomerular filtration rate (GFR) This is considered the best index to assess renal function. It measures kidney filtration capacity using renal clearance of an exogenous marker (inulina or I-iothalamate) or more often an endogenous marker, i.e., serum creatinine. GFR is generally estimated on the basis of serum creatinine levels.

Glomerulosclerosis This is a disease characterized by scarring and hardening of glomeruli. If only a part of glomeruli are affected, then it is defined as focal segmental glomerulosclerosis. It can be idiopathic or due to known causes (HIV, obesity, diabetes, drugs, lupus, etc).

Heart failure with preserved ejection fraction (HFpEF) This term is used to define patients with signs and symptoms of heart failure but who have a normal or mildly reduced ejection fraction, no left ventricle dilatation, abnormalities of cardiac structure, and/or diastolic dysfunction. HFpEF has a higher prevalence among the elderly, females, and patients with hypertension.

Heart failure with reduced ejection fraction (HFrEF) This term is used to define patients with signs and symptoms of heart failure but who have a reduced ejection fraction. The most frequent cause is coronary artery disease. Other possible causes are myocarditis, alcohol abuse, chemotherapy, idiopathic forms, etc.

Pulse arterial pressure This is the difference between the systolic pressure and the diastolic pressure. It represents the maximal change in aortic pressure during systole. It depends on the compliance of the aorta and on cardiac output.

Pulse wave velocity This is the best index of aortic stiffness. It is usually measured by determining the propagation time of the pulse pressure from the carotid to femoral artery. Different methods are used in order for measurement, among which the most common are mechanical methods requiring specific devices. However, it may also be easily measured using high reproducible, noninvasive ultrasound methods. A threshold of >10 m/s has been suggested by a recent expert consensus statement as an index of an altered arterial distensibility in hypertensive patients.

Renal blood flow (RBF) This is the amount of blood that passes through the kidney in a specific time unit. Mathematically, it is the difference between the aortic pressure and renal venous pressure, divided by renal vascular resistance. The normal value is 1,200 ml/min.

Worsening renal function (WRF) In patients affected by cardiovascular diseases, this is generally defined as an increase in serum creatinine of >0.3 mg/dl and/or >25 % between two time points. It has been shown to be associated with a worse prognosis, hospitalizations for heart failure and higher mortality.

Introduction

The kidney is a profusely vascularized organ, and, unlike other organs, renal blood flow (RBF) is not mainly influenced by oxygen demand but is determined by reflex (myogenic effect and tubular-glomerular feedback) and neurohormonal mechanisms that regulate the tone of afferent and efferent arterioles as well as of the major resistance vessels in the kidneys (Braam et al. 2012). Moreover, arterial renal resistances are also influenced by a number of other factors, such as an increased arterial stiffness, arterial atherosclerosis, renal parenchymal relevant pathophysiological abnormalities, and increased central venous pressure. These factors can cause a permanent increase in vascular resistance due to microvascular remodeling and capillary rarefaction caused by vasoconstriction-related ischemia, endothelial dysfunction, and the production of inflammatory cytokines and fibrosis (Chade 2013). Therefore, a parameter that provides information on functional and permanent changes in RBF by reflecting abnormalities in renal arterial resistances could offer an incremental value to better characterize renal function.

This potential use is even more obvious when considering the limitations of the parameters currently used to assess renal function. The estimation of renal function is generally based on the calculation of glomerular filtration rate (GFR) by serum creatinine levels (Stevens et al. 2006). While creatinine serum levels and estimated GFR represent the cornerstone in the evaluation of renal function and its worsening, they are limited by several factors, such as between-person and within-person variability, age, diet, gender and body mass, the active creatinine tubular secretion (Damman et al. 2012), drug interference, and the loss of muscle mass frequent in the end stages of systemic diseases (Smilde et al. 2006). Finally, it is worth noting that the kidneys use only part of their filtering capacity. A normal GFR does not exclude an impairment in filtration capacity; in other words, a normal GFR in kidneys with a reduced renal reserve which increases the filtration capacity of residual nephrons (Bosch et al. 1995) could be observed. Due to the limitations of creatinine, new biomarkers have been proposed to detect early glomerular dysfunction (cystatin C) or identify tubular damage (neutrophil gelatinase-associated lipocalin, *N*-acetyl-beta-glucosaminidase, and kidney injury molecule) preceding the drop in GFR.

In this clinical setting, a parameter reflecting RBF alterations could be useful because, by detecting abnormalities, patients at a higher risk of renal disease onset and/or its progression could be better identified (Fig. 1). This more accurate evaluation of renal function could be relevant not only for the treatment of chronic kidney disease (CKD) patients, but also for a better management of patients affected by cardiovascular diseases.

Over the last few years, more attention has been focused on the link between the kidney and the heart. These two organs are characterized by many common pathophysiological mechanisms which can negatively affect each other, and, consequently, the term "cardiorenal syndrome" has recently been introduced. This term indicates a condition characterized by an acute or chronic dysfunction of one of the two organs which may induce the acute or chronic dysfunction of the other (Ronco et al. 2008).

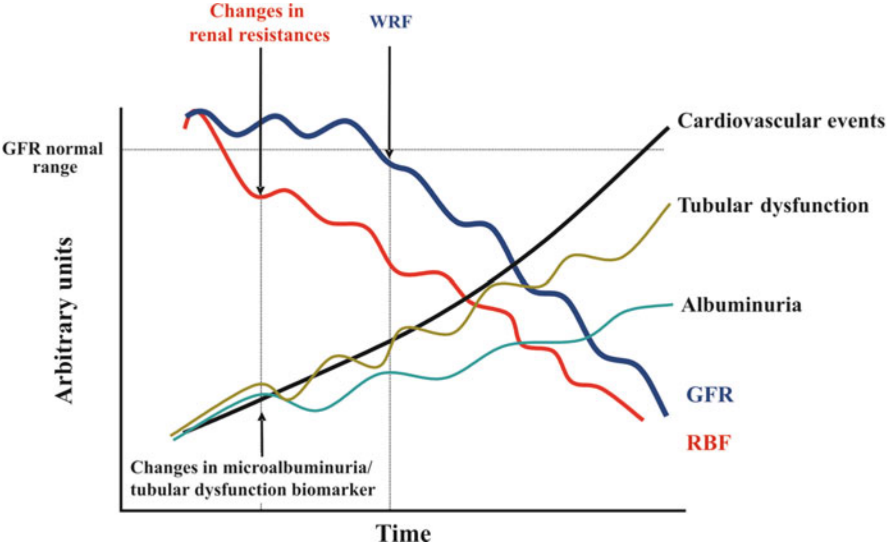

Fig. 1 **Changes in renal blood flow preceding the drop in glomerular filtration rate**. The pathophysiological background of possible clinical usefulness of renal resistances (Modified by Damman et al. 2010). The glomerular filtration rate drops when the kidneys are no longer able to compensate for the loss of nephrons by enhancing the filtration of those remaining. Renal resistance index, as well as microalbuminuria and tubular dysfunction biomarkers, could provide early detection of functional and structural renal changes preceding the fall in GFR. *GFR* glomerular filtration rate, *RBF* renal blood flow, *WRF* worsening of renal function

In patients affected by chronic cardiovascular diseases and especially in those with heart failure (HF), CKD as well as worsening renal function (WRF), i.e., type 2 cardiorenal syndrome, represents a clinical condition associated with a poor prognosis (Hillege et al. 2000; Smith et al. 2006; Damman et al. 2009).

The aim of this chapter is to focus on the possible clinical role of renal arterial resistances in patients with renal and cardiovascular diseases.

Renal Circulation and Renal Arterial Resistances

Twenty-two percent of cardiac output is directed to the kidney. It has a terminal-type circulation, without anastomosis (Fig. 2). At the level of the hilum branch, the renal arteries branch out into the segmental, arcuate, and interlobular arteries. The afferent arterioles derive from the interlobular arteries leading into the glomerular capillaries that coalesce to form the efferent arterioles. Efferent arterioles give rise to a second capillary network, i.e., peritubular capillaries that are followed by the venous system (Chade 2013).

In order to control arterial pressure and water and sodium reabsorption, reflex mechanisms (such as myogenic effect and tubular-glomerular feedback) and neuro-hormonal mechanisms (sympathetic system, renin-angiotensin-aldosterone system,

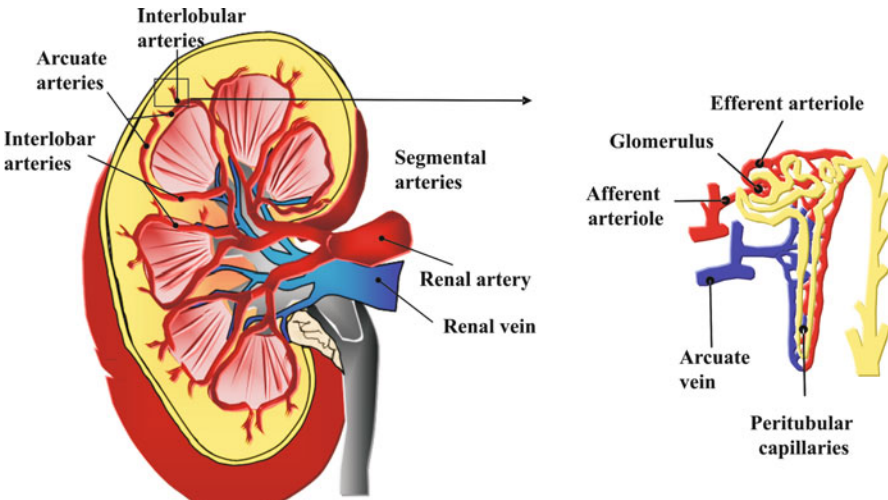

Fig. 2 **Anatomy of renal circulation**. The renal arterial and vein anatomy is shown

endothelin, nitric oxide, prostaglandins, bradykinin, and natriuretic peptides) modulate the arteriolar tone, thus affecting renal resistance.

The myogenic effect is a fast mechanism consisting in the vasoconstriction of kidney small vessels when wall tension increases. The tubuloglomerular feedback is a slower mechanism of vasoconstriction of the afferent arteriole that occurs when an increase in GFR produces a rise in NaCl concentration, which is then sensed by the macula densa, and a vasoconstrictor mediator (probably adenosine) is secreted. Of the neurohormonal mediators, the most important are catecholamines and the renin-angiotensin-aldosterone system (RAAS) which determine vasoconstriction and an increased reabsorption of water and sodium (Braam et al. 2012).

The RBF self-regulation maintains the renal perfusion constant for arterial pressure changes between 70 and 180 mmHg. The abovementioned mechanisms regulate RBF and GFR in order to keep them constant when there are fluctuations in renal perfusion pressure both in physiological situations and pathological conditions.

A depletion or an overstimulation of these systems, for example, in heart failure, could determine an increase in intrarenal resistances and a dissociation between cardiac output and RBF. This is the consequence of a disproportional decrease in renal perfusion pressure and RBF in reduced cardiac output (Braam et al. 2012).

The arterial renal resistances are also influenced by a number of other relevant pathophysiological factors such as increased arterial stiffness, arterial atherosclerosis, renal parenchymal abnormalities, and increased central venous pressure (Fig. 3). Moreover, endothelin, nitric oxide (NO), prostaglandins, bradykinin, and natriuretic peptides are also involved in the regulation of arteriolar tone.

Among these, the decreased availability of NO seems to play a pivotal role in determining abnormalities in renal circulation. A reduction, mediated by oxidative stress (reactive oxygen species, ROS), promotes vasoconstriction. Moreover,

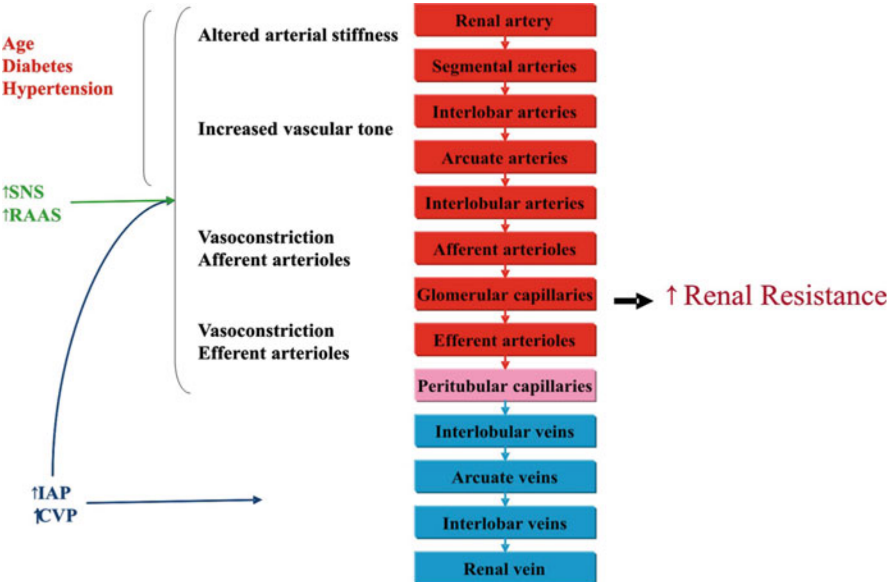

Fig. 3 Factors influencing renal circulation. The factors influencing renal perfusion and renal resistances are summarized. The myogenic reflex and the tubular-glomerular feedback regulate the tone of afferent and efferent arterioles. However, arterial renal resistances are also influenced by a number of other pathophysiological factors, such as neurohormonal mechanisms, arterial stiffness, arterial atherosclerosis, renal parenchymal abnormalities, and intra-abdominal and central venous pressure. *CVP* central venous pressure, *IAP* intra-abdominal pressure, *RAAS* renin-angiotensin-aldosterone system, *SNS* sympathetic nervous system

ROS-mediated inflammation further reduces NO availability thus perpetuating a vicious circle leading to permanent changes in renal vasculature.

In fact, the ischemia related to endothelial dysfunction and the inflammatory cytokines can cause fibrosis, vascular remodeling, and rarefaction (Chade 2013). This latter condition is generally due to changes in renal microcirculation, which are also defined as microvascular disease, and is a frequent feature of CKD. When it occurs, a permanent increase in renal resistances and a further decline in renal function can be observed.

Doppler Evaluation of Arterial Resistances

Arterial pulsed Doppler has been proposed as a useful tool to estimate the arterial resistance of an organ. The parameter generally used is the Doppler resistive index (RI), i.e., a measure obtained from pulsed wave Doppler velocity curves of the peripheral arteries according to the Pourcelot equation ([peak systolic velocity – end-diastolic velocity]/peak systolic velocity) (Pourcelot 1974).

The first studies on RI were aimed at evaluating its role in the assessment of the grade of stenosis of internal carotid artery lesions (Pourcelot 1974). Several studies

have subsequently suggested the possible usefulness of RI also in the evaluation of other vascular beds, among which the kidney.

Assessment of renal resistance index. The examination of kidney and renal arteries is usually performed by an anterior approach by means of a convex ultrasound probe. The patient should be adequately prepared to avoid intestinal gases.

Nonetheless, it is possible to evaluate the renal resistance index (RRI) at the end of an echocardiogram also using the echocardiographic probe (Ciccone et al. 2014). With the patient in a lateral or sitting position, the scan images of the kidney are obtained from a posterior approach. The use of Color or Power Doppler helps to localize the vessels.

RRI slightly decreases from the hilum toward the renal cortex. Usually, pulsed Doppler volume sample is placed at the level of the segmental arteries. It is necessary to achieve the best alignment between the ultrasound beam and flow direction to record Doppler velocities maximizing waveforms gain and size, to obtain peak systolic velocity and end-diastolic velocity (Fig. 4). Three to five reproducible waveforms should be evaluated.

In healthy adult subjects, the RRI mean value is around 60 with nonsignificant differences between the two kidneys (Pontremoli et al. 1999). The index is affected by extreme bradycardia and tachycardia and pathologically increases with severe aortic valve insufficiency and decreases with severe aortic valve stenosis. It is less reliable during arrhythmias (Krumme et al. 2007).

An RRI greater than 70 is generally considered abnormal in adults, while it could physiologically exceed this cutoff in the first years of life (Tublin et al. 2003).

According to a recent meta-analysis (Lubas et al. 2014), measures taken by expert staff showed an intraobserver variability ranging from 2.07 % to 5.1 % and an interobserver variability from 3.61 % to 6.2 %. Therefore, overall RRI has a good reproducibility and repeatability

RRI and renal resistances. The relationship between RRI and renal resistance is not actually linear. RRI changes have been evaluated in an ex vivo system in which vascular compliance and resistance could have been separately modified (Bude et al. 1999). The study authors concluded that RRI is influenced by a combination of compliance and resistance. For fixed compliance, the RRI increases with increasing resistance.

In vivo conditions are even more complicated than this because many factors play a role in perfusion pressure regulation. Also, considering that the majority of published studies have enrolled nonhomogeneous study populations, this preamble explains why the literature on RRI sometimes produced discordant results that have caused some skepticism about the routine use of this parameter in clinical practice.

Renal artery stenosis and RRI. Besides the exploration of the entire renal artery, also the examination of the intrarenal arteries is extremely important in the diagnosis of renal artery stenosis (RAS) (Fig. 5). The RRI, together with the acceleration time index, is in fact an indirect parameter of stenosis. In the presence of a significant RAS, reduction in blood flow and pulsatility occur leading to a tardus-parvus pattern Doppler waveform characterized by a longer time needed to achieve the peak systolic velocity. In addition, the vasodilatation of the distal capillary bed produces

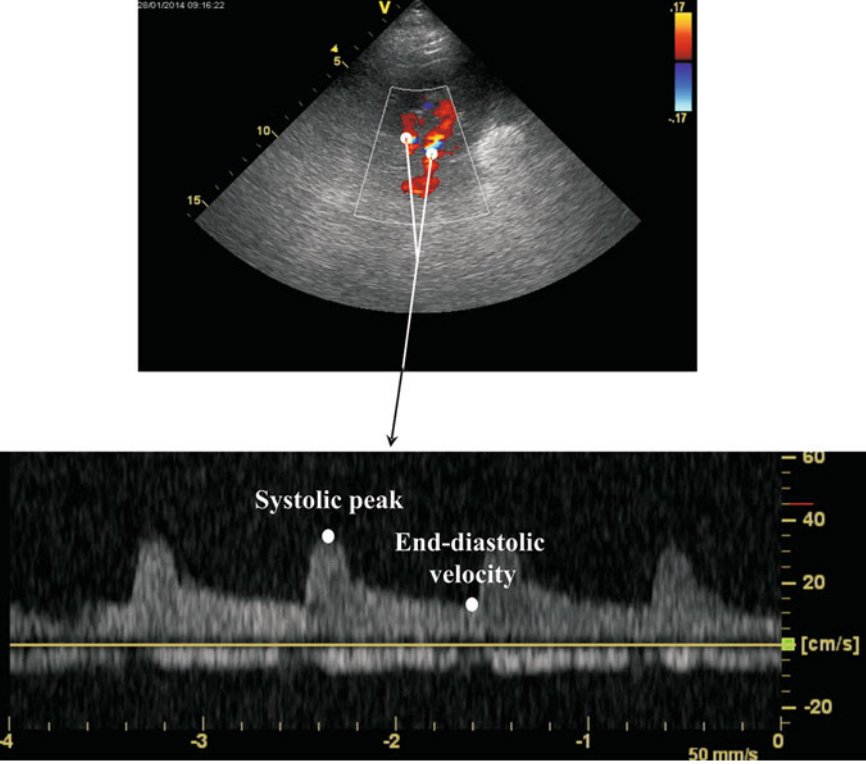

Fig. 4 **Renal resistance index calculation**. Calculation of the renal resistance index. The renal arterial Doppler was performed using a 4 MHz probe with the patient in the sitting position and using a posterior approach to visualize the kidney. The course of the segmental arteries is visualized by color Doppler flow, and, at the middle tract level of the best one visualized, pulsed wave Doppler is performed. Peak systolic velocity and end-diastolic velocity are used to calculate the renal arterial resistance index according to Pourcelot formula

a reduced peak systolic velocity and a relatively increased end-diastolic velocity, reducing overall the poststenotic RRI.

Zeller and Schwerck have proved, in different studies, the accuracy of a side-to-side difference in an RRI >5 to detect RAS, while an AT >100 ms was found to be reliable for the diagnosis, with an 89 % sensitivity and a 91 % specificity (Zeller et al. 2008).

Factors Influencing RRI

In the absence of a significant renal artery stenosis, RRI is influenced by a number of physiological and pathophysiological factors able to modify arterial and venous RBF.

Aging and arterial stiffness. As previously mentioned, RRI could be influenced by changes in arterial compliance and resistance (Bude et al. 1999).

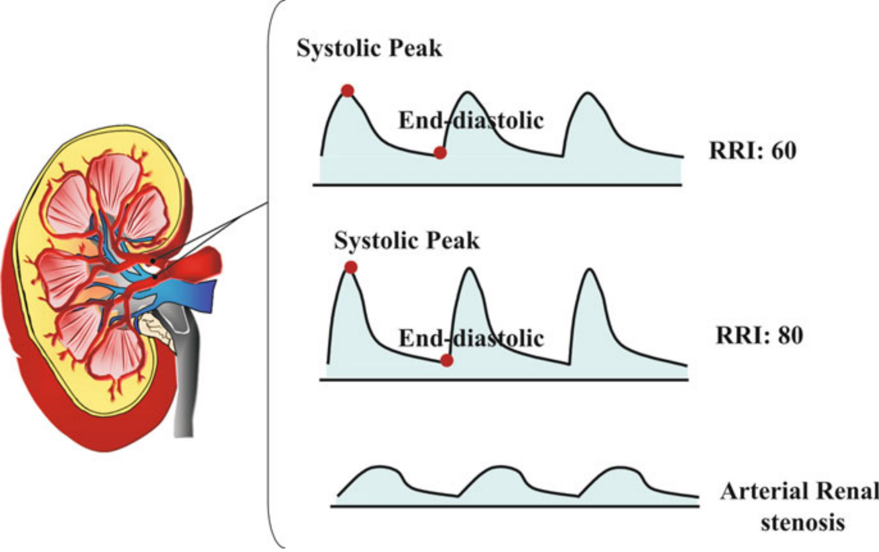

Fig. 5 **Patterns of renal resistance index**. Examples of possible alterations of the renal Doppler pattern. The figure shows a normal subject at the *top*, a subject with increased renal resistances in the *middle*, and a subject with renal arterial stenosis at the *bottom*

Consequently, RRI is influenced by all physiological and pathophysiological factors which change arterial compliance and resistance. It has been widely demonstrated that RRI positively correlates with age, pulse pressure, pulse wave velocity (Schwenger et al. 2006; Lubas et al. 2014; Ohta et al. 2005), and ambulatory arterial stiffness index (Ratto et al. 2006), which are all markers of reduced arterial compliance.

In a population of renal transplanted patients, RRI was correlated to the recipient's ankle-brachial blood pressure index (Heine et al. 2005) and to subject's age, rather than the age of the grafted kidney (Krumme et al. 1997).

All cardiovascular risk factors which can modify arterial stiffness, such as diabetes and essential hypertension, are also able to modify RRI. RRI has been found to be significantly correlated to daytime systolic blood pressure variability (Kawai et al. 2012). Similarly, RRI was also independently associated with blood glucose level (Otha et al. 2008) and insulin resistance (Afsar et al. 2010). These two risk factors affect the RRI not only by modifying arterial stiffness but also by determining parenchymal renal lesion and/or promoting oxidative stress.

Parenchymal renal diseases. Platt et al. (1990) first showed the utility of RRI to differentiate between isolated glomerular disease and vascular or interstitial disease as assessed by renal biopsies. Patients with glomerulopathies had normal RI values (mean value: 58), whereas the others had markedly elevated RI values (mean values are 87 in vascular alterations and 75 in interstitial fibrosis).

Numerous studies have subsequently confirmed the association of RRI with tubulointerstitial injury (Bigè et al. 2012; Sugiura et al. 2004; Boddi et al. 2005) and arteriolosclerosis. In particular, one found that RRI was correlated to renal involvement in patients with progressive sclerosis (Aikimbaev et al. 2001).

It is probable that parenchymal diseases, involving any parenchymal component, produce a scarring process that, in turn, leads to a reduction in the intrarenal vessels area and to an increase in interstitial pressure, which decreases vascular compliance and increases resistance to blood flow.

Endothelial dysfunction. As previously mentioned, endothelial dysfunction and the related NO imbalance play a key role in the vasomotor nephropathy. Intrarenal NO regulates glomerular hemodynamics, tubular transport, and tubuloglomerular feedback (Majid et al. 2001). The net result is increased renal and glomerular perfusion, natriuresis, and diuresis.

Changes in NO availability could influence RRI by modifying both afferent and efferent arteriole tone as well as renal medullary blood flow. Moreover, the ischemia related to reduced NO and the related increase in inflammatory cytokines can cause fibrosis, vascular remodeling, and rarefaction (Chade 2013).

Although these are potential effects, there are limited data about the relationship between RRI and endothelial dysfunction (Bruno et al. 2011).

Intra-abdominal and central venous pressure. Another important determinant of renal resistances is renal venous pressure. Its changes influence RBF more than changes in arterial blood pressure. It has been demonstrated experimentally that RBF decreases when renal venous pressure increases (Winton 1931).

The mechanisms influencing renal venous pressure include the obstruction of renal vein (thrombosis, neoplastic mass, inferior vena cava syndrome), an increase in abdominal pressure (ascites and hemorrhage), and a rise in central venous pressure (CVP) (HF and pulmonary hypertension) (Braam et al. 2012).

An increased renal venous pressure causes a decrease in arteriovenous gradient with a consequent reduction in RBF. Moreover, it determines a rise in efferent arterioles and end glomerular capillary pressures, thus inducing a decrease in net filtration pressure and in GFR and an increase in arterial renal resistances (Jessup and Costanzo 2009). Finally, besides the effects on gradients of renal vasculature, an increased renal venous pressure causes a rise in interstitial pressure. This is due to the low compliance of interstitium and to the tight capsule of the kidney (Braam et al. 2012).

As a consequence, in the clinical setting of heart failure, venous congestion is the other determinant mechanism responsible for the decrease in renal perfusion together with reduced cardiac output. This has recently been supported by a study evaluating a series of CHF outpatients in which a high CVP was independently associated with higher RRI values (Ciccone et al. 2014).

This is even more interesting considering that both in patients with acute and chronic heart failure (CHF) an increased CVP has been found to be associated with a WRF and a progression of cardiorenal syndrome (Damman et al. 2007; Winton 1931; Mullens et al. 2009; Iacoviello (a) et al. 2013).

Potential Applications to Prognosis

Arterial Hypertension

Several data show that a high RRI in hypertensive patients is also associated to organ-damage markers such as left ventricular hypertrophy and carotid intima-media thickness (Florczak et al. 2009; Kawai et al. 2012; Parolini et al. 2009; Otha et al. 2008; Doi et al. 2012).

Independently from the presence of overt nephropathy, hypertensive patients show higher vascular resistance and a higher RRI (Raff U et al. 2010).

In 426 hypertensive patients, Doi and coll. (2012) demonstrated that RRI >73 for males and >72 for females was predictive of a combined end point of cardiovascular and renal events (i.e., all-cause death, myocardial infarction, stroke, congestive heart failure requiring hospitalization, aortic dissection, and end-stage renal failure requiring regular hemodialysis).

Although RAAS inhibitors seem to decrease RRI values, showing a renoprotective effect, in some studies the stratification of the population on the basis of the use of these drugs did not alter the prognostic results (Sugiura and wada 2009; Doi et al. 2012).

Diabetes

As in arterial hypertension, diabetes is a systemic disease with a multiorgan target. Diabetic nephropathy is characterized by nodular and diffuse forms of intercapillary glomerulosclerosis, afferent and efferent glomerular arteriolar hyalinization, tubular atrophy, and interstitial fibrosis (Ohta et al. 2005), although glomerulosclerosis is the histological hallmark of the disease.

It has been demonstrated that in diabetic patients, high RRI values are significantly correlated to the observation of microvascular diabetic complications, including nephropathy, retinopathy, or sensory neuropathy (Liu et al. 2012). Moreover, in patients with renal diseases, RRI is higher in diabetic nephropathy than in chronic glomerulonephritis and/or nephrosclerosis (Ohta et al. 2005). Finally, higher RRI in diabetic patients is also associated with left ventricular diastolic dysfunction (MacIsaac et al. 2008), thus highlighting that type 2 diabetes leads to multiorgan damage (including simultaneous injury to the heart and kidneys).

In advanced CKD (eGFR <30 ml/min/1.73 m2), however, the RRI difference between diabetic and nondiabetic patients loses statistical significance (Kawai et al. 2011), thus suggesting the prevalent relevance of the advanced renal disease in affecting the value of the parameter.

Renal Diseases

More data are available for RRI in renal diseases.

Chronic kidney disease. In this clinical setting, several studies have shown that the RRI assessed at enrollment is predictive of WRF (Radermacher et al. 2002; Ikee et al. 2005; Splendiani et al. 2002; Sugiura et al. 2009; Parolini et al. 2009; Hanamura et al. 2012).

Splendiani et al. (2002) demonstrated a positive correlation between the initial value of RRI and the percentage of serum creatinine variation.

Radermacher and coll. (2002) found that RRI >80 can identify patients with a worse prognosis. In a multivariate regression analysis, only proteinuria and RRI were independent predictors of progressive renal dysfunction. In a study conducted by Hanamura and coll. in patients with CKD (Hanamura et al. 2012), a RRI >70 was an independent risk factor for WRF, and it was considered a marker of organ dysfunction, histological damage, and renal prognosis and a possible determinant for steroid therapy.

Bigè and coll. (2012) studied 58 patients affected by CKD, defined according to the KDOQI definition, and undergoing renal biopsy. In this series, a RRI >70 was predictive of a reduction in renal function and identified patients at higher risk of end-stage renal disease independently from GFR baseline values.

Also the studies conducted on renal transplanted patients showed very interesting conclusions. A RRI >80 was predictive of allograft failure. However, it did not permit identification of a specific cause of failure, but could raise the suspicion of vascular complications associated with transplantation, such as arteriovenous fistula or vein thrombosis. What is more important, however, is that the higher values of RRI were associated with patient deaths, also in cases of functioning grafts (Radermacher et al. 2003).

Acute kidney injury. Very recent studies suggest the usefulness of RRI in predicting acute kidney injury (AKI) in intensive care unit patients (namely, patients with polytrauma and sepsis or patients who underwent cardiac surgery) (Schnell et al. 2012; Bossard et al. 2011; Dewitte et al. 2012). Moreover, (Darmon et al. 2011) found even higher values of RRI in patients with persistent AKI.

Renal artery stenosis. Radermacher and coll. (2001) suggested RRI as a marker to predict survival after therapeutic intervention (angioplasty or surgery) for RAS. Patients with a RRI >80 showed no improvement in renal function, hypertension, or renal survival even after a successful procedure. Two subsequent studies (Yuksel et al. 2012; Cianci et al. 2010) confirmed similar results while another disproved them (Zeller et al. 2008).

Heart Failure

Renal impairment in CHF patients is very common and is associated with higher morbidity and mortality (Hillege et al. 2000; Smith et al. 2006; Damman et al. 2009). Therefore, its accurate characterization plays a key role in the management of these patients. The role of RRI in the clinical setting of CHF is a topic of interest since, reflecting kidney vascular and parenchymal abnormalities (Fig. 6), it adds information about renal impairment and the outcome of these patients (Ennezat et al. 2011; Ciccone et al. 2014).

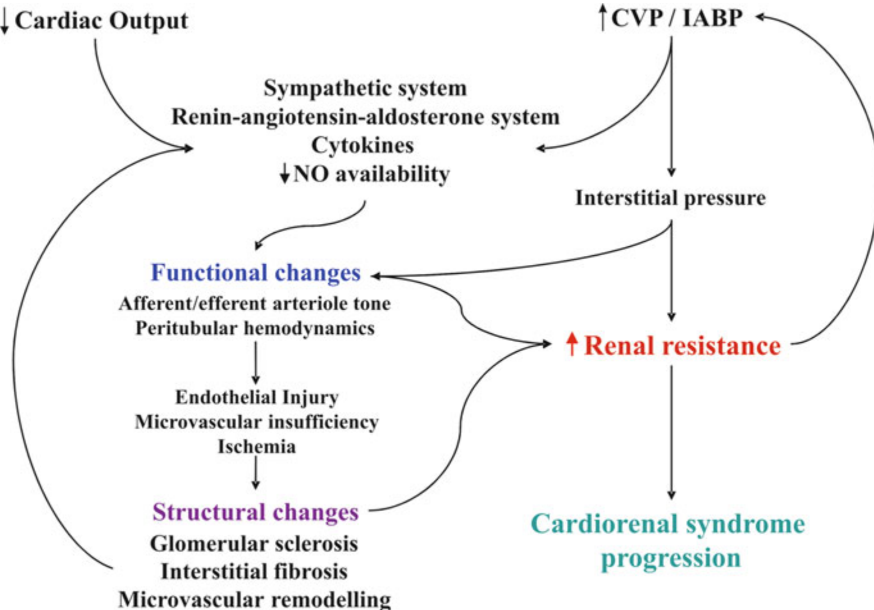

Fig. 6 Pathophysiology of renal resistance index in cardiorenal syndrome. The pathophysiological factors influencing the renal resistances and the consequent prognostic impact on cardiorenal syndrome are summarized. The combination of hemodynamic and neurohormonal factors leads to functional and structural changes responsible for the increase in renal resistance index. This further promotes the activation of neurohormonal systems as well as sodium and water retention, thus favoring the progression of cardiorenal syndrome

The first association between high RRI and worse outcome has been demonstrated in a series of patients affected by HF with preserved ejection fraction (HFpEF) (Ennezat et al. 2011). Subsequently, this association has been found in a series of CHF outpatients mainly affected by HF with reduced ejection fraction (HFrEF). In this series, RRI was an independent predictor of both a composite end point reflecting HF progression (i.e., death hospitalization due to HF worsening) (Ciccone et al. 2014) and mortality for all causes (Monitillo et al. 2014).

Also in CHF patients, the different pathophysiological conditions underlying the increase in renal vascular resistance and, as a consequence, in RRI can explain the relevant prognostic information carried by this parameter. This is confirmed by the independent association that has been found in CHF patients between RRI and the variables reflecting the presence of atherosclerosis and/or an increased arterial stiffness (i.e., age, diabetes, and pulse pressure) as well as an increased CVP.

This pathophysiological background underlying RRI values can also explain its incremental value when added to GFR. A high RRI could identify patients at higher risk of HF progression both in patients with GFR above and below 60 ml/min*1.73 m^2. On the other hand, patients with low RRI were characterized by a

similar risk of events also when dichotomized according to the presence or not of reduced GFR (Ciccone et al. 2014).

But it is also worth noting that RRI is not only associated with HF progression but also with WRF in CHF outpatients. In fact a RRI >70 is independently associated with a 1-year increase in creatinine >0.3 mg/dl (Citarelli et al. 2014).

Finally, in CHF outpatients, RRI was also demonstrated to be independently associated with high doses of loop diuretics as well as with their increase during a midterm follow-up (Iacoviello (b) et al. 2015). The increased intrarenal resistance could, in fact, cause a reduction in filtration pressure and lead to a reduced delivery of diuretic molecules at the level of Henle's loop and a reduced response (Paul 2002). As a result, an increased RRI can allow the detection of patients with an altered diuretic dose-response curve who may develop diuretic resistance.

These data suggest that this parameter could be used together with GFR,in the clinical setting of CHF in order to obtain a better characterization of kidney function. This is further strengthened by the fact that RRI evaluation has been found to be easy, fast, and highly reproducible (Ciccone et al. 2014).

Summary Points

- Renal resistances play a key role in renal function as well as in CKD onset and progression. It has been shown that RRI, a Doppler-derived parameter, reflects renal arterial resistances and is a noninvasive evaluation tool.
- An altered RRI reflects the many factors which can modify renal resistances, such as increased arterial stiffness, arterial atherosclerosis, oxidative stress, endothelial dysfunction, renal parenchymal abnormalities, renal microvascular disease and vascular rarefaction, intra-abdominal and central venous pressure, and neurohormonal activation.
- In patients with renal diseases, RRI is not a marker for a specific disease because it increases in different clinical conditions. However, it certainly could be an index to indicate renal disease progression as it reflects the abnormalities of both the systemic and local vascular bed.
- In renal diseases, an increased RRI is associated with a higher risk of CKD progression and a worse outcome.
- In cardiovascular diseases, RRI seems particularly relevant in patients with arterial hypertension and in those with CHF. In the latter group, an increased RRI has been found to be independently associated both with HF progression and mortality. Moreover, it offers incremental prognostic information when added to GFR and is able to detect patients prone to developing WRF.

References

Afsar B, Elsurer R, Sezer S, Ozdemir FN. Insulin resistance is associated with increased renal resistive index independent of other factors in newly diagnosed type 2 diabetes mellitus and hypertensive patients. Metabolism. 2010;59:279–84.

Aikimbaev KS, Canataroglu A, Ozbek S, Usal A. Renal vascular resistance in progressive systemic sclerosis: evaluation with duplex Doppler ultrasound. Angiology. 2001;52:697–701.

Bigé N, Lévy PP, Callard P, et al. Renal arterial resistive index is associated with severe histological changes and poor renal outcome during chronic kidney disease. BMC Nephrol. 2012;13:139.

Boddi M, Cencioni I, Poggesi L, et al. Renal Resistive Index early detects chronic tubulointerstitial nephropathy in normo and hypertensive patients. Am J Kidney Dis. 2005;46:603–9.

Bosch JP. Renal reserve: a functional view of glomerular filtration rate. Semin Nephrol. 1995;15:381–5.

Bossard G, Bourgoin P, Corbeau JJ, Huntzinger J, Beydon L. Early detection of postoperative acute kidney injury by Doppler renal resistive index in cardiac surgery with cardiopulmonary bypass. Br J Anaesth. 2011;107:891–8.

Braam B, Cupples WA, Joles JA, Gaillard C. Systemic arterial and venous determinants of renal hemodynamics in congestive heart failure. Heart Fail Rev. 2012;17(2):161–75.

Bruno RM, Daghini E, Landini L, Versari D, Salvati A, Santini E, Di Paco I, Magagna A, Taddei S, Ghiadoni L, Solini A. Dynamic evaluation of renal resistive index in normoalbuminuric patients with newly diagnosed hypertension or type 2 diabetes. Diabetologia. 2011;54:2430–9. doi:10.1007/s00125-011-2148-y.

Bude RO, Rubin JM. Relationship between the resistive index and vascular compliance and resistance. Radiology. 1999;211:411–7.

Chade AR. Renal vascular structure and rarefaction. Compr Physiol. 2013;3:817–31.

Cianci R, Martina P, Cianci M, et al. Ischemic nephropathy: proteinuria and renal resistance index could suggest if revascularization is recommended. Ren Fail. 2010;32:1167–71.

Ciccone MM, Iacoviello M, Gesualdo L, et al. The renal arterial resistance index: a marker of renal function with an independent and incremental role in predicting heart failure progression. Eur J Heart Fail. 2014;16:210–6.

Citarelli G, Iacoviello M, Monitillo F, Leone M, et al. A high renal arterial resistance index is associated to one year worsening of renal function in heart failure outpatients. Eur J Heart Fail. 2014;16 Suppl 2:126.

Damman K, Navis G, Smilde TD, Voors AA, van der Bij W, vanVeldhuisen DJ, et al. Decreased cardiac output, venous congestion and the association with renal impairment in patients with cardiac dysfunction. Eur J Heart Fail. 2007;9:872–8.

Damman K, Jaarsma T, Voors AA, Navis G, Hillege HL, van Veldhuisen DJ, COACH Investigators. Both in- and out-hospital worsening of renal function predict outcome in patients with heart failure: results from the Coordinating Study Evaluating Outcome of Advising and Counseling in Heart Failure (COACH). Eur J Heart Fail. 2009;11:847–54.

Damman K, Kalra PR, Hillege H. Pathophysiological mechanisms contributing to renal dysfunction in chronic heart failure. J Ren Care. 2010;36:18–26. doi:10.1111/j.1755-6686.2010.00172.x.

Damman K, Voors AA, Navis G, van Veldhuisen DJ, Hillege HL. Current and novel renal biomarkers in heart failure. Heart Fail Rev. 2012;17:241–50.

Darmon M, Schortgen F, Vargas F, Liazydi A, Schlemmer B, Brun-Buisson C, Brochard L. Diagnostic accuracy of Doppler renal resistive index for reversibility of acute kidney injury in critically ill patients. Intensive Care Med. 2011;37:68–76.

Dewitte A, Coquin J, Meyssignac B, et al. Doppler resistive index to reflect regulation of renal vascular tone during sepsis and acute kidney injury. Crit Care. 2012;16:R165. doi:10.1186/cc11517.

Doi Y, Iwashima Y, Yoshihara F, et al. Renal resistive index and cardiovascular and renal outcomes in essential hypertension. Hypertension. 2012;60:770–7.

Ennezat PV, Maréchaux S, Six-Carpentier M, et al. Renal resistance index and its prognostic significance in patients with heart failure with preserved ejection fraction. Nephrol Dial Transplant. 2011;26:3908–13.

Florczak E, Januszewicz M, Januszewicz A, et al. Relationship between renal resistive index and early target organ damage in patients with never-treated essential hypertension. Blood Press. 2009;18:55–61.

Hanamura K, Tojo A, Kinugasa S, Asaba K, Fujita T. The resistive index is a marker of renal function, pathology, prognosis, and responsiveness to steroid therapy in chronic kidney disease patients. Int J Nephrol. 2012;2012:139565. doi:10.1155/2012/139565.

Heine GH, Gerhart MK, Ulrich C, Kohler H, Girndt M. Renal Doppler resistance indices are associated with systemic atherosclerosis in kidney transplant recipients. Kidney Int. 2005;68:878–85.

Hillege HL, Girbes AR, de Kam PJ, Boomsma F, de Zeeuw D, Charlesworth A, Hampton JR, van Veldhuisen DJ. Renal function, neurohormonal activation, and survival in patients with chronic heart failure. Circulation. 2000;102:203–10.

Iacoviello M (a), Puzzovivo A, Monitillo F, Saulle D, Lattarulo MS, Guida P, Forleo C, Gesualdo L, Favale S. Independent role of high central venous pressure in predicting worsening of renal function in chronic heart failure outpatients. Int J Cardiol. 2013;162:261–3.

Iacoviello M (b), Doronzo A, Paradies V et al. The independent association between altered renal arterial resistance and loop diuretic dose in chronic heart failure outpatients. IJC Heart & Vasculature 2015;7:119–123.

Ikee R, Kobayashi S, Hemmi N, et al. Correlation between the resistive index by Doppler ultrasound and kidney function and histology. Am J Kidney Dis. 2005;46:603–9.

Jessup M, Costanzo MR. The cardiorenal syndrome: do we need a change of strategy or a change of tactics? J Am Coll Cardiol. 2009;53:597–9.

Kawai T, Kamide K, Onishi M, et al. Usefulness of the resistive index in renal Doppler ultrasonography as an indicator of vascular damage in patients with risks of atherosclerosis. Nephrol Dial Transplant. 2011;26:3256–62.

Kawai T, Ohishi M, Kamide K, et al. The impact of visit-to-visit variability in blood pressure on renal function. Hypertens Res. 2012;35:239–43.

Krumme B, Hollenbeck M. Doppler sonography in renal artery stenosis—does the Resistive Index predict the success of intervention? Nephrol Dial Transplant. 2007;22:692–6.

Krumme B, Grotz W, Kirste G, Schollmeyer P, Rump LC. Determinants of intrarenal Doppler indices in stable renal allografts. J Am Soc Nephrol. 1997;8:813–6.

Liu KH, Chu WC, Kong AP, et al. Intrarenal arterial resistance is associated with microvascular complications in Chinese type 2 diabetic patients. Nephrol Dial Transplant. 2012. doi:10. 1093/ndt/gfs471 59.

Lubas A, Kade G, Niemczyk S. Renal resistive index as a marker of vascular damage in cardiovascular diseases. Int Urol Nephrol. 2014;46:395–402. doi:10.1007/s11255-013-0528-6.

MacIsaac RJ, Thomas MC, Panagiotopoulos S, et al. Association between intrarenal arterial resistance and diastolic dysfunction in type 2 diabetes. Cardiovasc Diabetol. 2008. doi:10.1186/1475-2840-7-1560.

Majid DS, Navar LG. Nitric oxide in the control of renal hemodynamics and excretory function. Am J Hypertens. 2001;14:74S–82.

Monitillo F, Iacoviello M, Citarelli G, Leone M, et al. Independent and incremental role of renal resistance index in predicting mortality among heart failure outpatients. Eur J Heart Fail. 2014;16 Suppl 2:126.

Mullens W, Abrahams Z, Francis GS, Sokos G, Taylor DO, Starling RC, Young JB, Tang WH. Importance of venous congestion for worsening of renal function in advanced decompensated heart failure. J Am Coll Cardiol. 2009;53:589–96.

Ohta Y, Fujii K, Ibayashi S, et al. Renal and carotid vascular resistance assessed with Doppler sonography. J Clin Ultrasound. 2008;36:85–90.

Ohta Y, Fujii K, Hisatomi A. Increased renal resistive index in atherosclerosis and diabetic nephropathy assessed by Doppler sonography. J Hypertens. 2005;23:1905–11.

Parolini C, Noce A, Staffolani E, et al. Renal resistive index and long-term outcome in chronic nephropathies. Radiology. 2009;252:888–96.

Paul S. Balancing diuretic therapy in heart failure: loop diuretics, thiazides, and aldosterone antagonists. Congestive Heart Fail. 2002;8:307–12.

Platt J, Ellis J, Rubin J, et al. Intrarenal arterial Doppler sonography in patients with nonobstructive renal disease: correlation of resistive index with biopsy findings. AJR. 1990;154:1223–7.

Pontremoli R, Viazzi F, Martinoli C, et al. Increased renal resistive index in patients with essential hypertension: a marker of target organ damage. Nephrol Dial Transplant. 1999;14:360–5.

Pourcelot L. Velocimetrie ultrasonore Doppler. Seminaire INSERM. Paris: Edition INSERM; 1974. p. 213–40.

Radermacher J, Chavan A, Bleck J, et al. Use of Doppler ultrasonography to predict the outcome of therapy for renal artery stenosis. N Engl J Med. 2001;344:410–7.

Radermacher J, Ellis S, Haller H. Renal resistance index and progression of renal disease. Hypertension. 2002;39:699–703.

Radermacher J, Mengel M, Ellis S, et al. The renal arterial resistance index and renal allograft survival. N Engl J Med. 2003;349:115–24.

Raff U, Schmidt BM, Schwab J, Schwarz TK, Achenbach S, Bar I, Schmieder RE. Renal resistive index in addition to low-grade albuminuria complements screening for target organ damage in therapy-resistant hypertension. J Hypertens. 2010;28:608–14.

Ratto E, Leoncini G, Viazzi F, Vaccaro V, Falqui V, Parodi A, Conti N, Tomolillo C, Deferrari G, Pontremoli R. Ambulatory arterial stiffness index and renal abnormalities in primary hypertension. J Hypertens. 2006;24:2033–8.

Ronco C, Haapio M, House AA, Anavekar N, Bellomo R. Cardiorenal syndrome. J Am Coll Cardiol. 2008;52:1527–39.

Schnell D, Deruddre S, Harrois A, Pottecher J, Cosson C, Adoui N, Benhamou D, Vicaut E, Azoulay E, Duranteau J. Renal resistive index better predicts the occurrence of acute kidney injury than cystatin C. Shock. 2012;38:592–7.

Schwenger V, Keller T, Hofmann N, Hoffmann O, Sommerer C, Nahm AM, Morath C, Zeier M, Krumme B. Color Doppler indices of renal allografts depend on vascular stiffness of the transplant recipients. Am J Transplant. 2006;6:2721–4.

Smilde T, van Veldhuisen D, Navis G, Voors A, Hillege H. Drawbacks and prognostic value of formulas estimating renal function in patients with chronic heart failure and systolic dysfunction. Circulation. 2006;114:1572–80.

Smith GL, Lichtman JH, Bracken MB, Shlipak MG, Phillips CO, DiCapua P, Krumholz M. Renal impairment and outcomes in heart failure: systematic review and meta-analysis. J Am Coll Cardiol. 2006;47:1987–96.

Splendiani G, Parolini C, Fortunato L, et al. Resistive index in chronic nephropathies: predictive value of renal outcome. Clin Nephrol. 2002;57:45–50.

Stevens LA, Coresh J, Greene T, Levey AS. Assessing kidney function—measured and estimated glomerular filtration rate. N Engl J Med. 2006;354:2473–83.

Sugiura T, Wada A. Resistive index predicts renal prognosis in chronic kidney disease. Nephrol Dial Transplant. 2009;24:2780–5.

Sugiura T, Nakamori A, Wada A, et al. Evaluation of tubulointerstitial injury by doppler ultrasonography in glomerular diseases. Clin Nephrol. 2004;61:119–26.

Tublin ME, Bude RO, Platt JF. The resistive index in renal Doppler sonography: where do we stand? AJR. 2003;180:885–92.

Winton FR. The influence of venous pressure on the isolated mammalian kidney. J Physiol. 1931;72:49–61.

Yuksel UC, Anabtawi AG, Cam A. Predictive value of renal resistive index in percutaneous renal interventions for atherosclerotic renal artery stenosis. J Invasive Cardiol. 2012;24:504–9.

Zeller T, Bonvini RF, Sixt S. Color-coded duplex ultrasound for diagnosis of renal artery stenosis and as follow-up examination after revascularization. Catheter Cardiovasc Interv. 2008;71:995–9.

Pulmonary Pressure as a Novel Prognostic Biomarker in Renal Patients

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Abstract

Renal patients are notoriously at high risk for cardiovascular complications, but such risk is not fully explained by traditional and chronic kidney disease (CKD)-related risk factors. New prognostic biomarkers are therefore needed to refine outcome prediction in this population. High pulmonary pressure (PP; also known as pulmonary hypertension) is remarkably prevalent among persons with CKD,

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particularly in hemodialysis patients. High PP is a powerful and independent predictor of death in the general population and in subjects with heart or lung diseases. In renal patients, there is now evidence showing that PP may hold the same prognostic utility. High PP predicts adverse cardiovascular outcomes in dialysis and pre-dialysis populations. In kidney transplant recipients, high PP is associated with worse renal outcomes. In this chapter, we will focus on high PP in the CKD population, spanning from the main techniques for assessing PP to the pathophysiology of pulmonary hypertension (PH) in renal patients. Prognostic implications of PH in CKD patients for risk stratification and therapeutic management will also be discussed.

Keywords

Pulmonary pressure • Pulmonary hypertension • Chronic kidney disease • End-stage kidney disease • Dialysis • Cardiovascular risk

Abbreviations

ACEi	Angiotensin-converting enzyme inhibitor
ADMA	Asymmetric dimethylarginine
APAH	Associated forms of pulmonary arterial hypertension
ARBs	Angiotensin receptor blockers
AVF	Arteriovenous fistula
CKD	Chronic kidney disease
CKD-5D	Chronic kidney disease stage 5 on dialysis
CKD-ND	Chronic kidney disease not on dialysis
COPD	Chronic obstructive pulmonary disease
CV	Cardiovascular
E/e′	Early trans-mitral flow velocity [E]/early mitral annular tissue velocity [e′]
EGD	Early graft dysfunction
eGFR	Estimated glomerular filtration rate
ePP	Estimated pulmonary pressure
ERS	European Respiratory Society
ESC	European Society of Cardiology
ESKD	End-stage kidney disease
FPAH	Familial pulmonary arterial hypertension
HD	Hemodialysis
HIV	Human immunodeficiency virus
ILD	Interstitial lung disease
IPAH	Idiopathic pulmonary arterial hypertension
KDOQI	Kidney Disease Outcomes Quality Initiative
LV	Left ventricular
mPP	Measured pulmonary pressure
NO	Nitric oxide
NYHA	New York Heart Association
PAH	Pulmonary arterial hypertension

PAWP	Pulmonary artery wedge pressure
PD	Peritoneal dialysis
PH	Pulmonary hypertension
PP	Pulmonary pressure
PVR	Pulmonary vascular resistance
RAP	Right atrial pressure
RHC	Right heart catheterization
Vmax	Maximum tricuspidal jet velocity
WHO	World Health Organization

Key Facts of Pulmonary Circulation

- The pulmonary circulation is a low-resistance, low-impedance, high-capacitance, and high-flow circuit.
- This circuit has hemodynamic and structural characteristics that are deeply different from those of the systemic circulation.
- Pressure values in the pulmonary arteries are roughly one fourth to one sixth of those of the systemic circulation.
- Medial thickening of the major branches of the pulmonary artery is lower than that of systemic arteries, being comparable to the structure of large veins.
- The normal pulmonary circulation consists of highly compliant vessels and a vast capillary network with large recruitment capability.

Key Facts of Pulmonary Hypertension

- Pulmonary hypertension (PH) is a condition characterized by a pathological increase in pulmonary artery pressure (PP).
- Such increase may result from an increase in pulmonary artery resistances (“pre-capillary” forms) or by a passive congestion of the venous side of the pulmonary circulation (“post-capillary” forms) that is usually secondary to left heart dysfunction.
- PH may arise during several lung or systemic diseases and, particularly, in the presence of left heart dysfunction. In rare cases, PH manifests as a primary disease.
- The overall, estimated prevalence of asymptomatic PH in the general population ranges from 5 % to 10 %. Precapillary forms are much more rare with an estimated prevalence of about 15 cases per million and an annual incidence of about 2–3 per million.
- Echocardiography may be useful as a screening test to identify patients at risk of PH.
- Invasive maneuvers (right heart catheterization) are needed for a clear-cut diagnosis of PH, for the etiological framework, and for an optimal therapeutic planning (vasoreactivity test).
- Prognosis and response to treatment are highly variable, being mostly dependent on the type of PH.

- Subjects with pre-capillary PH (particularly primary forms) are usually scarcely responsive to treatments and may need lung transplant.

Definitions

Arteriovenous fistula (AVF) Artificial vascular shunt surgically created by connecting a peripheral artery and a vein in the elbow or in the wrist of patients that need to start chronic hemodialysis treatment. AVFs serve as a high-flow vascular access for performing the dialysis procedure.

Chronic kidney disease (CKD) A pathological condition characterized by a reduced renal function with consequent alterations in fluid regulation, blood pressure control, waste product elimination, and metabolic alterations. CKD may progress, more or less rapidly, to end-stages.

End-stage kidney disease (ESKD) The final stage of CKD, also known as terminal uremia. Residual renal function is not anymore sufficient to control the body homeostasis so that patients need to start in due course chronic hemodialysis treatment or be transplanted.

Hemodialysis (HD) Chronic or acute therapy for replacing renal function. ESKD patients usually undergo chronic hemodialysis thrice a week. Each HD session lasts 3.5–4 h. During the HD session, fluid and electrolyte excess and waste products are removed from the blood circulation.

Peritoneal dialysis (PD) Alternative technique to hemodialysis consisting of using the patient's own peritoneal membrane as an exchange surface for balancing fluid, electrolyte, and waste homeostasis. The peritoneal cavity is accessed via a permanent catheter placed in the patient's abdomen that is linked to an external machine. Exchanges are induced and regulated according to diffusion and convection principles by fluid bags with established content.

Right heart catheterization (RHC) Invasive maneuver consisting of reaching the right side of the heart via a small catheter inserted from a peripheral vein (e.g., femoral, subclavian, or jugular). This procedure allows diagnostic measurements (e.g., right heart pressures, pulmonary artery pressure) as well as interventional procedures.

Introduction

The incidence of chronic kidney disease (CKD) and end-stage kidney disease (ESKD) is on the rise. Currently, it has been estimated that over 50 million people are affected by CKD and over 2 million persons need chronic dialysis for ESKD

(Eggers 2011). This amount is expected to increase by 60 % by 2020 (Gilbertson). CKD ranks now as one of the main risk factors for cardiovascular (CV) mortality and morbidity with a substantial impact on health resources. In the USA, annual costs attributable to manage CV complications of CKD patients rank from 1,700 (KDOQI CKD stage 1) to 12,700 (CKD stage 4) dollars (Honeycutt et al. 2013). Although a large percentage of patients with CKD have traditional CV risk factors such as diabetes, hypertension, and lipid abnormalities, interventions targeting these factors have failed to significantly decrease CV mortality and morbidity. Similarly, normalization of other nontraditional risk factors peculiar of CKD including anemia, microalbuminuria, inflammation, oxidative stress, and altered mineral metabolism was not totally effective in improving event-free survival.

High pulmonary pressure (PP) has recently emerged as a novel CV risk factor in the general population. In a surveillance from 1980 to 2002 (Hyduk et al. 2005), the Centers for Disease Control and Prevention identified increasing rates of hospitalization associated with high PP and stable death rates ranging from 5.2 to 5.4 per 100.000 persons. Conversely, during the last decade, an increasing trend in mortality was documented with an estimated age-adjusted death rate of 4.5–12.3 per 100.000 (George et al. 2014). High PP rarely presents as an idiopathic condition, being more frequently associated with systemic, cardiac, or lung disorders which may affect the pulmonary vascular circuit.

There is now accruing evidence indicating that masked, non-symptomatic high PP is exceedingly prevalent in CKD persons, particularly in ESKD patients on chronic renal replacement therapy (Bolignano et al. 2013). Several underlying conditions, such as volume overload, the presence of high-flow arteriovenous fistulas, breath disorders, and sympathetic hyperactivation have been postulated to explain such a high frequency. Nevertheless, PP is emerging as a novel, important prognostic biomarker in the renal population. In fact, the presence of high PP in CKD is generally associated with poor outcomes, spanning from increased mortality to higher rate of CV events and delayed graft function in renal transplanted patients (Bolignano et al. 2013). The evaluation of PP might therefore represent an additional, helpful tool for risk assessment and risk stratification of renal patients.

Pulmonary Circulation and Pulmonary Pressure Assessment

Less known to nephrologists, who are more familiar with the systemic circulation, the pulmonary circulation is a delicate and exclusive low-resistance, low-impedance, high-capacitance, and high-flow circuit. Under physiological conditions, pressure levels in the pulmonary arteries are roughly one fourth to one sixth of those normally found in the systemic circulation (Naeije 2013). Medial thickening of the major pulmonary arteries is notably lower than that of systemic arteries, being more similar to the structure of large veins. The normal pulmonary circulation therefore consists of highly compliant pulmonary arteries and a vast capillary network with large recruitment capability which is able to accommodate large increases in blood flow

without significant increases in PP, e.g., during sustained exercise or in the presence of left-to-right congenital intra-cardiac shunts.

How can PP be assessed in clinical practice? The gold standard of measurement is represented by right heart catheterization (RHC), an invasive procedure which consists in reaching the right heart with a catheter inserted via a peripheral vein (Badesch et al. 2009). The catheter can be moved until the right atrium or even further, reaching the right ventricle and the main pulmonary artery branches. As long as the catheter proceeds through the right heart to the pulmonary circulation, this gets characteristic pressure responses, very similar to a sequence of spikes, peaking at about 20–25 mmHg. When in the distal branch of the pulmonary artery, values of the measured PP of about 14 $\pm$ 3 mmHg are considered as normal. The assessment of the so-called pulmonary artery wedge pressure (PAWP), that is, the pressure measured by wedging a Swan-Ganz catheter with an inflated balloon into a small pulmonary arterial branch, may give additional information, particularly in the presence of pathological PP values (see below). In fact, PAWP allows to assess the pulmonary vascular resistance (PVR), expressed as the ratio between the difference of mean PP and PAWP and the cardiac output. RHC is crucial for assessing PP, but as it is an invasive procedure, it may be associated with an increased risk of dangerous complications. Therefore, with some exceptions, cardiac catheterization in daily practice is usually not considered as the first-line approach to evaluate PP. In most cases, PP is firstly estimated by non-invasive procedures, like a simple transthoracic Doppler echocardiography. PP estimation with such technique is based on the eventual finding of tricuspidal regurgitation, a phenomenon that can be minimally present also in apparently healthy subjects. If tricuspidal regurgitation is present, the echocardiography instrument can automatically assess the maximum tricuspidal jet velocity (Vmax). This parameter is important to finally estimate the PP (ePP) according to the so-called Bernoulli's equation as the product of the square of the Vmax by 4 (4x $Vmax^2$) (Rudski et al. 2010). However, this equation often gives a too much rough estimate of ePP that can be further refined by implementing also information about the estimated right atrial pressure (RAP) in the "modified" Bernoulli's equation (4x $Vmax^2$+ RAP) (Yock and Popp 1984). RAP is supposed to range from 10 to 30 mmHg in case of absence or presence of relevant inferior vena cava collapse.

Diagnosing High PP

A recent joint guideline (Galie et al. 2009) made by the European Society of Cardiology (ESC) and the European Respiratory Society (ERS) has established the main criteria for the definition of "pathological" PP values, finally making clearness in a very controversial and debated topic.

High, pathological PP (a condition also known as pulmonary hypertension (PH)) is defined by a documented increase in the measured PP (mPP) $\geq$25 mmHg at rest.

However, although not diagnostic, ePP values 35–49 mmHg (roughly corresponding to Vmax values of 2.8–3.4 m/s) can be considered suggestive of PH

Fig. 1 Differential diagnosis of high pulmonary pressure in relation to the anatomic site of disease. *COPD* chronic obstructive pulmonary disease, *ILS* interstitial lung syndrome, *PAWP* pulmonary artery wedge pressure, *PP* pulmonary pressure, *PVR* pulmonary vascular resistance

while values ≥50 mmHg are strongly indicative of the true presence of PH. Echocardiography estimation can then be useful as screening test for selecting patients who deserve more invasive exams for a clear-cut diagnosis of pathological PP. Even though it is of foremost importance to be sure about the true diagnosis/presence of pathological PP, it is also important to identify the underlying cause of such alteration.

As briefly alluded to before, mPP or ePP values alone are not sufficient to make a differential diagnosis of PH and additional parameters, such as PAWP and PVR, are required.

According to the ESC-ERS guideline, which has recently been endorsed by a WHO document, different types of PH exist, each one with its peculiar natural history and clinical approach. The WHO classification (McGlothlin 2012) of the different types of PH mostly looks at the pathogenesis and the anatomic location of the primary alteration responsible of increased PP (Fig. 1).

As mentioned, the pulmonary circulation consists of an arterial side that mostly recalls the characteristics of systemic veins in terms of compliance and sectional structure and a venous side bringing the oxygenated blood back to the heart and, from there, to the systemic circulation. The capillary barrier, that is, ideally in the middle between the two sides, can be useful to distinguish pathological conditions

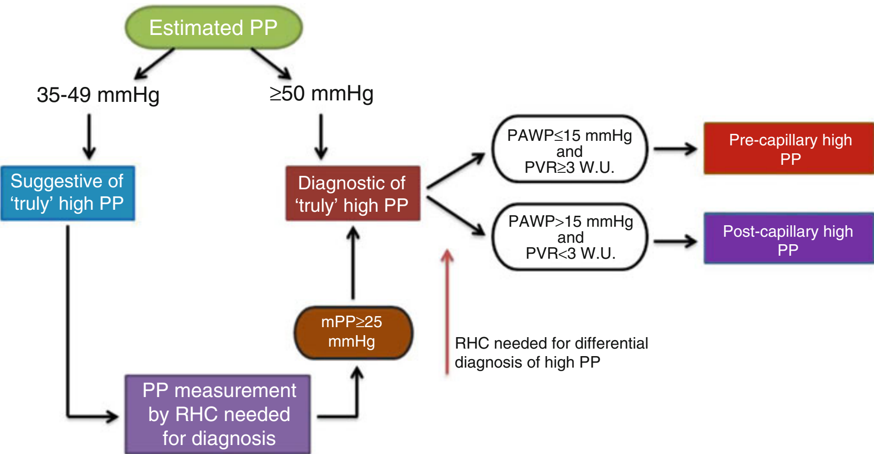

Fig. 2 Diagnostic algorithm for approaching patients with suspected high pulmonary pressure. *mPP* measured pulmonary pressure, *PAWP* pulmonary artery wedge pressure, *PP* pulmonary pressure, *PVR* pulmonary vascular resistance, *RHC* right heart catheterization

characterized by a primary increase in pulmonary artery resistances, the so-called "pre-capillary" pulmonary hypertension, from those mostly secondary to a passive venous congestion ("post-capillary"). PAWP and PVR can be helpful in discriminating pre-capillary from post-capillary forms of high PP. In pre-capillary forms, PAWP is low (<15 mmHg) while PVR is expectedly high (>3 Woods units); conversely, in post-capillary forms, PAWP values are increased (>15 mmHg) with usually low PVR (<3 Woods units) (Badesch et al. 2009). Pre-capillary forms of PH include the so-called pulmonary arterial hypertension (PAH) which encompasses idiopathic (IPAH), familial (FPAH), and forms associated (APAH) to congenital heart disease, connective tissue diseases, drugs and toxins, HIV infection, portal hypertension, or pulmonary veno-occlusive disease. In addition, pre-capillary PH can arise as a consequence of chronic thromboembolism such as in the presence of obstruction of pulmonary arterial vessels (proximal or distal) by thromboemboli, tumors, or foreign bodies. Post-capillary forms are most frequently found as associated to left heart disorders such as systolic or diastolic dysfunction or valve diseases (mitral and/or aortic).

High PP can be found also in the presence of other conditions primarily affecting the lungs (e.g., chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), sleep apnea) or as manifestation of several systemic diseases (McGlothlin 2012). These two latter cases can present either with a prevalent pre-capillary or post-capillary involvement so that a proper differential diagnosis based on PAWP and PVR is not always possible. Figure 2 depicts a diagnostic algorithm that can be useful for approaching patients with suspected pathological PP. As said, the first approach would be to perform a non-invasive assessment of PP by echocardiography. ePP of 35–49 mmHg or Vmax 2.8–3.4 m/s are suggestive of PH but would require RHC for the final diagnosis. ePP>50 mmHg or Vmax >3.4 m/s might

be considered diagnostic of PH, but RHC is needed for further characterization of PH (pre- or post-capillary) on the basis of PAWP and PVR values.

Significance of High PP in the General Population and in Renal Patients

What is the epidemiological impact of elevated PP? In other words, what do studies and registries report about the penetrance and diffusion of this condition? Recently, evidence is accruing showing that high PP is much more prevalent in the general population than expected (Simonneau et al. 2009). This condition might remain undetected because of the absence of symptoms in the early phases and suspected only when clinical signs of right ventricular dysfunction (dyspnea, fatigue, non-productive cough, angina pectoris, syncope, peripheral edema, and, rarely, hemoptysis) are manifested (Badesch et al. 2009). In the Olmsted County study (Lam et al. 2009), a general population study conducted in a random sample of the same county, the prevalence of high PP defined by a Doppler-derived PP >35 mmHg was about 5 % in individuals older than 45 years. Most cases of high PP detected in this population were secondary to concomitant left heart disorders, and the presence of high PP was predicted by diastolic dysfunction as measured by the E/e′ (early transmitral flow velocity [E]/early mitral annular tissue velocity [e′]) ratio and by the presence of systemic hypertension and high pulse pressure.

In a survey on 4.579 patients undergoing echocardiographic examinations (Strange et al. 2012), the prevalence of high PP (>40 mmHg) was 10.5 %. Among the 483 cases with elevated PP, 78.7 % had left heart disease, 9.7 % had lung diseases, 4.2 % had primary pulmonary artery hypertension, and 0.6 % had pulmonary thromboembolism. In another study (Ghio et al. 2001), the prevalence of PH in patients with chronic heart failure increased with the progression of NYHA class. Up to 60 % of patients with severe LV systolic dysfunction and up to 70 % of patients with isolated LV diastolic dysfunction had pathologically high PP.

Pre-capillary forms of PH are less frequent with an annual incidence of about 2–3 per million and an estimated prevalence of about 15 cases per million (Simonneau et al. 2009). Adult females are almost three times more likely to present with PAH than males. In children, the presence of PAH is equally split along gender lines. But what happens if we refer specifically to renal patients? Do things change in this particular population?

Although epidemiological data are scarce and sparse and mainly based on retrospective studies, high PP appears to be exceedingly prevalent among renal patients and not only confined to connective tissue and systemic diseases (Fig. 3).

Among pre-dialysis patients, the prevalence of PH is about two to eight times higher than in the general population, ranging from 9 % to 39 % (Bolignano et al. 2013). PH prevalence is higher in the dialysis population (CKD-5D) than in CKD-ND patients. With regard to dialysis modality, the prevalence of PH is lower in patients on peritoneal dialysis (from 0 % to 42 %) than in hemodialysis patients (from 18.8 % to 68.8 %) (Bolignano et al. 2013). Unfortunately, there is a lack of

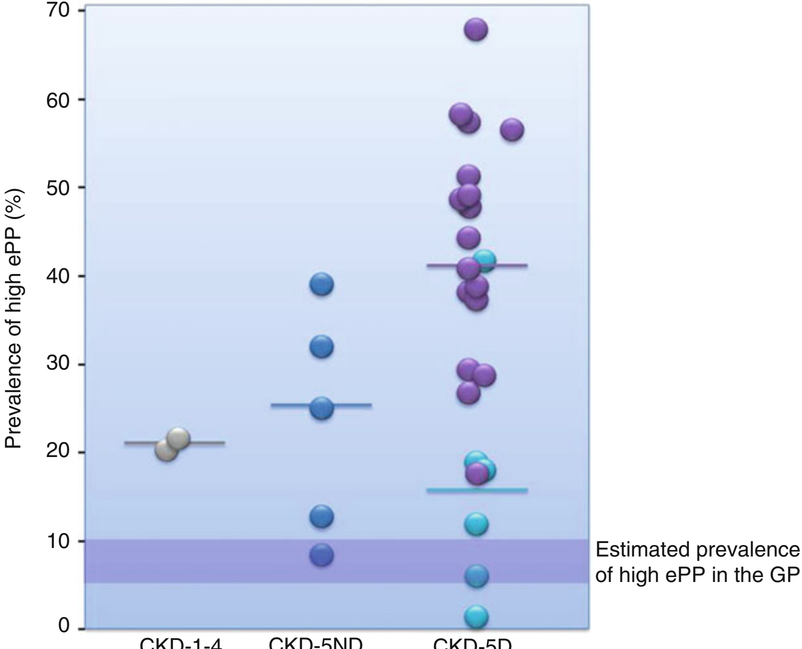

Fig. 3 Reported prevalence of high estimated pulmonary pressure (ePP) in renal patients. Each dot represents a single prevalence reported in a different study cohort (see text). Gray dots indicate prevalence of high ePP in CKD stage 1–4 populations; blue dots in CKD stage-5 not on dialysis; purple dots in CKD stage-5 on chronic hemodialysis treatment; azure dots in CKD stage-5 on chronic peritoneal dialysis treatment. Correspondent lines indicate the median (calculated) value of high ePP prevalence in a given CKD class. *ePP* estimated pulmonary pressure, *CKD* 1–4 chronic kidney disease stage 1–4, *CKD 5ND* chronic kidney disease stage 5 not on dialysis, *CKD 5D* chronic kidney disease stage 5 on dialysis, *GP* general population

uniformity among studies with respect to the ePP cut-offs considered as "pathological" (ranging from 25 to ≥45 mmHg). Such a variability in the diagnostic criteria explains the wide range of PH prevalence reported in CKD patients and hampers the possibility to perform rough comparisons between studies and to provide reliable overall estimates of the frequency of high PP among renal patients.

But what kind of PH do renal patients have? Understanding the type of PH would be useful to better understand the pathophysiology of this condition and, eventually, to plan also the best treatment in such patients. Unfortunately, as stressed before, the only way to characterize the nature/origin of high PP is to perform RHC to measure PP but also to assess PAWP and PVR.

In the only study measuring PP by RHC (Pabst et al. 2012), PH was present in 81 % of HD and 71 % of pre-dialysis patients. The prevalence of (pre-capillary) high PP was 6 % in CKD stage 4–5 patients and 13 % in HD patients, and the prevalence of post-capillary PH was 71 % and 65 %. These observations, although partly biased by the strict inclusion criteria of the study population (all subjects underwent RHC

for characterization of an unexplained dyspnea after exclusion of other potential causes), seem to indicate that high PP is mostly post-capillary also in the renal population.

Risk Factors of High PP in Renal Patients

As alluded to before, post-capillary high PP is likely to depend on the presence of LV disorders. Heart dysfunction is prevalent in the renal population, particularly in chronic hemodialysis patients. This condition can trigger volume overload but can be aggravated itself by fluid excess, therefore generating a vicious circle that can contribute to pulmonary congestion and high PP. Nevertheless, other risk factors/conditions may trigger and/or exacerbate PH in renal patients (Fig. 4). Arteriovenous fistula (AVF) is known to induce a decrease in systemic vascular resistances, which increase venous return and cardiac output to maintain proper blood flow to peripheral tissues. These hemodynamic effects might increase pulmonary blood flow and set the stage for high PP. Accordingly, high PP are more prevalent among HD than PD patients or CKD patients not on dialysis; furthermore, PP rises in strict parallelism with AVF creation (Abassi et al. 2006) and tends to worsen overtime in the HD population (Havlucu et al. 2007; Fabbian et al. 2011). However, evidence that renal transplantation may revert to normal PP in patients who still have a functioning AVF (Nakhoul et al. 2005) indicates that other risk factors are involved in the genesis of high PP in CKD. Endothelial dysfunction is a major determinant of PH in the general population

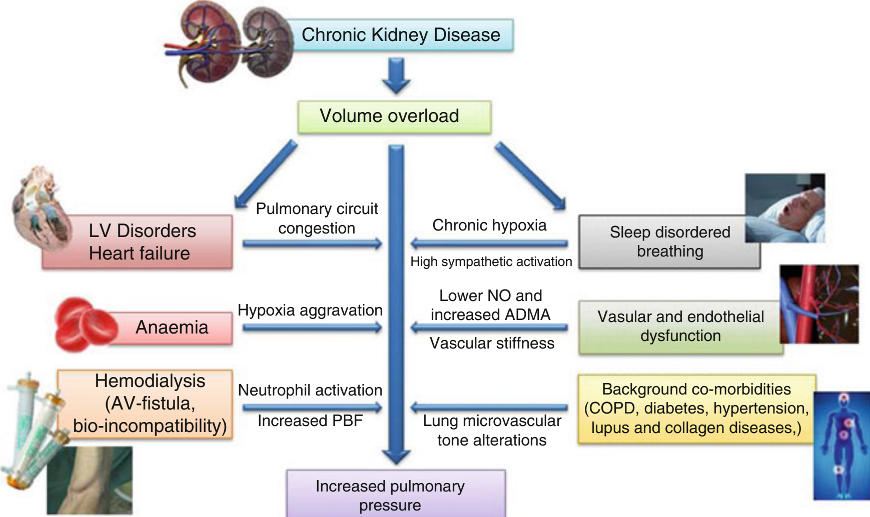

Fig. 4 Main pathophysiological mechanisms leading to an increase in pulmonary pressure in renal patients. *ADMA* asymmetric dimethylarginine, *AV fistula* arteriovenous fistula, *COPD* chronic obstructive pulmonary disease, *LV* left ventricular, *NO* nitric oxide, *PBF* pulmonary blood flow

(Giaid 1998) and is exceedingly frequent in renal patients (Zoccali 2007). Plasma levels of the powerful, endothelial-derived, vasodilator nitric oxide (NO) are more reduced in HD patients with higher PP (Yigla et al. 2004). Furthermore, asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase which is copiously synthesized at lung level (Arrigoni et al. 2003), has been strongly involved in experimental (Sasaki et al. 2007) and in primary forms (Kielstein et al. 2005) of PH and accumulates in subjects with renal function impairment (Zoccali et al. 2001). Interestingly, ADMA is increased in patients with sleep breathing disorders (Barcelo et al. 2009). Sleep breathing disorders, particularly sleep apnea, are highly pervasive in both pre-dialysis (Sakaguchi et al. 2011) and dialysis patients (Zoccali et al. 2002), and nocturnal hypoxemia by sleep apnea is a strong trigger of high PP by enhancing sympathetic activation (Ward and McMurtry 2009; Sica et al. 2000).

Chronic exposure of blood to dialysis membranes causes reversible neutrophil sequestration in the lung and neutrophil activation (Craddock et al. 1977) which may contribute to microvascular lung disease and PP increase in HD patients (Kiykim et al. 2010). The control of microvascular tone in the lung might also be affected by other diseases, such as diabetes and connective, liver, infectious, and hematologic diseases. Severe anemia is a recognized cardiovascular risk factor in renal patients and its impact on the cardiovascular system includes direct effects to the pulmonary circulation. Indeed, anemia could worsen PH by aggravating hypoxia (Buemi et al. 2007).

As a consequence of the overall dysfunction in mineral metabolism, arterial rigidity is increased in renal patients and calcium deposits have been found even in the pulmonary artery of CKD patients (Nitta et al. 2003). These findings are in line with observations in the general population showing that stiffening of the pulmonary artery is significantly correlated to high PP (Lam et al. 2009).

Potential Applications to Prognosis, Other Diseases, or Conditions

What is the prognostic impact of high PP? Do we have to fear the presence of PH in our patients?

A large US survey (Hyduk et al. 2005), which collected data on PH at the community level over a 22-year period (1980–2002), documented a stable death rate in patients with pathologically high PP, ranging from 5.2 to 5.4 per 100.000. Conversely, over the last 10 years, an increasing trend in mortality was documented with an estimated age-adjusted death rate of 4.5–12.3 per 100.000 (George et al. 2014). In addition, high PP was also associated with steadily increasing rates of hospitalizations (Hyduk et al. 2005). With this background in mind, one would easily argue that this trend simply reflects that one of “common” cardiovascular or pulmonary diseases; in this view, PH would be nothing more than a simple complication of traditional heart or lung disorders. However, a milestone study on PH in cardiopathic subjects has promptly rejected this hypothesis (Kjaergaard et al. 2007). The authors studied over 400 patients with known or presumed heart failure. After echocardiographic assessment of PP and LV ejection fraction, patients were followed

for up to 5.5 years. Patients presenting with a substantial increase in PP (>39 mmHg) had a significantly increased mortality in the long term (5 years) and even in the short term (1 year) with respect to those with lower PP values. Interestingly, these observations remained unaffected if patients were stratified according to the presence of a conserved or impaired LV function (≤50 % or >50 %). Multiple Cox analyses apportioned a 9 % increase in mortality per each 5 mmHg increase in PP, independent of age and presence of chronic lung disease, heart failure, and impaired renal function. These solid findings indicate that the prognostic impact of high PP is not influenced by traditional risk factors, including heart dysfunction. Similar observations have been extended to other populations, such as patients with primary PH or PH mostly secondary to chronic lung disease (Lau et al. 2014).

What happens in renal patients? Does high PP hold the same prognostic power even in this particular, high-risk population? A study in 2003 (Yigla et al. 2003) tested for the first time this possibility in a cohort of chronic hemodialysis patients. Indeed, at unadjusted Kaplan-Meier survival curves, patients with ePP ≥35 mmHg showed poorer survival with respect to those with lower ePP over a 5-year follow-up. These preliminary observations prompted the authors to carry out an observational study (Yigla et al. 2009) on a wider cohort of 127 ESKD patients initiating chronic renal replacement therapy. Patients' survival was assessed over a longer follow-up period (7 years), and subjects were stratified according to the absence of PH (ePP <45 mmHg) or to the presence of prevalent PH (ePP >45 mmHg already present before starting HD treatment) or incident PH (ePP >45 mmHg developed after starting HD). Overall, the presence of high PP conferred a higher risk of death (hazard ratio 2.1 and 3.6 for incident and prevalent PH, respectively), and patients already having abnormal PP values before starting dialysis had poorer survival as compared to those developing PH after dialysis initiation. Furthermore, the prognostic power of high PP was independent of gender, age at onset of HD, and presence of diabetes mellitus, left ventricular dysfunction, and clinically relevant valve disease. In this study, each 10 mmHg increase in ePP was associated with a fully adjusted increased risk of mortality of 50 %.

Similar findings were reported in another cohort of 278 patients on chronic renal replacement therapy who were followed for 24 months (Li et al. 2013). In this series, the prevalence of ePP >35 mmHg was as high as 35 %. Subjects with baseline PH had a hazard ratio of cardiovascular death of 2.36 with respect to those with "normal" ePP. This increased risk was fully independent of several potential confounders, such as gender, age at HD onset and duration of HD, pre-dialysis diastolic blood pressure, serum phosphorus, urea levels, presence of diabetes, systolic dysfunction, and history of CV events. Of note, the presence of high ePP was also a powerful predictor of non-fatal cardiovascular events (hazard ratio 2.27), even after adjustment for the above-mentioned risk factors plus medication with ACEi or ARB, dialysis adequacy, and C-reactive protein.

In another study of 288 chronic HD patients (Agarwal 2012), high ePP (>35 mmHg) was found in 38 % of subjects and its presence predicted by left atrial dysfunction, urea removal ratio, and use of vitamin D receptor activators. During follow-up (median 2.15 years), patients with higher ePP had significantly worsen

cumulative hazard estimates of cardiovascular fatal events (53 %, crude mortality rate 168.9/1,000 patient-years vs. 22 %, crude mortality rate 52.5/1,000 patient-years). After adjustment for race, age, vascular access, serum albumin, and history of CV disease, the presence of ePP >35 mmHg still conveyed a higher risk of death (hazard ratio 2.17) in this population.

Another study (Ramasubbu et al. 2010) found abnormal ePP values (defined as Vmax >2.5 m/s) in 42/90 HD patients (47 %). Subjects were followed over 12 months to assess all-cause mortality and hospitalizations. There was a statistically significant parallelism among severity of PH (defined as low, Vmax <2.5 m/s; mild, Vmax 2.6–2.9 m/s; or severe, Vmax >3 m/s) and mortality trend, but no differences were found in all-cause hospitalizations.

Recently, the prognostic impact of PP was extended also to persons with early CKD (KDOQI stages 2–4) (Bolignano et al. 2015). In a series of 468 subjects with early renal impairment, the estimated prevalence of PH according to an ePP cut-off of 35 mmHg was 23 % and the presence of high ePP was predicted by age and left atrial volume. Patients were followed over time (median 4 years) to assess the incidence of a combined endpoint including cardiovascular death, acute decompensated heart failure, coronary artery disease events, and cerebrovascular and peripheral artery events. In a Cox multivariate model adjusting for age, eGFR, hemoglobin, left atrial volume, left ventricular mass, and presence of diabetes mellitus and background CV disease, high ePP predicted a high risk for the combined cardiovascular endpoint (hazard ratio 1.75). Thus, PH is remarkably prevalent also in patients with non-advanced CKD and holds its prognostic capacity with respect to adverse CV outcomes independently of classical and CKD-specific risk factors.

Accruing evidence indicates that the prognostic utility of PP might be extended also to outcomes of kidney transplant recipients. In a retrospective, 3-year observational study (Zlotnick et al. 2010), 55 patients undergoing renal transplantation were studied with respect to the incidence of early graft dysfunction (EGD), defined either as delayed or slow graft function. The percentage of patients developing EGD was higher within the subgroup of patients with high ePP (>35 mmHg). Furthermore, this increased risk (odds ratio 15.0) was fully independent from other traditional risk factors such as age of recipient, history of coronary artery disease, left ventricular ejection fraction, pre-transplant HD, presence of functional AVF, age of donor, and cold ischemia time. Another retrospective study (Issa et al. 2008) analyzed the clinical course of 215 transplant candidates. The authors stratified these subjects into three different ePP categories (<35 mmHg, 35–59 mmHg, and >60 mmHg). Despite most of them having ePP apparently falling within normal values, more than one third presented with frankly pathological values. Roughly 10 % of the study cohort had even seriously high ePP (>60 mmHg). The severity of ePP was directly correlated to dialysis vintage, and the presence of a severe PH (ePP >50 mmHg) was a significant predictor of death after transplantation even after adjustment for age, reduced left ventricular ejection fraction, serum albumin, and delayed graft function.

Table 1 summarizes the main findings from prognostic studies of high PP in renal patients.

Table 1 Main prognostic studies of high PP in renal patients

Study/year	Country	Population	High ePP prevalence	ePP cut-off	Follow-up	Results
Studies looking at mortality and CV outcomes						
Yigla et al. 2003	Israel	58 HD	39.7 % HD	≥35 mmHg	>5 years	At unadjusted Kaplan-Meier survival curves, patients with ePP ≥35 mmHg had poorer survival with respect to those with lower ePP
		5 PD	0 % PD			
Yigla et al. 2009	Israel	127 HD	29.1 %	≥45 mmHg	7 years	High PP conferred a higher risk of death (HR 2.1 and 3.6 for incident and prevalent PH, respectively) with a fully adjusted HR of mortality of 1.5 (1.2–1.9) for each 10 mmHg increase in ePP; ($p = 0.0007$)
Li et al. 2013	China	278 HD	64.7 %	≥30 mmHg	2 years	Subjects with baseline high PP had a HR of CV death of 2.36 with respect to those with normal ePP. High ePP was also an independent predictor of non-fatal CV events (HR 2.27)
Agarwal 2012	USA	288 HD	38 %	≥35 mmHg	2.15 years (median)	The presence of ePP >35 mmHg conveyed a higher risk of death (HR 2.17) after adjustment for race, age, vascular access, serum albumin, and history of CV disease
Ramasubbu et al. 2010	USA	90 HD	47 %	Vmax ≥2.5 m/s	>1 year	Statistically significant parallelism between severity of PH (defined as low, Vmax <2.5 m/s; mild, Vmax 2.6–2.9 m/s; or severe, Vmax >3 m/s) and mortality trend but no differences in all-cause hospitalizations
Bolignano et al. 2015	Italy/ Germany	468 CKD	23 %	≥35 mmHg	4 years (median)	In a Cox multivariate model adjusting for age, eGFR, hemoglobin, left atrial volume, LVM, and presence of diabetes mellitus and background CV disease, high ePP predicted a high risk for a combined CV endpoint (HR 1.75)

(continued)

Table 1 (continued)

Study/year	Country	Population	High ePP prevalence	ePP cut-off	Follow-up	Results
Studies looking at mortality and CV outcomes						
Studies looking at renal outcomes in kidney transplant recipients						
Zlotnick et al. 2010	USA	55 HD	38 %	$\geq$35 mmHg	3 years	Higher percentage of patients developing EGD within the subgroup with high ePP (>35 mmHg) with a fully adjusted OR of 15.0
Issa et al. 2008	USA	215 CKD/HD/PD	32 %	$\geq$35 mmHg	2.5 years	The presence of a severe PH (ePP >50 mmHg) was a significant predictor of death after transplantation, after adjustment for age, reduced left ventricular ejection fraction, serum albumin, and delayed graft function

CKD chronic kidney disease, *CV* cardiovascular, *EGD* early graft dysfunction, *eGFR* estimated glomerular filtration rate, *ePP* estimated pulmonary pressure, *HD* hemodialysis, *HR* hazard ratio, *LVM* left ventricular mass, *OR* odds ratio, *PD* peritoneal dialysis, *PH* pulmonary hypertension, *PP* pulmonary pressure, *Vmax* maximum tricuspidal jet velocity

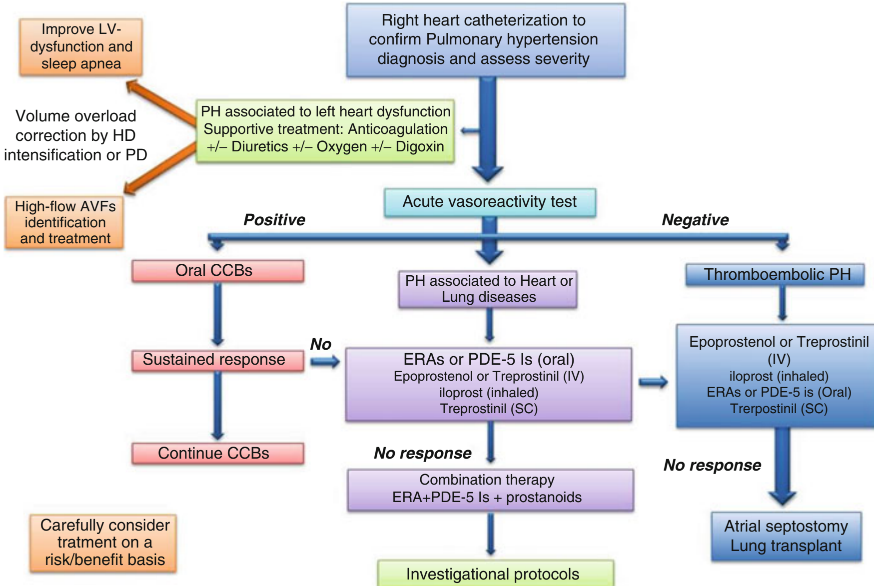

Fig. 5 Potential therapeutic approach to high pulmonary pressure in renal patients. *AVFs* arterio-venous fistulas, *CCBs* calcium-channel blockers, *ERAs* endothelin-receptor antagonists, *HD* hemo-dialysis, *IV* intravenous, *LV* left ventricular, *PDE-5 Is* phosphodiesterase-5 inhibitors, *PD* peritoneal dialysis, *PH* pulmonary hypertension, *SC* subcutaneous

Although few doubts exist on the prognostic usefulness of PP in this population, one question still remains open: is pathologically high PP also as a modifiable factor that deserves correction? Unfortunately, to date there are no specific studies of PH treatment in CKD patients. In the absence of such evidence, one can refer to the therapeutic algorithm proposed by current guidelines on PH (Galie et al. 2009) (Fig. 5). In the general population, the first step is basically to confirm the presence of high PP and to assess the severity and possible type of PH by right heart catheterization. The second step would be to tailor the best treatment to the patient according to the (invasive) vasoreactivity test and the individual response to drug. Nevertheless, given the peculiar characteristics of renal patients, is there any additional approach that could be particularly useful in the CKD setting? Of course, the ideal solution would be to encourage kidney transplantation since accumulating (although not univocal) evidence indicates that PP may revert to normal values after receiving a renal transplant. Yet, as briefly alluded to before, a higher risk of death may persist in kidney transplant recipients with previous PH even after normalization of PP (Issa et al. 2008). Volume overload correction by ultrafiltration intensification or peritoneal dialysis might improve LV dysfunction and sleep apnea. The identification and surgical treatment of very high flow AVF would probably be helpful as well. Finally, when a pharmacological approach is needed, this should be always weighted on a risk/benefit balance, considering that most drugs for PH treatment can be dangerous in patients with impaired renal function or on dialysis.

Conclusions

Evaluation of pulmonary pressure might represent an additional prognostic tool for risk stratification of renal patients. Pulmonary hypertension is highly prevalent in CKD patients, particularly in stage 5 patients on chronic HD. PH in CKD is a potentially reversible process because it may regress after kidney transplantation. However, in dialysis patients with established PH, the excess risk for death may persist after kidney transplantation. Accruing evidence indicates that high PP predicts a high risk of death, particularly in ESKD patients, and worse outcomes in kidney transplant recipients. Large prospective studies adopting well-standardized criteria of PP measurement, such as right heart catheterization, are needed to clarify the exact risk of PH in persons with CKD.

Summary Points

- This chapter focuses on pulmonary pressure (PP), that is, the pressure in the arterial side of the pulmonary circulation, as a novel prognostic biomarker in nephrology.
- In renal patients, PP assessment is now receiving growing attention because high PP (also known as pulmonary hypertension (PH)) is exceedingly prevalent in such population.
- Several factors have been called into question to explain high PP in renal patients, including left heart dysfunction, volume overload, breath disorders, and the presence of high-flow arteriovenous fistulas.
- High PP portends a risk excess for mortality and adverse outcomes in the general population that is fully independent of traditional heart and lung risk factors.
- Similar observations have been reported in renal patients, particularly in dialysis patients.
- In addition, PH predicts adverse renal outcomes in kidney transplant recipients.
- Future studies are eagerly awaited to clarify the exact risk of high PP in renal patients and to demonstrate whether normalization of PP translates into better outcomes.

References

Abassi Z, Nakhoul F, Khankin E, Reisner SA, Yigla M. Pulmonary hypertension in chronic dialysis patients with arteriovenous fistula: pathogenesis and therapeutic prospective. Curr Opin Nephrol Hypertens. 2006;15:353–60.

Agarwal R. Prevalence, determinants and prognosis of pulmonary hypertension among hemodialysis patients. Nephrol Dial Transplant. 2012;27:3908–14.

Arrigoni FI, Vallance P, Haworth SG, Leiper JM. Metabolism of asymmetric dimethylarginines is regulated in the lung developmentally and with pulmonary hypertension induced by hypobaric hypoxia. Circulation. 2003;107:1195–201.

Badesch DB, Champion HC, Sanchez MA, Hoeper MM, Loyd JE, Manes A, Mcgoon M, Naeije R, Olschewski H, Oudiz RJ, Torbicki A. Diagnosis and assessment of pulmonary arterial hypertension. J Am Coll Cardiol. 2009;54:S55–66.

Barcelo A, De La Pena M, Ayllon O, Vega-Agapito MV, Pierola J, Perez G, Gonzalez C, Alonso A, Agusti AG. Increased plasma levels of asymmetric dimethylarginine and soluble CD40 ligand in patients with sleep apnea. Respiration. 2009;77:85–90.

Bolignano D, Rastelli S, Agarwal R, Fliser D, Massy Z, Ortiz A, Wiecek A, Martinez-Castelao A, Covic A, Goldsmith D, Suleymanlar G, Lindholm B, Parati G, Sicari R, Gargani L, Mallamaci F, London G, Zoccali C. Pulmonary hypertension in CKD. Am J Kidney Dis. 2013;61:612–22.

Bolignano D, Lennartz S, Leonardis D, D'Arrigo G, Tripepi R, Emrich IE, Mallamaci F, Fliser D, Heine G, Zoccali C. High estimated pulmonary artery systolic pressure predicts cardiovascular outcomes in stage 2–4 chronic kidney disease. Kidney Int. 2015. doi:10.1038/ki.2015.27.

Buemi M, Senatore M, Gallo GC, Crasci E, Campo S, Sturiale A, Coppolino G, Bolignano D, Frisina N. Pulmonary hypertension and erythropoietin. Kidney Blood Press Res. 2007;30:248–52.

Craddock PR, Fehr J, Brigham KL, Kronenberg RS, Jacob HS. Complement and leukocyte-mediated pulmonary dysfunction in hemodialysis. N Engl J Med. 1977;296:769–74.

Eggers PW. Has the incidence of end-stage renal disease in the USA and other countries stabilized? Curr Opin Nephrol Hypertens. 2011;20:241–5.

Fabbian F, Cantelli S, Molino C, Pala M, Longhini C, Portaluppi F. Pulmonary hypertension in dialysis patients: a cross-sectional italian study. Int J Nephrol. 2011;2011:283475.

Galie N, Hoeper MM, Humbert M, Torbicki A, Vachiery JL, Barbera JA, Beghetti M, Corris P, Gaine S, Gibbs JS, Gomez-Sanchez MA, Jondeau G, Klepetko W, Opitz C, Peacock A, Rubin L, Zellweger M, Simonneau G. Guidelines for the diagnosis and treatment of pulmonary hypertension. Eur Respir J. 2009;34:1219–63.

George MG, Schieb LJ, Ayala C, Talwalkar A, Levant S. Pulmonary hypertension surveillance: United States, 2001 to 2010. Chest. 2014;146:476–95.

Ghio S, Gavazzi A, Campana C, Inserra C, Klersy C, Sebastiani R, Arbustini E, Recusani F, Tavazzi L. Independent and additive prognostic value of right ventricular systolic function and pulmonary artery pressure in patients with chronic heart failure. J Am Coll Cardiol. 2001;37:183–8.

Giaid A. Nitric oxide and endothelin-1 in pulmonary hypertension. Chest. 1998;114:208S–12.

Gilbertson DT. Projecting the ESRD population to 2020 2007 [cited 2014 18 July]. Available from: http://www.usrds.org/2007/pres/GILBERTSON_ASN_PRESENTATION_11_2_07_tmp.swf.

Havlucu Y, Kursat S, Ekmekci C, Celik P, Serter S, Bayturan O, Dinc G. Pulmonary hypertension in patients with chronic renal failure. Respiration. 2007;74:503–10.

Honeycutt AA, Segel JE, Zhuo X, Hoerger TJ, Imai K, Williams D. Medical costs of CKD in the Medicare population. J Am Soc Nephrol. 2013;24:1478–83.

Hyduk A, Croft JB, Ayala C, Zheng K, Zheng ZJ, Mensah GA. Pulmonary hypertension surveillance – United States, 1980–2002. MMWR Surveill Summ. 2005;54:1–28.

Issa N, Krowka MJ, Griffin MD, Hickson LJ, Stegall MD, Cosio FG. Pulmonary hypertension is associated with reduced patient survival after kidney transplantation. Transplantation. 2008;86:1384–8.

Kielstein JT, Bode-Boger SM, Hesse G, Martens-Lobenhoffer J, Takacs A, Fliser D, Hoeper MM. Asymmetrical dimethylarginine in idiopathic pulmonary arterial hypertension. Arterioscler Thromb Vasc Biol. 2005;25:1414–8.

Kiykim AA, Horoz M, Ozcan T, Yildiz I, Sari S, Genctoy G. Pulmonary hypertension in hemodialysis patients without arteriovenous fistula: the effect of dialyzer composition. Ren Fail. 2010;32:1148–52.

Kjaergaard J, Akkan D, Iversen KK, Kjoller E, Kober L, Torp-Pedersen C, Hassager C. Prognostic importance of pulmonary hypertension in patients with heart failure. Am J Cardiol. 2007;99:1146–50.

Lam CS, Borlaug BA, Kane GC, Enders FT, Rodeheffer RJ, Redfield MM. Age-associated increases in pulmonary artery systolic pressure in the general population. Circulation. 2009;119:2663–70.

Lau EM, Humbert M, Celermajer DS. Early detection of pulmonary arterial hypertension. Nat Rev Cardiol. 2014;12(3):143–155.

Li Z, Liu S, Liang X, Wang W, Fei H, Hu P, Chen Y, Xu L, Li R, Shi W. Pulmonary hypertension as an independent predictor of cardiovascular mortality and events in hemodialysis patients. Int Urol Nephrol. 2013;46(1):141–9.

Mcglothlin D. Classification of pulmonary hypertension. Heart Fail Clin. 2012;8:301–17.

Naeije R. Physiology of the pulmonary circulation and the right heart. Curr Hypertens Rep. 2013;15:623–31.

Nakhoul F, Yigla M, Gilman R, Reisner SA, Abassi Z. The pathogenesis of pulmonary hypertension in haemodialysis patients via arterio-venous access. Nephrol Dial Transplant. 2005;20:1686–92.

Nitta K, Akiba T, Uchida K, Kawashima A, Yumura W, Kabaya T, Nihei H. The progression of vascular calcification and serum osteoprotegerin levels in patients on long-term hemodialysis. Am J Kidney Dis. 2003;42:303–9.

Pabst S, Hammerstingl C, Hundt F, Gerhardt T, Grohe C, Nickenig G, Woitas R, Skowasch D. Pulmonary hypertension in patients with chronic kidney disease on dialysis and without dialysis: results of the PEPPER-study. PLoS One. 2012;7:e35310.

Ramasubbu K, Deswal A, Herdejurgen C, Aguilar D, Frost AE. A prospective echocardiographic evaluation of pulmonary hypertension in chronic hemodialysis patients in the United States: prevalence and clinical significance. Int J Gen Med. 2010;3:279–86.

Rudski LG, Lai WW, Afilalo J, Hua L, Handschumacher MD, Chandrasekaran K, Solomon SD, Louie EK, Schiller NB. Guidelines for the echocardiographic assessment of the right heart in adults: a report from the American Society of Echocardiography endorsed by the European Association of Echocardiography, a registered branch of the European Society of Cardiology, and the Canadian Society of Echocardiography. J Am Soc Echocardiogr. 2010;23:685–713. quiz 786-8.

Sakaguchi Y, Shoji T, Kawabata H, Niihata K, Suzuki A, Kaneko T, Okada N, Isaka Y, Rakugi H, Tsubakihara Y. High prevalence of obstructive sleep apnea and its association with renal function among nondialysis chronic kidney disease patients in Japan: a cross-sectional study. Clin J Am Soc Nephrol. 2011;6:995–1000.

Sasaki A, Doi S, Mizutani S, Azuma H. Roles of accumulated endogenous nitric oxide synthase inhibitors, enhanced arginase activity, and attenuated nitric oxide synthase activity in endothelial cells for pulmonary hypertension in rats. Am J Physiol Lung Cell Mol Physiol. 2007;292: L1480–7.

Sica AL, Greenberg HE, Ruggiero DA, Scharf SM. Chronic-intermittent hypoxia: a model of sympathetic activation in the rat. Respir Physiol. 2000;121:173–84.

Simonneau G, Robbins IM, Beghetti M, Channick RN, Delcroix M, Denton CP, Elliott CG, Gaine SP, Gladwin MT, Jing ZC, Krowka MJ, Langleben D, Nakanishi N, Souza R. Updated clinical classification of pulmonary hypertension. J Am Coll Cardiol. 2009;54:S43–54.

Strange G, Playford D, Stewart S, Deague JA, Nelson H, Kent A, Gabbay E. Pulmonary hypertension: prevalence and mortality in the Armadale echocardiography cohort. Heart. 2012;98:1805–11.

Ward JP, Mcmurtry IF. Mechanisms of hypoxic pulmonary vasoconstriction and their roles in pulmonary hypertension: new findings for an old problem. Curr Opin Pharmacol. 2009;9:287–96.

Yigla M, Nakhoul F, Sabag A, Tov N, Gorevich B, Abassi Z, Reisner SA. Pulmonary hypertension in patients with end-stage renal disease. Chest. 2003;123:1577–82.

Yigla M, Keidar Z, Safadi I, Tov N, Reisner SA, Nakhoul F. Pulmonary calcification in hemodialysis patients: correlation with pulmonary artery pressure values. Kidney Int. 2004;66:806–10.

Yigla M, Fruchter O, Aharonson D, Yanay N, Reisner SA, Lewin M, Nakhoul F. Pulmonary hypertension is an independent predictor of mortality in hemodialysis patients. Kidney Int. 2009;75:969–75.

Yock PG, Popp RL. Noninvasive estimation of right ventricular systolic pressure by Doppler ultrasound in patients with tricuspid regurgitation. Circulation. 1984;70:657–62.

Zlotnick DM, Axelrod DA, Chobanian MC, Friedman S, Brown J, Catherwood E, Costa SP. Non-invasive detection of pulmonary hypertension prior to renal transplantation is a predictor of increased risk for early graft dysfunction. Nephrol Dial Transplant. 2010;25:3090–6.

Zoccali C. The endothelium as a target in renal diseases. J Nephrol. 2007;20 Suppl 12:S39–44.

Zoccali C, Bode-Boger S, Mallamaci F, Benedetto F, Tripepi G, Malatino L, Cataliotti A, Bellanuova I, Fermo I, Frolich J, Boger R. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. Lancet. 2001;358:2113–7.

Zoccali C, Mallamaci F, Tripepi G. Nocturnal hypoxemia predicts incident cardiovascular complications in dialysis patients. J Am Soc Nephrol. 2002;13:729–33.

Estimation of Glomerular Filtration Rate 51

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Abstract

We describe the principles of estimating glomerular filtration rate (GFR) with an emphasis on the role of creatinine and cystatin C in the calculation of estimated GFR (eGFR). We present a list of selected equations for eGFR calculation together with a critical evaluation of their role in the diagnostics, classification, and monitoring of kidney function.

History: The first part briefly describes the history of GFR assessment.

Pathophysiology: The second part deals with the physiology of GFR, the Starling equation, and the regulation of GFR.

Creatinine, cystatin C, and urea: The third part describes the pathophysiology, analytical details on the measurements, interferences, reference intervals, and important features of creatinine, cystatin C, and urea, together with data on intraindividual and interindividual variability of these compounds, desirable accuracy, and precision as well as data on real analytical quality, indexes of individuality, and reference change values.

Estimation of GFR: The fourth part describes the principle of renal clearance, creatinine clearance and the Cockcroft and Gault equation, eGFR based on cystatin C, urea clearance and the mean of urea and creatinine clearance, the MDRD formula, the CKD-EPI equations, the Lund-Malmö equations, and equations used in children. Comparison of inulin clearance with other equations for eGFR is also given.

Interpretation: The fifth part deals with the weaker aspects and sources of errors that may occur when using creatinine and cystatin C in the estimation of GFR. The relation of age and body surface area to GFR is also described.

Clinical use of eGFR: The sixth part describes the importance of eGFR in the classification of chronic kidney disease, diabetes mellitus, and acute kidney injury, with an emphasis on recommendations in recent guidelines.

Keywords

Glomerular filtration rate • Chronic kidney disease • CKD-EPI equation • Creatinine • Cystatin C • Urea

Abbreviations

ACR	Albumin-to-creatinine ratio
ADA	American Diabetes Association
ADQI	Acute Dialysis Quality Initiative
AKI	Acute kidney injury
AKIN	Acute Kidney Injury Network
BMI	Body mass index
BSA	Body surface area
BUN	Blood urea nitrogen
CCr	Clearance of creatinine
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
GFR	Glomerular filtration rate
HPLC	High-performance liquid chromatography
ID-MS	Isotope dilution mass spectrometry
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
KDIGO	Kidney Disease: Improving Global Outcomes
LBM	Lean body mass
MDRD	Modification of Diet in Renal Diseases
NDF	Net driving force (net filtration pressure)
NIST SRM	National Institute of Standards and Technology, Standard Reference Material
PCR	Protein-to-creatinine ratio
RCV	Reference change value, critical difference
RIFLE	Risk, Injury, Failure, Loss of function, End-stage kidney disease
RRT	Renal replacement therapy
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

Key Facts

Glomerular Filtration Rate: Key Facts

- Doctors require information on two principal kidney properties: function of glomeruli and function of tubuli.
- Glomerular filtration rate (GFR) is a measure of the filtration ability of the kidney.
- Glomerular filtration rate can be measured, or – much more frequently – estimated (eGFR).
- Doctors use eGFR for diagnostics and staging of kidney diseases according to internationally accepted guidelines.

Assessment of Glomerular Filtration Rate: Key Facts

- The gold standard for direct measurement of GFR is inulin clearance. Other slightly less precise but acceptable methods also include iohexol or iothalamate clearance.
- Direct measurement of GFR is impractical and time consuming; therefore, GFR is estimated from the concentration of serum biomarkers.
- Two serum biomarkers are used: creatinine and cystatin C, where the higher the creatinine or cystatin C, the lower the GFR.
- Laboratories provide physicians with information on eGFR by means of calculations based on serum creatinine and/or serum cystatin C.
- Reference ranges for estimated GFR (eGFR) are not specified, because of the necessity to evaluate kidney function in the context of the clinical condition of the patient; however, values lower than 90 mL/min per 1.73 m^2 (1.5 mL/s per 1.73 m^2) are considered decreased.

Creatinine and Cystatin C in the Assessment of GFR: Key Facts

- Creatinine and cystatin C are endogenous compounds eliminated by the kidneys, mainly via glomerular filtration. Both are widely used in medical laboratories.
- There are a number of internationally accepted equations for eGFR calculation.
- Creatinine and cystatin C have some disadvantages: creatinine is influenced by a high-protein diet, muscle mass changes, and muscle catabolism; tubular secretion of creatinine can occur; some methods interfere with body substances and drugs; cystatin C is influenced by corticosteroids, in diabetes mellitus and diseases of the thyroid gland.

Definitions

CKD-EPI equations The recommended equations for estimating glomerular filtration rate, based on serum creatinine (the 2009 CKD-EPI creatinine equation), serum cystatin C (the 2012 CKD-EPI cystatin C equation), or both (the 2012 CKD-EPI cystatin C and creatinine equations).

Cockcroft-Gault equation Not recommended. An obsolete formula for estimating glomerular filtration rate, based on sex, weight, and serum creatinine.

Creatinine A metabolic product of muscle creatine, produced by the body at a constant rate. Elimination is influenced by kidney glomerular function, and increased concentration in blood is found in patients with decreased glomerular filtration rate.

Creatinine clearance Not recommended. A method of estimating glomerular filtration rate, based on the measurement of creatinine concentration in serum and urine together with the collection of urine during a specified time interval. Creatinine clearance has been replaced by more modern equations based on serum concentrations of creatinine and/or cystatin C.

Cystatin C A protein inhibitor of cysteine proteases, produced by all nucleated cells at a constant rate. Elimination is influenced by kidney filtration, and increased concentration in blood is found in patients with decreased glomerular filtration rate. In contrast to creatinine, a very low amount of cystatin C is excreted by the kidney, because of tubular reabsorption of this compound.

Glomerular filtration rate The volume of plasma ultrafiltrate (primary urine) generated by the kidneys per unit of time. Values are expressed in ml/min (or ml/s) per standardized body surface area (1.73 m^2).

MDRD equation Not recommended. A formula that was used to estimate glomerular filtration rate between 1999 and 2009.

Urea A metabolic product of body proteins, synthesized by the liver. Urea represents the main route of nitrogen elimination from the body. Increased concentration in the blood occurs during hypercatabolism of proteins or kidney failure.

Introduction

Estimation of glomerular filtration rate (GFR) is a basic method of assessing kidney function. Decreased GFR, present for more than 3 months, is one of the important diagnostic criteria for chronic kidney disease (CKD). Furthermore, CKD is classified based on the GFR category (KDIGO 2012 Guideline 2013). Gold-standard methods (renal or plasma inulin clearances) are precise, but time consuming. Creatinine has been used as a surrogate marker in GFR assessment for years, but physicians are more interested in function (GFR) than concentration (creatinine in plasma). Therefore, the reliable conversion of plasma biomarker concentration to eGFR is used for practical reasons. Among the available biomarkers, creatinine and cystatin C are mostly recommended to calculate eGFR, provided they are traceable to international calibration and suitable equations are used. Due to the plethora of eGFR equations, it is necessary to develop practical guidelines for international application and support their use in clinical practice.

Historical Background

Max Eduard Jaffé published his paper on the estimation of creatinine in 1886 (Jaffé 1886). Many modifications appeared because of the interference of what became known as Jaffé-positive substances. However, the Jaffé reaction with alkaline

picrate is used even today, and enzymatic determination of creatinine is only slowly becoming the main method for the determination of creatinine in routine clinical laboratories. Inulin clearance was described in 1935 by Shannon and Smith (Shannon and Smith 1935), and the recent KDIGO 2012 Guideline (KDIGO 2012 Guideline 2013) recognizes inulin clearance as a gold standard of GFR assessment. The Cockcroft and Gault equation was published in 1976 (Cockcroft and Gault 1976). This equation was derived from samples of 249 patients aged 18–92. The MDRD equation was published by Andrew Levey (Levey et al.1999), and the same author published the CKD-EPI equation in 2009 (Levey et al. 2009). The best and most recent recommendation describing the use of creatinine, cystatin C, and estimations of GFR was published in 2013 in the "KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease."

Physiology of Glomerular Filtration Rate

Approximately 80 % of plasma volume entering afferent renal arterioles passes to efferent arterioles, and the remaining 20 % of plasma volume is filtered. Filtration is illustrated by the Starling equation.

The general Starling equation describes the rate of fluid through the capillary wall:

$$J_V = K_F A[(P_C - P_T) - \delta(\pi_C - \pi_T)]$$

where

J_V = rate of fluid
K_F = capillary filtration constant
A = area for fluid exchange
P_C and P_T = capillary and tissue hydrostatic pressure
π_C and π_T = capillary and tissue oncotic pressure
δ = coefficient describing possible transfer of proteins

A simplified equation describing net filtration pressure (or net driving force, NDF) in the glomeruli is as follows:

$$NDF = (P_C - P_T) - (\pi_C - \pi_T)$$

where

P_C = hydrostatic pressure of plasma (blood pressure)
P_T = fluid pressure in the Bowman's capsule
π_C = oncotic pressure of plasma proteins
π_T = oncotic pressure of plasma ultrafiltrate in the Bowman's capsule

Filtration appears where NDF > 0. Filtration is influenced by the filtration area (e.g., 50 % in patients with one kidney), the quality of the glomerular membrane (changed in acidemia or in kidney disease), and the ability of various proteins to enter the Bowman's capsule (molecular weight, shape of the molecule, isoelectric point of the protein). Approximately 120 mL/min (~170 L per day) of ultrafiltrate is generated every day, while about 1 mL/min (~1.5 L per day) of urine is excreted daily. This means that 99 % of the ultrafiltrate is reabsorbed (i.e., the fractional excretion of water is about 1 %). Glomerular filtration rate (GFR) is regulated by myogenic stretch and tubuloglomerular feedback. Tubuloglomerular feedback represents the autoregulation system, where GFR is regulated according to tubular urine flow. The aim of the system is to decrease loss of water and ions in situations with increased glomerular filtration rate. In case of increased GFR, the delivery of the chloride ion to the thick ascending limb of the loop of Henle is increased, which results in the constriction of afferent arterioles and the decrease of GFR. Sympathetic regulation, angiotensin II, prostaglandins, and natriuretic peptides contribute to the regulation of GFR.

Creatinine, Cystatin C, and Urea

Creatinine

Pathophysiology

Muscle creatine is converted to creatinine by a spontaneous, nonenzymatic reaction at a constant rate; however, ingested meat is a source of creatine and hence of creatinine. Creatinine is eliminated by glomerular filtration, but proximal secretion of creatinine exists; proximal secretion of creatinine increases with decreasing GFR. Also, a small amount of creatinine is reabsorbed by tubuli.

Analytical Remarks

International calibration is based on NIST SRM 967 (human liquid serum, traceable to the ID-MS (isotope dilution mass spectrometry) reference method). Two groups of methods are used throughout the world: the group of Jaffé methods with alkaline picrate and the group of enzymatic methods. Two key enzymes are used: creatinine deaminase (creatinine iminohydrolase, EC 3.5.4.21) and creatininase (creatinine amidohydrolase, EC 3.5.2.10). Other methods, e.g., HPLC, high-performance liquid chromatography, are not used routinely. The reference method is ID-MS.

Interferences

The Jaffé reaction is specific not only to creatinine, but many compounds – known as "Jaffé-positive chromogens" – can falsely increase concentration of creatinine, when measured in plasma or serum. Among them, glucose, protein, fatty acids, acetone, acetoacetate, pyruvate, and cephalosporins are the most important compounds. The measurement in urine is less influenced by these compounds. Other substances can falsely decrease creatinine concentration, e.g., ascorbic acid and bilirubin.

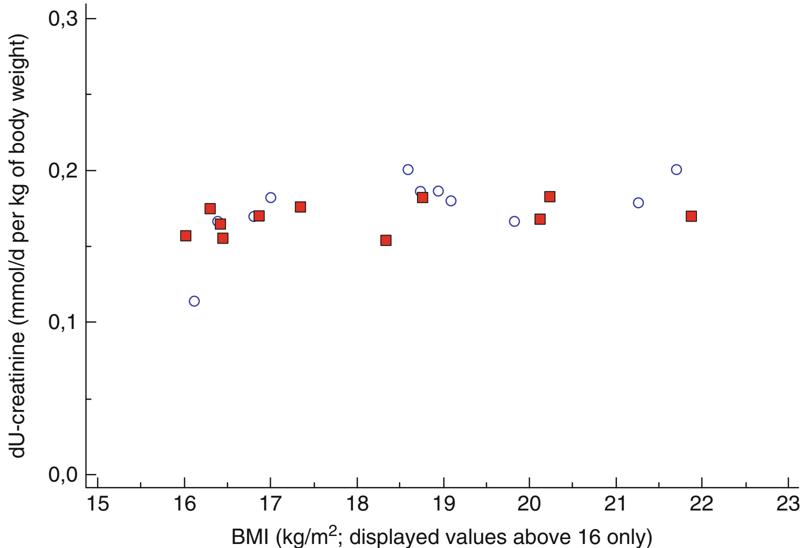

Fig. 1 The relationship between BMI and creatinine output over 24 h calculated per kilogram of the total body weight in adolescents (Based on data published by Remer 2002**).** As can be seen from the Figure, urinary output (dU) of creatinine (recalculated per kg of the body weight) is constant over a wide range of body mass index (*BMI*). Two outliers were excluded. *Circle* = boys, *square* = girls

Enzymatic methods can be influenced by bilirubin, hemoglobin, monoclonal immunoglobulin M, and some drugs, e.g., dobutamine.

Reference Intervals

Reference intervals are based on the abovementioned international calibration. Reference intervals for plasma (serum) creatinine are always different for both sexes due to the difference in muscle mass. Reference intervals for plasma (serum) are 64–104 μmol/L (0.72–1.18 mg/dL) in men and 49–90 μmol/L (0.55–1.02 mg/dL) in women.

Output of Creatinine

In order to predict the output of creatinine, a great number of equations for estimating glomerular filtration rate (eGFR), e.g., the Cockcroft-Gault equation, are based on the patient's weight. Therefore, this equation fails in individuals with fluid retention or obesity. Output of creatinine calculated per kilogram of the body weight is expected to be constant in adults without fluid retention, excessive amount of body fat, or muscle atrophies. Figure 1 shows body mass index (BMI) 16–22 kg/m^2, where creatinine output calculated per kilogram of the body weight is practically independent of BMI. Output of creatinine ranges from 0.15 to 0.18 mmol/day/kg of the body weight (median 0.17 mmol/day/kg of body weight, 10th percentile 0.13, 90th percentile 0.20 mmol/day/kg of body weight).

If a constant amount of creatinine is excreted from one kilogram of body mass (except for individuals with muscle atrophies, fluid retention, or excessive obesity), we may then check the output of creatinine in adults in comparison to body mass. Table 1 indicates the reference limits of daily creatinine excretion in the urine according to ARUP's Laboratory Test Directory (http://ltd.aruplab.com/Tests/Pub/0020473).

If values are recalculated based on body weight, i.e., we want to calculate the body weight in relation to the amount of excreted creatinine (a coefficient of 0.17 mmol of creatinine based on one kilogram of the total body weight per day was used), unrealistic values are obtained in some cases. Maximum creatinine output is derived from the maximum possible GFR (150 mL/min, i.e., 2.5 mL/s). When diuresis is 1,500 mL/day, plasmatic concentration of creatinine is 90 μmol/L, and concentration of creatinine in the urine is 13 mmol/L, then creatinine clearance is 2.508 mL/s and creatinine output is 19.5 mmol/day (Schück, 2012, personal communication). Nevertheless, the maximum possible output of creatinine does not represent the reference limit. Therefore, we recommend the following reference ranges for creatinine output in the urine over 24 h:

Men	10–16 mmol/day (1.13–1.81 g/day)
Women	8–14 mmol/day (0.90–1.58 g/day)

Note: If the coefficient of 0.17 mmol/day per kilogram of the body weight is used, the corresponding weights are 59–94 kg in men and 47–82 kg in women.

Ratios of Urinary Compounds to Creatinine

Ratios of urinary compounds to creatinine are becoming increasingly popular as tools suitable for evaluating substances eliminated through the urine, based on the principle that the concentration of creatinine reflects diuresis (Fig. 2). It is obvious that the reliability of the estimation of diuresis from creatinine concentration decreases with decreasing creatinine concentration.

The most used ratios are PCR (protein-to-creatinine ratio) and ACR (albumin-to-creatinine ratio). As can be seen from Fig. 2, lower concentrations of urinary creatinine are rather poor predictors of urine volume. On the other hand, interpreting ratios is practical and much better than evaluation of concentration alone. However, timed urine samples can provide the physician with more accurate protein and albumin excretion values.

Cystatin C

Pathophysiology

Cystatin C (cystatin 3, gamma-trace protein) is a member of cystatin superfamily II, gen CST3, locus 20p11.2. Cystatin C is a basic (isoelectric point, pI, 9.3) protein composed of a single polypeptide chain of 120 amino acid residues with a molecular weight of 13.4 kDa (13,359 g/mol). Cystatin C is synthesized at a constant rate by all nucleated cells. The synthesis is not influenced by inflammation, catabolism, or diet.

Table 1 The reference limits of creatinine output indicated in the literature (ARUP's Laboratory Test Directory)

Age	Men (mmol/day)		Women (mmol/day)		Men (g/day)		Women (g/day)	
	Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit
3–8 years	1.2	6.2	1.2	6.2	0.14	0.70	0.14	0.70
9–12 years	2.7	11.5	2.7	11.5	0.31	1.30	0.31	1.30
13–17 years	4.4	20.3	3.5	14.1	0.50	2.30	0.40	1.60
18–50 years	8.8	22.1	6.2	14.1	1.00	2.50	0.70	1.60
51–80 years	7.1	18.6	4.4	12.4	0.80	2.10	0.50	1.40
>81 years	5.3	17.7	3.5	11.5	0.60	2.00	0.40	1.30

Daily output of creatinine increases with increasing muscle mass. However, the upper limit of creatinine daily output is unrealistic. For example, the upper limit of creatinine in the group of men aged 18–50 years (22.1 mmol/day) corresponds to 130 kg of the body weight, when a factor of 0.170 mmol/kg of the body weight is used. The error is due to an incorrect collection of urine during experiments when defining reference ranges

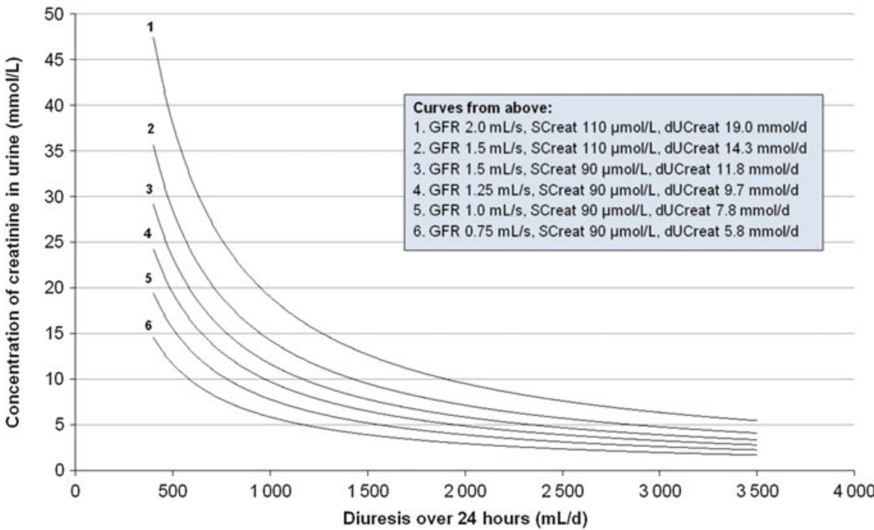

Fig. 2 The relationship between diuresis and concentration of creatinine in the urine. Creatinine is excreted at a constant rate. Therefore, concentration of creatinine in the urine decreases with increasing volume of urine. However, kidney function influences the balance between serum and urine creatinine. Higher concentrations of creatinine in a lower volume of urine have an acceptable low error estimate. On the other hand, lower concentrations of creatinine in the urine may represent different volumes of urine, and the estimation of urinary volume is imprecise. For example, concentration of creatinine of 20 mmol/L means that the volume of urine is between approximately 500 and 1,000 mL/day. In contrast, concentration of creatinine of about 5 mmol/L means that the volume of urine is between 1,200 and 3,500 mL/day for different kidney functions. Nevertheless, ratios of urinary compounds to creatinine are better estimates for interpretation purposes than concentrations of these compounds without any correction

Measurable concentrations of cystatin C are found in the plasma (about 1 mg/L), urine (0.2 mg/L), cerebrospinal fluid (3–14 mg/L), seminal plasma (40–60 mg/L), milk (2 mg/L), saliva (0.4–5 mg/L), tears (1 mg/L), and amniotic (1 mg/L) and synovial fluid (2 mg/L). With regard to molecular weight and pI, cystatin C is freely filtered and completely reabsorbed and degraded in the proximal tubuli. Therefore, the plasma concentration of cystatin C indirectly reflects glomerular filtration rate, and increased urine concentration reflects tubular injury.

Analytical Remarks

International calibration is based on CRM ERM-DA471/IFCC. Immunoassay is a dominant principle of cystatin C measurement.

The Use of Cystatin C in Nephrology

The measurement of cystatin C is recommended in situations where eGFR based on serum creatinine is less accurate and also for confirmation of CKD in adult patients with mildly to moderately decreased eGFR (based on creatinine), i.e., 45–59 mL/min per 1.73 m^2 (G3a) (KDIGO 2012 Guideline 2013). As cited from KDIGO 2012

Guideline: “1.4.3.2: We suggest using additional tests (such as cystatin C or a clearance measurement) for confirmatory testing in specific circumstances where eGFR based on serum creatinine is less accurate. (2B).”

Cystatin C, Cardiovascular Mortality, and Morbidity

Shlipak (Shlipak et al. 2005) published a relation between increased concentrations of cystatin and the annual rate of death due to all causes and also death due to cardiovascular causes. While the relation between cystatin C and the rate of death was almost exponential (the higher the level of cystatin C, the higher the hazard ratio), association of creatinine categories and mortality revealed a J-shaped curve. One important result was that within each quintile of creatinine concentration, higher quintiles of cystatin C were associated with increased risk of mortality due to all causes. Cystatin C was further assessed as a cardiovascular risk factor (Taglieri et al. 2009; Woitas et al. 2013) and is classified as a cardiorenal biomarker (Chowdhury et al. 2013).

Reference Intervals

Reference intervals based on the abovementioned international calibration are 0.31–0.99 mg/L in men and 0.4–0.99 mg/L in women.

Urea

Pathophysiology

Urea is an end product of protein catabolism, synthesized in the liver. About 90 % of urea is excreted by the kidneys. Free filtration in the glomeruli is followed by several stages of passive tubular transport, which results in the reentering of the plasma compartment. Clearance of urea is lower than true GFR. Impaired kidney function, dehydration, increased protein catabolism (including catabolism of protein in gastrointestinal bleeding), a high-protein diet, administration of cortisol, and obstruction of the urinary tract are the main reasons for increased urea concentration in plasma. Creatinine rises more slowly than urea in prerenal failure or in cases of decreased renal blood flow.

Analytical Remarks

Older nonenzymatic methods of urea determination are used in exceptional cases, and the majority of laboratories use the enzymatic method with urease (EC 3.5.1.5) and glutamate dehydrogenase (EC 1.4.1.3). International calibration is based on NIST SRM 912a.

Reference Intervals

Increased concentrations of urea are found in older populations and in men. Different reference values can be found in the literature. Reference intervals in men up to 50 years of age are 3.2–7.4 mmol/L and 3.0–9.2 mmol/L in men above 50 years of age. The intervals are 2.5–6.7 mmol/L in women up to 50 years of age and 3.5–7.2 mmol/L in women above 50 years of age.

Output of Urea

Output of urea is influenced by protein catabolism. The normal concentration of urea in the urine is 250–433 mmol/L. If daily volume of urine is about 1,350 mL/day, daily production of urea is calculated at 340–585 mmol/day.

Biological and Analytical Variability of GFR Measures and Related Tests

Intraindividual (variability within a subject) and interindividual (variability between subjects) biological variabilities are important characteristics required for accurate interpretation of laboratory tests. Desirable precision, maximum tolerable bias, and total allowable error are calculated according to biological variability, and they define the requirements for laboratory tests. The index of individuality is calculated as a ratio of intraindividual biological variability and analytical variability. Reasonable analytical variability is expressed as a coefficient of variability (CVa, given in %) and calculated from intermediate precision (long-term analytical variability assessed over a period of several weeks) in the clinical laboratory. The reference change value (RCV, or critical difference, given in %) is calculated from intraindividual variability (CVi) and analytical variability (CVa) as

$$\mathrm{RCV} = 2.77 * \left(\mathrm{CVa}^2 + \mathrm{CVi}^2\right)^{1/2}$$

and expresses changes (in %) in the concentration of the analyte, which represents a significant departure from the basal value. The absolute RCV is calculated for the selected basal value. Required parameters for accurate interpretation of laboratory tests are given in Table 2.

Estimation Methods of GFR

The Principle of Renal Clearance

Renal clearance is defined as "the volume of plasma from which the substance is completely cleared by the kidneys per unit of time" (Burtis 2006). For a substance with specific properties (stable rate of synthesis, stable plasma concentration, freely filtered, no influence of renal tubuli in terms of reabsorption, secretion, synthesis, or catabolism), the amount of the filtered substance is the same as the amount of the excreted substance:

$$\mathrm{GFR} * \mathrm{PSubst} = \mathrm{USubst} * \mathrm{V}$$

where GFR is the glomerular filtration rate,

Table 2 Required parameters for accurate interpretation of laboratory tests (S/P = serum or plasma, dU = output per day)

	S/P creatinine	dU creatinine	S/P cystatin C	S/P urea	dU urea
Intraindividual biological variability	6.0 %	11.0 %	4.6 %	12.3 %	17.4 %
Interindividual biological variability	14.7 %	23.0 %	13.0 %	18.3 %	25.4 %
Desirable precision	3.0 %	5.5 %	2.3 %	6.2 %	8.7 %
Maximum tolerable bias	4.0 %	6.4 %	3.5 %	5.5 %	7.7 %
Total allowable error	8.9 %	15.4 %	7.2 %	15.7 %	22.1 %
Index of individuality	0.41	0.48	0.35	0.67	0.69
Reasonable CVa	2.0 %	2.7 %	2.0 %	3.4 %	2.6 %
Reference change value (%)	17.5 %	31.4 %	13.9 %	35.3 %	48.7 %
Reference change value// for baseline value	17.5 μmol/L// 100 μmol/L	3.77 mmol/d// 12 mmol/d	0.14 mg/L// 1 mg/L	3.54 mmol/L// 10 mmol/L	171 mmol/d// 350 mmol/d

Intraindividual variability corresponds to the fluctuation of the concentration “within” a person, while interindividual variability corresponds to the difference “between” persons. Analytical quality must reflect the parameters of biological variability: the lower the biological variability, the better analytical performance required. Analytical quality derived from biological parameters is represented by desirable imprecision, maximum tolerable bias, and total allowable error. It is essential to use the reference change value in order to properly interpret two consecutive measurements and to achieve a significant difference: the higher the biological (CVi) and analytical (CVa) variability, the higher the difference needed between the two consecutive values to be significant

PSubst and USubst are plasma and urine concentrations of that substance, respectively, and V is the volume of urine per unit of time.

Then we have

$$GFR = (USubst * V)/PSubst$$

The problem consists in the properties of an "ideal" substance: creatinine is far from ideal due to proximal tubular secretion; inulin, ^{51}Cr-EDTA, and iohexol display extrarenal clearance; the use of ^{51}Cr-EDTA, ^{125}I-iothalamate, and ^{99m}Tc-DTPA is connected with the risk of ionizing radiation; other markers are neither sensitive nor specific for the assessment of GFR (beta-2-microglobulin, alpha-1-microglobulin, urea, and retinol-binding protein).

Creatinine Clearance and the Historical Cockcroft and Gault Equation

Creatinine is supposed to be filtrated freely by the glomeruli and excreted in the urine without any tubular secretion or reabsorption. Unfortunately, this is not the case since creatinine is secreted in the proximal tubuli and this secretion increases with decreasing GFR. Renal clearance of creatinine (CCr) is calculated by the following simple equation:

$$eGFR \sim CCr = (UCreat * V)/SCreat \tag{1}$$

where UCreat is the concentration of creatinine in the urine, V is the volume of urine per unit of time, and SCreat is the plasma (serum) concentration of creatinine. Volume is expressed in mL, time in minutes (SI units: in seconds), and concentrations of creatinine must be in the same units (both urine and plasma in mg/dL, or both urine and plasma in mmol/L). The product (UCreat * V) in Eq. 1 represents the rate of creatinine elimination in the urine, e.g., in g/day (SI: mmol/day). Creatinine is supposed to be synthesized at a constant rate, where the amount of creatinine produced daily is a function of body muscle mass. Because of the correlation between the muscle mass and body weight, one can assume that the rate of creatinine elimination can be deduced from

$$eGFR \sim CCr = X/SCreat \tag{2}$$

where X represents the amount of creatinine excreted per day. For example, here is the Cockcroft and Gault formula

$$eGFR = [(140 - Age) * Weight]/(48,8 * SCreat) * F \tag{3}$$

where Age is in years, Weight is in kg, SCreat is in μmol/L, factor F is 1.0 for men and 0.85 for women, and eGFR is in mL/s.

Cockcroft and Gault derived this formula in 1976 based on data from 249 healthy volunteers. This formula is not recommended any more. However, there have been

instances where this formula has been used among clinical pharmacists; also, some pharmacokinetic programs still use the obsolete equation. A similar principle is used in other equations, where the estimation of creatinine output is calculated from body measurements (height in children, weight in adults, etc.).

eGFR Based on Cystatin C

There are many equations based on the assumption that cystatin C is produced at a constant rate:

$$\mathrm{eGFR} = \mathrm{X}/\mathrm{SCystC}$$

where X is an estimate of the daily output of cystatin C.

However, certain extrarenal clearance of cystatin C exists, and therefore, new equations are based on the regression between cystatin C and the "gold" standard.

In 2005, Grubb derived a cystatin C-based equation for use in adults and children (Grubb 2005):

$$\mathrm{eGFR} = 84.69 * \mathrm{SCystC}^{-1.680} * 1.384(\mathrm{if} < 14\mathrm{years})$$

or with gender factor

$$\mathrm{eGFR} = 87.62 * \mathrm{SCystC}^{-1.693} * 1.376(\mathrm{if} < 14\mathrm{years}) * 0.940(\mathrm{if\ female})$$

Results are in mL/min per 1.73 m^2.

A review of other cystatin C-based equations in children was published by Andersen (Andersen et al. 2009) and Filler (Filler et al. 2012).

The CKD-EPI formula based on cystatin C was published in 2013 (KDIGO 2012 Guideline 2013). It should be noted, however, that Grubb published a new cystatin C-based equation in 2014:

$$\mathrm{eGFR} = 130 * \mathrm{SCystC}^{-1.3069} * \mathrm{Age}^{-0.117} - 7\left(\mathrm{mL/min\ per\ 1.73m^2}\right)$$

This equation disregards sex and race and can be used as an assay-independent calculation (Grubb 2014).

Urea Clearance

When compared with GFR measured by inulin clearance, urea clearance underestimates GFR, while creatinine clearance overestimates GFR. Therefore, an equation was derived as a mean of urea and creatinine clearance (listed in the European Best Practice Guidelines for Haemodialysis (ERA-EDTA 2002)).

Mean of Urea Clearance and Creatinine Clearance

This equation is based on the assumption that urea clearance underestimates “true” GFR and creatinine clearance (due to the proximal secretion of creatinine, increased with decreasing GFR) overestimates GFR:

$$\mathrm{eGFR} = \mathrm{V}/(2 * \mathrm{t}) * (\mathrm{UUrea}/\mathrm{SUrea} + \mathrm{UCreat}/\mathrm{SCreat}) * 1.73/\mathrm{BSA}$$

V = volume of urine in mL per time
t = time in minutes or seconds
UUrea and UCreat = urinary concentrations of urea and creatinine in mmol/L
SUrea nad SCreat = serum concentrations of urea and creatinine in mmol/L,
BSA = body surface area in m^2

$$\text{eGFR is then in mL/min per } 1.73\mathrm{m}^2 \text{or mL/s per } 1.73\mathrm{m}^2.$$

MDRD Formula

MDRD (Modification of Diet in Renal Diseases) was published by Levey (Levey et al. 1999). Three equations were derived for chronic kidney diseases, the first based on age, gender, and serum creatinine (and race); the second on age, gender, serum creatinine, and serum urea (and race); and the third on age, gender, serum creatinine, serum urea, and serum albumin (and race). The equations were derived from a sample of 1,070 patients and validated using a sample of 558 patients with chronic kidney disease. Renal clearance of ^{125}I-iothalamate was selected as the “gold” standard; creatinine was measured using a kinetic alkaline picrate method. The first equation was enhanced by improvements in international creatinine standardization: the “new” MDRD formula (called the “175 MDRD equation”) replaced previous versions (Levey et al. 2007):

$$\mathrm{eGFR(MDRD)} = 175 * \left(\mathrm{SCreat}^{-1.154}\right) * \mathrm{Age}^{-0.203} * \mathrm{F}$$

where SCreat is in mg/dL, Age is in years, and F is 1.0 for men and 0.742 for women. Another factor is used for race (1,210 if African American). eGFR (MDRD) is in mL/min per 1.73 m^2.

For the SI unit, the MDRD equation is

$$\mathrm{eGFR(MDRD)} = 2.9167 * \left[(\mathrm{SCreat} * 0.0113)^{-1.154}\right] * \mathrm{Age}^{-0.203} * \mathrm{F}$$

where SCreat is in μmol/L, Age is in years, and the other factor as above. eGFR (MDRD) is in mL/s per 1.73 m^2.

CKD-EPI Equations

The CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation was published by Levey (Levey et al. 2009), and the authors recommend using this equation instead of the older MDRD equation. The 2009 CKD-EPI is based on creatinine in serum, derived from a sample of 8,254 participants and validated using a data set of 3,896 participants. Iothalamate clearance was selected as the "gold" standard. The KDIGO CKD 2012 Guideline strictly recommends using CKD-EPI equations. The use of other creatinine-based equations is possible only when improved accuracy is proved, compared with the 2009 CKD-EPI creatinine equation. When recording eGFR, the equation used should be specified (KDIGO 2012 Guideline 2013).

There are three recently recommended CKD-EPI equations:

- 2009 CKD-EPI creatinine-based equation
- 2012 CKD-EPI cystatin C-based equation
- 2012 CKD-EPI cystatin C- and creatinine-based equation

These equations are described in Table 3.

Lund-Malmö Equations

These equations were derived from a population of Swedish Caucasians (derivation set from Lund, $N = 436$; validation set from Malmö, $N = 414$); clearance of iohexol was used as the "gold" standard. The entire cohort of 850 patients was within an age interval of 26–85 years; plasma creatinine was 45–545 μmol/L, and iohexol clearance was 9–121 mL/min per 1.73 m^2 (Björk et al. 2007). All equations (Table 4) used creatinine, age in years, and sex; adjustment to lean body mass can be applied. Lean body mass (LBM) can be calculated according to James (see Björk et al. 2007) as follows:

$$\text{Women : LBM} = 1.07 * \text{Weight} - 148 * (\text{Weight}/\text{Height})^2$$

$$\text{Men : LBM} = 1.1 * \text{Weight} - 120 * (\text{Weight}/\text{Height})^2$$

where Weight is in kg, Height in cm, and LBM is in kg.

One of the advantages of the Lund-Malmö equations lies in the fact that the equation estimates GFR using a broad age range and predicts GFR in patients with "normal" serum creatinine. A further advantage is its derivation from a European population. Björk published a revised version of the Lund-Malmö equations in 2011 (Björk et al. 2011). Nyman published a better performance of the revised Lund-Malmö eGFR than MDRD or CKD-EPI in a population of 2,847 adults (Nyman et al. 2014). However, CKD-EPI is now the standard method of GFR estimation.

Table 3 CKD-EPI creatinine equations

Type of equation	Sex	Condition	Equation
2009 CKD-EPI creatinine equations	Women	SCreat $\leq$62 μmol/L	$2.4 * (\mathrm{SCreat}/61.9)^{-0.329} * 0.993^{\mathrm{Age}}$ (* 1.159 if black)
		SCreat >62 μmol/L	$2.4 * (\mathrm{SCreat}/61.9)^{-1.209} * 0.993^{\mathrm{Age}}$ (* 1.159 if black)
	Men	SCreat $\leq$80 μmol/L	$2.35 * (\mathrm{SCreat}/79.6)^{-0.411} * 0.993^{\mathrm{Age}}$ (* 1.159 if black)
		SCreat >80 μmol/L	$2.35 * (\mathrm{SCreat}/79.6)^{-1.209} * 0.993^{\mathrm{Age}}$ (* 1.159 if black)
2012 CKD-EPI cystatin C equation	Women and men	SCystC $\leq$0.8 mg/L	$2.217 * (\mathrm{SCystC}/0.8)^{-0.499} * 0.996^{\mathrm{Age}}$ (*0.932 in women)
		SCystC >0.8 mg/L	$2.217 * (\mathrm{SCystC}/0.8)^{-1.328} * 0.996^{\mathrm{Age}}$ (*0.932 in women)
2012 CKD-EPI creatinine-cystatin C equations	Women	SCreat $\leq$62 μmol/L SCystC $\leq$0.8 mg/L	$2.17 * (\mathrm{SCreat}/61.9)^{-0.248} * (\mathrm{SCystC}/0.8)^{-0.375} * 0.995^{\mathrm{Age}}$ (* 1.08 if black)
		SCreat $\leq$62 μmol/L SCystC >0.8 mg/L	$2.17 * (\mathrm{SCreat}/61.9)^{-0.248} * (\mathrm{SCystC}/0.8)^{-0.711} * 0.995^{\mathrm{Age}}$ (* 1.08 if black)
		SCreat >62 μmol/L SCystC $\leq$0.8 mg/L	$2.17 * (\mathrm{SCreat}/61.9)^{-0.601} * (\mathrm{SCystC}/0.8)^{-0.375} * 0.995^{\mathrm{Age}}$ (* 1.08 if black)
		SCreat >62 μmol/L SCystC $\geq$0.8 mg/L	$2.17 * (\mathrm{SCreat}/61.9)^{-0.601} * (\mathrm{SCystC}/0.8)^{-0.711} * 0.995^{\mathrm{Age}}$ (* 1.08 if black)

(*continued*)

Table 3 (continued)

Type of equation	Sex	Condition	Equation
	Men	SCreat ≤80 μmol/L SCystC ≤0.8 mg/L	$2.25 * (\mathrm{SCreat}/79.6)^{-0.207} * (\mathrm{SCystC}/0.8)^{-0.375} * 0.995^{\mathrm{Age}} (* 1.08 \text{ if black})$
		SCreat ≤80 μmol/L SCystC >0.8 mg/L	$2.25 * (\mathrm{SCreat}/79.6)^{-0.207} * (\mathrm{SCystC}/0.8)^{-0.711} * 0.995^{\mathrm{Age}} (* 1.08 \text{ if black})$
		SCreat >80 μmol/L SCystC ≤0.8 mg/L	$2.25 * (\mathrm{SCreat}/79.6)^{-0.601} * (\mathrm{SCystC}/0.8)^{-0.375} * 0.995^{\mathrm{Age}} (* 1.08 \text{ if black})$
		SCreat >80 μmol/L SCystC >0.8 mg/L	$2.25 * (\mathrm{SCreat}/79.6)^{-0.601} * (\mathrm{SCystC}/0.8)^{-0.711} * 0.995^{\mathrm{Age}} (* 1.08 \text{ if black})$

Creatinine should be in μmol/L, age in years, and cystatin C in mg/L. Results are in mL/s per 1.73 m^2

SCreat is given in μmol/L; therefore, the original factors used for creatinine should be recalculated. Example: $0.7 * 88.4 = 61.88 = 61.9$

Note: CKD- EPI equations are not intended for use in children

Table 4 Lund-Malmö equations

Type of equation	Condition	Equation
Lund-Malmö equation without correction to LBM	SCreat <150 μmol/L	$e^{((4.62-0.0112*\text{SCreat})-0.0124*\text{Age}+0.339*(\ln(\text{Age}))-0.226*\text{Sex})}/60$
	SCreat ≥150 μmol/L	$e^{((8.17+0.0005*\text{SCreat}-1.07*\ln(\text{SCreat}))-0.0124*\text{Age}+0.339*(\ln(\text{Age}))-0.226*\text{Sex})}/60$
Lund-Malmö equation with correction to LBM	SCreat <150 μmol/L	$e^{((4.95-0.011*\text{SCreat})-0.00587*\text{Age}+0.00977*\text{LBM})}/60$
	SCreat ≥150 μmol/L	$e^{((8.58+0.0005*\text{SCreat}-1.08*\ln(\text{SCreat}))-0.00587*\text{Age}+0.00977*\text{LBM})}/60$

Creatinine should be in μmol/L and age in years. Results are in mL/s per 1.73 m^2. Sex is 0 for men and 1 for women. For LBM calculation, see the section "Lund-Malmö Equations"

Estimation of GFR in Children

The Schwartz formula (Schwartz et al. 1976) has been used for years, based on serum creatinine and body height:

$$\text{eGFR} = 41.3 * (\text{Height}/\text{SCreat})$$

where Height is in meters and SCreat is in mg/dL.

This equation was recommended in the KDIGO 2012 Guideline as the "bedside" Schwartz formula.

In 2009, Schwartz published a new formula based on height, serum creatinine, cystatin C, and BUN (Schwartz et al. 2009):

$$\text{eGFR} = 39.1 * [(\text{Height}/\text{SCreat})^{0.516} * 1.8/\text{SCystC}^{0,294} * 30/\text{BUN}(\text{mg/dL})^{0.169} * 1.099(\text{if male}) * \text{Height}/1.4]^{0,188}$$

where Height is in meters, SCrea in mg/dL, SCystC in mg/L, and BUN in mg/dL.

Schwartz used plasma iohexol clearance as the "gold" standard. Other equation is

$$\text{eGFR} = 40.7 * (\text{Height}/\text{SCreat})^{0.64} * (30/\text{BUN})^{0.202}$$

where Height is in meters, SCreat and BUN are in mg/dL, and results are in mL/min per 1.73 m^2. This equation was recommended in the 2012 KDIGO CKD (KDIGO 2012 Guideline 2013).

However, Nyman tested the use of the Lund-Malmö equation in children and found this equation performed well in 85 pediatric Caucasian patients, aged 0.3–17 years. The Lund-Malmö equation performed better when not corrected for lean body mass (Nyman et al. 2008).

Table 5 Measured and calculated values

Variable	Value	Unit	Value	Unit
Serum creatinine	0.1 (100)	mmol/L (μmol/L)	1.13	mg/dL
Serum urea	5.0	mmol/L	5.0	mmol/L
Serum cystatin C	1.1	mg/L	1.1	mg/L
Urine creatinine	7	mmol/L	0.79	g
Urine urea	250	mmol/L	250	mmol/L
BSA	1.94	m^2	1.94	m^2
Clearance of creatinine	1.08	mL/s per 1.73 m^2	64.9	mL/min per 1.73 m^2
Clearance of urea	0.77	mL/s per 1.73 m^2	46.4	mL/min per 1.73 m^2
Mean of urea and creatinine clearance	0.93	mL/s per 1.73 m^2	55.7	mL/min per 1.73 m^2
eGFR (2009 CKD-EPI creatinine)	1.254	mL/s per 1.73 m^2	75.2	mL/min per 1.73 m^2
eGFR (2012 CKD-EPI cystatin C)	1.189	mL/s per 1.73 m^2	71.3	mL/min per 1.73 m^2
eGFR (2012 CKD-EPI creatinine + cystatin C)	1.217	mL/s per 1.73 m^2	73.0	mL/min per 1.73 m^2
eGFR (MDRD)	1.14	mL/s per 1.73 m^2	68.6	mL/min per 1.73 m^2

The differences among various GFR estimations are natural and caused by using different populations when deriving the respective equations. Some equations perform well during the interval of "normal" GFR values, and others perform better in pathology

Example: Male, white, 50 years, 24-h collection of urine (1,440 min.), volume of collected urine 1,500 mL/day. Weight 75 kg, height 180 cm. Measured and calculated values are given in Table 5.

The use of an exogenous filtration marker is recommended in situations where more accurate GFR values are needed to make better treatment decisions. Inulin clearance is recognized as the "gold" standard for GFR assessment (KDIGO 2012 Guideline 2013). There are two principles of inulin clearance measurement: "renal" clearance of inulin (continuous infusion of inulin with measurement of plasma and urine inulin concentrations under steady-state conditions, where timed collection of urine is necessary) and "plasma" clearance of inulin (measurement of several plasma inulin concentrations after an intravenous bolus of inulin). There are several models of "plasma" clearance of inulin (simple exponential analysis, area under curve calculation, the Jung model using early and late plasma concentrations, the biexponential model, etc.).

Table 6 shows our own experience with plasma inulin clearance, based on the Jung model (Jung et al. 1991). Results of other equations are compared.

Table 6 Comparison of "plasma" inulin clearance (the Jung model of calculation) with other eGFR equations

eGFR	Absolute bias (mL/min)	Relative bias (%)	Correlation coefficient	Percentage ± 30 % (accuracy)
2009 CKD-EPI creatinine	−5.1	−2.0	0.504	80
Clearance of creatinine	9.7	14.7	0.380	46
"Plasma" clearance of inulin (monoexponential model)	10.5	14.0	0.959	87
2012 CKD-EPI cystatin C	−7.4	−9.1	0.627	73
MDRD equation	−11.8	−10.9	0.461	71
2012 CKD-EPI cystatin C + creatinine	−7.5	−7.6	0.602	76

The table shows the "gold" standard (inulin clearance) compared with various equations for estimating GFR. As can be seen, the creatinine clearance and MDRD equations perform poorly while the CKD-EPI equation performs better. The different mathematical inulin clearance model performs well for obvious reasons (calculation is based on the same variables)

Interpretation of eGFR

Specific Problems with Cystatin C Measurement and Interpretation

Sources of errors in GFR estimation using creatinine and cystatin C (KDIGO 2012 Guideline, modified) are shown in Table 7. Synthesis, tubular processes, and extrarenal elimination are listed as "non-GFR variables that differ from derivation sets of patients" in KDIGO 2012 Guideline.

GFR and Age

GFR decreases with age; however, values below 60 mL/min per 1.73 m^2 (1 mL/s per 1.73 m^2) are rather unusual in individuals without renal pathology. Mathew (Mathew 2007) used data from Sikaris (personal communication) to elucidate this relationship. More than 97.5 % of the mixed population (more than 300,000 examinations in a large private pathology, exclusive of creatinine outliers for each decade) have an eGFR above 60 mL/min per 1.73 m^2 (1 mL/s per 1.73 m^2) at ages ≤57 years, and 80 % of the mixed population have an eGFR above 60 mL/min per 1.73 m^2 (1 mL/s per 1.73 m^2) at ages ≤80 years. Similar data are available in the KDIGO 2012 Guideline, where inulin clearance is above 60 mL/min per 1.73 m^2 in older healthy men and women.

Table 7 Factors influencing the interpretation of creatinine, cystatin C, and eGFR. According to the KDIGO 2012 Guideline, modified

Source of error	Creatinine	Cystatin C
Non-steady state	AKI	AKI
Synthesis	Increased with great muscle mass, high-protein diet, creatine supplements, ingestion of cooked meat, muscle hypercatabolism, African American race	Increased by administration of corticosteroids
	Decreased in malnutrition, marasmus, muscle-wasting diseases, amputations, liver disease, vegetarian diet	Diseases of thyroid glands
		Diabetes, adiposity
Tubular processes	Drug-induced inhibition of tubular secretion (trimethoprim, cimetidine, fenofibrate)	
Extrarenal elimination	Decreased after dialysis	Increased by severe decrease in GFR
Higher GFR	Higher biological variability in non-GFR determinants relative to GFR, higher measurement error	Higher biological variability in non-GFR determinants relative to GFR, higher measurement error
Interferences	Jaffé method	Heterophile antibodies
	Decreased: ascorbic acid, bilirubin, hemoglobin (neonates)	
	Increased: acetone, acetoacetate, albumin, cephalosporins (cefoxitin), glucose, methyldopa pyruvate, trimethoprim, uric acid	
	Enzymatic method	
	Decreased: dobutamine, dopamine,[a] bilirubin increased: calcium dobesilate	

The important part of the interpretation lies in the fact that both creatinine and cystatin C can be influenced by a variety of processes and compounds (both of exogenous and endogenous origin). Creatinine is influenced mainly by changes in muscle mass while cystatin C is influenced by diseases. Both creatinine and cystatin C are also influenced by interference. The "classic" Jaffé reaction with alkaline picrate for measuring creatinine produces a lot of interference. Enzymatic measurement of creatinine and immunoanalytic methods for measuring cystatin C can also be influenced by interference
[a]Note: Dobutamine and dopamine only interfere with supratherapeutic concentrations, e.g., contamination of the sample

Glomerular Filtration Rate and Body Surface Area

Results of all "new" equations for eGFR are always expressed in standardized format, i.e., in mL/min per 1.73 m^2 (or mL/s per 1.73 m^2). However, for drug dosing it is necessary to recalculate the standardized results here to reflect the actual filtration of the patient (in mL/min or mL/s).

The following simple equation is used:

$$\text{GFR} = \text{eGFR}/1.73 * \text{BSA}$$

where BSA (in m^2) is calculated according to the DuBois and DuBois formula (DuBois and DuBois 1916)

$$\text{BSA} = 0.007184 * \text{Weight}^{0.425} * \text{Heigth}^{0.725}$$

where Weight is in kg and Height is in cm. GFR is then in mL/min or mL/s.

Specific Clinical Situations

Chronic Kidney Disease (CKD)

"Chronic kidney disease (CKD) is defined as abnormalities of kidney structure or function, present for more than 3 months, with implications for health and CKD is classified based on cause, GFR category, and albuminuria category (CGA)" (KDIGO 2012 Guideline 2013). This completely new definition reflects the need for both a more "clinical" definition and a more precise "laboratory" classification. GFR categories together with recommended terms are listed in Table 8.

Lower values of eGFR correlate with worse prognoses: the age-standardized rate of hospitalization, rate of death, and rate of cardiovascular events (per 100 person-years) increase almost exponentially with decreasing eGFR (Levey et al. 2006).

Table 8 GFR categories in CKD (KDIGO 2012 Guideline 2013)

GFR category	GFR (mL/min per 1.73 m^2)	GFR (mL/s per 1.73 m^2)	Terms
G1	≥1.50	≥90	Normal or high
G2	1.00–1.49	60–89	Mildly decreased[a]
G3a	0.75–0.99	45–59	Mildly to moderately decreased
G3b	0.50–0.74	30–44	Moderately to severely decreased
G4	0.25–0.49	15–29	Severely decreased
G5	<0.25	<15	Kidney failure

The importance of the KDIGO 2012 Guideline for chronic kidney disease lies in the combination of the GFR and albuminuria categories for staging and prognostication. Terminology is also clearly recommended for each GFR category. GFR categories G1 and G2 do not fulfill the criteria for CKD unless evidence of kidney damage is present

[a]Note: Relative to a young adult level

Diabetes Mellitus

Pathophysiology and the Development of Diabetic Nephropathy

In type 1 diabetes mellitus (T1DM), five stages of diabetic nephropathy can be distinguished:

(a) Early hypertrophy and hyperfunction, lasting anywhere from months to years. At this stage, eGFR is increased of about 20–40 %, and hyperfiltration is supposed to be a risk factor.
(b) Clinically latent stage of renal impairment, where eGFR can be increased, but the basal membrane is thickened and mesangial expansion can be found.
(c) Incipient nephropathy, with positive albuminuria; eGFR is sometimes increased, but a thickened basal membrane and mesangial expansion is more common.
(d) Manifest nephropathy with proteinuria and decreased eGFR; GFR decreases at a rate of 1.2 mL/min per year (0.2 mL/s per year).
(e) Chronic kidney disease with severely decreased GFR or kidney failure.

Only 25 % of patients with type 2 diabetes (T2DM) mellitus have similar renal impairment, as is common in T1DM, frequently as a result of decompensated diabetes mellitus. About 40 % of patients with T2DM display slight or moderate histological findings with borderline renal function. About 35 % display tubulointersticial changes or glomerulosclerosis.

Guidelines

Recently American Diabetes Association Standards of Medical Care in Diabetes (ADA 2016) describes consequences of decreased GFR in the following chapters: Cardiovascular Disease and Risk Management and Microvascular Complications and Foot Care. Estimated GFR should be used as a screening tool at least once a year in patients with type 1 diabetes mellitus lasting five or more years and in all patients with type 2 diabetes mellitus. Serum creatinine with MDRD formula or preferably CKD-EPI formula is recommended for eGFR assessment of diabetes mellitus. The five stages of CKD are defined in the 2016 ADA Standards of Medical Care in Diabetes (Table 9). In contrast to the KDIGO 2012 Guideline, stage 3a and 3b are not distinguished here.

Both eGFR and albuminuria (and serum/plasma potassium) are measured together. Screening based on albuminuria alone is not sufficient. It should be stressed, however, that albuminuria is measured repeatedly due to its high biological variability. The new term "increased urinary albumin excretion" can be used only if two of three urine specimens collected within a 3- to 6-month period contain $\geq$30 mg/g creatinine ($\geq$3.0 g/mol creatinine). The use of the term "microalbuminuria" (30–299 mg/g creatinine, 3–29.9 g/mol creatinine) should no longer be used; similarly, the term "macroalbuminuria" (or "clinical albuminuria", $>$299 mg/g creatinine or 30 g/mol creatinine) is also inappropriate.

The ADA Standards (ADA 2016) recommends actions for the management of CKD. Patients in stages 4 and 5 should always be referred to a nephrologist, as well

Table 9 Stages of CKD from the ADA Standards of Medical Care in Diabetes 2016

Stage	Description	GFR mL/min per 1.73 m^2	GFR mL/s per 1.73 m^2	Comparison with KDIGO 2012
1	Kidney damage with normal or increased GFR	$\geq$90	$\geq$1.5	Normal or high in KDIGO 2012 without remarks on damage
2	Kidney damage with mildly decreased GFR	60–89	1–1.49	Same in KDIGO 2012, without remarks on damage
3	Moderately decreased GFR	30–59	0.50–0.99	Mildly to severely decreased GFR
4	Severely decreased GFR	15–29	0.25–0.49	Same in KDIGO 2012
5	Kidney failure	$<$15 or dialysis	$<$0.25 or dialysis	Dialysis is not mentioned in KDIGO 2012

The American Diabetes Association uses a similar GFR classification to the KDIGO Guideline. In contrast to the KDIGO 2012 Guideline, stages 3a and 3b are not distinguished here

as patients with eGFR 45–60 mL/min per 1.73 m^2 (0.75–1.0 mL/s per 1.73 m^2) and suspect of nondiabetic kidney disease. More frequent monitoring of an expanded set of laboratory tests is recommended in patients with eGFR 30–44 mL/min per 1.73 m^2 (0.5–0.74 mL/s per 1.73 m^2).

Estimated GFR (or creatinine measurement) should also be monitored (together with potassium levels in serum/plasma) in diabetic patients using ACE inhibitors, angiotensin receptor blockers, loop diuretics, hydrochlorothiazide, or chlorthalidone. Hydrochlorothiazide and chlorthalidone should be avoided with eGFR levels under 30 mL/min per 1.73 m^2 (0.5 mL/s per 1.73 m^2).

AKI and Intensive Care

Definition and Staging of Acute Kidney Injury

Acute kidney injury (AKI) is present when

- Serum creatinine increases by 26.5 μmol/L or more (0.3 mg/dL and more) within 48 h, or
- Serum creatinine increases by at least 50 % above the baseline (at least 1.5 times above the baseline) within 7 days, or
- The volume of urine is lower than 0.5 mL/kg per hour for 6 h (oliguria).

There are two staging systems – the older RIFLE system (Risk, Injury, Failure, Loss of function, End-stage kidney disease) and the newer AKIN system (Acute Kidney Injury Network) (Ronco 2013). Comparison of the RIFLE and AKIN systems is given in Table 10. Both systems can be used, but a fraction of the patients classified in the respective stages is not equal.

Table 10 Comparison of the RIFLE and AKIN systems

RIFLE staging	SCreat/GFR criteria	Urine output criteria	AKIN staging	SCreat criteria	Urine output criteria
Risk	Increased SCreat >50 % or GFR decreases >25 %	Urine output <0.5 mL/kg per hours within 6 h	Stage 1	Increased SCreat > +50 % to +100 % (i.e., 1.5–2.0-fold from the baseline) or > increase of 26.5 μmol/L (0.3 mg/dL)	Urine output <0.5 mL/kg per hour within 6 h
Injury	Increased SCreat >100 % or GFR decreases >50 %	Urine output <0.5 mL/kg per hour for more than 12 h	Stage 2	Increased SCreat +101 % to +200 % (i.e., 2.01–3.0-fold from the baseline)	Urine output <0.5 mL/kg per hour for more than 12 h
Failure	Increased SCreat >200 % or GFR decreases >75 % or SCreat >354 μmol/L (4 mg/dL) (with acute rise of 44 μmol/L or more (0.5 mg/dL and more)	Urine output <0.3 mL/kg per hour for 24 h or anuria for 12 h	Stage 3	Increased SCreat +200 % and more (more than 3.0-fold from the baseline) or SCreat higher than 354 μmol/L (4.0 mg/dL) with an acute increase of at least 44 μmol/L (0.5 mg/dL)	Urine output <0.3 mL/kg per hour for 24 h or anuria for 12 h

Staging of AKI is based on changes of serum creatinine and urine volume (similar in both the RIFLE and AKIN classifications) or GFR changes (RIFLE only). Loss of function and end-stage renal disease (*ESRD*) do not have a parallel stage in the AKIN system and were removed from the staging system in 2007 (Mehta et al. 2007). Patients receiving renal replacement therapy (*RRT*) are considered to meet the criteria for Stage 3 in the AKIN system (irrespective of the stage before RRT)

Several methods for GFR estimation are discussed among intensivists with strong emphasis on frequent monitoring of plasma (serum) creatinine; however, creatinine clearance based on a 2-h collection of urine is also admitted, and iohexol or iothalamate is classified as acceptable and realistic by the ADQI group. GFR can be used for staging of acute kidney injury as part of the RIFLE (but not the AKIN) classification.

Monitoring of creatinine concentration is an essential tool for GFR assessment in intensive care. Plasma (serum) creatinine is not a precise predictor of GFR under acute circumstances. On the other hand, dynamic changes in creatinine concentrations are similar to the dynamic changes in GFR. In cases of unknown basal creatinine concentration, it is possible to assess creatinine concentration using the CKD-EPI equation. This principle was recommended for the MDRD equation by Bellomo et al. (2004). They used "basal" GFR of 75 mL/min per 1.73 m^2 (1.25 mL/s per 1.73 m^2). Table 11 is based on the 2009 CKD-EPI

Table 11 Estimated baseline creatinine from the 2009 CKD-EPI equation for "basal" GFR of 75 mL/min per 1.73 m^2 (1.25 mL/min per 1.73 m^2) (According to Bellomo et al. 2004)

Age (years)	Male baseline creatinine (mg/dL)	Male baseline creatinine (μmol/L)	Female baseline creatinine (mg/dL)	Female baseline creatinine (μmol/L)
20	1.35	119	1.06	94
30	1.28	113	1.01	89
40	1.20	106	0.95	84
50	1.13	100	0.89	79
60	1.07	95	0.85	75
70	1.01	89	0.80	71
80	0.95	84	0.76	67

Changes in serum creatinine are used for the diagnosis and staging of acute kidney injury. In acute settings, physicians do not know the basal value of serum creatinine prior to the development of acute kidney injury. Because of the relation between age, creatinine, and GFR, basal creatinine can be estimated from the expected GFR before injury (here it is at least 75 mL/min (1.25 mL/s) per 1.73 m^2 of body surface area)

creatinine equation with "basal" GFR of 75 mL/min per 1.73 m^2 (1.25 mL/s per 1.73 m^2).

Potential Applications to Prognosis, Other Diseases, or Conditions

The relation between eGFR and prognosis is given in the KDIGO 2012 Guideline for CKD. Four variables should be taken into account: cause of CKD, GFR category (G1–G5), albuminuria category (A1–A3), and complications (other risk factors, comorbidities). eGFR is used to diagnose and monitor other diseases and conditions: increased GFR can be expected during pregnancy, with a high-protein diet and in conditions where there is increased output of osmotically active substances (osmotic diuresis, e.g., after mannitol, or in hyperglycemic situations). For extrarenal reasons, decreased eGFR can occur during and after cases of dehydration, e.g., hemorrhagic shock.

Summary

- Estimated glomerular filtration rate (eGFR) is used for the diagnostics, staging, and monitoring of kidney function.
- Estimation is based on serum creatinine and/or cystatin C concentration.
- Methods of determining creatinine and cystatin C should be traceable to international calibrators; enzymatic determination of creatinine is preferred to the Jaffé (alkaline picrate) method.

- The gold-standard method for GFR assessment (inulin clearance) is preferred to other methods (^{125}I-iothalamate, iohexol).
- The CKD-EPI equations (Chronic Kidney Disease – Epidemiology Collaboration) are recent equations for calculating eGFR.
- Three CKD-EPI equations are recommended: the 2009 CKD-EPI creatinine, 2012 CKD-EPI cystatin C, and 2012 CKD-EPI cystatin C and creatinine equations, respectively.
- The MDRD equation or Cockcroft-Gault formula should not be used for eGFR assessment.
- The KDIGO 2012 Guidelines (both for chronic kidney disease and acute kidney injury) and ADQI recommendations (Acute Dialysis Quality Initiative) provide suggestions for health care that positively impact on patient benefit and safety.

References

American Diabetes Association (ADA). Standards of medical care in diabetes-2016. Diabetes Care. 2016;39 Suppl 1:S1–112.

Andersen TB, Eskild-Jensen A, Frøkiaer J, et al. Measuring glomerular filtration rate in children; can cystatin C replace established methods? Rev Pediatr Nephrol. 2009;24:929–41.

ARUP's Laboratory Test Directory. http://ltd.aruplab.com/Tests/Pub/0020473. Accessed February 2016.

Bellomo R, Ronco C, Kellum JA, et al. Acute renal failure – definition, outcome measures, animal models, fluid therapy and information technology needs: the second international consensus conference of the Acute Dialysis Quality Initiative (ADQI) group. Crit Care. 2004;8:R204–12.

Björk J, Bäck SE, Sterner G, et al. Prediction of relative glomerular filtration rate in adults: new improved equations based on Swedish Caucasians and standardized plasma-creatinine assays. Scand J Clin Lab Invest. 2007;67:678–95.

Björk J, Grubb A, Sterner G, et al. Revised equations for estimating glomerular filtration rate based on the Lund-Malmö study cohort. Scand J Clin Lab Invest. 2011;71:232–9.

Burtis CA, Ashwood ER, Bruns DE. Tietz texbook of clinical chemistry and molecular diagnostics. 4th ed. St. Louis: Elsevier Saunders; 2006. p. 818.

Chowdhury P, Kehl D, Choudhary R, et al. The use of biomarkers in the patient with heart failure. Curr Cardiol Rep. 2013;15:372.

Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron. 1976;16:31–41.

DuBois D, DuBois EF. A formula to estimate the approximate surface area if height and weight be known. Arch Intern Med. 1916;17:863–71.

European Renal Association-European Dialysis, Transplant Association (ERA-EDTA). European best practice guidelines for haemodialysis (part 1). Nephrol Dial Transplant. 2002;17 Suppl 7:7–9.

Filler G, Huang SH, Yasin A. The usefulness of cystatin C and related formulae in pediatrics. Clin Chem Lab Med. 2012;50:2081–91.

Grubb A, Nyman U, Björk J, et al. Simple cystatin C-based prediction equations for glomerular filtration rate compared with the modification of diet in renal disease prediction equation for adults and the Schwartz and the Counahan-Barratt prediction equations for children. Clin Chem. 2005;51:1420–31.

Grubb A, Horio M, Hansson LO, et al. Generation of a new cystatin C-based estimating equation for glomerular filtration rate by use of 7 assays standardized to the international calibrator. Clin Chem. 2014;60:974–86.

Jaffé M. Über den Niederschlag, welchen Pikrinsäure in normalen Harn erzeugt und über eine neue Reaction des Kreatinins. Z Physiol Chem. 1886;10:391–400.

Jung K, Henke W, Schulze BD, et al. Single-injection inulin clearance using only one blood sample as a suitable procedure to measure glomerular filtration rate. Nephron. 1991;59:694–5.

KDIGO, CKD Work Group. KDIGO clinical practice guideline for the evaluation and management of chronic kidney disease. Kidney Int Suppl. 2013;3:1–150.

Levey AS, Bosch JP, Lewis JB, et al. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of diet in renal disease study group. Ann Intern Med. 1999;130:461–70.

Levey AS, Stevens LA, Hostetter T. Automatic reporting of estimated glomerular filtration rate-just what the doctor ordered. Clin Chem. 2006;52:2188–93.

Levey AS, Coresh J, Greene T, et al. Expressing the Modification of Diet in Renal Disease Study equation for estimating glomerular filtration rate with standardized serum creatinine values. Clin Chem. 2007;53:766–72.

Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009;150:604–12.

Mathew TH, Johnson DW, Jones GR, et al. Chronic kidney disease and automatic reporting of estimated glomerular filtration rate: revised recommendations. Med J Aust. 2007;187:459–63.

Mehta RL, Kellum JA, Shah SV, et al. Acute kidney injury network: report of a initiative to improve outcomes in acute kidney injury. Crit Care. 2007;11:R31.

Nyman U, Björk J, Lindström V, et al. The Lund-Malmö creatinine-based glomerular filtration rate prediction equation for adults also performs well in children. Scand J Clin Lab Invest. 2008;68:568–76.

Nyman U, Grubb A, Larsson A, et al. The revised Lund-Malmö GFR estimating equation outperforms MDRD and CKD-EPI across GFR, age and BMI intervals in a large Swedish population. Clin Chem Lab Med. 2014;52:815–24.

Remer T, Neubert A, Maser-Gluth C. Anthropometry-based reference values for 24-h urinary creatinine excretion during growth and their use in endocrine and nutritional research. Am J Clin Nutr. 2002;75:561–9.

Ronco C. ADQI consensus on AKI biomarkers and cardiorenal syndromes. Contrib Nephrol Basel Karger. 2013;182:5–12.

Schwartz GJ, Haycock GB, Edelmann Jr CM, et al. A simple estimate of glomerular filtration rate in children derived from body length and plasma creatinine. Pediatrics. 1976;58:259–63.

Schwartz GJ, Munoz A, Schneider MF, et al. New equations to estimate GFR in children with CKD. J Am Soc Nephrol. 2009;20:629–37.

Shannon JA, Smith HW. The excretion of inulin, xylose and urea by normal and phlorizinized man. J Clin Invest. 1935;14:393.

Shlipak M, Sarnak MJ, Katz R, et al. Cystatin C and the risk of death and cardiovascular events among elderly persons. N Engl J Med. 2005;352:2049–60.

Taglieri N, Koenig W, Kaski JC. Cystatin C and cardiovascular risk. Clin Chem. 2009;55:1932–43.

Woitas RP, Kleber ME, Meinitzer A, et al. Cystatin C is independently associated with total and cardiovascular mortality in individuals undergoing coronary angiography. The Ludwigshafen Risk and Cardiovascular Health (LURIC) study. Atherosclerosis. 2013;229:541–8.

Red Blood Cell Distribution Width: Useful Predictor for Treatment Response in Primary Glomerular Diseases

52

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Abstract

Red blood cell distribution width (RDW) shows the variation in size of erythrocytes in the circulation and it is obtained easily by automated blood cell counters. RDW is associated with systemic inflammation. Clinical or subclinical inflammation is usually present in pathogenesis of primary glomerular diseases (PGDs). Control of many of PGDs can be achieved by immune modulator agents and this supports the underlying systemic inflammatory process. Treatment response cannot be achieved in some patients. There is neither a standard dose nor a standard dose reduction protocol for immunosuppressive treatment in PGDs. In this aspect, use of predictive biomarkers for treatment response can protect patients from side effects of unnecessary immunosuppressive drugs. New

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biomarkers are essential in prediction of treatment response and prognosis of PGDs. So, studies have been focused to investigate potential clinical importance of RDW in prediction of prognosis and clinical outcome of PGDs. RDW is assessed as part of the complete blood count which is a quite cheap test, and therefore, it may be a useful novel marker for evaluation of treatment response in nephrotic syndrome due to PGDs.

Keywords

Biomarker • Immunosuppressive drugs • Nephrotic syndrome • Primary glomerular diseases • Red blood cell distribution width • Treatment response

Abbreviations

Anti-PLA2R	Phospholipase A2 receptor
CRP	C-reactive protein
FSGS	Focal segmental glomerulosclerosis
FSP1+	Fibroblast-specific protein 1-positive
IgAN	IgA nephropathy
IgG	Immunoglobulin G
IgM	Immunoglobulin M
L FABP	L fatty-acid-binding protein
MCD	Minimal change disease
MG	Membranous glomerulopathy
MPGN	Type 1 membranoproliferative glomerulonephritis
MPV	Mean platelet volume
NAG	*N*-acetyl-beta-glucosaminidase
NHANES	National health and nutrition examination survey
NS	Nephrotic syndrome
PGDs	Primary glomerular diseases
RBC	Red blood cell
RDW	Red blood cell distribution width
SOCS	Suppressors of cytokine signaling
α1M	α1-microglobulin
β_2M	β_2-microglobulin

Key Facts

- NS due to PGDs is an immunoinflammatory condition.
- Clinical course of PGDs varies widely from complete remission to end-stage renal disease.
- Control of many of PGDs can be achieved by conservative treatment and immune modulator agents.
- Predictive markers of therapeutic response are useful especially in determining the treatment plan and follow-up of PGDs
- Use of predictive biomarkers for treatment response can protect patients from the side effects of unnecessary immunosuppressive drugs.

- Most of classical predictors are weak, unpractical in use, and also they are quite expensive, so their use is limited in most centers.
- RDW may be associated with systemic inflammation and may predict prognosis and clinical outcome of PGDs

Definitions

Antibodies An immunoglobulin, a specialized immune protein, produced due to the introduction of an antigen into the body which possesses the significant capability to combine with the antigen that triggers its production. The production of antibodies is a major function of the immune system and is carried out by a type of white blood cell called a B cell (B lymphocyte).

Biomarkers A predictor that can be used to measure the presence or progress of a disease or the effects of treatment. For example, prostate-specific antigen is a biomarker for prostate cancer.

End-stage renal disease End-stage renal disease is the stage of kidney impairment which is irreversible, cannot be controlled by conservative management alone, and requires dialysis or kidney transplantation to maintain life.

Inflammation A protective tissue response of the organism to injury which serves to remove injurious insult and to mediate the healing process.

Primary glomerular diseases Primary glomerular diseases consist of a group of disorders characterized by pathologic alterations in normal glomerular structure and function, independent of systemic disease courses. This distinction is important because the clinical presentation and pathologic findings of glomerulopathy secondary to systemic diseases may mimic primary glomerular disorders, yet the correct diagnosis of the underlying systemic disease may significantly alter the treatment of the patient.

Proteinuria The presence of abnormally large amounts of protein in the urine, usually resulting from kidney diseases, but sometimes results from fever, excessive exercise, and other abnormal conditions. It is often defined as an amount in excess of 300 mg per day.

Introduction

Clinical course of primary glomerular diseases (PGDs) varies widely from complete remission to end-stage renal disease. Besides conservative treatment, clinicians need to use immunosuppressive drugs which have serious side effects. However, unfortunately only some of the patients respond to the treatment. For this reason, some patients may use these therapeutic agents though treatment response would never be achieved and they face serious side effects of the treatment regimen.

Table 1 Universal characteristics of any biomarker. These features of new biomarkers are essential for diagnosis, prediction of treatment response, and prognosis in patients with primary glomerular disease (Data are from Biomarkers Definitions Working Group (2001))

They should be noninvasive, easily measured, and inexpensive
They should be from readily available sources, such as blood or urine
They should have a high sensitivity, allowing early detection, and no overlap in values between diseased patients and healthy individuals
They should have a high specificity, being greatly upregulated (or downregulated) specifically in the diseased samples and unaffected by comorbid conditions
Biomarker levels should vary rapidly in response to treatment
Biomarker levels should aid in risk stratification and possess prognostic value in terms of real outcomes
Biomarkers should be biologically plausible and provide insight into the underlying disease mechanism

Renal biopsy is still the gold standard method for diagnosis of glomerular diseases. However, it is not only an invasive procedure which cannot be performed in every clinic, but it also results in important complications, and pathologic evaluation may be time-consuming. Although serial renal biopsies might improve treatment, they are often difficult to justify because of the risk, discomfort, and expense. New methods are needed to identify the cause of renal diseases and prognosis without a biopsy. On the other hand, various biomarkers which have been used for years in order to investigate the etiology and progression rate of glomerular diseases are available. However, their low selectivity and specificity limit their use.

The National Institutes of Health Biomarkers Definitions Working Group has defined a biological marker (biomarker) as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group 2001). Universal characteristics for any ideal biomarker are listed in Table 1 (Biomarkers Definitions Working Group 2001). New biomarkers with such these features are essential in diagnosis, prediction of treatment response, and prognosis of PGDs. In this section, we discussed markers which have been still used to predict treatment response in PGDs like minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous glomerulopathy (MG), type 1 membranoproliferative glomerulonephritis (MPGN) and IgA nephropathy (IgAN). We focused on red blood cell distribution width (RDW) which we thought can predict treatment response in PGDs.

Pathogenesis of Primary Glomerular Diseases

Proteinuria due to glomerular diseases was first described 200 years ago by Richard Bright (1836). During this period, besides the developments in histopathologic evaluation, many investigations about underlying etiologic factors, genetic studies,

studies on experimental models, studies about mediators effecting the development of tissue injury, and studies investigating the role of immune response were all held to understand more about glomerular injury. Although many studies focusing to identify nephritogenic factors were carried out in last 50 years, we still do not know exactly what the precipitating factors are and how can we prevent development of PGDs. Understanding of immune response caused by environmental factors (especially infections, drugs etc.) and genetic features can allow development of new biomarkers for PGDs.

Nowadays, it has been suggested that nephrotic syndrome (NS) due to PGDs is an immunoinflammatory condition (Camici 2007). Many PGDs are associated with positive staining for immunoglobulins, often with components of the complement system, and with the presence of immune complexes. Infiltration by inflammatory cells is largely determined by the site of immune complexes. Some of the authors thought that specific or nonspecific infections which can be acute or chronic may play a role in PGDs' pathogenesis (Couser and Johnson 2014). Glomerular diseases can develop as a consequence of infection with organisms releasing super antigens that cause a polyclonal activation of B cells. The main organism responsible for super antigen-associated glomerular injury is *Staphylococcus aureus*. Once an immune response is initiated, the local injury may lead to the release of additional antigens that may further increase the immune response. Normal immune response is a reaction only against the pathogen. As deletion of self-reactive T cells which arise during development of thymus occurs in adulthood, autoimmune response is not observed (central tolerance). If self-reactive T and B cells are not deleted for any reason and pass into the systemic circulation, mechanisms may be activated to detect and either delete or suppress them (peripheral tolerance). Loss of tolerance for various reasons (genetic susceptibility and problems, T-regulator cell dysfunctions, antigenic similarity, persistence of self-reactive cells, structural changes in epitopes, increase in number of similar epitopes, development of antibodies and immune complexes) can initiate autoimmune injury (Couser and Johnson 2014). Generally, this process occurs silently and remains subclinical.

Twenty thousand five hundred of 1,000,000 microbial derived genes in humans are inherited and pass to future generations (Pillai 2013). Microorganisms were suggested to trigger autoimmune mechanism in type 1 diabetes mellitus and autoimmune arthritis (Couser and Johnson 2014). Sufficient amount of data supporting the role of infectious agents in development of PGDs is actually present in literature (post-streptococcal glomerulonephritis, etc.). Systemic inflammatory response due to infectious agents play an important role in the pathogenesis of PGDs MCD, FSGS, MG, type 1 MPGN, and IgAN (Couser and Johnson 2014). Furthermore, antibody-mediated or immune complex-related glomerular injuries due to microorganisms are evident in IgAN, MG, and type 1 MPGN (Couser and Johnson 2014).

On the other hand, there are non-antibody-mediated injury mechanisms modulated by microorganisms such as problems in T-cell regulation in MCD and problems in parts of immune system like complement pathway in C3 nephropathies (Couser and Johnson 2014). In contrast to the immune complex mechanisms, certain glomerular diseases develop primarily through cell-mediated immunity. A direct role

for T cells in mediating glomerular injury and crescent formation has been shown in some experimental crescentic nephritis (Atkins et al. 1996).

All these processes lead to a clinical or subclinical inflammation in PGDs. Besides control of many of these diseases can be achieved by immune modulator agents, and this supports the underlying systemic inflammatory process (Camici 2007). So, studies have been focused to investigate potential clinical importance of RDW which is directly associated with systemic inflammation in prediction of prognosis and clinical outcome of PGDs (Turgutalp et al. 2014).

Prognostic Factors in Primary Glomerular Diseases

There are many factors affecting the prognosis and outcome of PGDs. Predictive markers of therapeutic response are useful especially in determining the treatment plan and follow-up of PGDs.

Prognosis of MG and FSGS are usually better in women than men probably due to lower proteinuria and blood pressure. However, women lose the advantage at higher levels of proteinuria (Cattran et al. 2008). Older age is one of the other poor prognostic parameters in MG. However, some studies declared that prognosis is similar in elderly and young idiopathic MG patients. Indeed, elderly patients have higher risk for infection due to immunosuppressive drugs (Yamaguchi et al. 2014).

Impaired renal function, higher blood pressure, and higher proteinuria are associated with worse prognosis in membranous nephropathy (Troyanov et al. 2006). Basal level of proteinuria is important in predicting the prognosis of MG (Caro et al. 2014). Titer of the antibodies against the M-type phospholipase A2 receptor (anti-PLA2R) is also correlated with the clinical activity and the response to immunosuppressive treatment in patients with MG (Segarra-Medrano et al. 2014). Depletion of anti-PLA2R autoantibodies can predict proteinuria response in MG (Beck et al. 2011). It has been documented that the amount of low-, medium-, and high-molecular-weight urinary proteins may be related with the rate of response to the treatment in patients with MG (Irazabal et al. 2013). Selective urinary biomarkers such as α1-microglobulin (α1M), β_2-microglobulin (β_2M), immunoglobulin G (IgG), and M (IgM) are used to predict both the outcome and the treatment response in MG (Irazabal et al. 2013). Urinary complement levels, *N*-acetyl-beta-glucosaminidase (NAG) and L fatty-acid-binding protein (L FABP), are some of the other predictive urinary markers for progression of MG. High urinary concentration of C5b9, a complement activation marker, is associated with poor prognosis (Brenchley et al. 1992). NAG, a lysosomal enzyme of proximal tubular cells, is a marker of tubular cell injury like β_2M. β_2M was found superior in predicting progression of renal disease when compared with NAG (Hofstra et al. 2008). These markers can be measured by noninvasive methods and can be repeated during the follow-up. However, their efficacy is still controversial. Particular histologic findings like tubulointerstitial damage, glomerular, and vascular sclerosis are also associated with poor prognosis in MG (Troyanov et al. 2006). Detection of specific protein expression in renal biopsy specimen may help to predict the rate of

progression. Interstitial alpha smooth muscle actin staining (Badid et al. 1999) and interstitial infiltration of CD68-positive cells with MCP-1/CCR2 expression are associated with progression to end-stage renal disease among patients with MG (Yoshimoto et al. 2004).

Severity of proteinuria, elevated serum creatinine, and black race are clinical risk factors for poor outcome in FSGS. Collapsing variant and tubulointerstitial fibrosis are histopathologic features of poor prognosis (Appel and D'Agati 2015). Urinary fractional excretion of IgG, and α 2-macroglobulin/creatinine ratio were documented as powerful predictors of outcome and responsiveness to steroids and cyclophosphamide (Bazzi et al. 2013). Low β_2M may suggest a good prognosis without immunosuppressive therapy in FSGS (Deegens and Wetzels 2007). Urinary-retinol binding protein, a marker of proximal tubular dysfunction, was studied in patients with FSGS, MCD, or mesangial proliferative glomerulonephritis and higher levels were correlated with treatment unresponsiveness (Mastroianni et al. 2000). Urinary NAG may be an indicator of relapse in both FSGS and MCD (Dillon et al. 1998).

Elevated serum creatinine concentration at presentation, higher blood pressure, and persistent and severe proteinuria are signs of poor prognosis in IgAN. Reduced proteinuria is associated with better renal function (Shimizu et al. 2009). Urinary β_2M levels are correlated with renal function and proteinuria in IgAN (Shin et al. 2014). Urinary excretion of low molecular weight proteins is not superior to total proteinuria and serum creatinine in predicting prognosis in patients with IgAN (Peters et al. 2009). Higher IgA/C3 ratio is correlated with worse prognosis (Ishiguro et al. 2002). Urinary interleukin 6 (IL 6) has prognostic value in patients with IgAN and was found higher in progressors (Harada et al. 2002). The ratio of epidermal growth factor (EGF) to monocyte chemotactic peptide 1 (MCP 1) in the urine was also used as a prognostic marker for patients with IgAN (Torres et al. 2008). Glomerular changes with segmental glomerulosclerosis, crescents, mesangial hypercellularity, and tubulointerstitial changes are poor histological parameters for IgAN (Shin et al. 2014). As the glomerular changes are related to hypertension, serum creatinine levels, and proteinuria levels, glomerular grading system may be useful in predicting the prognosis of IgAN (Shin et al. 2014). Renal biopsy can also help distinguishing crescentic variant of IgAN which has a worse prognosis than other noncrescentic variants. In crescentic IgAN, ratio of fractional excretion of IgG to surviving glomeruli could inform us about progression. Its predictive value was found higher when it was evaluated with serum creatinine (Bazzi et al. 2009). Renal biopsy requirement was its disadvantage. On the other hand, immunohistochemical evaluation is useful in prediction of outcome. Positive staining of GMP-17, a protein found on cytotoxic T lymphocytes, is associated with progression of IgAN (van Es et al. 2008). Fibroblast-specific protein 1-positive (FSP1+) fibroblasts are associated closely with the interstitial fibrosis and tubulointerstitial fibrosis correlates with renal survival; therefore, a number of (FSP1+) cells may predict response to the treatment with corticosteroids (Harada et al. 2008). Smoking, hyperuricemia, gross obesity, long duration of preceding symptoms, and increasing age are the other poor prognostic factors (Feehally and Floege 2015).

Elevated serum creatinine, nephrotic range proteinuria, severe hypertension, and crescents (50 %) or marked interstitial fibrosis on biopsy are the risk factors for progression of MPGN. Patients with nephritic syndrome and high C1q staining in glomerular deposits are poor prognostic parameters for MPGN (Schena and Alpers 2015).

1-β glycoprotein fragmentation was documented as a powerful urinary biomarker for steroid resistance in pediatric NS (Piyaphanee et al. 2011), whereas NPHS2 allele combination was reported as another marker of steroid resistance in patients with NS (Santín et al. 2011). Serum IgG level and IgG/IgM ratio were found to be significantly lower in children who had corticosteroid-resistant NS (Roy et al. 2009). Plasma levels of suppressors of cytokine signaling (SOCS) 3 and SOCS 5 might predict initial resistance to steroids in NS patients (Ostalska-Nowicka et al. 2011). Fractional excretion of magnesium was found to be correlated with various renal histopathologic findings in children and it was reported that it might be an early predictor of clinical outcome in steroid-resistant NS (Rumana et al. 2014). Mean platelet volume (MPV) was found higher on admission in therapy-resistant patients. In the same study it was documented that MPV levels were significantly correlated with the proteinuria level during a yearly follow-up (Kocyigit et al. 2013).

Most of the predictors are weak, impractical in use, and also they are quite expensive, so their use is limited in most centers. Ultimately, some diseases are likely to require the use of combinations of markers in useful clinical assays.

Immunosuppressive drugs are the mainstay in the treatment of patients with PGDs who are unresponsive to conservative treatment. On the other hand, it is well known that immunosuppressive drugs have many short- and long-term side effects like increase in malignant diseases, metabolic complications, sleep and mood disorders, gastrointestinal discomfort, hypertension, and life-threatening infections. Immunosuppressive drugs should be given in appropriate doses only for an appropriate duration to reduce these side effects. There is neither a standard dose nor a standard dose reduction protocol in PGDs. In this aspect, use of predictive biomarkers for treatment response can protect patients from side effects of unnecessary immunosuppressive drugs (Turgutalp et al. 2014). Studies have been focused to investigate potential clinical importance of RDW which is directly associated with systemic inflammation in prediction of prognosis and clinical outcome of PGDs (Turgutalp et al. 2014).

What Is RDW?

RDW shows the variation in size of erythrocytes in the circulation. The first quantitative assessments of variation in red blood cell diameter measurements in fixed stained peripheral blood smear were reported by Price-Jones in normal individuals and the patients with anemia (Price-Jones 1910). He postulated that difference in size and range variation might be diagnostically useful, but his method was tedious, was time-consuming, and had some limitations. Currently, RDW value is obtained easily by automated blood cell counters.

Higher RDW values indicate higher difference in size of circulating erythrocytes (Simel et al. 1988). RDW is a semi-quantitative statistical value which is routinely obtained in a complete blood cell count. Automated cell counters estimate red blood cell (RBC) volume cell by cell, sampling millions of RBCs in the process. It is a simple, cheap, and easily repeatable parameter. RDW is calculated with the following formula:

$$\text{RDW} = (\text{Standard deviation of MCV}/\text{mean MCV}) \times 100$$

Normal RDW values are 11.6–14.6 % in adults (Vajpayee et al. 2011). If RDW is measured in EDTA anticoagulated blood sample instead of citrated blood, false high results can be obtained. Prolonged storage of the specimen at room temperature may result in decrease in RDW values. Except such this condition, there is not any clinical situation in which RDW values are below normal. Therefore RDW value is either in normal range or high.

Clinical Importance of Increased RDW

It has been used in differential diagnosis of anemia for many years. Deficiency of iron, folate, or vitamin B12 can lead to higher RDW values, whereas RDW is in normal range in anemia with homogeneous structure in erythrocytes like thalassemia syndromes. However, the finding of an increased RDW is not specific for any one abnormality. On the other hand, a normal RDW does not mean that the main population of red cells is normal.

Furthermore, elevated RDW value has been associated with poor prognosis in an increasingly large number of non-hematological clinical conditions, particularly in disorders with underlying inflammatory processes. Systemic inflammatory process and oxidative stress can affect iron metabolism negatively, can suppress erythrocyte maturation, can shorten erythrocyte survival, can lead to early escape of reticulocytes into the blood circulation, and therefore can increase the RDW values (Spiropoulos et al. 2010; Pierce and Larson 2005; Ghaffari 2008). RDW has a positive, independent linear correlation with acute phase reactants like C-reactive protein (CRP) and IL-6 (Lippi et al. 2009; Veeranna et al. 2013). For this reason, the value of RDW started to be investigated in detail in clinical disorders other than hematologic problems. RDW was firstly evaluated and found increased in diseases with underlying inflammatory mechanisms (hearth failure, inflammatory bowel disease, malnutrition, etc.) (Song et al. 2012; Ozcan et al. 2013; Huang et al. 2014). Furthermore, meta-analysis reports showed that RDW could predict mortality and morbidity in disorders with underlying inflammatory processes like hearth failure (Yu et al. 2011).

Recent studies have documented that circulating erythrocytes participate in coagulopathy and development of atherosclerotic vascular disease (Yu et al. 2011). It was suggested that erythrocyte deformability is reduced in people with high RDW values (Patel et al. 2013), therefore blood flow slows, blood viscosity increases, and

Table 2 Clinical conditions for which predictive value of RDW was tested. RDW is a predictive marker for prognosis in many different clinical situations

Hematological
Multiple myeloma
Hairy cell leukemia
Lymphoma
Cardiovascular
Acute coronary syndrome
Stable angina pectoris
Acute/chronic heart failure
Stroke
Peripheral artery disease
Pulmonary embolism
Eisenmenger syndrome
Connective tissue diseases
Rheumatoid arthritis
Systemic lupus erythematosus
Takayasu's arteritis
Systemic sclerosis
Malignancies
Breast cancers
Prostate cancer
Pancreatic cancer
Malignant mesothelioma
Lung cancer
Miscellaneous
Nonalcoholic steatohepatitis/hepatic fibrosis
Hip fracture
Crohn's disease
Diabetes complications
Acute pancreatitis
Critically ill patients
Preeclampsia
Trauma patients
Sarcoidosis

this can increase aggregation and can lead to arterial and venous thrombosis (Rezende et al. 2014). RDW is also a predictor for acute coronary syndrome in general population without cardiovascular disease (Skjelbakken et al. 2014). These findings suggested subclinical inflammatory process as the leading factor for "out-of-sight" or undefined clinical problems in these people. Besides, meta-analysis reports documented that RDW could predict acute coronary syndrome in patients with documented coronary artery disease (Su et al. 2014). Some clinical conditions that have investigated the predictive value of RDW are listed in Table 2. Absence of randomized controlled trials is the limited side of these reports.

These findings support the importance of RDW as a predictive marker for prognosis in many different clinical situations. However, underlying mechanisms of this important relationship is not clear enough.

RDW in Patients with Proteinuria

As both RDW and microalbuminuria are found to be related with cardiovascular morbidity and mortality, it can be hypothesized that a link between RDW and microalbuminuria may exist (Arbel et al. 2014; Schmieder et al. 2014; Deveci et al. 2010). The cross-sectional analysis of the National Health and Nutrition Examination Survey (NHANES) data from 1999 to 2006 showed that RDW increases significantly as the degree of the urinary albumin ratio increases (Afonso et al. 2011). The same study also showed that the risk of microalbuminuria increases as RDW values increase, which is independent of the potential comorbidities such as hypertension, diabetes, and obesity. It was suggested that common interactions between inflammation, oxidative stress, endothelial dysfunction, and neurohumoral overactivity may explain the association between RDW and microalbuminuria (Afonso et al. 2011). However, in another study by Mathew, RDW was shown to be unrelated with microalbuminuria among patients with diabetes mellitus and hypertension (Mathew et al. 2014).

Relation between RDW and preeclampsia, which is a disorder associated with proteinuria, is controversial. It was suggested that RDW level is correlated with both the presence and the severity of preeclampsia; however, in another study, it was found that there is no association between RDW and preeclampsia (Abdullahi et al. 2014; Keskin et al. 2013).

So, there may be a relation between RDW and disorders associated with microalbuminuria or proteinuria. But, there are very few studies and further studies should be performed to display whether there is a direct correlation between RDW and proteinuria or it is the common characteristics of the disorders associated with increased RDW and microalbuminuria/proteinuria, such as inflammation, oxidative stress, or endothelial dysfunction.

Potential Role of RDW in Primary Glomerular Diseases

There are insufficient data on the potential role of RDW in PGDs. Only one study was carried out to investigate the importance of RDW in PGDs. In this study it was hypothesized that higher RDW levels can represent resistance to the treatment of PGDs. It was designed to show the predictive value of RDW, a simple, inexpensive biomarker, for the treatment response in patients with PGDs. Then, a prospective study including 176 patients with NS due to biopsy-proven PGDs (MCD, $n = 42$; MG, $n = 47$; FSGS, $n = 41$; type 1 MPGN, $n = 46$) was performed (Turgutalp et al. 2014). Patients were grouped according to their response to the treatment. All subjects had been followed for 12 months. Group 1 was composed of 55 patients

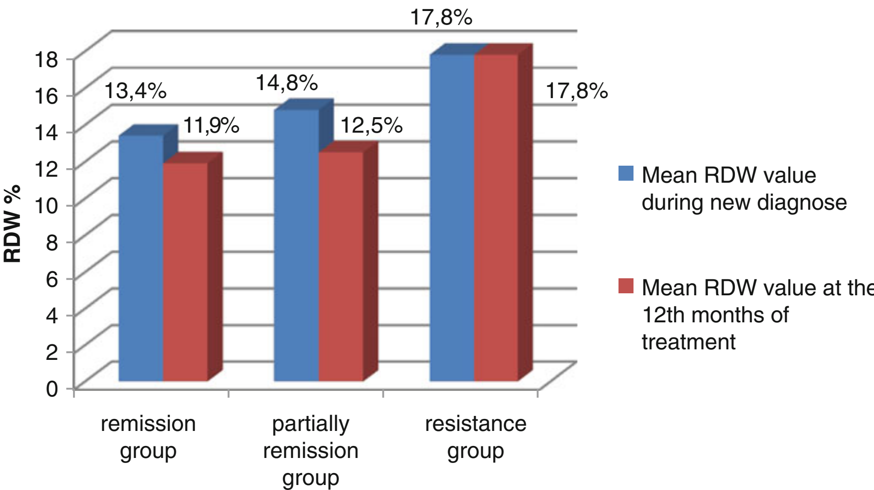

Fig. 1 Mean RDW values of all the groups and their comparisons both before and at the end of the treatment. While baseline mean RDW value was low in group 1 patients, it was higher in group 2 and group 3 subjects. RDW values when evaluated before treatment in all groups, the highest mean RDW level was found in group 3 patients ($p < 0.05$), the lowest mean RDW level was found in group 1 patients ($p < 0.05$). In group 1 and group 2, there was a significant decrease in mean RDW levels after treatment comparing with baseline levels ($p < 0.05$). In group 3 patients, there was no decrease in RDW levels after treatment comparing with first admission levels ($p > 0.05$) (Data are from Turgutalp et al. (2014))

with complete remission whereas group 2 was composed of 53 patients with partial remission and group 3 was composed of 68 patients who were resistant to therapy at the end of treatment (12th month). Mean RDW values of the all groups and their comparisons both before and at the end of the treatment are shown in Fig. 1.

According to the treatment response, mean RDW values of patients with different types of PGDs are shown in Table 3. Statistically significant difference was not detected between histological types of PGDs (Turgutalp et al. 2014). Additionally, they reported that sensitivity (ability to determine of treatment sensitive patients) and specificity (ability to determine of treatment resistance patients) of RDW was 98.2 % and 81.8 %, respectively.

In the same study, most of the patients with complete remission had a baseline RDW value $\leq$14 % (90 %), and most of the patients who were resistant to the treatment had a baseline RDW value >15 % (86.1 %). Similarly, most of the patients with partially remission had baseline RDW values between 14.1 % and 15 % (78.7 %) (Fig. 2) (Turgutalp et al. 2014) (Table 4).

These results suggested that serum RDW level may be a useful predictive biomarker for estimating the response to the therapy and may reflect increased inflammatory response in NS. RDW level can be seen as a part of the complete blood count which is a cheap test and therefore it may be a cost-effective novel marker for evaluation of treatment response in NS due to PGDs. However, it should

Table 3 Mean RDW values (%) in patients with different types of PGDs according to response to the therapy

Groups	MCD	MG	FSGS	MPGN	p**
Remission (1)	11.82 ± 0.21	11.80 ± 0.20	12.15 ± 0.19	12.14 ± 0.15	NS
Partial remission (2)	12.3 ± 1.3	12.4 ± 1.3	12.6 ± 1.4	12.7 ± 1.5	NS
Resistant (3)	17.7 ± 2.0	17.7 ± 2.1	17.8 ± 2.1	17.9 ± 2.2	NS
p*	<0.05	<0.05	<0.05	<0.05	

Mean RDW value in patients with different types of PGDs according to response to the therapy. Statistically significance was not detected between histological types (for all $p < 0.05$) (Data are from Turgutalp et al. (2014))
RDW red cell distribution width, *MCD* minimal change disease, *MGN* membranous nephropathy, *FSGS* focal segmental glomerulosclerosis, *MPGN* membranoproliferative glomerulonephritis (Multiple comparisons: Dunn test was applied for variables which showed non-normal distribution)
p* Difference between group 1, 2, and -3 in same histopathological type of PGDs, p** difference between histopathological types of PGDs in the same group

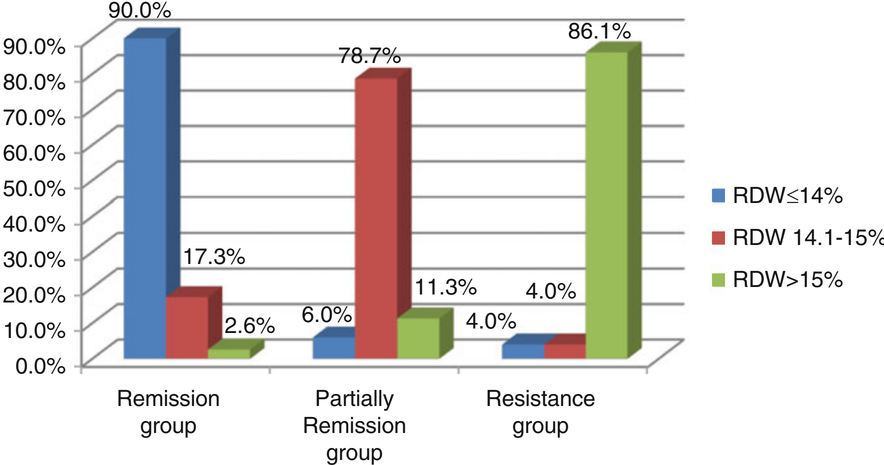

Fig. 2 Proportion of response to treatment according to initial RDW value. Most of the patients with complete remission had a baseline RDW value ≤14 % ($n = 45$, 90 %) ($p < 0.001$, Kendal Tau: −0.86), and most of the patients who were resistant to the treatment had a baseline RDW value >15 % ($n = 68$, 86.1 %) ($p < 0.001$, Kendal Tau: −0.87). Similarly, most of the patients with partial remission had baseline RDW values between 14.1 % and 15 % ($n = 37$, 78.7 %) ($p < 0.001$, Kendal Tau: −0.85) (Data are from Turgutalp et al. 2014)

be considered that RDW value may also increase due to the other comorbid conditions. Therefore it may be confusing whether the increase in RDW value is associated with PGDs or comorbid conditions. Cutoff values for RDW may be useful in differential diagnosis. Therefore new studies may be designed to assess these cutoff values. Further research is also required to clarify the association between the factors that have a role in the pathogenesis of different histopathological types of PGDs and RDW (Turgutalp et al. 2014).

Table 4 Determinants of proteinuria levels in the entire study population as evaluated by ANCOVA analysis. The ANCOVA results showed that RDW was a strong predictor of proteinuria independent of age, albumin, CRP values. The coefficient of determination (R^2) shows that RDW as an independent variable predicts 65.3 % of the changes in proteinuria by regression analysis. Both the coefficients of the regression equation (slope and intercept) are significant at $p < 0.001$ level. The independent variable RDW is a significant predictor of the change in proteinuria

Source	Mean square	F value	*p*
Age	167,404.965	0.056	>0.05
Albumin	2,460,952.409	0.826	>0.05
RDW	746,972,702.509	250.628	<0.001
hs-CRP	349,886.563	0.117	>0.05
eGFR	279,617.394	0.094	>0.05

RDW red cell distribution width, *hs-CRP* high sensitive C-reactive protein, *eGFR* estimated glomerular filtration rate (Data are from Turgutalp et al. (2014))

Summary Points

- This chapter focuses on RDW which may be a novel biomarker predicting the response to the therapy in patients with PGDs.
- RDW shows the variation in size of erythrocytes in the circulation and it is obtained easily by automated blood cell counters.
- Studies have been focused to investigate potential clinical importance of RDW which is directly associated with systemic inflammation in prediction of prognosis and clinical outcome of PGDs.
- RDW level can be seen as a part of the complete blood count.
- RDW is a cheap test and therefore it may be a cost-effective novel marker for evaluation of treatment response in NS due to PGDs.

References

Abdullahi H, Osman A, Rayis DA, et al. Red blood cell distribution width is not correlated with preeclampsia among pregnant Sudanese women. Diagn Pathol. 2014;9:29.

Afonso L, Zalawadiya SK, Veeranna V, et al. Relationship between red cell distribution width and microalbuminuria: a population-based study of multiethnic representative US adults. Nephron Clin Pract. 2011;119:277–82.

Appel GB, D'Agati VD. Primary and secondary (non-genetic) causes of focal and segmental glomerulosclerosis. In: Johnson RJ, Feehally J, Floege J, editors. Comprehensive clinical nephrology. 5th ed. Philadelphia: Elsevier Saunders; 2015. p. 218–30.

Arbel Y, Weitzman D, Raz R, et al. Red blood cell distribution width and the risk of cardiovascular morbidity and all-cause mortality. A population-based study. Thromb Haemost. 2014;111:300–7.

Atkins RC, Nikolic-Paterson DJ, Song Q, et al. Modulators of crescentic glomerulonephritis. J Am Soc Nephrol. 1996;7:2271–8.

Badid C, Desmoulière A, McGregor B, et al. Interstitial alpha-smooth muscle actin: a prognostic marker in membranous nephropathy. Clin Nephrol. 1999;52:210–7.

Bazzi C, Rizza V, Raimondi S, et al. In crescentic IgA nephropathy, fractional excretion of IgG in combination with nephron loss is the best predictor of progression and responsiveness to immunosuppression. Clin J Am Soc Nephrol. 2009;4:929–35.

Bazzi C, Rizza V, Casellato D, et al. Urinary IgG and α2-macroglobulin are powerful predictors of outcome and responsiveness to steroids and cyclophosphamide in idiopathic focal segmental glomerulosclerosis with nephrotic syndrome. Biomed Res Int. 2013:941831. doi: 10.1155/2013/941831. Epub 2013 Sep 4.

Beck Jr LH, Fervenza FC, Beck DM, et al. Rituximab-induced depletion of anti-PLA2R autoantibodies predicts response in membranous nephropathy. J Am Soc Nephrol. 2011;22:1543–50.

Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69:89e95.

Brenchley PE, Coupes B, Short CD, et al. Urinary C3dg and C5b-9 indicate active immune disease in human membranous nephropathy. Kidney Int. 1992;41:933–7.

Bright R. Tabular view of the morbid appearances in 100 cases connected with albuminous urine. Guy's Hosp Rep. 1836;1:338–79.

Camici M. The nephrotic syndrome is an immunoinflammatory disorder. Med Hypotheses. 2007;68:900–5.

Caro J, Gutiérrez-Solís E, Rojas-Rivera J, et al. Predictors of response and relapse in patients with idiopathic membranous nephropathy treated with tacrolimus. Nephrol Dial Transplant 2015 Mar;30(3):467–74.

Cattran DC, Reich HN, Beanlands HJ, Genes, Gender and Glomerulonephritis Group, et al. The impact of sex in primary glomerulonephritis. Nephrol Dial Transplant. 2008;23:2247–53.

Couser WG, Johnson RJ. The etiology of glomerulonephritis: roles of infection and autoimmunity. Kidney Int. 2014;86:905–14.

Deegens JK, Wetzels JF. Fractional excretion of high- and low-molecular weight proteins and outcome in primary focal segmental glomerulosclerosis. Clin Nephrol. 2007;68:201–8.

Deveci OS, Kabakci G, Tulumen E, et al. The relationship between microalbuminuria and the presence and extent of coronary atherosclerosis. Angiology. 2010;61(2):184–91.

Dillon SC, Taylor GM, Shah V. Diagnostic value of urinary retinol-binding protein in childhood nephrotic syndrome. Pediatr Nephrol. 1998;12:643–7.

Feehally J, Floege J. IgA nephropathy and henoch-schönlein nephritis. In: Johnson RJ, Feehally J, Floege J, editors. Comphrensive clinical nephrology. 5th ed. Philadelphia: Elsevier Saunders; 2015. p. 266–77.

Ghaffari S. Oxidative stress in the regulation of normal and neoplastic hematopoiesis. Antioxid Redox Signal. 2008;10:1923–40.

Harada K, Akai Y, Kurumatani N, et al. Prognostic value of urinary interleukin 6 in patients with IgA nephropathy: an 8-year follow-up study. Nephron. 2002;92:824–6.

Harada K, Akai Y, Yamaguchi Y, et al. Prediction of corticosteroid responsiveness based on fibroblast-specific protein 1 (FSP1) in patients with IgA nephropathy. Nephrol Dial Transplant. 2008;23:3152–9.

Hofstra JM, Deegens JK, Willems HL, et al. Beta-2-microglobulin is superior to N-acetyl-beta-glucosaminidase in predicting prognosis in idiopathic membranous nephropathy. Nephrol Dial Transplant. 2008;23:2546–51.

Huang YL, Hu ZD, Liu SJ, et al. Prognostic value of red blood cell distribution width for patients with heart failure: a systematic review and meta-analysis of cohort studies. PLoS One. 2014;18 (9):e104861.

Irazabal MV, Eirin A, Lieske J, et al. Low- and high-molecular-weight urinary proteins as predictors of response to rituximab in patients with membranous nephropathy: a prospective study. Nephrol Dial Transplant. 2013;28:137–46.

Ishiguro C, Yaguchi Y, Funabiki K, et al. Serum IgA/C3 ratio may predict diagnosis and prognostic grading in patients with IgA nephropathy. Nephron. 2002;91:755–8.

Keskin KR, Aras Z, Silfeler DB, et al. Relationship of red cell distribution width with the presence and severity of preeclampsia. Clin Appl Thromb Hemost 2015 Mar; 21(2):128–31.

Kocyigit I, Yilmaz MI, Simşek Y, et al. The role of platelet activation in determining response to therapy in patients with primary nephrotic syndrome. Platelets. 2013;24:474–9.

Lippi G, Targher G, Montagnana M, et al. Relation between red blood cell distribution width and inflammatory biomarkers in a large cohort of unselected outpatients. Arch Pathol Lab Med. 2009;133:628–32.

Mastroianni Kirsztajn G, Nishida SK, Silva MS, et al. Urinary retinol-binding protein as a prognostic marker in the treatment of nephrotic syndrome. Nephron. 2000;86:109–14.

Mathew R, Pai VR, Vijayakumar T. Comparison of microalbuminuria with biochemical and hematological parameters as a marker for renal involvement in patients at high risk for chronic kidney disease-Apilot study. Int J Sci Eng Res. 2014;5:97–100.

Ostalska-Nowicka D, Smiech M, Jaroniec M, et al. SOCS3 and SOCS5 mRNA expressions may predict initial steroid response in nephrotic syndrome children. Folia Histochem Cytobiol. 2011;49:719–28.

Ozcan F, Turak O, Durak A, et al. Red cell distribution width and inflammation in patients with non-dipper hypertension. Blood Press. 2013;22:80–5.

Patel KV, Mohanty JG, Kanapuru B, et al. Association of the red cell distribution width with red blood cell deformability. Adv Exp Med Biol. 2013;765:211–6.

Peters HP, van den Brand JA, Wetzels JF. Urinary excretion of low-molecular-weight proteins as prognostic markers in IgA nephropathy. Neth J Med. 2009;67:54–61.

Pierce CN, Larson DF. Inflammatory cytokine inhibition of erythropoiesis in patients implanted with a mechanical circulatory assist device. Perfusion. 2005;20:83–90.

Pillai S. Rethinking mechanisms of autoimmune pathogenesis. J Autoimmun. 2013;45:97–103.

Piyaphanee N, Ma Q, Kremen O, et al. Discovery and initial validation of α 1-B glycoprotein fragmentation as a differential urinary biomarker in pediatric steroid-resistant nephrotic syndrome. Proteomics Clin Appl. 2011;5:334–42.

Price-Jones C. The variation in sizes of red blood cells. Br Med J. 1910;2:1418.

Rezende SM, Lijfering WM, Rosendaal FR, et al. Hematologic variables and venous thrombosis: red cell distribution width and blood monocyte count are associated with an increased risk. Haematologica. 2014;99:194–200.

Roy RR, Roy E, Rahman MH, et al. Serum immunoglobulin G, M and IgG:IgM ratio as predictors for outcome of childhood nephrotic syndrome. World J Pediatr. 2009;5:127–31.

Rumana J, Hanif M, Muinuddin G, et al. Correlation of fractional excretion of magnesium with steroid responsiveness in children with nephrotic syndrome. Saudi J Kidney Dis Transplant. 2014;25:830–6.

Santín S, Tazón-Vega B, Silva I, FSGS Spanish Study Group, et al. Clinical value of NPHS2 analysis in early- and adult-onset steroid-resistant nephrotic syndrome. Clin J Am Soc Nephrol. 2011;6:344–54.

Schena FP, Alpers CE. Membranoproliferative glomerulonephritis and cryoglobulinemic glomerulonephritis. In: Johnson RJ, Feehally J, Floege J, editors. Comphrensive clinical nephrology. 5th ed. Philadelphia: Elsevier Saunders; 2015. p. 253–60.

Schmieder RE, Schutte R, Schumacher H, ONTARGET/TRANSCEND Investigators, et al. Mortality and morbidity in relation to changes in albuminuria, glucose status and systolic blood pressure: an analysis of the ONTARGET and TRANSCEND studies. Diabetologia. 2014;57:2019–29.

Segarra-Medrano A, Jatem-Escalante E, Carnicer-Cáceres C, et al. Evolution of antibody titre against the M-type phospholipase A2 receptor and clinical response in idiopathic membranous nephropathy patients treated with tacrolimus. Nefrologia. 2014;34:491–7.

Shimizu A, Takei T, Uchida K, et al. Predictors of poor outcomes in steroid therapy for immunoglobulin A nephropathy. Nephrology (Carlton). 2009;14:521–6.

Shin JR, Kim SM, Yoo JS, et al. Urinary excretion of β2-microglobulin as a prognostic marker in immunoglobulin A nephropathy. Korean J Intern Med. 2014;29:334–40.

Simel DL, DeLong ER, Feussner JR, et al. Erythrocyte anisocytosis. Visual inspection of blood films vs automated analysis of red blood cell distribution width. Arch Intern Med. 1988;148:822–4.
Skjelbakken T, Lappegård J, Ellingsen TS, et al. Red cell distribution width is associated with incident myocardial infarction in a general population: the Tromsø Study. J Am Heart Assoc. 2014;18(3):e001109.
Song CS, Park DI, Yoon MY, et al. Association between red cell distribution width and disease activity in patients with inflammatory bowel disease. Dig Dis Sci. 2012;57:1033–8.
Spiropoulos A, Goussetis E, Margeli A, et al. Effect of inflammation induced by prolonged exercise on circulating erythroid progenitors and markers of erythropoiesis. Clin Chem Lab Med. 2010;48:199–203.
Su C, Liao LZ, Song Y, et al. The role of red blood cell distribution width in mortality and cardiovascular risk among patients with coronary artery diseases: a systematic review and meta-analysis. J Thorac Dis. 2014;6:1429–40.
Torres DD, Rossini M, Manno C, et al. The ratio of epidermal growth factor to monocyte chemotactic peptide-1 in the urine predicts renal prognosis in IgA nephropathy. Kidney Int. 2008;73:327–33.
Troyanov S, Roasio L, Pandes M, et al. Renal pathology in idiopathic membranous nephropathy: a new perspective. Kidney Int. 2006;69:1641–8.
Turgutalp K, Kıykım A, Bardak S, et al. Is the red cell distribution width strong predictor for treatment response in primary glomerulonephritides? Ren Fail. 2014;36:1083–9.
Vajpayee N, Graham SS, Bem S. Basic examination of blood and bone marrow. In: McPherson RA, Pincus MR, editors. Henry's clinical diagnosis and management by laboratory methods. 22nd ed. Philadelphia: Elsevier/Saunders; 2011. p. 30.
Van Es LA, de Heer E, Vleming LJ, et al. GMP-17-positive T-lymphocytes in renal tubules predict progression in early stages of IgA nephropathy. Kidney Int. 2008;73:1426–33.
Veeranna V, Zalawadiya SK, Panaich S, et al. Comparative analysis of red cell distribution width and high sensitivity C-reactive protein for coronary heart disease mortality prediction in multi-ethnic population: findings from the 1999–2004 NHANES. Int J Cardiol. 2013;168:5156–61.
Yamaguchi M, Ando M, Yamamoto R, et al. Patient age and the prognosis of idiopathic membranous nephropathy. PLoS One. 2014;9:e110376.
Yoshimoto K, Wada T, Furuichi K, et al. CD68 and MCP-1/CCR2 expression of initial biopsies reflect the outcomes of membranous nephropathy. Nephron Clin Pract. 2004;98:25–34.
Yu FT, Armstrong JK, Tripette J, et al. A local increase in red blood cell aggregation can trigger deep vein thrombosis: evidence based on quantitative cellular ultrasound imaging. J Thromb Haemost. 2011;9:481–8.

Part VI

Resources

Recommended Resources on Biomarkers in Kidney Disease

53

Rajkumar Rajendram, Vinood B. Patel, and Victor R. Preedy

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Abstract

Biomarkers of kidney disease are substances that identify damage to the renal tract and may reflect renal function. They may be released from the kidney or result from a specific response to damage to the renal tract or changes in renal function.

Serum creatinine is currently the most widely used marker of kidney disease and renal function in clinical practice. However as it is affected by several non-renal factors it is unreliable and so better biomarkers are urgently required.

Several potentially relevant novel biomarkers of kidney disease have been discovered through omic technologies such as genomics, and proteomics. These

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novel biomarkers could be used to predict the risk of kidney disease, diagnose renal disease after an acute event, suggest the likely outcome (prognosis) in the absence of treatment, and predict the likely response to treatment. Keeping up-to-date with the rapid pace of the developments in this field is difficult. When attempting to do so it is difficult to know which of the myriad of available resources are reliable. To assist our colleagues we have therefore written this chapter which includes tables containing reliable, up-to-date resources. The experts who assisted with the compilation of these tables of resources are acknowledged.

Keywords
Biomarkers • Kidney Disease • Evidence • Resources • Books • Journals • Regulatory bodies • Professional societies

Key Points

- Biomarkers are of significant value in modern nephrology.
- Biomarkers are used in screening for kidney disease.
- Biomarkers are used in risk stratification, diagnosis, prognostication, directing initial therapy, monitoring response to treatment, and guiding the choice of further treatments.
- This chapter lists the most up-to-date resources on the regulatory bodies, journals, books, professional bodies, and websites that are relevant to an evidence-based approach to the use of biomarkers of renal disease.

Introduction

As defined at the beginning of this millennium, biomarkers are objective measures that indicate normal biological processes, pathogenic processes, or pharmacologic responses to a health care intervention (Atkinson et al. 2001). Biomarkers have significant scientific and clinical value in kidney diseases which are common causes of morbidity and mortality (Brown et al. 2015; Findlay et al. 2015; Panocchia et al. 2016).

A renal disease biomarker is a substance that indicates the presence of kidney disease. It could be released from the kidney or result from a specific response to kidney disease. Ideally, such a biomarker could be assayed in noninvasively collected fluids (e.g., urine, blood or serum).

Serum creatinine is currently the most commonly used marker of renal function in routine clinical practice. It is used to estimate the glomerular filtration rate (GFR), but is unfortunately an unreliable indicator of kidney function, for many reasons. For

Table 1 **Regulatory bodies and organizations.** This table lists the regulatory bodies and organizations involved with various aspects of biomarkers

Organization	Website
Biomarkers Consortium	biomarkersconsortium.org
Biomarker, Imaging and Quality of Life Studies Funding Program, National Cancer Institute, USA.	cancer.gov/aboutnci/organization/ccct/funding/BIQSFP
Biomarker Qualification Program US Food and drug administration	fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/ucm284076.htm
Canadian Coordinating Office for Health Technology Assessment	www.ccohta.ca
Centers for Disease Control and Prevention (CDC)	www.cdc.gov/globalhealth/countries/egypt
CKD Biomarkers Consortium	www.ckdbiomarkersconsortium.org/
European Medicines Agency	ema.europa.eu/ema/index.jsp?curl=pages/special_topics/general/general_content_000349.jsp
George Institute for Global Health	www.georgeinstitute.org/publications/biomarkers-in-kidney-fibrosis-are-they-useful
International Federation of Clinical Chemistry and laboratory Medicine (IFCC)	www.ifcc.org
KIDNEY, Swiss National Centre of Competence in Research	www.nccr-kidney.ch/index.php?nav=43GEN Exclusives
Medicines and Healthcare products Regulatory Agency (MHRA)	mhra.gov.uk
National Center for Environmental Research	www.epa.gov/ncer/rfa/2006/2006_star_pbk_modeling.html
National Institutes of Health	www.nlm.nih.gov/medlineplus/ency/anatomyvideos/000023.htm
National Research Council Canada	www.nrc-cnrc.gc.ca/eng/transparency/access_information/infosource.html
U.S. Food and Drug Administration	www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/ucm437988.htm

example, serum creatinine is affected by several nonrenal factors such as age, gender, muscle mass, and hydration.

In acute kidney injury (AKI), serum creatinine does not reflect the actual decrease in the GFR and takes several hours or days to reach a new steady state. In fact, serum creatinine may not increase until over half of the patient's renal function is lost because there is a significant renal reserve.

As a result of the limitations of serum creatinine, several possible biomarkers of kidney disease have been studied over the past decade. These include urinary and serum proteins, molecules, and, most recently, microRNAs. The driver for this line

Table 2 Professional societies. This table lists the professional societies involved with biomarkers and/or kidney disease

American Association for Clinical Chemistry	www.aacc.org
American Society of Nephrology (ASN)	www.asn-online.org
Association for Clinical Biochemistry and Laboratory Medicine	www.acb.org.uk
Australasian Association of Clinical Biochemists	www.linkedin.com/company/australasian-association-of-clinical-biochemists
Australian and New Zealand Society of Nephrology (ANZSN)	www.nephrology.edu.au
Biomarkers Consortium	www.biomarkersconsortium.org
Carriel Institute for Medical Research	www.carriel.org
Deutsche Gesellschaft für Nephrologie	www.dgfn.eu
European Renal Association – European Dialysis and Transplant Association	www.era-edta.org
European Society of Endocrinology (ESE)	www.ese-hormones.org
High Blood Pressure Research Council of Australia	www.hbprca.com.au
Hypertension, Dialysis and Clinical Nephrology	www.hdan.com
Institute of Biomedical Science	www.ibms.org
International Society of Hypertension	ish-world.com
International Society of Nephrology	www.theisn.org
Japanese Society for Biomedical Mass Spectrometry	www.jsbms.jp/english
Mass Spectrometry Society of Japan	www.mssj.jp/en/index.html
National Kidney Foundation	www.kidney.org
Sociedad Portugesa de Nefrolagia	www.spnefro.pt
Société Française de Biologie Clinique (SFBC)	www.sfbc.asso.fr
Société française d'endocrinologie (SFE)	www.sfendocrino.org
Society of Medical Biochemists of Serbia	www.dmbj.org.rs
Society of Nephrologists of Serbia	www.udruzenjenefrologa.com
Viapath – Clinical Biochemistry Laboratory at King's College Hospital	www.viapath.co.uk/departments-and-laboratories/clinical-biochemistry-laboratory-at-kings

of investigation is that prompt specific intervention may prevent permanent renal damage if AKI is detected at any early stage.

These novel biomarkers could be used to predict the risk of kidney disease, diagnose renal disease after an acute event, suggest the likely outcome (prognosis) in the absence of treatment, and predict the likely response to treatment. Thus, there are four main potential uses for biomarkers in renal disease:

Table 3 Journals publishing on kidney disease. This table lists the top 25 journals publishing original research and review articles related to kidney disease. The list was generated from SCOPUS (www.scopus.com) using general descriptors. The journals are listed in descending order of the total number of articles published in the past 5 years. Of course, different indexing terms or different databases will produce different lists so this is a general guide only. For example, journals associated with biomarker discovery will produce a different list (see Table 4)

Plos One
Nephrology Dialysis Transplantation
Transplantation Proceedings
Kidney International
American Journal of Kidney Diseases
Journal of Urology
Transplantation
American Journal of Physiology Renal Physiology
Pediatric Nephrology
Urology
American Journal of Transplantation
Renal Failure
BMJ Case Reports
Nature Reviews Nephrology
International Urology and Nephrology
Clinical and Experimental Nephrology
Saudi Journal of Kidney Diseases and Transplantation an Official Publication of the Saudi Center for Organ Transplantation Saudi Arabia
Nephrology
Journal of Endourology
BMC Nephrology
Clinical Nephrology
BJU International
New England Journal of Medicine
American Journal of Nephrology
International Journal of Cardiology

Risk stratification
Diagnosis
Prognostication
Monitoring response to treatment

Several potentially relevant renal biomarkers have been discovered through omic technologies such as genomics and proteomics. The use of emerging high-throughput technologies to integrate biomarkers into clinical practice will allow "personalization" of disease management in the future.

Table 4 Journals publishing on kidney disease and biomarkers. This table lists the top 25 journals publishing original research and review articles related to kidney disease and biomarkers. The list was generated from SCOPUS (www.scopus.com) using general descriptors. The journals are listed in descending order of the total number of articles published in the past 5 years. Of course, different indexing terms or different databases will produce different lists so this is a general guide only

Journal
Plos One
Nephrology Dialysis Transplantation
Kidney International
Transplantation Proceedings
Pediatric Nephrology
Renal Failure
Transplantation
American Journal of Physiology Renal Physiology
American Journal of Kidney Diseases
Nephrology
BMC Nephrology
Journal of Nephrology
Saudi Journal of Kidney Diseases and Transplantation an Official Publication of the Saudi Center for Organ Transplantation Saudi Arabia
Clinical Nephrology
Clinical and Experimental Nephrology
International Urology and Nephrology
Journal of Urology
American Journal of Nephrology
Tumor Biology
Nature Reviews Nephrology
Critical Care
Nephron Clinical Practice
American Journal of Transplantation
Clinica Chimica Acta
International Journal of Cardiology

Biomarker discovery in relation to kidney disease is also important for several reasons other than the patient's physical domains. Kidney diseases impact on quality-of-life measures (Brown et al. 2015; Griva et al. 2016; Moreira et al. 2015). Furthermore, one needs to consider the global impact of kidney diseases (Jha et al. 2013; Mills et al. 2015). Kidney diseases are estimated to have a global prevalence of 8–16 % and are driven by risk factors such as obesity and hypertension, though other less well-defined factors may also contribute (Weaver et al. 2015). Kidney diseases are ranked within the top 20 causes of all global deaths. There are about half a billion people with chronic kidney disease alone (Mills et al. 2015) though less precise figures have been obtained for acute kidney disease. The Lancet highlighted the importance of kidney disease as being on par with diabetes in terms

Table 5 Relevant books on biomarkers. This table lists books on biomarkers

Aptamers in Bioanalysis. Mascini M. Wiley-Interscience, 2009, USA.
Aptamer Handbook: Functional Oligonucleotides and Their Applications. Klussmann S (editor). Wiley-VCH, 2006, Germany.
Biomarkers in Bone Disease. Preedy VR, Patel VB. Springer, 2017, Netherlands.
Biomarkers in Cardiovascular Diseases. Tousoulis D, Stefanadis C. CRC Press, 2013, UK.
Biomarkers for CKD: Glomerular Filtration and Progression of Kidney Disease (CD). Dalton R. American Association of Clinical Chemists, 2012, USA.
Biomarkers in Kidney Disease. Edelstein CL, Elsevier, 2010, USA.
Biomarkers in Renal Disease. Rosner MH, Okusa M. Nova Publisher, 2008, USA.
Handbook of Biomarkers. Kewal KJ. Lippincott, 2010, USA.
Biomarker Guide. Peters KE, Walters CC, Moldowan JM. Cambridge University Press, 2010, USA.
Role of Novel Biomarkers in Chronic Kidney Disease: Renalase. Desir GV. Springer, 2010, Italy.

Table 6 Relevant books on kidney disease. This table lists books on kidney disease

Brenner and Rector's the Kidney. Brenner BM. Saunders, 2008, Philadelphia.
Comprehensive Clinical Nephrology. Johnson RJ, Feehally J, Flaege J, Elsevier, 2014, USA
Diagnostic Pathology: Kidney Diseases, 2nd Edition. Colvin R, Chang A. Elsevier, 2015, USA
Kidney Disease and Laboratory Medicine. Lamb E, Delaney M. ACB Venture Publications 2009, UK.
Pathophysiology. McCance KL, Brashers VL, Rote NS. Mosby Elsevier, 2010, USA.
Uremic Toxins. Niwa T (Editor). John Wiley & Sons, 2012, USA
Urinalysis and Body Fluids, 6th Edition. Strasinger SK, Di Lorenzo MS. Elsevier, 2011, USA

of burden (Anon 2013). Other areas of the causes and impact of kidney-related disease are highlighted in this book.

It is now difficult even for experienced scientists and clinicians to remain up-to-date. For those new to the field, it is difficult to know which of the myriad of available sources are reliable. To assist colleagues who are interested in understanding more about biomarkers of kidney disease, we have therefore produced tables containing reliable, up-to-date resources in this chapter. The experts who assisted with the compilation of these tables of resources are acknowledged below.

Examples of the definitions, measurement, and applications of biomarkers can be found in this book and also via the recommended resources in the tables below.

Tables 1, 2, 3, 4, 5, 6, 7, and 8 list the most up-to-date information on the regulatory bodies (Table 1), professional bodies (Table 2), journals on cardiovascular disease (Table 3), journals on biomarkers (Table 4), books on biomarkers (Table 5), books on cardiovascular disease (Table 6), emerging techniques and platforms (Table 7), and websites (Table 8) that are relevant to an evidence-based use of biomarkers in cardiovascular disease.

Table 7 Sources and resources for emerging techniques and platforms. This table lists some emerging sources, resource platforms in biomarker discovery, and application

Biobanking and Biomolecular Resources Research Infrastructure	bbmri.eu
Clinical Center of Vojvodina, Novi Sad, Serbia	www.kcv.rs/rs
Eurolab	www.eurolab.org
GEN Exclusives	www.genengnews.com/gen-articles/biomarker-discovery-methods-evolving/4097/
Human genome variation society	www.hgvs.org
Fondation PremUP	www.premup.org
Intech, open science, open minds	www.intechopen.com/books/latest-research-into-quality-control/quality-control-of-biomarkers-from-the-samples-to-data-interpretation
Laboratory for emerging technologies	Opto.brown.edu
MediBEACON	www.medibeacon.com/products/nephrology/renal-function-system/
Medical Faculty, Novi Sad, Serbia	www.mf.uns.ac.rs
NanoINK, Nano biodiscovery	www.marketwired.com/press-release/nanoink-platform-proven-reproducibly-detect-protein-biomarkers-from-dried-blood-spot-1562611.htm
Quintiles, American Heart & Quintiles	www.quintiles.com/library/white-papers/biomarkers-recent-advances-in-their-application-to-the-treatment-of-hematologic-malignancies
University of Zurich Progenetix database	progenetix.org/cgi-bin/pgHome.cgi

Table 8 Relevant internet resources. This table lists some Internet resources on biomarkers and kidney disease

Acute Dialysis Quality Initiative	www.adqi.org
Biomarkers Test (BMT)	www.biomarkers.it
Biomed Central (BMC) Biomarkers	biomarkerres.org
Broad Institute	www.broadinstitute.org/scientific-community/science/platforms/proteomics/biomarkers
Insightomics – Biomarkers discovery	www.insightomics.com/biomarker-discovery/?gclid=CJvJ9–Sp8gCFYUIwwodnNQIwQ
Kidney Health Australia	www.kidney.org.au
Medscape	www.medscape.com
Mercer’s institute for successful aging (MISA)	www.misa.ie/research/clinical/biomarkapd
Military Medical Academy, Belgrade, Serbia	www.vma.mod.gov.rs
News medical	www.news-medical.net/health/What-is-a-Biomarker.aspx

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References

Anon. The global issue of kidney disease. Lancet. 2013;382(9887):101.

Atkinson AJ, Colburn WA, DeGruttola VG, DeMets DL, Downing GJ, Hoth DF, Oates JA, Peck CC, Schooley RT, Spilker BA, Woodcock J, Zeger SL, NCI-FDA Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharm Ther. 2001;69:89–95.

Brown MA, Collett GK, Josland EA, Foote C, Li Q, Brennan FP. CKD in elderly patients managed without dialysis: survival, symptoms, and quality of life. Clin J Am Soc Nephrol. 2015;10 (2):260–8.

Findlay M, Donaldson K, Robertson S, Almond A, Flynn R, Isles C. Chronic kidney disease rather than illness severity predicts medium- to long-term mortality and renal outcome after acute kidney injury. Nephrol Dial Transplant. 2015;30(4):594–8.

Griva K, Goh CS, Kang WCA, Yu ZL, Chan MC, Wu SY, Krishnasamy T, Foo M. Quality of life and emotional distress in patients and burden in caregivers: a comparison between assisted peritoneal dialysis and self-care peritoneal dialysis. Qual Life Res. 2016;25(2):373–84.

Jha V, Garcia-Garcia G, Iseki K, Li Z, Naicker S, Plattner B, Saran R, Wang AYM, Yang C-W. Chronic kidney disease: global dimension and perspectives. Lancet. 2013;382(9888):260–72.

Mills KT, Xu Y, Zhang W, Bundy JD, Chen C-S, Kelly TN, Chen J, He J. A systematic analysis of worldwide population-based data on the global burden of chronic kidney disease in 2010. Kidney Int. 2015;88(5):950–7.

Moreira JM, Bouissou Morais Soares CM, Teixeira AL, Simoes e Silva AC, Kummer AM. Anxiety, depression, resilience and quality of life in children and adolescents with pre-dialysis chronic kidney disease. Pediatr Nephrol. 2015;30(12):2153–62.

Panocchia N, Tazza L, Di SE, Liberatori M, Vulpio C, Giungi S, Lucani G, Antocicco M, Bossola M. Mortality in hospitalized chronic kidney disease patients starting unplanned urgent haemodialysis. Nephrology. 2016;21(1):62–7.

Weaver VM, Fadrowski JJ, Jaar BG. Global dimensions of chronic kidney disease of unknown etiology (CKDu): a modern era environmental and/or occupational nephropathy? BMC Nephrol. 2015;16(1):1–8.

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