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).

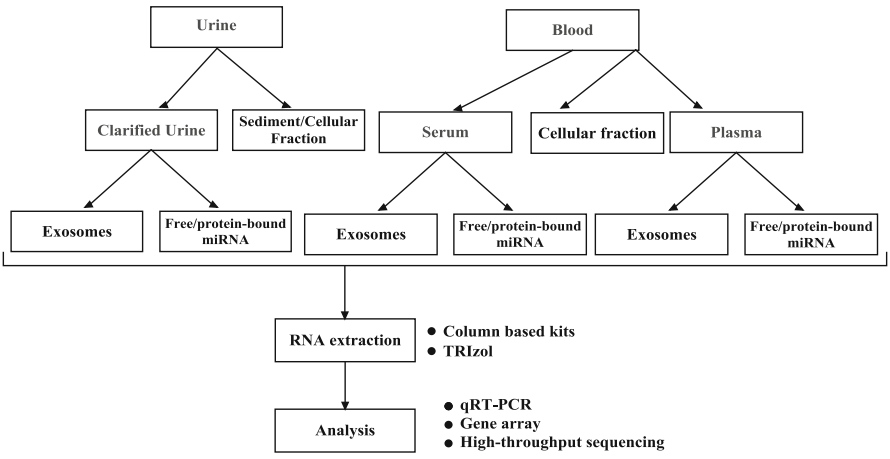

Fig. 1 Depiction of the various samples obtainable for miRNA analysis from both blood and urine and the methods by which they may be analyzed

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.

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