Diabetic Nephropathy

Pathophysiology and Clinical Aspects

Joris J. Roelofs
Liffert Vogt
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Preface

Over the past 30 years, the world has witnessed a dramatic increase in the prevalence of diabetes and its many complications. One of these, diabetic nephropathy, is currently the leading cause of end-stage renal disease worldwide, at a tremendous human and economic cost. Comprehensive understanding of the many complex pathophysiological mechanisms and their mutual interrelationship will be mandatory to facilitate the development of novel preventive and therapeutic regimens for diabetic nephropathy.

This book aims to cover diabetic kidney disease in the most comprehensive fashion. We have chosen a broad viewpoint, zooming in from global and population level to molecular mechanisms, providing both a historical and future-oriented perspective. We believe that this approach has resulted in the most complete volume on diabetic nephropathy currently available. Written by renowned experts, the 29 chapters cover the current understanding of pathophysiology, research tools, and clinical features and summarize the current treatment options for diabetic nephropathy. In addition, closely related areas such as *diabesity*, diabetic eye disease, and macrovascular involvement in diabetes are addressed.

We expect the book to be of great use for basic researchers and clinicians studying and treating patients with diabetes, as well as medical students, fellows, and public health professionals. Since it is estimated that by the year 2040, about 1 in 9 individuals around the world will be living with diabetes, a large proportion of which will experience renal complications, we hope this book will eventually contribute to reducing the burden of diabetic nephropathy in the future.

Amsterdam, The Netherlands — Joris J. Roelofs
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Part I
Diabetic Nephropathy: Scope of the Problem

Chapter 1
A Historical Overview of Diabetic Nephropathy

Garabed Eknoyan

The history of diabetes dates back to the very beginnings of civilization when rudimentary efforts at the accrual and transmission of medical knowledge were first being undertaken. It begun at a time when it was the external manifestations of an illness that defined it and diseases were classified broadly by their presentations as fevers, epidemics, dropsies, icterus, etc. It is not unexpected then that polyuria soon attracted attention as a specific disease entity that was termed diabetes and attributed to an impaired retentive capacity of the kidney for fluids. In fact, of all diseases to acquire a name diabetes is one of the, if not the, oldest, which throughout most of its history was considered a disease of the kidneys. It was only in the latter part of the nineteenth century, when chemistry and physiology emerged and medicine begun its change from an observational bedside to an investigative laboratory science that diabetes first came to be defined as a metabolic, and shortly thereafter as an endocrine disease, whose therapy with insulin was to close the chapter of diabetes as a disease of the kidneys [1–8]. Ironically, it was the successful treatment of this new endocrine disorder as diabetics began to survive the acute fatal complications of their disease that would lead to the emergence of diabetes as a cause of kidney disease [9–11]. A complication that has assumed epidemic proportions over the recent past as a result of parallel but independent external changes in increased longevity in general, allowing time necessary for the progression of the renal lesions of diabetes, and increasing caloric consumption resulting in obesity with its altered insulin disorders. As a result, diabetic kidney disease is now a leading cause of chronic kidney disease, of kidney failure, and a major risk factor for the shortened life expectancy of diabetics [12, 13]. The biography of the changing concepts of the kidney in diabetes mellitus within the dynamic construct of the history of evolving medical knowledge is shown in Fig. 1.1.

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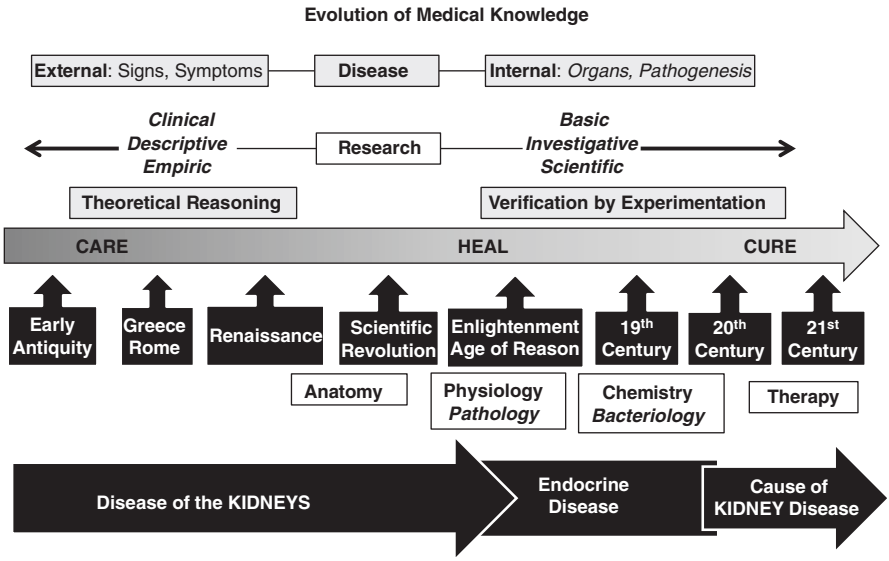

Fig. 1.1 A timeline of the history of diabetic kidney disease in relation to that of the evolution of medical knowledge. The upper part of the figure shows the conceptual evolution of diseases in general from a descriptive empiric stage based on external findings to an investigative scientific basic research of organ involvement and clinical course of a disease, in essence, a paradigm shift from theoretical deductive reasoning to an analytic one verified by experimentation. The long arrow in the center represents a timeline of the historical periods of progress shown below it in black boxes. The emergence of the basic sciences during each period is shown in the white boxes appended to each historical period. The bottom black boxed timeline shows the corresponding conceptual changes in the history of diabetes mellitus from a disease of the kidneys to an endocrine disease that is a cause of kidney disease

Diabetes: A Disease of the Kidneys

Beginning in antiquity, when what constituted evidence of an illness were the external manifestations and abnormal symptoms with which patients presented, polyuria soon attracted attention and was identified as a specific disorder. Most extant medical texts of antiquity devote sections of variable length and detail to its description and treatment. However, polyuria is a symptom of varied etiology, and it is impossible to discriminate clearly what some of those ancient writings referred to or what the treatments described were directed at. Notwithstanding the justifiable objection of interpreting the past in light of what we know now, the fact is that most clinical features of diabetes mellitus as we know them were noted in polyuric patients of old, and most of the extant descriptions given in ancient medical texts are quite consistent with diabetes mellitus [4–8]. Notable among those are Ayurvedic texts, dated to the fifth century B.C., that refer to cases of excessive urine (*meha*), coupled with thirst and emaciation, in whom the urine is described as "*kshoudra*" (honey) or "*madhu*" (sweet), a disease (*kshoudrameha, madhumeha*) said to affect the rich and affluent who consumed large quantities of rice, cereals, and sweets [14].

Demetrius of Apamea (first or second century B.C.) is credited with introducing the term "diabetes," which derives from the Ionic verb (*diabainein)* meaning "to pass or run through" and its subsequent Latin meaning of "siphon" [4, 15]. It corresponds to the prevailing notion that the large volumes of urine of these patients were due to the passage of ingested fluids through the body unchanged as if through a tube. It is under the term "diabetes" that Aretaeus of Cappadocia (early second century A.D.) wrote what is considered the first accurate clinical description of diabetes. He describes it as "an affliction that is not very frequent... being a melting down of the flesh and limbs into the urine... life is short, disgusting and painful… thirst unquenchable… the kidneys and bladder never stop making water... it may be something pernicious, derived from other diseases, which attack the bladder and kidneys." Another rare cause Aretaeus mentions is the bite of a serpent (*dipsas*) that "kindles up an unquenchable thirst" [4, 6, 16].

While Aretaeus attributed diabetes to a disease of the bladder and the kidneys, his more famous contemporary, Galen (129–200 AD) considered it specifically a disease of the kidneys and not of the bladder. In Chap. 3 on diseases of the kidneys of his *On Affected Parts* he states: "This condition impresses me as an ailment of the kidneys, whereas other physicians call it 'dropsy of the chamber pot', or 'urinary diarrhea' others define it as 'diabetes'; but some call it 'dipsakos' (violent thirst). It is a very rare disease, which I observed only twice until now. These patients had an immoderate thirst. For this reason they drank abundantly and passed all the water they consumed after a short time in the same condition (sic) as they took it" [17]. Making reference to his elegant series of experiments on dogs showing the kidneys as the source of urine [18], he continues: "It also has been shown that the kidneys attract the watery substance of the blood, but that the urinary bladder does not attract anything... the kidneys send discharged matter to the bladder through the ureters... Someone could therefore blame the failure to retain the urine for any period of time on a weakness of the kidneys but not the other organs through which the ingested fluid has to pass" [17].

Of particular relevance to our current understanding of changes in serum tonicity and intravascular blood volume in diabetes, which only began to be elucidated in the 1950s [19], is Galen's visionary explanation, speculative but yet so perceptive, that "diuresis starts slowly, but when it becomes more intense it draws the serum of the blood first from the veins without us being aware of it. When all the serum has been released and the blood in the vein appears to have lost its moisture, the dried up blood vessels will attract new moisture from the liver, and later from the bowels and stomach; but when the veins of the opening of the stomach are dried up, the patient craves fluid, since he becomes aware of this condition. Then, when fluids have been supplied, the parched veins from the liver to the stomach rapidly seize the entire amount which flows from these veins to adjoining vessels until it reaches the kidneys." Concluding that "Diabetes is a disease specific to the kidneys, the thirst analogous to ravenous hunger has its seat in the opening of the gastric cavity, and is combined with a weakness (atonia) of the retentive faculty of the kidneys... the lack of a weakened retentive faculty would not allow a rapid elimination of urine" [17]. Galen had his circulation confused and also seems to have erroneously placed the

site of osmoregulation in the stomach, but his deductive physiological reasoning is certainly impressive. This rational reasoning coupled with the dogma that Galen's authority came to be accepted dominated the prevailing concepts of diabetes as a disease of the kidneys over the next 1500 years (Fig. 1.1).

Over time, additional clinical features of diabetes were described. Avicenna (980–1037), who termed the disease *aldulab* or water wheel and *zalkh el kuliah* or diarrhea of the kidneys, terms that Galen and others had used, added to the complications of the disease those of mental troubles, impotence, gangrene, and furunculosis [4, 6]. Avicenna is said to have more clearly differentiated diabetes associated with emaciation from other causes of polyuria and prescribed a treatment that subsequent limited trials in Tunis have been reported as effective in five cases of diabetes [20].

With expanding reference to diabetes in the medical literature, one gets a sense of its increasing prevalence over time. In fact, it is in Arabic medicine that the first extant treatise dedicated to diabetes (*Fil marad allahi yusamma diabeta*, On the Disease Called Diabetes) was written by a contemporary of Maimonides (1138–1204), one Abdel Latif el Baghdadi (1162–1231) in 1225, during the turbulent years of the crusades [3, 4, 21].

Diabetes: A Disease of the Kidneys?

The first paradigm shift in the conceptual evolution of diabetes comes from the studies of the Swiss Renaissance physician Paracelsus (1493–1541), who described it as a constitutional disease that *irritates the kidneys* and provokes excessive urination. Having evaporated the urine from a diabetic patient, Paracelsus reported an excessive residue, which he called *sal urinae*, and described diabetes as an affection of the blood which "being involved with salt Particles, do run forth through the most open passages of the Reins" [4, 6, 8, 22]. A provocative conceptual analysis that Ralph Major (1884–1970) characterized as "… although couched in somewhat fantastic language, is clear and, if we substitute sugar for salt, has a surprisingly modern ring" [16]. Actually, this is no exaggeration or wishful thinking because in the budding chemistry of Paracelsus, using combustion, evaporation, distillation, and precipitation, the four humors of Galen were replaced by his analysis by fire of all matter as composed of *sulfur* or the combustible flames, *mercury* the precipitating vapors, and *salt* the final dry residue of the combustion. Essentially, as a founder of iatrochemical thought, Paracelsus was not using the term "salt" for its taste but for the crystalline nature of the white urine residue he obtained. It would be another century, in 1674, before a graduate of Oxford and a founding member of the Royal Society of London, Thomas Willis (1621–1675) would characterize the "salt" of Paracelsus as being sweet, "as if imbued with honey (quasi melle) and sugar." It was then that the attention of the profession was finally directed to the specificity of saccharine character to diabetic urine that would provide the basis of the subsequent differentiation of diabetes mellitus from other causes of polyuria [4, 6, 16, 23].

As important as the observation of Willis was, it was not a new one. As mentioned earlier, the sweetness of the urine was described in Ayurvedic texts. Moreover, tasting urine was an acclaimed and a well-known part of its regular examination. In the opening scene of his 1645 play *Le Médecin Volant* (The Flying Doctor) Molière (1622–1673) has his doctor boastingly claim that "unlike ordinary physicians who are satisfied by just looking at the urine, as an extraordinary physician I taste it, for it is the taste of urine that allows for discerning the cause and outcome of a disease" [24]. In fact, both Avicenna and Paracelsus advise tasting the urine and Paracelsus refers to its sweetness (*dulcet urinae*) in other contexts (6). Why the sweetness of urine had failed to be linked to diabetes until then is most likely due to the faulty understanding of kidney function perpetuated by Galen. Pertinent in this regard is the description of diabetes by Morgagni (1635–1683) as "what is drunk should be discharged by the urinary passages, without the least change whatever(sic), preserving the same colour, consistence, taste (sic) and smell as when take in" [25]. In fact, Avicenna in the first book of the Canon states that "when the urine of diabetics is left to stand in ambient air, it leaves a residue that is particularly sticky and tastes sweet as honey" [20]. It would have been unusual for Paracelsus, a man known for his rough and crude ways, not to have tasted the residue of the diabetic urine that he analyzed. It is fair to conclude then that sweetness of the urine was observed in the past but had been attributed to the fact that ingested nutrients, including sweet ones among others, passed *without the least change whatever* into the urine along with the fluids that had been consumed and hence the sweet taste of urine. Essentially, it was the *weakened retentive faculty* of the kidneys described by Galen that accounted for the non-specific appearance of saccharine matter, along with other nutrients, in the urine. Moreover, as we know it now the glycosuria of diabetics depends on the level of sugar in the blood and occurs only when the filtered glucose exceeds the reabsorptive capacity of the tubule; as such, individuals with diabetes do not always spill sugar in their urine, even if regularly tasted as in old days or tested as done nowadays; essentially a causal link was missed because diabetic urine did not always have a sweet taste. A quantitative relationship between the magnitude of hyperglycemia and the degree of glycosuria would not be established until 1862 by the British physiologist Frederick Pavy (1829–1911), a trainee of Claude Bernard (1813–1878) and coworker of Richard Bright (1789–1858) at Guy's Hospital [26, 27].

What makes the observation of Willis a transforming landmark is that unlike his predecessors, he not only highlighted the specificity of the sweetness of urine in diabetes, or the *Pissing Evil* as he calls it, but also proposed that the disease is an affliction of the blood arguing that the sweetness appears first in the blood and then in the urine. An argument buttressed by the discovery of the circulation in 1628 by William Harvey (1578–1657), which Willis uses to condemn Galen's notion of impaired retentive powers of the kidney function, "It in no way pleases us that some do assign for the cause of Diabetes the attracting force of the Reins: because the Blood is not drawn to the Reins but driven thither by the motion of the Heart. Further neither doth Serum seem to be drawn or emulged from Blood washing through them, but to be separated (as we have already more clearly shewed) partly by

straining, and partly by fusion or a certain kind of precipitation: wherefore we believe the Diabetes to be rather and more immediately an affection of the Blood than the Reins" [16].

Two Principal Forms of Diabetes

Nevertheless, it was the rather simple observation of Willis that set the stage for the appropriate naming of the disease "diabetes mellitus" and for the differentiation of the two principal forms of diabetes: mellitus (*diabetes vera*) and insipidus (*diabetes spuria*) (Fig. 1.1) [23]. Actually, it would be yet another century before Willis's argument of diabetes as *an affection of the blood* was substantiated by the demonstration of sugar in the blood and urine of diabetics by Robert Wyatt in 1774 and 2 years later by the more thorough studies of Matthew Dobson (1732–1784) of Liverpool [28]. Dobson had a fairly good of knowledge of chemistry but also consulted his Scottish colleague and famed chemist William Cullen (1710–1780) [4, 6]. In 1776, Dobson documented that the sweetness of urine is due to sugar, which he quantified and showed it to be subject to alcohol and acetate fermentation, and that its appearance in the urine is preceded and accompanied by a similar sweetness and sugar in the residue of blood, albeit not in the larger quantities detected in the urine [6, 29]. To quote Dobson, "It appears …that a considerable quantity of saccharine matter is passed off by the kidneys, in this case of diabetes, and probably does so in every imbalance of this disease, where the urine has a sweet taste …It further appears that this saccharine matter is not formed in this secretory organ (kidney) but previously existed in the blood" [29, 30]. This was a transformative paradigm shift from the theretofore prevailing concept of diabetes as a disease of the kidneys, whose etiology remained to be defined.

It was just about then that Cullen first called attention to diabetic urine that is insipid in taste and added the descriptive adjective "mellitus" to the disease described by Willis and Dobson [3, 23, 29]. As reflected in his correspondence with Dobson it is with some hesitation that Cullen also first described a case of polyuria he had encountered in which the urine taste was insipid rather than saccharine, "…I have only to add that I wish you would examine both by taste and evaporation what might be called the Urina Potus or that copius limpid urine which runs in some people after drinking largely of water or watery liquors" [6, 16].

The *urina potus* to which Cullen refers is a vestigial remnant of Medieval uroscopy in which the urine to be "looked at" was classified as that after eating (*urina cibis*), after sleep (*urina sanguinis*), and after drinking (*urina potus*). The use of *urina potus* interchangeably with that of excessive urination (polyuria, diabetes) remained in use well into the 1930s even after chemical analysis and osmometry of urine to diagnose diabetes insipidus had come into use [23, 31–33]. *Potus* is also the origin of the clinical entity termed "beer potomania" that was to be described much later [34].

Diabetes Mellitus: A Metabolic Disorder

By the close of the eighteenth century and the end of the Enlightenment (Fig. 1.1), diabetes mellitus had come to be viewed as a metabolic disorder of digestion and assimilation of nutrients from which sugar is generated, so called *mal-assimilation of sugar*, which then accumulates in the blood and is excreted in the urine [33]. This was to launch a whole new approach for the dietary treatment of diabetics and with it a shift to the digestive system as the site of the disease, specifically in the handling of absorbed potential "saccharine matter." The series of studies on the dietary treatment of diabetics that followed are best exemplified in the early and pioneering work of a military surgeon, John Rollo (1749–1809), with the assistance of a military chemist and apothecary, William Cruickshank (d. 1810), both from the Royal Military Academy in Woolwich, now a district within the borough of Greenwich in London [6]. Their initial longitudinal study of one Captain David Meredith on a diet rich in protein and fat but poor in starches resulted in elimination of his weight loss and the reversal of his glycosuria and hyperglycemia [35, 36]. After their study of another officer, they summarized their studies in a monograph titled *An Account of Two Cases of Diabetes Mellitus* published in 1797 [37]. Of note to the story of albuminuria in diabetes is Cruickshank's mention of the appearance of *albumen* in the urine of some, but not all, diabetic subjects he had studied.

As much of this early work was done in England during the Napoleonic era, the disease came to be dubbed in France facetiously as *diabetes anglicus*. Yet, much of the subsequent clinical and basic research on diabetes came from the continent. Notable among them are those of Apollinaire Bouchardat (1800–1886) and Raphael Lépine (1840–1919) from France, and Friedrich Theodor Frerichs (1819–1885) and Bernhard Naunyn (1839–1925) from Germany [3, 6, 38].

The sugar in the blood and urine was identified as glucose by the French chemist Michel Eugene Chevreul (1786–1889) in 1815. Efforts to quantify glucose in the urine continued to be refined, and by the second half of the nineteenth century, the disease could be diagnosed from the chemical analysis of urine. Although diabetes was now acknowledged as one of blood composition, it continued to be attributed to the kidneys whose weakened retentive powers resulted in the passage of sugar with water, rather than the mere passage of just water in the urine much as Galen had first implied [9, 18]. It is interesting in this regard that Rollo and Cruickshank, for whatever reason, in addition to the dietary management of the diabetes of Captain Meredith also made two incisions, the size of half a crown, on his back opposite each kidney [6, 36, 39]!

Still, a digestive disorder characterized by increased glucose absorption as the source of hyperglycemia continued to be debated and explored until 1855, when the French physiologist Claude Bernard, after 10 years of determined work, demonstrated the glycogenic properties of the liver and established glucose as the first internal secretion released by the liver [6, 40]. This led Bernard to his unsuccessful efforts to detect abnormalities of liver structure at postmortem of diabetics. Nevertheless, it was his observation of sugar as an internal secretion that over the

next 50 years would launch the discipline of endocrinology [41] and thereby paved the way to the discovery of the role of the pancreas as the source of insulin. Actually, Bernard was made aware of the then available preliminary evidence of abnormal pancreatic function as a cause of diabetes, but rejected it because of his own 1856 experimental studies on the role of pancreatic juice in digestion during which he had injected a mixture of bile and olive oil into the pancreatic duct of dogs who had died of malnutrition without developing polyuria or hyperglycemia [6].

Diabetes Mellitus: A Disease of the Pancreas

Ever since its recognition the pancreas (*pan* = all; *creas* = flesh) had been considered as merely a supportive fleshy cushion on which the surrounding viscera rested. With the advent of anatomical studies (Fig. 1.1), its ducts were identified and as studies in nutrition progressed its important role in the digestion of fatty matter came to be recognized. In 1683, the Swiss anatomist Johann Conrad Brunner (1653–1727), of duodenal Brunner's glands fame, had removed the pancreas in seven dogs, two of which experienced polyuria and polyphagia but all survived with no major sequelae [6, 42, 43]. Brunner had met Willis and was familiar with his work on diabetes, but failed to make the link of diabetes to the pancreas in his two polyuric dogs. As a result, for the subsequent 200 years, the pancreas came to be considered a non-vital gland of external secretion whose principal function was to digest fatty matter and to convert starch into sugar [6, 42].

Postmortem examination of patients with bulky and oily stools (steatorrhea) who had a diseased pancreas established the vital role of the pancreas in absorption. Among those publications is an account of eight cases reported by Richard Bright in 1832 [44]. The first case he describes had presented with diabetes mellitus but succumbed to his pancreatic ailment. In his discussion, Bright considered a possible link of the pancreas to diabetes but dismissed it because "I have seen a great number(sic) of diabetic cases, in which this symptom (fatty stools) did not occur" and "because diabetes was not detected, nor even suspected, in the other (seven) cases of this evacuation (fatty stools) which I have related." To the chagrin of any nephrologist he concludes that "there is no essential connection between the two diseased actions" [44]. While not referenced by Bright, he may have been refuting earlier observations among others by a British surgeon, Thomas Cawley (d. 1799), who in 1788 had described a case of pancreatic calcification and calculi that was associated with diabetes [45]. Cawley was the first to suggest a relationship between the pancreas and diabetes, an association subsequently confirmed in various other diseases of the pancreas [46, 47]. With accruing evidence a convincing argument for the role of the pancreas in diabetes was made in 1880 by the Paris Academician Etienne Lancereaux (1829–1910), who went on to coin the term "pancreatic diabetes" (*diabète pancreatique*) and to classify diabetics into two types: lean (*diabète maigre*) and obese (*diabète gras*) [47, 48]. These clinical observations were confirmed experimentally in 1889, when Oskar Minkowski (1858–1931) and Josef von

Mering (1849–1908) of Strasbourg showed that pancreatectomized dogs developed diabetes that could be reversed by the subcutaneous implantation of pancreatic fragments but not the injection of a pancreatic saline extract [6, 46, 49].

The specific role of the pancreas in diabetes was refined further in 1869 when as an undergraduate student working under the guidance of Rudolph Virchow (1821–1902) in Berlin, Paul Langerhans (1847–1888) described the unique morphologic features of the pancreatic islands that were subsequently named after him [3, 6, 49]. It would be 40 years later that the American Eugene L. Opie (1873–1971), a member of the first (1897) graduating class of Johns Hopkins Medical School, reported in 1909 hyaline degeneration of the islands of Langerhans in diabetic patients [50], a finding confirmed in a series of subsequent experimental studies that would lead Edward Sharpey-Schafer (1850–1935), then chair of physiology at Edinburgh and considered a founder of endocrinology, to hypothesize in 1916 that it was the islands of Langerhans that produced a glucose-regulating hormone that he termed *insulin*, coined from the Latin for island, *insula*.

The race for isolating the hypothesized hormone was now on. Among the many in its quest is the American pharmacologist John Jacob Abel (1857–1938), known for the introduction of his artificial kidney in 1913 [51]. But it was Frederick Banting (1891–1941), a frustrated orthopedic surgeon, and Charles Best (1899–1978), a medical student at the University of Toronto, who finally did so in 1922. They wanted to call it *isletin*, derived from the Old French *islet* for a small island, but John J.R. Macleod (1876–1935), in whose laboratory their work was done, insisted on using Sharpe-Schafer's term of insulin [6, 8, 49]. The endocrine nature of diabetes was now clearly established. The stage of diabetes as a disease of the kidneys was over. The stage of diabetes as a cause of kidney disease was yet to come (Fig. 1.1).

Diabetes as a Cause of Kidney Disease

For a disease long considered as one of the kidneys attempts to find an explanatory pathological abnormality in the kidneys had been futile. The resulting frustration is well expressed in an 1847 text on medicine: "Examination of the dead body throws little or no light upon the pathology of diabetes. We naturally look with interest to the kidneys. But we find nothing there to explain the symptoms noticed during life. I have noticed the deep purplish colour of kidneys which were veined and vascular, but not otherwise altered in texture. Others tell us that the kidneys are found hypertrophied in diabetes. But hypertrophy, and unnatural vascularity, are circumstances which we are not surprised at when we reflect upon the vastly increased quantity of work which the glands (sic) have been performing. They are the consequences rather than the cause of the morbid flow of urine" [52]. The large size and increased vascularity of the kidneys was long recognized (Fig. 1.2) and recorded in several other texts of the past in which the kidneys are described as being "rather fuller of blood than usual" and to "exhibit increased vascularity, often enlarged, soft" [52–54].

Fig. 1.2 A sick possibly diabetic lady with her physician. Top: The dropped urine flask (matula) indicates that the condition is hopeless. Bottom: The patient has died and a postmortem is in progress. Note the relative size of the kidneys, in the upper center part of the figure, relative to the size of the liver, in the hand of the prosector, and that of the lungs and heart, next to his left foot. The story told in this thirteenth-century illustration can be taken as an allegory of that of diabetes until the first part of the twentieth century when excessive urine that was sweet indicated a grave prognosis, and at autopsy the kidneys were found to be engorged and hypertrophied. From the Ashmole MS 399 folio 34. (Reproduced with permission of the Bodleian Library)

Ironically, the first abnormalities of glomerular lesions in diabetics that might lead to kidney failure were reported in 1859 by a German neurologist with an interest in the seizure disorders of diabetes, Wilhelm Griesinger (1817–1868), who actually had set up to examine Claude Bernard's suggestion of liver abnormalities as the cause of diabetes. What he found instead were the renal lesions of diabetes. In 32 of 64 autopsied cases of diabetes gleaned from the literature, including 4 of his own cases, he found renal changes that were consistent with Bright's disease leading to his conclusion that the notion of "the kidneys are infrequently affected in this disease and that renal changes of the kidney, if any, consist only of true hypertrophy" was wrong [11, 55]. Subsequent microscopic examinations of the kidney do not seem to have shed much light on the subject other than to confirm the presence of variable sclerotic changes in some of the diabetic kidneys and glomeruli and of vessels that were engorged with blood. Even the renal lesions observed at postmortem in diabetics with small sclerotic kidneys continued to be slighted and attributed to coexisting conditions notably arteriosclerosis, cardiovascular disease, and aging.

In 1881, Wilhelm Ebstein (1836–1912), acknowledging their prior recognition by the Neapolitan Luciano Armanni (1839–1903) in 1875, reported the hyalinization of tubular epithelial cells in diabetic kidneys. Hence, the acronym of Armanni-Ebstein of these lesions that came to be considered pathognomonic of diabetes [56, 57]. In 1883, the German 1908 Nobel laureate of Physiology and Medicine, Paul Ehrlich (1854–1915), determined the glycogenic nature of these tubular epithelial cell changes, which subsequently were shown to be related to the severity of the glycosuria, and with the advent of insulin therapy were to become rare and only occasionally observed [56].

Things were to change after 1936, when the German pathologist Paul Kimmelstiel (1900–1970) then at Harvard, and the English internist then on a Rockefeller Fellowship to Harvard, Clifford Wilson (1906–1997), reported on the presence of peculiar hyaline masses in the glomerular lobules of eight diabetic patients, aged 48–68 years none of whom was on insulin therapy, which they termed *intercapillary glomerulosclerosis* [58]. Similar lesions had previously been encountered, but as suggested by Kimmelstiel and Wilson were attributed to the *aging process of the glomerulus* that Kimmelstiel himself had previously attributed to arteriolosclerosis [59, 60]. What is critical in this landmark report is the accompanying symptom complex of diabetic kidney disease: nephrotic range proteinuria, hypertension, edema, and kidney failure. In retrospect, it is quite revealing that 21 centuries earlier Aretaeus of Cappadocia in his description of diabetes mentions literally the same features of the disease as "… others do not pass urine, nor is there any relief from what is drank. Wherefore, what from insatiable thirst, an overflow of liquids, and distention of the belly, the patients have suddenly burst." Essentially, what was "dropsy of the chamber pot" became the actual dropsical swellings of some patients when the region of discharge of ingested fluids changed from the kidney to the interstitium [16]. A complication vividly illustrated in 1694 by Frederick Dekkers of Leiden (1644–1720) in a line engraving of dropsy in one of his patients with

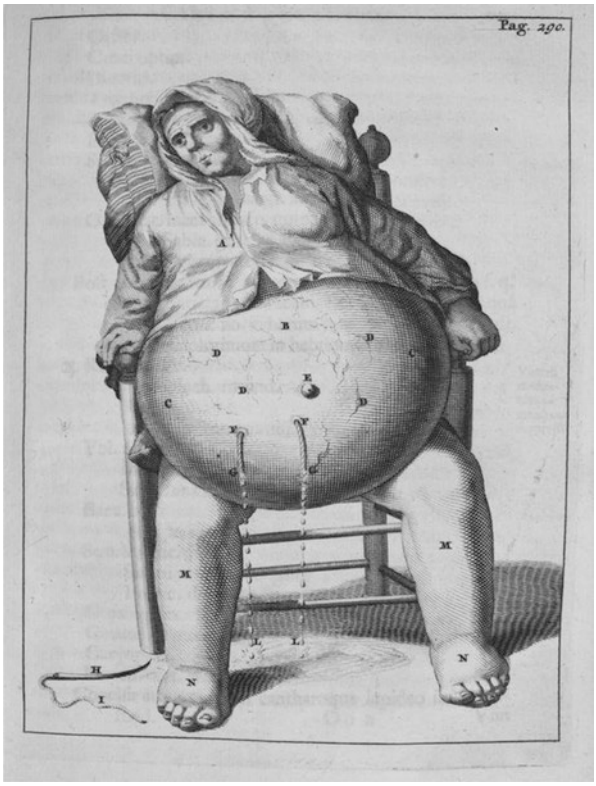

Fig. 1.3 A 40-year-old woman with dropsy being treated with indwelling drains inserted through a trocar developed by the Dutch physician from Leiden Frederik Dekkers (1648–1720). Reproduced from Dekker's 1694 book *Exercitationes Practicae* (Practical Exercises). (Image courtesy of the Hagsrömer Medico-Historical Library)

coagulable urine that likely tasted sweet as he had observed in some of his edematous patients (Fig. 1.3) [61].

What changed in the interim between the descriptions of Aretaeus, Dekkers, Kimmelstiel, and Wilson and what was to come was the prevalence of diabetes, the appreciation of proteinuria as a manifestation of diabetic kidney disease, the severity of proteinuria as a surrogate of progression to kidney failure, and the introduction of kidney biopsies as an investigative tool of kidney disease.

Albuminuria and Diabetic Kidney Disease

The story of proteinuria dating back to the Hippocratic aphorism linking bubbly urine to kidney disease has been told eloquently in excellent reviews [11, 62, 63]. However, it was not until 1827 that its link to kidney disease was established by Richard Bright in his now epic *Reports of Medical Cases* correlating dropsy and coagulable urine with kidney disease, a link that literally immediately assumed increasing worldwide interest. Within 10 years, what had been described theretofore as *milk of the kidneys*, *serum of the blood*, and *albumen* in the urine was termed albuminuria by the French academician Fernand Martin Solon (1795–1846) in his

De l'Albuminurie ou Hydropisie Causée par Maladie des Reins (On Albuminuria or Dropsy Caused by Disease of the Kidneys), with five colored palates published in 1838 [64, 65].

Notwithstanding the inaccuracy of the term albuminuria for what was actually being detected then was proteinuria, the simplicity of the procedure that emerged as the first readily available and simple clinical test for the diagnosis of kidney disease then classified as Bright's disease or nephritis. The term albumen and its derivative albuminuria was a result of progress in chemistry (Fig. 1.1) and of increasing interest in the analysis of organic matter by chemists such as the French Antoine Fourcroy (1775–1809) and Louis Nicolas Vauquelin (1763–1829) who in 1789 had identified albumin, along with fibrin and gelatin, in the soluble but coagulable matter circulating in serum as proteids, an archaic biochemical term derived from the Greek for first, *proteos*, that would be termed proteins in 1838 by the Swedish chemist Jons Jakob Berzelius (1779–1848) to convey their essential and primary (*proto*) role in nutrition [66, 67]. It would be another century before albumin would be isolated and quantified in the urine and as methods of analysis became more refined it became gradually possible to measure smaller amounts of albumin in the urine (*microalbuminuria*) to detect earlier markers of renal injury [62–68].

The story linking proteinuria to diabetes is of recent vintage. After the identification of diabetes as a metabolic disorder, the occurrence of proteinuria in diabetics was described in case reports by various authors and soon codified in medical texts such as the classic *Traité des Maladies des Reins* (in three volumes published 1839–1841) by Pierre Rayer (1793–1867) and considered in detail in a 733-page monograph by Jonas Louis Abeille (1809–1893) titled *Traité des Maladies a Urine Albumineuse et Sucrée* published in 1869 [69]. Subsequent progress made is reflected in the comment of William Osler (1849–1919) in his 1892 *The Principles and Practice of Medicine* that albuminuria in diabetics is "a tolerably frequent complication. The amount varies greatly, and, when slight, does not seem to be of much moment. It is sometimes associated with arteriosclerosis. It occasionally precedes the development of diabetic coma" [70]. A conclusion confirmed and clarified by Bernhard Naunyn in his 1898 treatise "*Der Diabetes Mellitus*" that mild proteinuria in diabetics is insignificant while that of heavy albuminuria is a bad prognostic sign [4, 6]. Still, proteinuric cases continued to be classified as Bright's disease well into the twentieth century, even after Hermann Senator (1834–1911) had argued rather convincingly that proteinuria was not always a sign of primary nephritis [71, 72].

It was the clinical pathologic correlation described by Kimmelstiel and Wilson that actually prompted the quest for the association of proteinuria with diabetic kidney disease. Within a very short period of time, a series of reports confirming the clinical picture, showing a closer association with another characteristic lesion of diffuse glomerulosclerosis, rather than just nodular, whose severity correlated with the duration of diabetes were published [73–77]. It was studies of this syndrome complex termed diabetic nephropathy [78, 79] that would elucidate the course of diabetic kidney disease. Initial evidence was marshaled from studies on juvenile diabetics who were surviving longer after the introduction of insulin. Subsequent studies of the clinical link of diabetes, albuminuria and kidney disease were strengthened

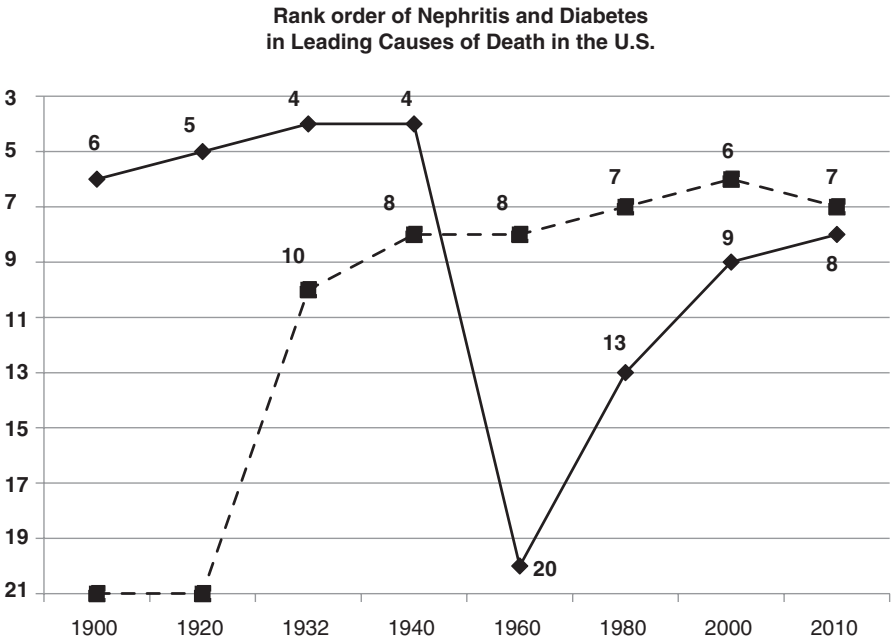

Fig. 1.4 Rank order of chronic kidney disease classified as nephritis (shown in continuous black line) and diabetes mellitus (shown in dashed black line) in leading causes of death in the United States. Diabetes does not register on the list until 1932, after the discovery of insulin. Until then, the presence of proteinuria was diagnosed and classified as nephritis. The precipitous drop of nephritis from rank order 4–20 likely reflects the identification of diseases other than nephritis (principally diabetes and hypertension) as causes of proteinuria. Its gradual re-emergence and rise to number 8 likely represent the introduction of renal replacement therapy, the emergence of nephrology as a discipline, and the definition and classification of chronic kidney disease. (Based on data available from the Center for Disease Control and Prevention)

by the advent of kidney biopsy in the 1950s, the emergence of nephrology as a discipline in the 1960s and refinements in the methodology of quantifying albuminuria [80, 81]. Independent but parallel increases in life expectancy and the prevalence of obesity account for the recent pervasive worldwide epidemic of diabetic kidney disease. The impact of this sequence of events on the leading causes of mortality in the United States is shown in Fig. 1.4. A detailed examination of the reasons for the evolving construct of diabetic kidney disease reviewed in this biographical recount is the subject matter of this book.

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Chapter 2
Clinical Features and Natural Course of Diabetic Nephropathy

Peter Rossing and Marie Frimodt-Møller

Background

Diabetic kidney disease is a major cause of morbidity and mortality in diabetes. Indeed, the excess mortality of diabetes occurs mainly in proteinuric diabetic patients and results not only from end-stage renal disease (ESRD) but also from cardiovascular disease, with the latter being particularly common in type 2 diabetic patients [1–3]. Clinically diabetic kidney disease is characterized by progressive kidney damage reflected by increasing albuminuria, impairment in renal function (decline in glomerular filtration rate (GFR)), elevated blood pressure, and excess morbidity and mortality due to cardiovascular complications. Diabetic kidney disease rarely develops in patients with type 1 diabetes before 10 years after diagnosis, whereas approximately 3% of patients with newly diagnosed type 2 diabetes already have overt nephropathy [4]. Diabetic kidney disease is the single most common cause of ESRD in many parts of the world including Europe, Japan, and the United States, with diabetic patients accounting for 25% to 45% of all patients enrolled in ESRD programs [5]. While other complications related to diabetes have been reported to decline in recent years, this has only to a smaller extent been the case for diabetic nephropathy, perhaps because people are surviving to end-stage renal disease as cardiovascular prognosis has improved, or because there is still an unmet need for better treatment [6].

Since not all persons with diabetes develop all complications, relevant systematic screening for occurrence of various complications has become a major part of

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diabetes care today. Detection of early stages of complications allows for more focused preventive treatment or even specific treatment that can delay further progression of an early manifestation of a complication. A major part of treatment for diabetes is preventive. In essence, the effort to reduce blood glucose and maintain glucose control is a preventive action in order to prevent classical micro- and macrovascular complications.

Screening, diagnosis, and treatment of diabetic kidney disease (DKD) have improved substantially over the last three decades, improving both time to diagnosis of DKD as well as life years gained after diagnosis [7, 8]. To further improve these variables, current research seeks to develop new methods for early detection of DKD as well as improved treatment.

Definition

Diabetic nephropathy (DN) is defined in both type 1 and type 2 diabetes as the presence of persisting severely elevated albuminuria of more than 300 mg/24 h (or >200 μg/min) or an albumin creatinine ratio > 300 mg/g creatinine, confirmed in at least two out of three samples, with concurrent presence of diabetic retinopathy and absence of signs of other forms of renal disease [9]. As such, it is a clinical diagnosis, requiring little more than basic clinical and laboratory evaluations. Normal value for albuminuria has been defined as <30 mg/g (or 30 mg/24 h), and abnormal values above 30, but albuminuria is a continuous measurement and increasing values within the normal and abnormal range are associated with elevated risk for renal and cardiovascular disease [10]. The presence of moderately elevated albuminuria (microalbuminuria) (between 30 and 299 mg/g) is widely regarded as a precursor of diabetic nephropathy, both indicating early risk and providing a target for intervention, although in some cases, microalbuminuria can display remission either spontaneously or due to treatment [11–13], an event that indicates better renal risk as compared to progression of albuminuria.

A broader term, "kidney disease in diabetes," is used for patients with chronic kidney disease (impaired renal function eGFR <60 ml/min/1.73m^2 or proteinuria) regardless of the background. Although impaired renal function with normal albuminuria (ACR <30 mg/g) is prevalent, particularly in elderly subjects, it is much less likely to progress if albuminuria is not present [14, 15].

The Italian RIACE study of over 15,000 type 2 diabetic subjects suggested that patients with elevated albuminuria display the typical microvascular phenotype, whereas the nonalbuminuric subjects with impaired renal function had a more cardiovascular or macrovascular phenotype [14].

For CKD in general, including diabetes, it has been recommended to stage the severity using a combination of etiology (if known), level of urinary albumin excretion, and eGFR stage (see Fig. 2.1) [16].

The National Kidney Foundation KDOQI work group for diabetes and CKD suggested that absence of retinopathy, fast deterioration of GFR, rapidly increasing

Prognosis of CKD by GFR and albuminuria category

Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30-300 mg/g 3-30 mg/mmol	>300 mg/g >30 mg/mmol
GFR categories (ml/min/ 1.73 m²) Description and range	G1	Normal or high	≥90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red, very high risk.

Fig. 2.1 Staging of CKD. (From Group KDIGOKCW [16])

albuminuria or nephrotic range albuminuria (>2500 mg/g), active urinary sediments, refractory hypertension, and signs or symptoms of other systemic diseases should raise suspicion of nondiabetic causes of CKD [17].

Pathology

If renal biopsies were feasible in all patients without any safety considerations, many patients would probably be diagnosed with early stages of DN. Morphological changes as mesangial expansion and thickening of the glomerular and tubular basement membrane, as well as the typical glomerulosclerosis with mesangial nodular lesions (Kimmelstiel Wilson nodular lesions) can be attributed to the impact of hyperglycemia and hyperfiltration. These changes can be present after only a few years of disease, but large variability is common, as patients with long-standing diabetes may only display minor changes. Since a renal biopsy is not without risk for complications, it is rarely used in routine clinical practice in uncomplicated cases, and often reserved for cases with severe albuminuria, fast decline in GFR or

in cases where differential diagnoses are considered. For more details on pathology, see Chap. 8 of this book. As biopsies today can be utilized for more than histopathology, for example, transcriptomic analyses, it has been argued that we should, at least for research purpose, use biopsies similarly to other specialties like oncology in order to obtain a better understanding of the molecular abnormalities and thus provide the basis for precision medicine.

Prevalence

The global DEMAND study [18], published in 2006, used a dipstick method to assess the presence of albuminuria in a referred cohort of >24,000 patients with type 2 diabetes without known albuminuria from 33 countries and found an overall global prevalence of macroalbuminuria of 10% with some variations between regions. Moreover, the presence of microalbuminuria was 39%, demonstrating incipient or overt diabetic nephropathy in approximately 50% of the population. Furthermore 22% had eGFR <60 ml/min/1.73m^2. Although the methodology cannot be regarded as robust, it provides one of few global pictures of global prevalence of diabetic nephropathy.

A number of population-based cohorts and data from clinical centers have provided more detailed and thorough descriptions of nephropathy in both type 1 and type 2 diabetes. In short, the prevalence of severely elevated albuminuria (macroalbuminuria) in type 2 diabetes clinics ranges from 5% to 48% (median 14%) and from 8% to 22% (median 15) in type 1 diabetes patients. Similarly, moderately elevated albuminuria (microalbuminuria) is prevalent in a median 13% and 20% of patients with type 1 and 2 diabetes, respectively [9]. Interestingly, however, the most recent publication from NHANES survey points to a declining temporal trend in albuminuria in the United States, which may be a result of more focused multifactorial treatment over the last decades [1].

Screening

To be able to detect abnormal and/or changing levels of albuminuria and renal function (eGFR) and thereby be able to initiate early renoprotective treatment, annual screening of all diabetic patients is recommended [17]. For screening and monitoring early morning spot urine collections are sufficient and most convenient for the patient [10, 19]. Due to large (30–40%) intraday variability, two out of three spot urine samples within 3–6 months must be above the threshold to ascertain the diagnosis. A 24-h collection has been considered gold standard for albuminuria assessment and can provide additional important information on sodium and protein intake, but complete collection is often difficult and is usually restricted to those with established diabetic kidney disease. Urinary albumin excretion may be elevated independent of kidney disease by factors such as severe exercise within 24 h, severe urinary tract infection, menstruation, heart failure, and marked hyperglycemia.

The second clinical variable to assess is glomerular filtration rate (GFR), in clinical practice most often done by estimation (eGFR) using serum creatinine-based formulas like the CKD-EPI [20]. This is currently the best validated equation. It has been suggested that improved estimation can be obtained with an equation including serum creatinine as well as serum cystatin C [21]. A more precise measurement of GFR requires the use of an external marker such as inulin. If untreated, the "natural" course of diabetic nephropathy displays a continuing annual decline in eGFR between 2 and 20 ml/min/1.73m^2 (mean 12 ml/min/1.73m^2), but proper treatment targeting glycemia and blood pressure, blocking the renin-angiotensin system, reducing cholesterol, and improving lifestyle factors can reduce progression to 2–5 ml/min/1.73m^2 per year, demonstrating the importance of screening and intervention.

Clinical Quality of Albuminuria Testing and Monitoring

Although screening for albuminuria and renal function in patients with diabetes for long has been part of guidelines, it remains difficult in many areas, to reach a reasonable fraction of patients that are being tested and continuously monitored. This is despite urinary testing being low cost, noninvasive, and relatively simple. In Denmark, the National Diabetes Register in 2014 demonstrated 85% of diabetic patients had been screened for albuminuria within a 2-year period if followed in general practice and 96% if followed in hospital-based outpatient clinics [22], and in the Swedish National Diabetes Register data from 2016 on albuminuria were available for 73% seen by general practitioners. The Scottish Diabetes Survey 2015 found 71% of type 2 diabetic patients had been screened within 15 months. All these registers most likely represent relatively successful areas with national monitoring of the quality. In the GIANTT cohort [23] of primary care patients in the Netherlands, 57% had albuminuria measured in 2009 and only 24% of patients had it measured annually. Similar or even lower levels of screening are found in other countries.

It is a limitation that methods for albuminuria assessment are not yet standardized, and there has been much focus on quantitative vs qualitative methods, timed or spot urine collections. The precision of estimates of GFR based on serum creatinine has also been extensively debated. The major limitation remains that systematic screening, regardless of the method, is often not implemented and diabetic kidney disease thus not detected.

Hyperfiltration

Prior to development of increasing albuminuria, approximately one third of type 1 diabetic patients will have a glomerular filtration rate (GFR) above the upper normal range for age-matched healthy nondiabetic subjects. The degree of hyperfiltration is less in type 2 diabetic patients, and hyperfiltration is even reported to be absent in some studies.

Longitudinal studies suggest that hyperfiltration is a risk factor for subsequent increase in urinary albumin excretion and development of diabetic nephropathy in type 1 diabetic patients, but conflicting results have also been reported. A meta-analysis based on 10 cohort studies following 780 patients found a hazard ratio of 2.71 (95% confidence interval 1.20–6.11) for progression to microalbuminuria (albuminuria grade A2) in patients with hyperfiltration [24]. The prognostic significance of hyperfiltration in type 2 diabetic patients is still debated, but in relation to the potential renoprotective effect of new medications like SGLT2 inhibitors and GLP1 receptor agonists which has been suggested to be mediated through a reduction in intraglomerular pressure and hyperfiltration, there has been renewed focus on this [25].

Prognosis

Most studies dealing with the natural history of diabetic nephropathy have demonstrated a relentless, often linear rate of decline in GFR. Importantly, this rate of decline is highly variable across individuals, ranging from 2 to 20 mL/min/yr, with a mean approximating 12 mL/min/year [26–28]. This contrasts a decline in healthy subjects above 40 years of approximately 1 ml/min/year. Type 2 diabetic patients with nephropathy display the same degree of loss in filtration function and in variability of GFR [29, 30].

Several putative promoters of progression in kidney dysfunction have been studied in patients with type 1 diabetes and type 2 diabetes with nephropathy (Fig. 2.2). A close correlation between blood pressure and the rate of decline in GFR has been documented in type 1 and type 2 diabetic patients [31, 32]. For many years it was

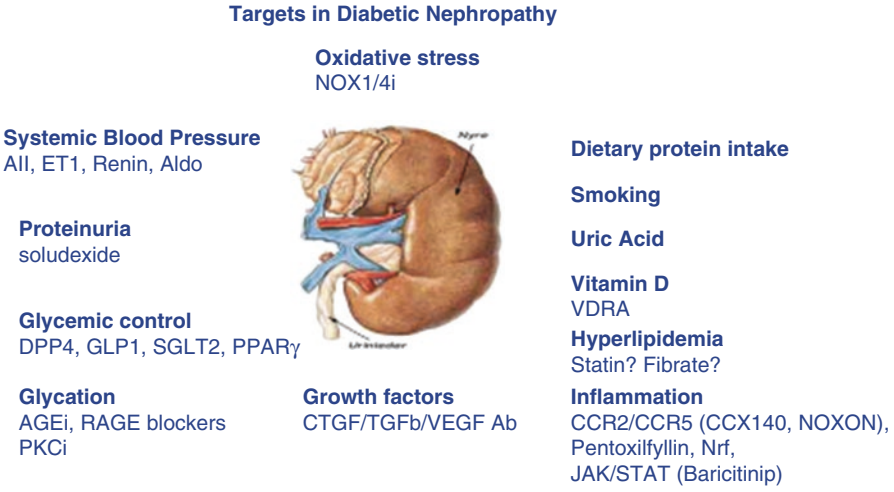

Fig. 2.2 Targets and putative interventions in diabetic nephropathy

believed that once albuminuria had become persistent, then glycemic control had lost its beneficial impact on kidney function and structure. This misconception was based on investigations involving a limited number of patients and studies that used inappropriate methods for monitoring kidney function (serum creatinine level) and glycemic control (random blood glucose level). Several more recent studies encompassing large numbers of type 1 diabetic patients have documented the important impact of glycemic control on the progression of diabetic nephropathy [3, 7].

Many studies in both type 1 and type 2 diabetic patients have demonstrated a correlation between serum cholesterol concentration and progression of diabetic nephropathy [31, 32], but intervention has not been able to slow decline in GFR. There is familial clustering of diabetic nephropathy [33]. Several gene variants have been investigated as candidate genes for risk factors for diabetic nephropathy. Genome-wide association studies have been performed in the search for genes linked to diabetic nephropathy, and although some areas of the genome have attracted attention, no major susceptibility genes have been identified so far [34, 35].

Early studies describing the prognosis for overt diabetic nephropathy observed a median patient survival time of 5–7 years after the onset of persistent proteinuria. End-stage renal failure was the primary cause of death in 66% of patients. When deaths attributed only to ESRD were considered, the median survival time was 10 years. All this was before patients were offered antihypertensive therapy. Long-term antihypertensive therapy was evaluated prospectively in 45 type 1 diabetic patients who developed overt diabetic nephropathy between 1974 and 1978. The cumulative death rate was 18% at 10 years after the onset of diabetic nephropathy, and the median survival time was around 16 years [36]. Fortunately, survival improved when aggressive antihypertensive medication was implemented with median survival time of 21 years after the onset of diabetic nephropathy as demonstrated in 2015 [37]. A recent study confirmed improved prognosis with a further 50% reduction in age adjusted mortality with better control of all risk factors [3, 7].

Recent Advances in Predictive Biomarkers

The classification of diabetic kidney disease based on albuminuria and eGFR level is simple (Fig. 2.1), provides prognostic information, and is helpful to guide therapeutic decisions, but not perfect. Not all patients with abnormal albuminuria progress to ESRD or cardiovascular disease, and many patients with impaired renal function (eGFR <60 ml/min/1.73 m^2) also do not progress. Therefore an intensive search for new biomarkers in blood or urine, that could improve diagnostic and prognostic precision in early or later stages of diabetic kidney disease, has been ongoing during the past decades. The underlying hypothesis is that the development from uncomplicated diabetes, to a stage with renal damage, subsequently a stage with impaired renal function and finally ESRD, cardiovascular events or death takes years, and that an increased risk for progression or early changes in structure or function are reflected by changes in the biomarkers [38]. Biomarkers may reflect cellular or systemic changes, changes

in different organs or compartments of organs such as: glomeruli, tubuli [39, 40] and can reflect different processes such as changes in extracellular matrix handling, fibrosis [41], inflammation [42], oxidative stress [43], glycemic damage, atherosclerosis [44], endothelial cell dysfunction, etc. Several studies including studies in type 1 and type 2 diabetes have found circulating TNF receptors to be associated with renal outcome although the underlying biology remains to be established [45–47].

It has recently been suggested that focus should be on patients with very fast decline in renal function corresponding to time to onset of ESRD of 2–6 years. These patients were characterized by elevated albuminuria and TNF receptor 1. This was based on observations from Joslin Diabetes Center in Boston, where a significant number of patients had very fast decline in eGFR (>15 ml/min/year) and 80% had a decline in eGFR >5 ml/min/year. These findings will have to be confirmed in other cohorts [48].

The search for biomarkers for increased risk for diabetic kidney disease have usually been hypothesis driven and have several markers have been suggested, but so far none of the markers have been implemented in clinical care, as validation, and confirmation of added value beyond the existing risk markers still has to be proven [49].

Extrarenal Complications in Diabetic Nephropathy

Diabetic retinopathy (see also Chap. 19 of this book) is present in virtually all type 1 diabetic patients with nephropathy, whereas only 50–60% of proteinuric type 2 diabetic patients have retinopathy. The absence of retinopathy should prompt further investigation for nondiabetic glomerulopathies. Blindness due to severe proliferative retinopathy or maculopathy is approximately five times more frequent in type 1 and type 2 diabetic patients with nephropathy than in normoalbuminuric patients. Macroangiopathies (e.g., stroke, carotid artery stenosis, coronary heart disease, and peripheral vascular disease) are two to five times more common in patients with diabetic nephropathy. Peripheral neuropathy is present in almost all patients with advanced nephropathy. Foot ulcers with sepsis leading to amputation occur frequently (>25% of cases), probably due to a combination of neural and arterial disease. Autonomic neuropathy may be asymptomatic and manifest simply as abnormal cardiovascular reflexes, or it may result in debilitating symptoms. Nearly all patients with nephropathy have grossly abnormal results on autonomic function tests.

Future Perspectives

Alternative approaches have used open hypothesis-free multiple-marker approaches to find new markers or combinations of markers associated with progression of diabetic kidney disease [50]. This involves the application of “omics” platforms, including genomics, transcriptomics, proteomics, and metabolomics as discussed in more details in Chap. 28.

Personalized Medicine

Diabetic kidney disease has many phenotypes in terms of rate of progression, degree of comorbidity, and response to interventions. As we learn more about the usefulness of the different omics-based markers, their value as well as their limitations, it is expected that data from multiple platforms can be integrated using systems medicine models, and thereby provide a better understanding of the underlying pathophysiology for the individual patient and this could thus lead to personalized medicine. This will obviously require that we will be able to identify specific subtypes of diabetic kidney disease and that treatment options can be targeted toward the relevant pathophysiology, whether this means increased blockade of the renin-angiotensin system, antifibrotic, anti-inflammatory, or other interventions. New techniques are more expensive to use than screening for albuminuria and eGFR; thus it has to be analyzed if the new methods are cost-effective by reducing renal and/or cardiovascular risk and thus delaying or even preventing ESRD.

Conclusions

Diagnosis of diabetic kidney disease relies on measurement and monitoring of urinary albumin excretion (ACR) and renal function (eGFR) in combination with clinical assessment. This guides classification, prognosis, and therapy. Although recommended in most guidelines, it is still lacking full implementation in global diabetes care. New markers and techniques have been suggested to improve diagnostic and prognostic precision and are currently being evaluated, but not yet fully validated and ready for use. The future may both hold an increased focus on early screening and a higher level of screening for diabetic kidney disease, as well as the implementation of new measures, with the promise of earlier and more precise renal and cardiovascular risk prediction.

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Chapter 3
Global, Regional, and Ethnic Differences in Diabetic Nephropathy

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Introduction

The International Diabetes Federation (IDF) in the 7th Edition of its Diabetes Atlas has projected that by the year 2040, about 642 million adults aged 20–79 years (about 1 in 9 individuals) around the world will be living with diabetes mellitus (DM) [1]. The estimated global population of adults with diabetes was 415 million (about 1 in 10 individuals) in 2015. In general, in the past two decades, there has been a remarkable increase in the global prevalence of DM which has thus emerged a chronic disease of global health dimensions. The increase in the burden of diabetes has been paralleled by an increase in the occurrence of its attendant chronic complications such as diabetic nephropathy (DN). DN has emerged as a leading cause of chronic kidney disease (CKD), as well as end-stage renal disease (ESRD). It is currently the commonest cause of ESRD in industrialized regions and is becoming a considerable cause of ESRD in low- and middle-income countries (LMIC) [2]. ESRD secondary to DN singularly contributes considerably to the additional

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health-care costs of the management of people with diabetes [3]. It has been estimated that strategies that could retard the progression of DN by 20% have the potential of reducing the economic costs of diabetes management by $39 billion over a decade [4]. DN is also associated with increased all-cause and cardiovascular mortality. The presence of kidney disease accounts for 21% and 11% of deaths among people with type 1 and 2 diabetes, respectively, while the annual mortality rates among DN patients are approximately 14 times higher than mortality rates among individuals without DN [5, 6].

DN, which is clinically defined by the presence of sustained albuminuria/proteinuria or a reduction in glomerular filtration rate or both, occurs in up to 40% and 20% of people with type 1 and 2 diabetes, respectively [7]. Type 2 diabetes-related nephropathy is more commonly encountered in the clinical setting due to the preponderance of type 2 DM which accounts for about 90% of the population with diabetes. There are recognized ethnic differences in the burden of both DM and DN, with certain ethnicities having a higher risk of developing diabetic complications such as DN, a greater tendency to expressing certain phenotypes of DN, as well as a higher propensity for progression of clinical disease [8–11]. There are likewise regional differences in the burden and impact of DN. These differences are in part explained by health-care-related factors such as poor availability and access, socioeconomic status, and low awareness, while the contributory role of genetic and epigenetic factors, though not completely elucidated, cannot be ignored.

The Global Dimensions of Diabetic Nephropathy

DN can occur as a chronic complication of both types 1 and 2 DM; however, the greater proportion of the worldwide burden of DN is accounted for by type 2 DM, a phenomenon explained by the higher prevalence of type 2 DM due to the increase in obesity and sedentary lifestyle associated with the global adoption of the Western lifestyle [12]. DN has globally become the cause of ESRD in 25–50% of patients commencing renal replacement therapy (RRT) with the estimated global prevalence of micro- and macroalbuminuria being approximately 40% and 10%, respectively [13, 14]. Within the current decade, Singapore, Malaysia, and the Mexican region of Jalisco, have reported the highest proportions of incident DN-related ESRD of between 58% and 66%, while incident proportions of less than 20% are being reported in Estonia, Romania, Italy, Switzerland, the Netherlands, Dutch-speaking Belgium, and Iceland [15]. Although DN has traditionally been known as the primary cause of ESRD in the Western world, there appears to be a shift in epidemiology with countries such as Austria, Dutch-speaking Belgium, Denmark, Finland, Iceland, and Sweden, reporting a decline in the incidence of DN-related ESRD in the period between 2001 and 2014 [15, 16]. The contrary has however been observed in countries such as Thailand, Russia, and the Philippines which have experienced exponential increases of 1447.9%, 980.6%, and 377.9%, respectively, in the incidence rates of DN-related ESRD in the period between 2001/2002 and 2013/2014 [15].

In the USA, even though an absolute decline in incidence rates has not yet been reported, there is now a reduction in the percentage change of annual incidence rates.

Regional and Ethnic Differences in the Risk of Diabetic Nephropathy and Associated End-Stage Renal Disease

In the USA, CKD and ESRD secondary to diabetes mellitus have been shown to be more prevalent among ethnic minorities such as African-Americans, Hispanics, Asians, and indigenous populations such as the Pima Indians than among White Americans. Differences in the prevalence rates of DM across various regions and/or ethnic groups do not completely explain the disproportionate prevalence of DN and ESRD in certain regions or ethnic groups over the others. ESRD risk among African-Americans with DN is 5.6-fold higher in comparison with other ethnic groups with this ethnic group constituting almost half the entire dialysis population in the USA. Overall, African-Americans have the highest incidence rate of ESRD from diabetes in the USA with as much as a threefold increase in the risk of ESRD in this racial group even after adjustment for the higher DM prevalence rates [10]. Similar higher rates of ESRD among African populations relative to other ethnicities have been observed in the UK [17]. Although large population-based data on DN among African populations are scarce, current data derived from predominantly urban area, clinic-based data suggest that as high as 95% of diabetics have DN (defined by proteinuria) 10 years after diabetes diagnosis and approximately 35% have ESRD by 5 years after diabetes diagnosis [18]. Poor availability of, and access to, DM-related health care in concert with genetic susceptibility may be contributory to the reported figures among African populations. Notably, in comparison with African-Americans and European Americans, DN is most prevalent among American Hispanics with as high as 67% of prevalent CKD patients being diabetics [19]. This group has also been shown to have greater degrees of albuminuria and proteinuria than other ethnic groups. Fifty-four percent of Hispanics in a multinational cohort of type 2 diabetics had prevalent micro- or macroalbuminuria [14]. Latest data from the US renal data system (USRDS) indicate that Hispanics still have a relatively higher incidence of ESRD with an incidence rate ratio of 1.3 (Hispanics versus non-Hispanics) [15]. These disproportionately high statistics are predominantly driven by the increased lifetime risk of diabetes among American Hispanics [20]. Lifetime diabetic risk is highest in this ethnic group compared to other ethnic groups in the USA.

The South Asian diabetic population has also been identified to have a relative higher risk for DN in comparison to Europeans. This increased risk for DN is true for South Asians both within the South Asian subregion and in the diaspora. In the Indian subcontinent, the prevalence of DN is significant with diabetes being the cause of CKD in a third of patients [21]. Prevalence rates are equally high in other South Asian countries with prevalence ranging between 15.7% and 45.5% when microalbuminuria is used to define DN [21]. In a multiethnic cohort of people with diabetes in the UK, the likelihood of DN among South Asians was 54% higher than

among Europeans (odds ratio 1.54 [95%]; CI, 1.26–1.88). South Asians are also more likely than Europeans to have the more severe stages of CKD (stages 4 and 5). This increase in prevalence among South Asians relative to Europeans has also been demonstrated in the Netherlands, where the risk of ESRD from DN was increased almost 40-fold among Indo-Asian migrants [22]. Migrant populations of the Southeast Asian and Indian subcontinent origins in Australia have also likewise shown this excess occurrence of ESRD from DN [23].

The increase in the global prevalence of DM over the next two decades is projected to occur predominantly within countries in the Middle East, sub-Saharan Africa, and the Indian subcontinent [24]. These regions will therefore bear a disproportionate higher burden of the complications of DM than other regions. Limited data out of the Gulf peninsula indicate a moderate to high prevalence of early DN (i.e., microalbuminuria). Prevalent microalbuminuria among T2DM patients was 27% and 61%, respectively, in Oman and the United Arab Emirates (UAE), respectively [25, 26]. In the UAE group, the prevalence of microalbuminuria was even higher at 75.7% among UAE nationals within the cohort. In the Kingdom of Saudi Arabia (KSA), overall prevalence of DN defined according to ADA criteria was 10.8%. Expectedly, prevalence is demonstrably higher with increasing age and increasing duration of diabetes. While 1.5% of a randomly selected cohort of type 2 diabetics from the Saudi National Diabetes Registry (SNDR) had prevalent ESRD, annual report data estimate that diabetics account for 40% of its dialysis population [27, 28].

Although type 2 diabetes-related kidney disease accounts for the majority of DN, kidney disease from type 1 diabetes is also a recognized complication with the classical timeline of DN of Mogensen being traditionally more easily demonstrable with type 1 DM-related DN. The occurrence of type 1 diabetes-related nephropathy also varies geographically. While the cumulative incidence of DN appears to be similar in Northwestern Europe at 13.0–13.7%, type 1 DM-related DN cumulative incidence among type 1 diabetics is much higher at 25% in the USA. While similar microalbuminuria rates have been reported from Western European countries, it would seem that incidence is higher among Mediterranean populations. In England and Denmark, annual incidence rates of microalbuminuria of 1.6% and 1.5% have been, respectively, reported; these rates are however higher in Southern Europe (Spain) with an annual incidence rate of 2.7% [29–31]. Although incidence rates may be broadly similar in Western Europe, it has been observed that rural dwellers in this region have as much as a twofold higher incidence rate in comparison to urban city dwellers [30]. Population-based data are scarce on the incidence of type 1 DM-related nephropathy in Africa; nonetheless, hospital-based data show high incidence and prevalence rates of DN. In the Arab regions of Africa, the cumulative incidence rate among Moroccan type 1 diabetics was 34.7% after 5 years of follow-up with prevalent micro- and macroalbuminuria of 48.6% and 36.1%, respectively; similar high prevalence rates of 32% microalbuminuria in type 1 diabetics have been reported in Ethiopia [32]. This is in contrast to lower prevalence rates of microalbuminuria reported in Central Africa (21% in Rwanda and 21.9% in the Democratic Republic of the Congo) [33, 34]. The epidemiology of type 1 DM-related DN from the Gulf States is largely unknown as data (community or clinic based) is scarce albeit a 31% frequency of T1DN has been described [35].

Differences in the Clinical Phenotype and Progression of Disease

The natural history of DN has been classically described as that in which microalbuminuria proceeds to macroalbuminuria/overt proteinuria and subsequent reduction in GFR. It is recognized that certain groups of patients present more commonly with microalbuminuria or proteinuria. South Asians have a higher prevalence of clinically significant proteinuria and a lower prevalence of microalbuminuria than Europeans [36]. It is also known that the likelihood of rapid progression from microalbuminuria to overt proteinuria is higher among South Asians relative to resident Europeans in the UK (odds ratio 2.17). The odds of proteinuria have also been found to be higher among Asians (Chinese, Filipino), American Hispanics, and African-Americans relative to White Americans in the USA [37]. Indeed, 20–40% of African-Americans have prevalent microalbuminuria at the time of diabetes diagnosis [10]. A similar pattern of more prevalent albuminuria is demonstrable in the Southern hemisphere among other ethnic groups relative to resident Europeans. The odds of macroalbuminuria among Pacific Islanders and East Asians relative to European New Zealanders is 3.7 and 2.9, respectively [38]. These preponderances of the micro-, macroalbuminuric, and proteinuric clinical phenotypes within certain ethnicities relative to Europeans have been observed despite comparable blood pressure control and use of renin-angiotensin system (RAS) blockade.

While clinical characteristics such as diabetes duration, the presence of other microvascular complications, glycemic control, blood pressure, and degree of albuminuria are some of the universally recognized correlates of DN progression (i.e., decline in GFR), it is worth highlighting that ethnic differences also exist with respect to the tendency for more rapid progression of disease in some ethnic groups relative to others. As it has been demonstrated in nondiabetic CKD, African-Americans have a faster decline of renal function/progression in DN CKD than White Americans [10, 11]. It is recognized that there is a genetic predisposition among African populations to renal function decline in CKD (DN inclusive). The APOL1 high-risk genetic variants (considered as copies of the G1 and G2 alleles of APOL1) account for this excess risk, resulting in as much as an annual decline of 4.3 $ml.min^{-1}.1.73\ m^{-2}$ among African diabetic people with CKD [39]. Among South Asians in the UK, faster rates of renal function decline have also been observed. South Asians have actually been shown to have the fastest progression rate (more than African-Americans and White Americans) in the USA with mean annual GFR decline of 1.01 $ml.min^{-1}.1.73\ m^{-2}$ (versus 0.73 $ml.min^{-1}.1.73\ m^{-2}$ and 0.72 $ml.min^{-1}.1.73\ m^{-2}$ in African-Americans and White Americans, respectively) [40]. When the presence of baseline proteinuria is factored into the risk profile for GFR decline, however, annual decline in function among African-Americans and South Asians was comparable and faster relative to decline in White Americans (2.51 $ml.min^{-1}.1.73\ m^{-2}$ and 2.11 $ml.min^{-1}.1.73\ m^{-2}$, respectively, versus 1.72 $ml.min^{-1}.1.73\ m^{-2}$ among Whites). There are unconfirmed suggestions that the rate of progression of DN might also be more rapid among Mexican-Americans [41].

Proteinuria is a strong predictor of progression in CKD with the anti-proteinuric action of ACEIs variously demonstrated to slow progression in DN independent of blood pressure control (i.e., reduction of proteinuria is renoprotective). The ethnic groups that have been identified with more rapid rates of renal function decline are also for the most part those that have a propensity toward the proteinuric phenotype of DN. Hypertension in African populations with DN contributes more significantly to the rapid progression of DN [42]. There are indications of a greater synergistic effect of hypertension and proteinuria on renal disease progression in African populations than in other ethnicities.

The Role of Genetics in Ethnic Differences in DN

DN is a composite disease in which clinical, environmental, and genetic factors each play a contributory role in its occurrence. Apart from the modifiable risk factors for DN such as glycemic control, duration of diabetes, blood pressure control, obesity, and dyslipidemia, there is an increased genetic susceptibility to kidney disease in DM, as discussed in great detail in Chap. 7. The familial aggregation of kidney disease in both types 1 and 2 DM offers evidence for the contributory role of genetics to the increased risk of DN within certain populations [43–45]. Genetics is also believed to play a role in the more rapid decline of renal function among certain ethnic groups such as African-Americans with DN. The discovery of DN risk and progression candidate genes has however been challenging with identified associations not widely reproducible across studies [46, 47].

The renin-angiotensin system is one of the notable pathogenetic pathways of DN. Plasma angiotensin-converting enzyme (ACE) levels are implicated in mediating hyperglycemia-related hyperfiltration and long-term hemodynamic changes leading to DN [48]. ACE plasma levels in individuals are genetically determined, and ethnic variations in ACE gene insertion/deletion (I/D) polymorphisms have been identified to play significant roles in the onset, progression, and response to treatment of DN [48]. ACE gene I/D polymorphisms have been extensively investigated in association with DN with the deletion of the rs179975 gene variant associated with an increased odds for DN among Asians but not Europeans (OR 1.28 [95% CI 1.10–1.49]) [49]. Indeed, homozygosity for the insertion polymorphism, i.e., I/I ACE gene polymorphism, confers a reduced risk of macroalbuminuria in Asians but not Europeans with type 2 DM. No other ACE SNP variants have been found to be associated with DN in pooled analytic studies. Data appear to be lacking with regards to the association between ACE gene variants and DN in African populations. Susceptibility loci for DN risk in African populations have however been identified on chromosomes 3q, 7p, and 18q. Similarly, chromosome 22 susceptibility loci involving single nucleotide polymorphisms (SNPs) of the MHY9, APOL1, SFI1, and LIMK2 genes have been identified to be associated with DN risk among African-Americans [50]. APOL1 high-risk variants are also associated with faster progression among African populations. Additionally identified genetic associations among Asians include APOE E2 (which increases the odds for DN) and the

inflammatory cytokine CCR5 gene polymorphisms. Among South Asian populations, single nucleotide polymorphisms in the carnosine dipeptidase 1 (CNDP1) and acetyl-coenzyme A carboxylase beta (ACACβ) genes are associated with an increased DN risk in this subpopulation of Asians. No definite genetic associations for DN risk or progression have been identified among Mexican-Americans [51]. In understanding genetic predisposition to DN, it is important to indicate that no single gene contributes exclusively to risk but that multiple genes act in concert to confer cumulative risk, i.e., genetic risk for DN and its progression are polygenetic [50].

Health-Care Access and Financing: A Driving Force for Regional and Ethnic Differences in DN

Global disparities in access (availability and affordability) to standard diabetes care contribute to the observed regional differences of DN. Optimization of glycemic control is a cardinal approach in diabetes management and the risk reduction of related complications. The availability of glucose-lowering agents however demonstrates diverse regional variability. In the WHO defined regions of Africa and Southeast Asia (SEA), insulin availability at the primary care level is significantly lower than that in Europe and the Americas with only 23% of low-income countries reporting availability in >50% primary care facilities [52]. Very few countries in low-income regions have sulfonylurea and metformin available at the same time in primary care facilities [52]. Essential skills and materials needed for the early detection and monitoring of diabetes and its complications (such as dipsticks for urine protein surveillance for DN) are also not comparably available globally. Dilated fundal examination as a means of microvascular complication surveillance and/or detection is only available in about 20% of low-income regions in comparison with approximately 90% availability in high-income regions [52].

In addition to glycemic control, other recommended CKD risk reduction approaches with respect to DN include adequate blood pressure control and renin-angiotensin axis blockade. These risk reduction strategies however come at great economic costs that are not equally affordable at national and individual levels across various regional health-care systems. For instance, no low-income or lower middle-income country is able to deliver complete public-funded provision of medications for the management of pre-ESRD CKD [53]. Global health expenditure for diabetes in 2010 was lowest in Africa and SEA with each region spending a paltry 1.3% and 3.0%, respectively, of the amount spent in Europe on diabetes in the same year [54]. In addition, in the low- and middle-income regions of the world, there is the further issue of out-of-pocket payment (OPP) for health as a barrier to accessing standard care. A study from Cameroon which demonstrates the OPP costs related to access to DM care puts this limitation (as it affects risk reduction) into clear perspective – it costs the equivalent of >18 days' wages to get 100 units of biphasic insulin and a 14 day wage to purchase the angiotensin-converting enzyme inhibitor (ACEi) Ramipril [55]. In India, the income per capita is in the region of 1300 Euro, while the annual cost per person for ambulatory DM care is

approximately 265 Euro [56]. OPP as a percentage of the total national health expenditure in India is 62.4%. As long as there is differential health-care spending across regions in the world, economic barriers will continue to influence the regional differences in diabetes and its complications such as DN, as are currently observed [57].

CKD Screening as a Strategy to Mitigating the Disparate Global Burden of DN

CKD screening among high-risk populations, such as people with diabetes, is a veritable tool for both primary prevention of DN and the secondary prevention of progression of early-stage DN to ESRD. These programs have the potential of identifying individuals at risk at the population/community level in addition to detecting earlier CKD stages of DN in the diabetic high-risk group. Early identification and detection would afford the opportunity to institute non-pharmacologic interventions such as lifestyle and dietary changes and the use of pharmacologic treatment (which is cheaper relative to the cost ESRD care) aimed at controlling known progression factors such as poor glycemic control, hypertension, and albuminuria. With patients on renal replacement therapies representing only the tip of the iceberg in the spectrum of CKD, the beneficial role of screening in addressing the disparate global burden of DN cannot be overlooked [58]. With the disproportionately high increase in prevalence projected for the coming decades in LMIC, screening programs are particularly needed in these economies for whom the financial costs of CKD and ESRD care are quite formidable and where primary health-care systems for the detection and treatment of CKD risk factors such as diabetes are poor [59]. The Kidney Early Evaluation Program (KEEP) is a screening program among populations at high risk for CKD initiated in the USA that has also been locally adapted for screening in Japan, Mexico, and Australia. These programs have so far given an insight into the magnitude of undiagnosed CKD (among diabetics as well as other high-risk groups) at the population level. Among rural high-risk patients screened in Mexico, the detected prevalence of CKD among diabetics was 35% with none of the patients in this rural population being aware of their CKD status prior to screening [60]. This proportion of patients detected at screening would have remained undiagnosed in the community and would only have become part of the health-care system at the point of advanced CKD or ESRD.

Conclusion

DM and its chronic complications such as DN are fast assuming global health proportions with profound current and future health-care cost implications. It has thus become imperative to understand on a global scale as well as at regional levels the epidemiology of DN so as to inform appropriate strategies to prevent the occurrence of DN as well as retard progression of microalbuminuria and decline in GFR.

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Chapter 4
Diabetic Nephropathy in Children and Adolescents

Petter Bjornstad

Introduction

Diabetic nephropathy (DN) continues to account for most cases of end-stage renal disease (ESRD) and dialysis in the Western world and remains a leading cause of mortality in type 1 (T1D) and type 2 diabetes (T2D) [1–3]. Markers of early DN, including elevated albumin excretion and renal hyperfiltration, are common in youth with T1D and T2D [4, 5]. The natural history of DN is typically defined by a long silent period without clinical signs and symptoms of nephropathy. By the time glomerular filtration rate (GFR) drops below 60 mL/min/1.73m^2, approximately 50% of renal function is lost, and renal structural changes are usually refractory to therapeutic interventions including improved blood pressure and glycemic control [6, 7]. This is further complicated by undertreatment of DN in youth, with only a third of participants <20 years in the T1D Exchange Clinic Registry with a diagnosis of elevated albumin excretion reported to receive ACE inhibitors (ACEi)/angiotensin receptor blockers (ARB) [8]. Finally, glycemic and blood pressure control may delay but do not stop the progression of DN [9, 10]. Accordingly, earlier identification of DN to decrease the rate of GFR loss and prolong the time to development of ESRD, and better understanding of the mechanisms contributing to early DN, is needed to develop new interventions that improve renal health and mortality for the estimated 422 million people with diabetes worldwide.

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The Pathogenesis and Natural History of Early Diabetic Kidney Disease

The conventional paradigm of DN is characterized by the development of progressive pathological changes over a long silent period without evidence of proteinuria, hypertension, or impaired GFR [11]. In this paradigm, during the clinically silent phase, a significant proportion of patients exhibit renal hyperfiltration secondary to elevated glomerular pressure resulting in a glomerular injury, followed by rapid GFR decline and elevated albumin excretion, eventually resulting in ESRD [12]. Renal hyperfiltration is the earliest hemodynamic abnormality seen in diabetes and likely contribute to the pathogenesis of DN through neurohormonal (e.g., renin-angiotensin-aldosterone system activation [RAAS]) and tubular (e.g., tubuloglomerular feedback) pathways. However, renal hyperfiltration alone cannot fully explain the development of early DN, since patients with other causes of renal hyperfiltration, such as unilateral nephrectomy, do not typically progress to nephropathy. This discrepancy may be ascribed to insulin resistance and the resultant susceptibility to renal hypoxia. Indeed, renal hypoxia stemming from a mismatch between renal oxygen utilization and consumption is increasingly proposed as a unifying pathway in the development of DN [13] (Fig. 4.1). The kidneys are metabolically active and have a high-energy requirement to sustain filtration, intrarenal hemodynamic function, and tubular reabsorption [14, 15], which is exacerbated in renal hyperfiltration. The elevated GFR and renal plasma flow associated with renal hyperfiltration result in higher renal oxygen consumption (Fig. 4.2). Diabetes animal models suggest that the process starts with renal hyperfiltration and glucosuria, which enhance sodium/glucose reabsorption through sodium/glucose cotransporter 2 (SGLT2). The resultant increased proximal tubular intracellular sodium

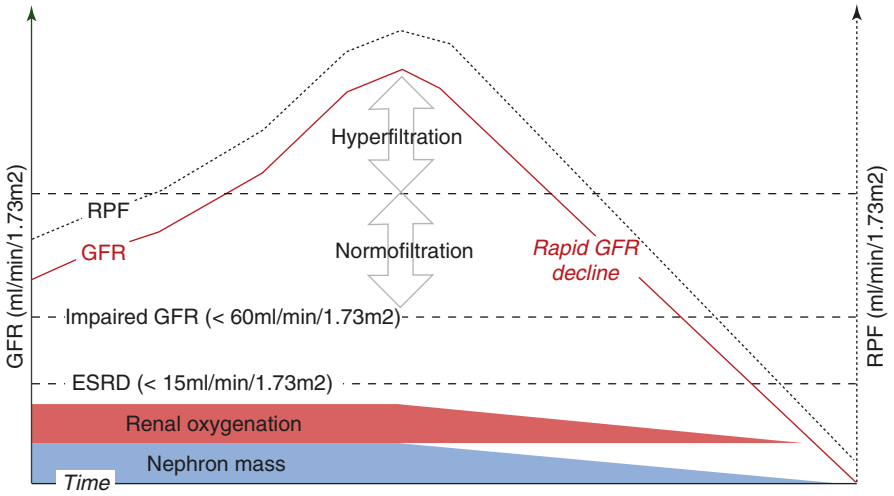

Fig. 4.1 Stages of diabetic nephropathy

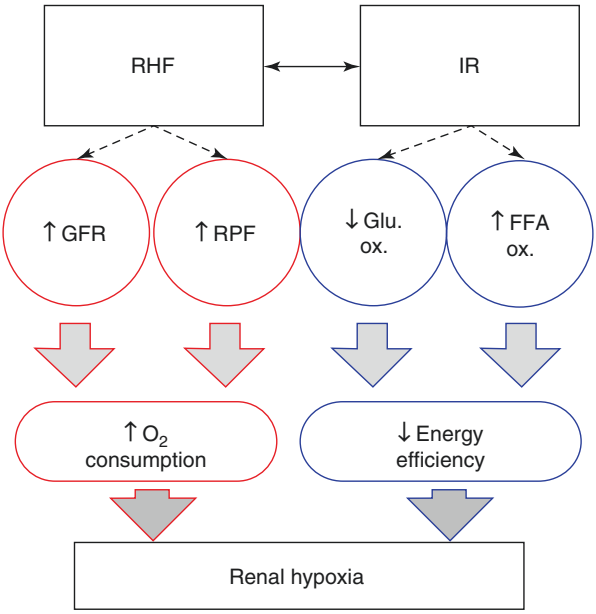

Fig. 4.2 Hyperfiltration and insulin resistance may contribute to renal hypoxia

concentration and activation of basolateral Na^+/K^+ ATPase lead to net increased renal oxygen consumption compared to nondiabetic models [14, 16–18] (Fig. 4.3). With increased renal oxygen consumption in DN, energy efficient fuel is required to meet the increased demand and prevent renal hypoxia. Emerging animal data show that organs prone to complications are unable to compensate for the effects of diabetes on fuel generation [16–18]. In fact, the insulin resistance is associated with impaired ability to synthesize ATP by inhibition of AMP-activated protein kinase [19–21]. In addition, hyperglycemia and insulin resistance results in mitochondrial dysfunction [22] and reduced electrolyte transport efficiency [23]. Accordingly, one may theorize that the energy profile of T1D and T2D cannot accommodate the renal hypermetabolism of early DN and that the consequent renal hypoxia may drive disease progression.

In addition to the above proposed pathways, diabetic tubulopathy is an important phenotype of DN which is often overlooked by researchers and clinicians. The tubules are just as susceptible to diabetic injury as the glomerulus, and diabetic tubulopathy is characterized by basement membrane thickening, tubular hypertrophy, epithelial-mesenchymal transition, glycogen accumulation, and interstitial inflammation [11]. Tubular injury is known to be more strongly associated with renal function than glomerular injury [24, 25] and may occur earlier in DN than glomerular injury [26, 27]. Glucosuria activates the polyol pathway which leads to increased intracellular fructose concentration, and the metabolism of the generated fructose results in increased intracellular uric acid concentration [25]. Murine studies showed that inhibiting uric acid production protects the kidney from tubular

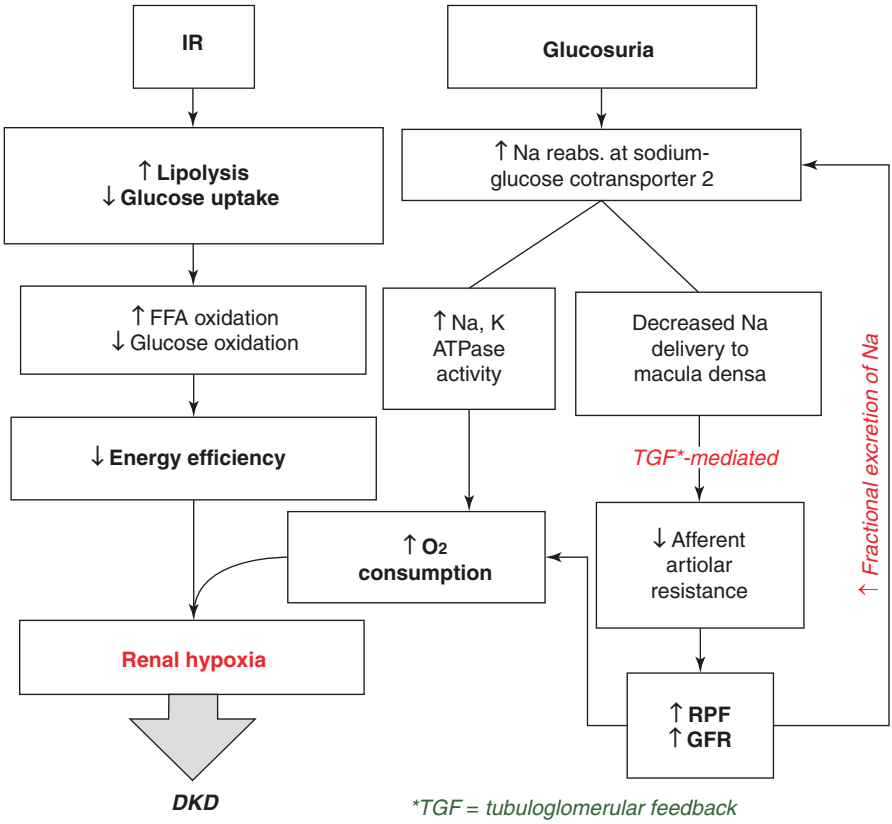

Fig. 4.3 Proposed roles of intrarenal hemodynamics and renal oxygenation in development of DN

injury, which may suggest a causal role for uric acid in the development of diabetic tubulopathy [28]. Such findings are not limited to murine models, as increased urine uric acid was recently found to promote apoptosis in human proximal tubular cells by oxidative stress and activation of NADPH oxidase NOX4 [29]. Finally, allopurinol has been shown to reduce the diabetic tubular injury associated with the KK-A(y)/Ta mouse model [30].

What Is the Risk in Youth with Type 1 Diabetes?

A quarter of T1D patients with DN progress to ESRD [31], and T1D accounts for approximately 20% of all patients with DN who enter ESRD programs [32]. Elevated albumin excretion, generally recognized as one of the earliest clinical phenotypes of DN, develops at a rate of around 2–3% annually with a cumulative lifetime incidence of approximately 50% in T1D [10]. In the Oxford Regional

Prospective Study of young people with T1D living in the UK, elevated albumin excretion was identified in 23% of samples [33]. Reported prevalence rates for elevated albumin excretion among youth with T1D in Australia range between 6% and 18% [34, 35] and 5.6% in Sweden [36]. Furthermore, elevated albumin excretion was reported in 4.4% of participants in the T1D Exchange Clinic Registry [8] and 5.8% in SEARCH for Diabetes in Youth (SEARCH) Study [37]. While elevated albumin excretion is traditionally recognized as the earliest clinical sign of DN, the paradigm of early DN in T1D changed after the demonstration that elevated albumin excretion does not necessarily imply progressive nephropathy and may in fact regress to normal albumin excretion in over one-third of cases [38, 39]. Other important phenotypes of early DN include renal hyperfiltration and rapid GFR decline. The prevalence of renal hyperfiltration in youth with T1D has been reported to exceed 50% when GFR is measured by inulin clearance [40] and between 13% and 31% when GFR is estimated by serum creatinine and serum cystatin C [5, 41]. The discrepancy in prevalence is likely attributed to the inaccuracy of estimated GFR in the normal to elevated GFR range [42]. Rapid GFR decline, an intermediate phenotype of DN, is thought to succeed hyperfiltration. In fact, hyperfiltration confers greater odds of experiencing rapid GFR decline in youth [41] and adults with T1D [43, 44]. Longitudinal data describing the prevalence and incidence of rapid GFR decline are needed to define the sequence of progression from hyperfiltration to rapid GFR decline and eventually impaired GFR.

What Is the Risk in Youth with Type 2 Diabetes?

DN remains a leading cause of morbidity and mortality in people with T2D [1–3] and accounts for almost 40% of all cases of ESRD from DN in the USA [32]. Early DN, including hyperfiltration and increased albumin excretion, is common in youth-onset T2D and progresses at an alarming rate. In fact, youth-onset T2D carries a particularly high risk of progressive DN, which is significantly greater than youth with T1D or adults with T2D of similar disease duration [33, 45–51]. In SEARCH for Diabetes in Youth (SEARCH) Study, 19.9% of youth with T2D had evidence of DN compared to 5.8% of youth with T1D, which translates to an odds ratio of 2.58 (95% 1.39–4.81) [37]. Furthermore, the risk of elevated albumin excretion is also reported to be twofold in youth with T2D compared to T1D [45, 48, 52]. In a small cohort of 46 adolescents with T2D, prevalence of hyperfiltration and elevated albumin excretion was reported to be 24% and 34%, respectively [4]. Longitudinal data from the Treatment Options for Type 2 Diabetes in Adolescents and Youth (TODAY) study demonstrate that the prevalence of elevated albumin excretion rose from 6.3% at baseline to 16.6% over an average follow-up of 3.9 years [48]. Similarly, a recent report by the TODAY study group found hyperfiltration in 7% of youth at baseline and 13.3% at 5 years, when hyperfiltration was defined as eGFR ≥99th percentile of a nationwide sample of healthy adolescents from (NHANES 1999–2002, [53, 54]). This strict definition likely underestimates the number of adolescents with T2D

with hyperfiltration. In fact, if one instead defined hyperfiltration classically as two standard deviations above the mean eGFR, over 50% of youth-onset T2D had hyperfiltration at 5-year follow-up [54]. There are limited longitudinal data available on the natural history of DN in youth with T2D [55], and longer TODAY and SEARCH follow-up will provide important insight into the progression of DN in youth-onset T2D. Registries and consortiums, including the Pediatric Diabetes Consortium, are also important to define prevalence and incidence of DN in youth-onset T2D.

Novel Risk Factors for Early DN

Extensive bench and translational research into the mechanisms of hyperglycemic injury and its modifiers [56] has to date not successfully translated into adjuvant therapeutics to supplement conventional intensive insulin in the prevention of DN. Accordingly, there is a need for novel modifiable risk factors. Although there are several promising risk factors, here I discuss four modifiable risk factors, including insulin resistance, serum uric acid, urine uric acid, and vasopressin activity.

Insulin Resistance

Insulin resistance is not a complication limited to youth-onset T2D. Indeed, reduced insulin sensitivity is an established metabolic component of both youth and adults with T1D. Furthermore, insulin resistance is increasingly recognized to contribute both to the initiation and progression of DN [57]. Although the mechanisms are still poorly understood, insulin resistance is thought to lead to important hemodynamic changes in the kidney, including increased sympathetic nervous system tone, hypertension, and accelerated atherosclerosis of the renal microvasculature. In addition, insulin resistance is associated with energy inefficiency with reduced glucose oxygenation and increased free fatty acid oxygenation rendering the kidney susceptible to renal hypoxia [16–18]. In youth with T2D, insulin resistance has been shown to be a stronger risk factor for DN in cross-sectional [4] and longitudinal studies [54] than traditional risk factors such as glycemic, blood pressure, and lipid control. A recent analysis in Treatment Options for Type 2 Diabetes in Adolescents and Youth (TODAY) study, one standard deviation in estimated insulin sensitivity carried a twofold greater risk of developing hyperfiltration over 5 years in youth with T2D. Similarly, in adults with T1D, reduced insulin sensitivity conferred greater odds of early DN, including incident elevated albumin excretion and rapid GFR decline over 6 years [44]. It is unclear whether lifestyle or therapeutic improvement in insulin sensitivity translates to attenuation of early DN. For example, the Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI 2D) study showed no benefit of an insulin-sensitizing strategy on DN in older adults with coronary

artery disease and T2D [58], and the use of metformin in adults with T2D and stage 5 CKD has been associated with a significantly increased risk of all-cause mortality [59]. Although these studies demonstrated no benefit of insulin sensitization on DN in adults T2D, the cohorts included older adults with multiple cardiovascular comorbidities and long-standing nephropathy. Established DN may be less responsive to changes in insulin sensitivity compared to early DN. Accordingly, strategies to improve insulin sensitivity in youth with T1D and T2D may still reduce risk of early DN and deserve further investigation. The REducing with MetfOrmin Vascular Adverse Lesions in Type 1 Diabetes (REMOVAL) study showed that 3 years of metformin therapy reduced maximal carotid intimal media thickness (cIMT), although progression of mean cIMT was not significantly reduced [60]. The recently completed Effects of Metformin on Cardiovascular Function in Adolescents with Type 1 Diabetes (EMERALD) will hopefully provide data on whether 3 month of metformin therapy improves cardiovascular and renal health in youth with T1D. Anderson et al. recently demonstrated that 12 months of metformin therapy improved glyceryl trinitrate-mediated brachial artery dilatation (vascular smooth muscle function) in youth with T1D (8–18 years of age) but found no effect on aortic IMT or cIMT [61].

Serum and Urine Uric Acid

Several studies link urine uric acid (UUA) and serum uric acid (SUA) to DN development [62–64]. Increasing amount of data suggest that lowering UUA and SUA impedes the development and progression of DN in animal models and in patients with type 2 diabetes [62–66]. To determine the effect of SUA lowering in adults with T1D, the multicenter double-blind randomized clinical trial Preventing Early Renal Function Loss in Diabetes (PERL) will test the hypothesis that lowering SUA with allopurinol will prevent rapid GFR decline measured by iohexol clearance [67]. If PERL shows a robust effect from allopurinol, studies examining the benefit of SUA lowering in youth with T1D and T2D are warranted.

Vasopressin

AVP has diverse actions in physiology which include direct effects on vascular hemodynamics, inflammation, lipid metabolism, ACTH secretion, and renin-angiotensin-aldosterone system (RAAS), in addition to sodium, volume, and osmolality regulation by the kidneys [68, 69]. The actions of AVP are mediated by at least three distinct G-protein-coupled receptor subtypes: V1a, V1b, and V2. Experimental data strongly support a causal and direct role of vasopressin in the pathogenesis of kidney disease through V2R activation [70, 71] (Fig. 4.4). In laboratory animals, V2R stimulation leads to afferent arteriolar dilation with increased RPF and GFR

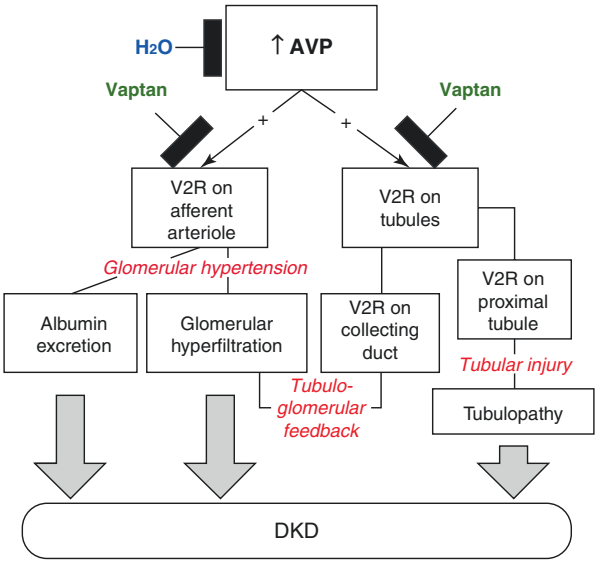

Fig. 4.4 The role of vasopressin overactivity in the pathogenesis of DN

[72], in addition to elevated albumin excretion and tubulopathy [73–75]. Measuring AVP is cumbersome due to its relatively small size and short half-life. Copeptin, a more stable peptide derived from the same precursor molecule as AVP, is recognized as a surrogate marker for AVP [74]. Data suggest that elevated copeptin is strongly associated with DN and CVD in adults with T1D in the CACTI study [76], T1DX Biobank registry [77], GENEDIAB, and GENESIS [78]. Similar data exist in adults with T2D, with elevated copeptin conferring greater risk of CVD events and mortality [79, 80] in addition to declining GFR [81, 82]. Studies in pediatric T1D and T2D are, however, lacking. The vasopressin system is not only a modifiable risk factor through increased water intake but also a promising therapeutic target with the availability of vaptans (vasopressin receptor antagonists).

Current Methods of Identifying Early DN

Albumin Excretion

The recommendation from the American Diabetes Association (ADA) is annual assessment of albumin excretion [83–85]. Moderately elevated albumin excretion (microalbuminuria) is defined as albumin excretion rate (AER) ≥20 ug/min or albumin creatinine ratio (ACR) ≥30 mg/g and severely elevated albumin excretion (macroalbuminuria) defined as albumin excretion rate (AER) ≥200 ug/min or albumin creatinine ratio (ACR) ≥300 mg/g. Although elevated albumin excretion is still an

important risk factor for cardiovascular disease, the implications of the phenotype as a marker of progressive nephropathy changed after the demonstration that a substantial proportion regress to normal albumin excretion in both T1D and T2D [86].

Glomerular Filtration Rate

The ADA also recommends annual determination of GFR to identify and monitor DN in adolescences [83]. In clinical research and practice, GFR is typically estimated by equations using endogenous filtration markers (serum creatinine and/or cystatin C). Although there are numerous equations available to estimate GFR in children and adolescents using these endogenous filtration markers, no single equation has been specifically developed or validated in adolescents with T1D and T2D. The most commonly used eGFR equations in pediatrics (e.g., Schwartz creatinine-based equation from 2009) are based on serum creatinine and generated from children and adolescents with chronic kidney disease and are thus most accurate in the impaired GFR range [87]. Such equations are likely inaccurate and imprecise in estimating GFR in youth with T1D and T2D who usually have GFR in the normal to elevated range [88–91]. Estimating equations based on serum cystatin C (e.g., Zappitelli, Larsson, Berg) and combined serum creatinine and cystatin C (CKiD, Zappitelli, Bouvet) demonstrate stronger agreement with measured GFR in the normal to elevated GFR range compared to creatinine equations [53, 87, 92–95]. Although eGFR calculated by serum cystatin C and/or creatinine are possibly superior to serum creatinine-based equations [96–98], there are no currently published equations validated against measured GFR in youth with T1D and T2D. Furthermore, data from adults demonstrate disagreement with eGFR and measured GFR in T1D and T2D. In DCCT/EDIC, changes in eGFR over a 3-year period did not reflect changes in measured GFR by iothalamate clearance [99, 100]. Similarly in an analysis by MacIsaac et al. in Australia, eGFR by serum creatinine significantly underestimated early decline in measured GFR in adults with T1D and T2D [101]. The dissociation between eGFR and measured GFR by inulin and iohexol clearance was also demonstrated in young adults and older adults with T1D, respectively (Maahs, Longevity). Data are not limited to adults with T1D and T2D; Perrin et al. reported that most GFR estimations severely underestimate hyperfiltration in adolescents and young adults with T1D compared to measured GFR and concluded that estimated GFR cannot replace measured GFR in T1D patients with hyperfiltration [102]. The inaccuracy and imprecision of eGFR in youth with T1D and T2D are concerning since hyperfiltration and rapid changes in GFR may be missed with a resultant delay in starting therapies [91, 103, 104]. Therefore, there is a clear need for improved methods of determining GFR in the clinical setting.

Several methods exist to *measure* GFR including inulin clearance, which is technically challenging and time-consuming [94, 105], and radioisotopes, such as ^{99m}Tc-DTPA, ^{51}Cr-EDTA, and ^{125}Iothalamate, which are problematic in clinical practice due to logistics, cost, and exposure to radiation, especially in asymptomatic young

adults and/or adolescents. Conversely, iohexol and nonradioactive iothalamate are nonionic, low-osmolar contrast agents that cannot be absorbed, metabolized, or secreted by the kidney, with a low toxicity profile [94, 105] and widely available for clinical practice. Furthermore, determining GFR by iohexol or iothalamate clearance using dried blood spots (DBS) is feasible, and studies in people with and without diabetes show that iohexol and iothalamate clearance using DBS provides GFR measurements comparable to plasma clearance [106–109]. The significantly reduced patient and staff time associated with GFR in DBS compared to GFR in plasma makes this a feasible method for clinical practice and research.

GFR can also be measured noninvasively by non-contrast MRI methods (e.g., blood oxygen level-dependent (BOLD) MRI and diffusion-weighted imaging (DWI) MRI) [110], but these techniques have yet to be validated in larger studies. The disadvantage of MRI-based GFR is the low accessibility and high cost which may prohibit these methods in clinical practice, but these factors may be offset in research by the ability to simultaneously quantify renal blood flow and oxygenation. Another promising method to accurately quantify GFR is fluorescent probe determination of GFR, which has the added advantage of measuring GFR in real time, rather than deferred determination of GFR, without the need for urine or blood collections. The disadvantages, in addition to the fact that these methods have primarily been tested in animal models [111, 112], with the exception of a few small human studies [113–115], include cost, availability of nuclear medicine, and radioactivity.

Intrarenal Hemodynamic Function

There remains a large unmet need for more accurate assessment of renal health in T1D and T2D that will enable the identification of patients at high risk of early DN at a time when the renal injury may be responsive to therapeutic intervention. Whereas determination of GFR provides important information about the filtration capacity of the kidney, it does not offer data on other parameters of intrarenal hemodynamic function, including renal plasma flow (RPF), afferent arteriolar resistance, efferent arteriolar resistance, glomerular pressure, filtration fraction, or oncotic pressure. To gain information about the human intrarenal hemodynamic function in vivo, mathematical equations were developed by Gomez et al. [116], using *measurements* of GFR, RPF, renal vascular resistance (RVR), hematocrit, and serum protein. Studies of intrarenal hemodynamic function have focused largely on adults with T1D and adult-onset T2D [116–118]. These studies have established that intrarenal hemodynamic function is abnormal in early DN [117, 119–121] and provided compelling evidence that intrarenal hemodynamic function can only be partially restored with therapeutic agents early in the course of DN [122, 123]. Yet no such data are available for youth-onset T2D, a disease that carries a significantly higher risk of progressive DN than T1D and adult-onset T2D [33, 37, 45–52, 124]. Further, no clinical data exist on the interaction between intrarenal hemodynamic function, renal

perfusion, and oxygenation, a probable unifying pathway in DN. The disadvantage with Gomez equation is that it requires accurate ascertainment of GFR and RPF. While changes in RPF are recognized to be one of the earliest changes in renal health, its measures are cumbersome and technically challenging, thereby limiting its broad use in clinical medicine and research [116, 125]. Para-aminohippurate (PAH) clearance is the gold standard method to quantify RPF [118], but like inulin and iohexol clearance, it is technically challenging and time-consuming [116]. There are, however, convincing data supporting strong agreement with RPF measured by phase-contrast (PC) MRI and RPF measured by PAH clearance in adults [126–129]. Such studies are needed in youth, and especially youth with T1D and T2D.

Pediatric Clinical Trial Data and What Progress Do We Need to Make in the Next 5 Years?

There is a shortage of DN clinical trials dedicated to youth with T1D and T2D; there are some important landmark studies worth highlighting. In the Diabetes Control and Complications Trial (DCCT) adolescent cohort, which included 195 pubertal adolescents, the intensive treatment arm carried lower risk and progression of microalbuminuria by 54% compared to the conventional therapy arm [130]. The recently published AdDIT study found that statin therapy corrected dyslipidemia, that ACE inhibition reduced elevated albumin excretion, and that both medications were safe in adolescents with T1D following standardized protocol [131]. In the TODAY study, metformin alone was only associated with durable glycemic control in 48.3% of participants with youth-onset T2D. The addition of rosiglitazone, but not an intensive lifestyle intervention, was superior to metformin alone in achieving glycemic control [132]. These data highlight that T2D is associated with a much more aggressive course in youth compared to adults, despite good medication compliance [133].

Priorities to impede the development of DN in youth with T1D and T2D are summarized in Table 4.1. A better differentiation of guidelines for youth with T1D vs. T2D is needed, as there are likely important pathophysiological differences contributing to DN risk in these populations. Accordingly, disease-specific preventive measures and therapeutic options are required to effectively impede the development of early DN. Data validating surrogate markers of DN are needed to determine both how well the markers predict renal events and how well they represent physiology (e.g., estimated GFR vs. measured GFR). Whereas yearly estimation of GFR is recommended by the ADA to screen for DN in youth with T1D and T2D, guidelines do not define hyperfiltration or rapid GFR decline and do not incorporate changes in GFR in the management algorithms. There is also a need for information to evaluate whether novel measures of renal vascular flow, oxygenation, and perfusion (e.g., MRI-based techniques) are superior markers of renal health. Although MRI-based methods and gold standard physiological measures will likely remain research tools given costs, mechanistic and experimental studies are needed to define the

Table 4.1 Priorities to impede the development of DN in youth with T1D and T2D

Areas of research priority
Better differentiation of guidelines for youth with T1D vs. T2D
Implementation of clinical guidelines and benchmarking of DN risk factors
Validation of the prediction performance of existing renal surrogate markers (e.g., albumin excretion, estimated GFR by serum creatinine and serum cystatin C)
Better methods to measure GFR in the ambulatory setting (e.g., iohexol or iothalamate clearance in dried blood spots)
Improved functional and structural evaluation of renal health by MRI methods (e.g., phase-contrast, 4D flow and XD flow, arterial spin labeling, and blood oxygen level-dependent MRI)
Mechanistic clinical trials with gold standard intrarenal hemodynamic function (measured GFR and RPF) as outcomes
Clinical trials evaluating treatment (lifestyle, pharmacotherapy, and technology) to impede the development of DN
Determination of appropriate screening guidelines for DN risk factors
Establishment of risk factor thresholds to initiate DN risk factor treatment (e.g., recommendations for hyperfiltration and rapid GFR decline)

pathophysiology of early DN and test novel therapeutic strategies. Randomized control trials evaluating therapies to stop or delay development of early DN in youth with T1D and T2D are also needed. To reduce long-term morbidity and mortality, we also need to address provider inertia and shorten the time lag from research results and guidelines to clinical implementation. The combination of mechanistic studies and randomized clinical trials will allow the pediatric research community to set well-defined risk factor targets and advance our understanding of optimal therapy to prevent the development and progression of DN in youth.

Conclusion

In conclusion, early DN is common in adolescents with T1D and T2D and contributes to greater risk for ESRD and CVD morality. Better methods to identify early abnormalities in renal health, in addition to early and specific interventions, offer the greatest potential to prevent the progression of early DN. Efforts to improve future DN outcomes and mortality must start in adolescence.

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Chapter 5
Renal Disease in Obesity, Metabolic Syndrome and Diabesity

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Introduction

Worldwide, more than 400 million subjects have type 2 diabetes (T2DM), and ~2 billion individuals are overweight or obese [1, 2]. These numbers are supposed to increase further, and by 2045 more than 600 million people are expected to have T2DM [2]. Also, obesity is particularly frequent in children and adolescence: in 2016, 18% of people from 5 to 19 years were overweight or obese [1].

This pandemic parallels the increase in metabolic syndrome (MS), which varies from 20% to 40% in diverse countries [3]. MS reflects the coexistence of hypertension, overweight/obesity, prediabetes, dyslipidemia and subclinical inflammation, among others [3, 4], indicating a deep interrelation between these metabolic diseases. Overweight and obesity are risk factors for T2DM [5]. Also, many patients

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with overweight or obesity may have prediabetes, when properly evaluated [6]. The common background of MS, which links all its components, is supposed to be insulin resistance, which is also a key factor in the pathogenesis of T2DM and obesity [7]. Accordingly, overweight, obesity, MS and T2DM may represent a continuum of metabolic alterations.

The pandemic of MS, obesity, and T2DM – *diabesity* – may portend severe consequences in public health increasing the risk for cardiovascular disease, chronic liver disease, certain types of cancer, and also renal disease [8–11]. All the components of MS are established risk factors for chronic kidney disease (CKD). However, there is scarce evidence on the pathogenesis, clinical evolution, and renal histology of renal disease in obesity, overweight, and MS. In this chapter we will review the available evidence on renal disease in extreme obesity, moderate obesity and overweight, and MS. Also, we will focus on common links between renal disease in obesity, MS, and diabetes.

Obesity and Renal Disease

Lessons from Extreme Obesity

In the 1970s, Weisinger and Cohen described for the first time the presence of focal and segmental glomerulosclerosis (FSGS) and glomerulomegaly in morbidly obese patients with nephrotic-range proteinuria [12]. Subsequently, this association was limited mainly to case or autopsy series [13]. In 2001, the group of Prof. Vivette D'Agati described, in a detailed clinical-histological analysis of 71 cases, the characteristics of obesity-related glomerulopathy (ORG), which include glomerulomegaly with or without FSGS. Most of the patients in this study had extreme obesity (BMI > 40 kg/m^2) and underwent a renal biopsy due to overt renal disease, i.e., proteinuria and/or CKD [14]. Interestingly, in a study in extremely obese patients without proteinuria or renal disease treated with bariatric surgery, FSGS was observed in 5% of the cases [15]. This finding may indicate that glomerular lesions precede the development of proteinuria or renal failure in extremely obese patients. Thus, glomerulomegaly and FSGS (ORG) are the major histological findings in patients with extreme obesity with and without clinical evidence of renal disease [14, 15]. Of note, ORG was also described in patients with lower degrees of obesity: grade 1, BMI 30–34.9 kg/m^2, or grade 2, BMI 35–39.9 kg/m^2 [14]. Little evidence is available on renal histology in patients with overweight.

Clinical Features of Renal Disease in Overweight and Obesity

Microalbuminuria is usually the first clinical manifestation of renal disease in obesity. Pinto-Sietsma in 7676 nondiabetic subjects observed that obesity, particularly central fat distribution, was a risk for microalbuminuria [16]. Also, the percentage

of microalbuminuria varied from 8.9% in thin to 15.9% in overweight and 21.2% in obese patients [16].

Isolated proteinuria with or without reduced renal function is the main manifestation of ORG. It is observed in 10–41% of patients and can be of low grade or even reach the nephrotic range [17, 18]. However, the presence of full nephrotic syndrome is the exception rather than the norm. This is important to differentiate other causes of renal disease in obese patients [17, 18]. Thus, the presence of edema, hypoalbuminemia, or severe hyperlipidemia may indicate not ORG but other cause of glomerular disease in a patient with obesity. Some authors also describe the presence of microhematuria between 5% and 26% of cases as part of ORG's clinical manifestations [19–21].

Renal function may vary from glomerular hyperfiltration in the early stages to renal impairment in more advanced stages. Finally, caution is needed in the interpretation of renal function in patients with obesity with the use of creatinine- or cystatin-c-based formulas. Formulas are known to reflect glomerular filtration rate with an average error of about ± 20–30% [22]. In fact, in a study of 600 patients with T2DM, most of them overweight or obese, creatinine-based formulas failed to detect glomerular hyperfiltration in about 70% of the cases [23]. Little information is available on the reliability of cystatin-c-based equations in obese subjects. Finally, the adjustment of GFR values according to body surface area artificially reduces renal function in obese patients and then, should be abandoned [24].

Clinical Evolution, Weight Reduction, and Renoprotection

There are few studies that evaluated the long-term evolution of ORG [17–21, 25, 26]. In general, renal progression of FSGS secondary to ORG with proteinuria or renal failure is slower than primary FSGS [17, 18, 21, 26, 27]. However, without intervention these patients can progress to ESRD, and renal survival ranges from 77% to 85% at 5 years and between 51% and 55% at 10 years [17, 18, 27]. In patients with ORG, a short-term improvement in proteinuria can be achieved through significant weight reduction by diet and physical exercise or bariatric surgery [26]. As expected, the improvement is more pronounced in patients undergoing bariatric surgery than in those with dietary and lifestyle interventions. In addition, drastic weight loss secondary to bariatric surgery in morbidly obese patients with ORG, normal preoperative renal function, and mild proteinuria resulted in excellent outcomes, as normal renal function was maintained, blood pressure was improved, and albuminuria was attenuated after 10 years of follow-up [21, 27].

Despite the evidence shown above, it has to be considered that most studies designed to evaluate the renoprotective effect of weight reduction are retrospective, observational, underpowered (~20–40 patients), with short follow-up (<12 months) and heterogeneous: including patients with extreme obesity treated with bariatric surgery and less severe obesity treated with drugs no longer available or diet [26]. Also, most studies used estimated and not measured GFR. Finally, few prospective clinicals in the field trials are available. Of note, the impact of weight reduction on

measured GFR is bimodal: an initial acute reduction is followed by a slower long-term GFR decline [22, 23, 28]. Moreover, the magnitude of the acute decrease of GFR determines a slower decline over time. These changes are not reflected by estimated GFR. These aspects should be taken into consideration when planning studies in patients with obesity and/or T2DM. Finally, little evidence is available on the impact of regular exercise alone or in combination with diet on the evolution of GFR over time in obesity and T2DM.

Metabolic Syndrome

Obesity in the Context of Metabolic Syndrome and Insulin Resistance

Several studies observed a clear association between MS and CKD [11]. This is not surprising since the components of MS have been individually related with renal disease. Major studies analyzed the impact of hypertension and obesity in renal dysfunction. In a cohort of >300.000 subjects, obesity (BMI >30 kg/m^2) determined a 3.6 relative risk for ESRD [20]. Interestingly, the risk increased in the stage of overweight (BMI 25–29 kg/m^2) indicating a continuous risk between BMI and CKD. In large cohort studies including >300.000 men or women both systolic and diastolic blood pressure were associated with increased risk for ESRD [29, 30]. High triglyceride levels, a well-known marker of insulin resistance, have been linked with faster decline of measured GFR in patients with metabolic syndrome [31]. Also, prediabetes, mainly impaired fasting glucose, has been related with glomerular hyperfiltration, a risk factor for future renal dysfunction [32].

However, the prevalence of CKD and ESRD does not match the prevalence of obesity, hypertension, dyslipidemia or prediabetes. This may indicate that the risk for CKD attributed to these metabolic alterations is not uniform. Eriksen et al. followed a cohort of 1627 subjects from the general nondiabetic population during about 5 years with repeated measurements of renal function (clearance of iohexol) and observed that moderate hypertension was not associated with faster GFR decline, even after adjusting for risk factors for CKD and antihypertensive medications [33]. Also, a meta-analysis of clinical trials in hypertensive patients showed no clear association between blood pressure reduction and the risk for ESRD [34]. These results are in contrast with the large studies cited above [29, 30].

A possible explanation of this phenomenon is that the individual presence of hypertension, obesity or other components of MS may not be sufficient to induce renal damage. In other words, the presence of more than one factor may be necessary to promote renal disease in patients with hypertension. In this line, Priscilla Kincaid-Smith proposed in 2004 that obesity and insulin resistance determine the risk for CKD in patients with hypertension [35]. Thus, hypertension without the context of obesity and insulin resistance would not be associated with renal damage.

In line with this hypothesis, in almost 75.000 patients followed for a median period of 21 years, blood pressure levels and BMI combined induced a higher risk for ESRD [36]. Also, the risk increased in prehypertensive subjects but only in those with obesity. In 2005, Gerald Reaven showed in 258 lean, overweight, and obese subjects without diabetes in whom insulin resistance was evaluated with a reference procedure that only 30% of the insulin-resistant subjects were overweight or obese [37]. These results clearly indicate that not all overweight/obese subjects are insulin resistant and, consequently, at risk for cardiovascular and renal diseases. Thus, insulin resistance may help to discriminate those obese and hypertensive patients at the highest risk for these complications. Further support to this hypothesis came for a Japanese study of 3136 participants followed during 8 years which evaluated the different impacts of obesity without (metabolically healthy obesity) or within the context of MS (metabolically unhealthy obesity) in the incidence of CKD [38]. Interestingly, only obesity within the context of MS was associated with a higher risk for CKD (RR 2.8). Thus, as shown for hypertension, in obesity, the risk for renal disease is promoted by other metabolic factors such as hypertension, dyslipidemia, prediabetes, etc.

In line with the above evidence, the risk for CKD increases with the number of components of the MS. Kurella et al, in about 10,000 nondiabetic subjects with normal kidney function followed during a mean period of 9 years, observed that MS portended a 40% increase in the risk for CKD [39]. The risk increased with the number of MS components, from an odd ratio of 1.16 (one component) to 1.75 (three components) and 2.45 (five components) [39]. Similar results were confirmed in a recent meta-analysis [11]. Then, for the relationship between obesity, hypertension, prediabetes, and dyslipidemia and renal disease, the sum of the parts (metabolic syndrome) is more important than the individual effect of each component.

Impact of Metabolic Syndrome in Diabetic Renal Disease

During the last 15 years, several studies reported that a subgroup of patients with T2DM may develop CKD (GFR < 60 ml/min) or even advanced renal disease (GFR < 30 ml/min) in the stages of normo- or microalbuminuria [40]. In accordance with these studies, about 50% of the cases with T2DM and CKD may not have proteinuria [41, 42]. Thus, renal function loss may start before the onset of proteinuria or even in the absence of proteinuria in patients with T2DM [40–42]. In example, in 600 diabetic patients with normo- or microalbuminuria followed with measured GFR every 6 months during a period of 4 years, mean GFR decline was 3.37 ml/min/year/1.73m^2 [23]. This rate of decline is three times faster than GFR decline in the general population, which averages 1 ml/min/y. This is in contrast with the classic definition of diabetic nephropathy in consecutive stages characterized by normoalbuminuria, microalbuminuria, and overt proteinuria, in which high levels of GFR (glomerular hyperfiltration) are expected during the first two stages and start to decline with the onset of proteinuria. So, based on recent studies, two possible

phenotypes of renal function loss in patients with T2DM can be defined: the proteinuric "classic" (diabetic nephropathy) and the non-proteinuric phenotype.

The pathogenesis of this non-proteinuric phenotype is not clearly understood. Similar to the observation for the nondiabetic population, overweight, obesity, systolic blood pressure, pulse pressure, and high triglyceride levels have been associated with the risk of CKD in patients with T2DM and without proteinuria [40]. Thus, the components of MS may also play a role in renal function loss in patients with T2DM. Interestingly, female gender has been consistently observed as a risk factor for CKD in patients with T2DM in the normo- or microalbuminuric stages [40]. This is in contrast with studies in nondiabetic renal diseases like membranous or IgA nephropathy, acquired dominant polycystic kidney disease, or even in the general population, in which men have faster GFR decline than women [43]. The pathogenic background of this association is unknown. However, it has to be noticed that in the context of T2DM, women have a cardiovascular risk profile that is exacerbated after menopause [44–47]. Diabetic women have higher post-load glucose levels after oral glucose tolerance test and dyslipidemia (high levels of triglyceride and low levels HDL cholesterol) than men. Also, the change in lipid accumulation after menopause changes from gluteal to abdominal deposits and is associated to increased insulin resistance and inflammation. In fact, the risk for cardiovascular disease highly increases after menopause [48, 49]. Thus, it is plausible that the changes in MS traits observed after menopause may promote accelerated renal function loss in women with diabetes in the absence of proteinuria. This new area of diabetes-related renal disease deserves further study.

Renal Histology

Obesity-Related Glomerulopathy (ORG)

In view of the epidemic proportion of overweight and obesity in recent decades, the glomerular changes in obese patients have called much attention. The numbers of ORG diagnosed have increased steadily over the last decades, and it has been detected in 2.5% renal biopsies in a recent retrospective study [14, 17], while only 0.2% of biopsies during 1986–1990 had ORG features. But the true prevalence of ORG is hard to guess because the percentage of the obese population subjected to renal biopsy is quite variable.

Pathological changes in the glomeruli of obese patients were first reported in autopsies [13] and consisted of glomerular hypertrophy and segmental sclerosis, which are still today the main features of ORG.

The diameter of a glomerulus in a normal adult is usually in the range 110–276 um [50]. In obese individuals with ORG, the diameter of glomeruli was determined to be about 30% larger (Fig. 5.1a, b), when compared to age- and gender-matched controls, while the glomerular volume was only modestly increased in the autopsy-examined kidneys from overweight or obese persons without renal disease [51]. In

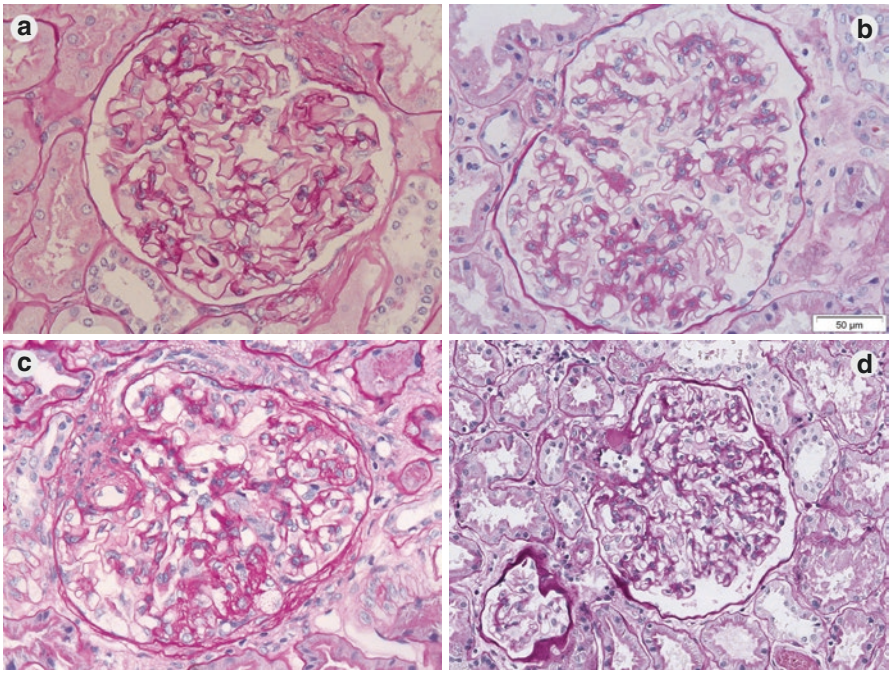

Fig. 5.1 Renal histology features in subjects with obesity and obesity-related glomerulopathy. Normal glomeruli (**a**), glomerulomegaly (**b**), focal and segmental sclerosis (**c**), and increased mesangial expansion "diabetoid changes" (**d**). (Courtesy of Rosa Rodriguez and Eduardo Salido)

addition, the number of glomeruli per area unit (glomerular density) was found diminished to 1.7 ± 0.6/mm^2 (vs. 3.1 ± 1.0 mm^2, in age-matched kidney donor controls). However, an analysis of autopsy cases without renal disease found no significant differences among nonobese, overweight, and obese individuals. In other words, glomerulomegaly and reduced glomerular density are not universal features of kidneys in obese patients but seem significant only in those obese patients that develop ORG.

Autopsy studies have shown a large variability in nephron number in normal populations [52]. A relationship between obesity and increased GFR has been known for years [53], and glomerular hyperfiltration in obese individuals has been proposed as the main driver of the typical ORG glomerular lesions (glomerulomegaly and FSGS) by a mechanism similar to that proposed for situations with reduced renal mass states [54]. Thus, a difference in original nephron number may be related to the pathogenesis of renal injury in ORG [55, 56].

High blood pressure is quite prevalent in the obese individuals [56], and the link between essential hypertension and lower glomerular density is well accepted [57]. In fact, hypertension has been detected in up to 65% of patients with ORG patients [51]. Thus, hypertension is somehow associated to the reduced glomerular density observed in ORG. Biopsies with ORG also tend to exhibit more severe arteriosclerosis, most

likely related to the prevalent high blood pressure in these patients, but fewer sclerotic glomeruli than control patients with idiopathic FSGS [14].

Segmental sclerosis in ORG is quite similar to that found in any other form of focal segmental glomerulosclerosis (FSGS) (Fig. 5.1c), but the severity of the lesions and the abundance of abnormal glomeruli are considerably milder than in primary FSGS or other types of secondary FSGS. As such, the segmental sclerosis found in ORG resembles the milder forms of FSGS, particularly the perihilar variant of the "Columbia classification" [58], with increased diameter of the afferent arteriole and perihilar hyalinosis. As it can be inferred from the current consideration of FSGS as a form of podocytopathy, podocyte damage or insufficiency is a central feature in ORG also. Podocyte numbers seem to be relatively fixed early on, and subsequent enlargement of glomerular volume cannot be matched by a corresponding increase in the number of podocytes, resulting in a relative deficiency of podocytes that could be the structural basis for loss of ultrafiltration capability. In ORG patients, the level of proteinuria has been associated with decreased podocyte number and podocyte density, and increased foot-process width [59]. However, podocyte effacement of foot processes is less severe in ORG than in other forms of FSGS [14].

Overweight and Metabolic Syndrome

Little evidence is available about renal histology in patients with MS, since renal biopsies are seldom performed in these patients because proteinuria is not a frequent marker of renal disease in MS. Alexander et al. analyzed 12 patients with and 12 without MS in whom a total nephrectomy was performed due to a kidney cancer [60]. The unaffected renal tissue was evaluated, and those patients with MS showed a higher prevalence of global and segmental sclerosis (although of borderline signification), tubular atrophy, interstitial fibrosis, and arteriolar hyalinosis than subjects without MS. Thus, it is plausible that tubular and interstitial damage play a role in renal disease and MS. However, the low number of patients limits the conclusion of this study. More evidence is needed to understand the renal histology and then the pathogenesis of renal disease in patients with MS.

"Diabetoid" Changes in Obesity: Common Links Between Obesity and Diabetic Nephropathy

Since the first description of ORG, it was observed that about 50% of the cases have an abnormal increase in mesangial matrix deposition (Fig. 5.1d) as well as thickening of glomerular and tubular basement membranes [14, 17]. These alterations are typical of diabetic nephropathy. However, most of the patients with ORG in the original studies did not have overt diabetes. Features of diabetic nephropathy can be observed in patients without diabetes, showing that renal disease may eventually

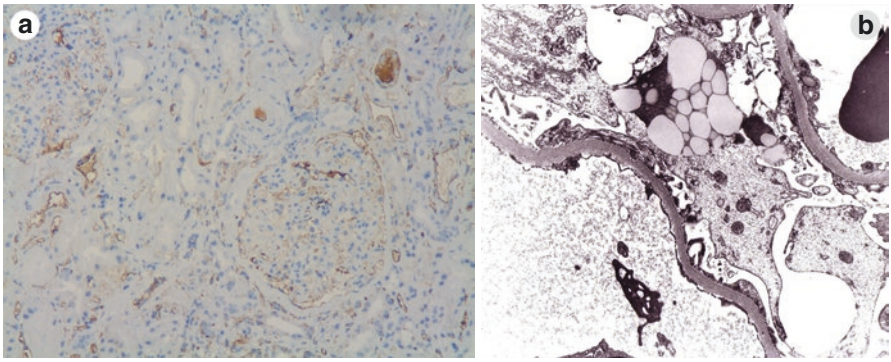

Fig. 5.2 Lipid deposits in renal tissue in subjects with obesity and obesity-related glomerulopathy. Lipid droplets in glomerular and tubular cells evaluated by adipophilin staining (**a**) and electronic microscopy (uranyl acetate and lead citrate stain, ×4000) Figure b also shows foot effacement in podocytes (**b**). (Courtesy of Maruja Navarro and Rosa Rodriguez)

precede the development of the disease [61]. Also, advanced renal histology like nodular sclerosis can be observed early in T2DM, in patients with normoalbuminuria [62]. These findings since may indicate common pathways of renal disease between obesity, overweight, MS, and T2DM. It is plausible to speculate that glomerular hyperfiltration, renal hemodynamic changes, inflammation, lipotoxicity, and albuminuria which are observed in MS and T2DM are implicated in renal disease in both entities, leading to similar patterns of renal histology. However, we acknowledge that there is not enough evidence for this hypothesis, which is worth investigating (Fig. 5.2).

Pathogenesis

Hemodynamic Changes

In the early stage of obesity, alterations in renal hemodynamics include hypercirculation and glomerular hyperfiltration, particularly in the presence of hypertension [53, 63]. Glomerulomegaly is the structural correlate of glomerular hyperfiltration which can be associated with glomerulosclerosis and proteinuria in obesity [64]. The mechanism by which obesity-related glomerular hyperfiltration occurs is not completely understood. Two diverse theories try to explain this phenomenon. The hemodynamic hypothesis postulates preglomerular vasodilation involving the afferent arteriole as the primary event [53]. Patients with severe obesity have increased renal plasma flow, compared with lean subjects, suggesting afferent arteriole vasodilation. Thus, the transmission of increased arterial pressure to the glomerular capillaries through a dilated afferent arteriole, particularly in the presence of hypertension, could account for a high transcapillary hydraulic pressure difference

resulting in an elevated glomerular filtration rate [53]. Dogs fed with a high-calorie diet had increased mean arterial blood pressure and glomerular hyperfiltration [65]. Moreover, in this model, histological changes including enlargement of Bowman's space, glomerular cell proliferation, thickening of glomerular and tubular basement membrane, increased glomerular mesangial matrix, and glomerular TGF-beta expression are probably a direct consequence of glomerular hyperfiltration [65]. In humans, obesity-related glomerular hyperfiltration is associated with proximal tubular epithelial hypertrophy and increased glomerular and tubular urinary space volume in subjects with proteinuria [64].

The other theory proposes that an altered glomerular-tubular feedback is caused by enhanced sodium reabsorption in the proximal tubule as the principal event that determines hyperfiltration [63, 66]. According to this theory, leptin, through activation of the sympathetic nervous system as well as direct effects on angiotensin II and insulin, could contribute to the increase in sodium reabsorption [63].

In animal models of early diabetic renal disease, proximal tubular hypertrophy an increased proximal tubular sodium reabsorption play a central role in the pathogenesis of glomerular hyperfiltration [67]. So, these similarities in the pathogenesis of glomerular hyperfiltration in diabetes and obesity support the theory that both entities may represent a continuum of metabolic alterations.

Some studies performed in obese nondiabetic patients in whom glomerular hyperfiltration was evaluated before and after undergoing bariatric surgery have shown that obesity-related hyperfiltration is reversible following weight loss [21, 25]. So, the improvement of this renal hemodynamic abnormalities following weight loss supports a cause-and–effect relationship between obesity and glomerular hyperfiltration.

Inflammation

The adipocyte is a source of several hormones (adipokines) that promote insulin resistance and vascular injury with potential effects in the kidney [68]. The deregulation of adipokines in obesity may mediate obesity-related comorbidities. Abdominal or central fat is closely associated with renal functional impairment possibly because it is the main source of adipokines compared with subcutaneous fat [17, 69]. In fact, central obesity is the most important factor that predisposes individuals to insulin resistance and hyperinsulinemia, favoring the development of metabolic syndrome and T2DM [70]. Proinflammatory adipokines such as interleukine-6, tumor necrosis factor-alpha, C-reactive protein, resistin, or leptin are elevated in obesity, whereas the levels of adiponectin or insulin-like growth factor-1 are reduced [55, 71, 72].

Leptin is synthesized mainly in adipocites, and its increase in obesity is proportional to the amount of adipose tissue [73]. Despite elevated levels of leptin, obese patients typically exhibit resistance to leptin. The kidneys contain leptin receptors that are present mainly in the medulla. Although the functions of these receptors are

not well understood, experimental studies have demonstrated that leptin exerts a fibrogenic effect by increasing the expression of glomerular transforming growth factor-beta-1 leading to glomerulosclerosis and proteinuria [74].

Adiponectin has insulin-sensitizing, anti-inflammatory and anti-atherogenic properties. Obesity is associated with hypoadiponectinemia, which has been linked to insulin resistance, cardiovascular disease and glomerular injury. Fetuin-A, which is a glycoprotein synthesized exclusively in the liver, that promotes insulin resistance and downregulation of adiponectin synthesis by the adipocytes [75]. Experimental studies have demonstrated that adiponectin deficiency in mice is associated with podocyte effacement [76]. These changes are accompanied by albuminuria and do not occur in mice without adiponectin deficit [77]. The administration of adiponectin to mice reduces podocyte damage and leads to the partial resolution of albuminuria [77]. Thus, all those therapeutic interventions that decrease leptin and increase of adiponectin, such as losing weight, will help to prevent or improve renal disease in obesity [7].

Another adipokine that is elevated in obesity is aldosterone, a hormone that regulates electrolyte metabolism on the distal tubule and is part of the renin-angiotensin axis. However, aldosterone is also secreted by adipocytes and can contribute ORG by inducing hyperfiltration and direct podocyte damage by the production of reactive oxygen species [77]. This has been demonstrated in a rat model of MS (spontaneously hypertensive rats) that develops obesity as a result of nonsense mutation in the leptin receptor gene [77]. These rats have high levels of aldosterone and podocyte damage evidenced by foot process effacement, induction of desmin, and attenuation of nephrin. Finally, the administration of eplerenone, an inhibitor of aldosterone in obese rats improved proteinuria and podocyte damage.

Insulin-like growth hormone-1 (IGF-1) levels are decreased in obesity, which may be related with ORG [78]. Wu and colleagues have demonstrated the differential expression of genes related to inflammatory cytokines, lipid metabolism, and insulin resistance in kidney biopsies from obese patients with proteinuria ORG [79]. This altered gene expression was not observed in biopsies from kidney donors. The evidence above may indicate the existence of a relationship between lipid dysmetabolism, insulin resistance, and subclinical inflammatory and ORG. However, further investigation is needed to elucidate the importance of inflammation in ORG.

Renal Lipotoxicity

One of the characteristics of obesity is ectopic lipid accumulation, which indicates lipid deposits in diverse organs different from fat tissue [80]. The classic example of ectopic fat accumulation is nonalcoholic fatty liver disease (NAFLD). Untreated, NAFLD may evolve into NASH (nonalcoholic steatohepatitis) and to cirrhosis. In fact, NAFLD is the most common cause of chronic liver disease worldwide [81]. In 1982, Moorhead proposed that fat deposition in the kidney may also induce renal damage and eventually CKD [82]. Since then, several studies, mainly from basic research, showed the consequences of lipid deposits in diverse renal cells [82, 83]. In

example, mesangial cells exposed to high concentrations of lipids may transform into foam cells and lose their contractile function [84, 85]. Considering that mesangial cells are pericytes, lipid accumulation may determine changes in the integrity of the capillary loop and promote glomerulomegaly. Lipid deposit in podocytes may induce podocyte insulin resistance and apoptosis by the activation of the mTOR pathway [86–89]. In obesity, there is an overflow of nonesterified fatty acids (NEFA) that will deposit in diverse tissues, including the kidney [80]. Importantly, the increased levels of NEFA that characterize obesity reach the tubular cell bound to albumin. Thus, tubular reabsorption of albumin implies also the reabsorption of NEFA, which are metabolized and stored in intracellular lipid droplets [90, 91]. The latter may be used as an accumulation of energy but eventually may induce lipotoxicity, inflammation, and fibrosis [92]. Evidence linking lipid deposits in human renal tissue and its consequences is, however, scarce. Bobulescu et al. observed an increased content of triglycerides in human renal cortex in obese subjects [92]. However, the link between lipid deposits in human renal tissue and evidence of chronic renal damage (atrophy and fibrosis) is lacking, which is worth investigating.

Conclusions

Obesity, particularly in the context of metabolic syndrome and insulin resistance, is a risk factor for accelerated renal function loss and CKD. The pathogenesis of this relationship is not completely known. ORG, which is characterized by glomerulomegaly with or without focal and segmental glomerular sclerosis, is the most common renal complication in obese subjects with CKD or proteinuria. Interestingly, half of the patients with ORG have diabetoid changes (mesangial expansion) in the absence of overt diabetes, indicating common pathways between diabetic nephropathy and renal disease in obesity. Less evidence is available on renal histology in patients with moderate obesity and metabolic syndrome.

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Part II
Pathophysiology and Clinical Pathology of the Diabetic Kidney

Chapter 6
Introduction to Pathogenetic Mechanisms of Diabetic Nephropathy

Liffert Vogt and Joris J. Roelofs

The natural history of diabetes consists of development of complications to various organ systems, including the kidney. Appearance of these diabetic complications becomes more likely as the years of diabetes duration have passed. Changes of the smallest blood vessels, i.e., microangiopathy, cause not only proteinuria and loss of kidney function (diabetic nephropathy) but also damage to the retinal vasculature (diabetic retinopathy) and neuronal damage due to changes of the endoneuronal and perineuronal vessels (diabetic neuropathy). Furthermore, premature atherosclerosis, i.e., macroangiopathy, causes a higher incidence of coronary artery disease, cerebrovascular disease, and peripheral artery disease, including renal artery stenosis, which in turn may affect the kidney. These phenomena occur both in type 1 diabetes mellitus and type 2 diabetes mellitus, but in the latter group, it is often seen that many of the late diabetic complications are already present when type 2 diabetes becomes manifest. This indicates that the duration of glucose intolerance is a common denominator in the development of damage to the various end organs.

Until today, however, no sound explanation for development of diabetic complications is present. The same applies for the risk of diabetic nephropathy develop-

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ment in the natural history of the disease. This chapter provides a general introduction to Part II, *Pathophysiology and Clinical Pathology of the Diabetic Kidney*, where all potential factors contributing to diabetic nephropathy in a systematic and comprehensive way will be discussed.

Epidemiology and Pathophysiology of Diabetic Nephropathy

Clinical observations have been demonstrated to be useful in the identification of causal factors leading to diabetic nephropathy. Only until a few decades ago, diabetic nephropathy caused end-stage renal failure and death within a mean duration of 7 years. Since then the importance of metabolic control of diabetes, treatment of the frequently present hypertension, and dietary measures, such as dietary protein and sodium restriction, have been recognized, as these have contributed to significant improvement of the prognosis of diabetic nephropathy patients. Indeed, the incidence of diabetic nephropathy has importantly diminished in the last decades. In the Netherlands, the incidence of diabetic nephropathy nowadays is estimated at approximately 10% of all diabetes patients.

The beneficial effects of improved metabolic control emphasize the role of elevated plasma glucose levels in development and progression of diabetic nephropathy. Indeed, both in animal and clinical research, high plasma glucose levels have been related to diabetic complications in general and to worse long-term outcomes. On the cellular level, hyperglycemia induces disturbances of cell metabolism via perturbation of the intracellular redox potential, activation of protein kinase C, and accumulation of diaglycerol. In addition, due to elevated glycosylation, the function of various proteins is altered in diabetes, and increased generation of advanced glycosylation end products is a feature of diabetes mellitus. All these derangements have been shown to contribute to the development of diabetic kidney disease.

In clinical practice, also other risk factors not related to glycemic control play an important role in diabetic nephropathy. For instance, smoking accelerates development and progression of both renal microangiopathy and macroangiopathy. Also, hypertension, which is present in 75% of the type 2 diabetes patients, is pivotal in both the development and prognosis of diabetic nephropathy. In type 1 diabetes, hypertension also contributes to worse outcomes but mostly presents itself when early features of diabetic nephropathy emerge. Disturbances of coagulation and hemostasis may relate to endothelial dysfunction as observed in diabetes but also contribute to (1) the progress of microangiopathic and macroangiopathic complications and subsequent renal damage and (2) the development of diabetic nephropathy in a direct causal fashion. This will be discussed in Chap. 17. Finally, dyslipidemia, again present in most type 2 diabetes patients, not only affects the renal artery but is also related to microangiopathy. In type 1 diabetes, dyslipidemia seems not very prevalent in uncomplicated diabetes, but becomes an important risk factor once diabetic nephropathy is present.

Pathology

The main histological findings in diabetic nephropathy consist of thickening of the glomerular basement membrane, followed by deposition of increasing amounts of extracellular matrix (ECM) in the mesangial areas, either in a diffuse or nodular fashion. ECM deposits presenting as nodular mesangial sclerosis are often described as Kimmelstiel-Wilson nodules. Another type of characteristic lesions of diabetic nephropathy results from accumulation of hyaline material, as a result of plasma protein exudation. These include arteriolar hyalinosis, typically of both the afferent and efferent arteriole. Further, hyaline deposits can occur in the inner side of Bowman's capsule, the so-called capsular drops. Later in the course of diabetic nephropathy, extensive tubulointerstitial lesions may develop. A detailed description of the histopathology of diabetic nephropathy, including a discussion about the mechanisms leading to the typical lesions in the kidney, will follow in Chap. 8. The finding in both animal experiments and clinical practice that many of the structural lesions disappear after pancreas transplantation or transplantation of Langerhans' islets emphasizes once again the importance of metabolic control in the development of diabetic nephropathy.

Genetic Factors of Diabetic Nephropathy

As mentioned above, it seems that diabetic nephropathy cannot develop without hyperglycemia. Yet, in many patients with uncontrolled diabetes, diabetic nephropathy may not be present, indicating that additional factors are of importance in developing diabetic kidney damage. For instance, the presence of a genetic predisposition contributes in the development of the disease, but diabetic nephropathy does not develop in the absence hyperglycemia. The genetic background will be discussed in detail in Chap. 7. While simple Mendelian inheritance does not occur, both familial clustering of diabetic nephropathy and the role of ethnic background in the susceptibility to diabetic nephropathy demonstrate the relevance of multigenetic predisposition in the pathogenesis of the disease. In addition, the contribution of various hereditary factors related to susceptibility to high blood pressure development as well as premature atherosclerosis to diabetic nephropathy shows that both hypertension and atherosclerosis are key factors in its pathogenesis. This will be discussed in Chaps. 20 and 22.

Observations from Interventional Studies in Type 1 Diabetes

The importance of hypertension in diabetic nephropathy was demonstrated for the first time by H.-H. Parving [1]. He showed that treatment of elevated blood pressure retards the progression of diabetic nephropathy. The monthly decrease

of GFR diminished from 0.94 mL/min to 0.10 mL/min. The observation that also proteinuria decreased in this study opened the way to study the causal role of proteinuria in the course of the disease. Generally, proteinuria in diabetic nephropathy is interpreted as a reflection of increased permeability of the glomerular filtration barrier, which consists of the glomerular endothelium, the glomerular basal membrane, and podocytes. Perturbations in the function of these cells, as well as mesangial cells, are discussed in Chaps. 9, 10, and 11. Proteinuria does also develop due to renal hemodynamic alterations, as described in Chap. 18. Due to both a disturbed autoregulatory response of the afferent glomerular arteriole in conjunction with an activated renin-angiotensin system (RAS), the intraglomerular hydrostatic pressure increases—a phenomenon often referred to as "hyperfiltration" that contributes to increased filtration of plasma proteins. In addition, glomerular inflammation, as reflected by increased macrophage invasion, is believed to play a causal role in progressive glomerular damage and proteinuria and is discussed in Chap. 12.

The reason why proteinuria contributes to progressive renal function loss is still subject to debate. Proteinuria leads to additional damage to the podocyte and the slit diaphragm between its foot processes, thus allowing progression from microalbuminuria to overt nephropathy coinciding with the loss of selectivity of proteinuria. Subsequent interactions with the downstream tubular cells elicit tubulointerstitial damage and loss of functioning nephrons. This prevailing concept, i.e., tubulotoxicity of proteinuria, is described in Chap. 13. Only very recent data indicate that this concept may need revision as diabetes-induced tubular damage by itself has been demonstrated to affect glomerular function and to contribute to proteinuria development [2]. The tubulo-glomerular communication may entail a paradigm shift in our understanding of the pathogenesis of diabetic nephropathy and is discussed in Chap. 14. Tubulointerstitial involvement is not only related to proteinuria, hyperglycemia, and glycated proteins but also to myofibroblast accumulation, excessive extracellular matrix deposition, and renal tubular destruction, i.e., the key features of tubulointerstitial fibrosis. A comprehensive overview of the mechanisms involved in tubulointerstitial fibrosis is given in Chap. 15.

In type 1 diabetic patients, microalbuminuria and proteinuria very frequently occur with hypertension, retinopathy, and neuropathy. This illustrates that microangiopathy is not limited to the kidneys in type 1 diabetes. Chapter 16 discusses the central role of microcirculatory changes in the origination and progression of diabetic nephropathy, and Chap. 19 describes the mechanisms that diabetic retinopathy and nephropathy share. In overtly proteinuric patients, the risk of cardiovascular death is three to ten times higher as opposed to non-proteinuric diabetic patients. This indicates that advanced kidney damage, in addition to classical cardiovascular risk factors, plays a causal role in the development of diabetic macroangiopathy.

Observations from Type 2 Diabetes

Data concerning the pathogenesis of type 2 diabetic nephropathy are less clear, perhaps explained by the highly multifactorial nature of type 2 as opposed to type 1 diabetes mellitus. Nevertheless, the crucial role of hypertension and proteinuria causing progression of type 2 diabetic nephropathy has been widely accepted. Unlike in type 1 diabetes, data on the natural course of type 2 diabetic nephropathy is, however, limited. An explanation for the lack of data is that many patients do not develop end-stage renal disease as they prematurely die from macroangiopathic events, such as myocardial infarction or stroke. In general, it is thought that GFR decline is similar to type 1 diabetic nephropathy and that microalbuminuria precedes development of overt nephropathy. The involvement of the RAS in progression of the disease is supported by clinical data showing that pharmacological RAS inhibition lowers the risk of end-stage disease, independent from blood pressure effects. Also, the importance of glycemic control has been widely accepted in type 2 diabetic nephropathy, and recent data support the notion that dyslipemia exerts a causal role in proteinuria and GFR decline. Macroangiopathic changes to the renal artery also affect the kidney, but do not always involve the typical pathogenetic mechanisms as observed in diabetic nephropathy. This is further discussed in Chap. 21.

Conclusive Remarks

This chapter has only briefly touched upon the complex nature of the pathogenetic mechanisms involved in diabetic nephropathy. Considering the burden of this disease worldwide, comprehensive understanding of each underlying mechanisms and downstream pathways as well as cross talks between these mechanisms is mandatory to prevention of diabetic nephropathy and development of novel therapeutic options. In the next chapters of Part II, the individual pathogenetic components are, therefore, discussed in more detail.

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Chapter 7
The Genetics of Diabetic Nephropathy

Marcus G. Pezzolesi and Andrzej S. Krolewski

Introduction

Diabetic nephropathy (DN) is the major late complication of diabetes that affects approximately 40% of all patients with diabetes and remains the leading cause of end-stage renal disease (ESRD) in the United States [1–3]. As the global incidence of diabetes continues to rise, so too has the incidence of DN and both the personal and societal burdens associated with this complication.

DN is a complex disease with a multifactorial etiology. Not all individuals with diabetes develop DN, however; this suggests that hyperglycemia in and of itself is not sufficient to elicit renal damage. In addition to known risk factors for DN, including glycemic control, blood pressure, lipid levels, obesity, and duration of diabetes, investigations on the familial clustering of DN and the heritability of this disease and its related traits provide compelling evidence that genetic factors also contribute to its susceptibility.

Over the past two decades, tremendous progress has been made in the identification of genetic factors for DN (Fig. 7.1). Family-based linkage studies, which test the co-segregation of DN with genetic markers across chromosomes, have mapped this phenotype to specific chromosomal regions, including loci on chromosomes 2p, 3q, 7p, 18q, and 22q. Fine-mapping has helped narrow the candidate regions at

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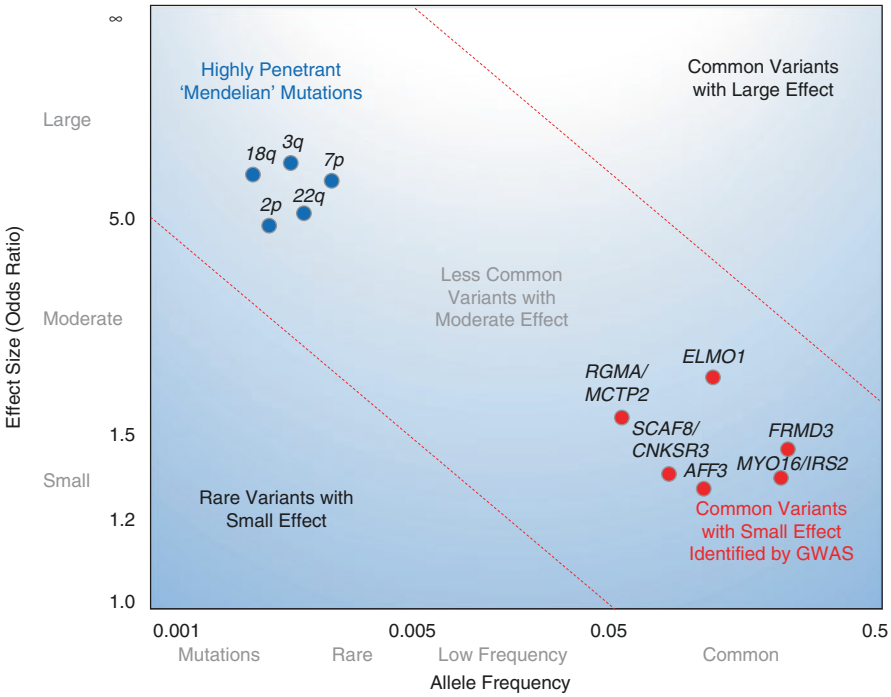

Fig. 7.1 Summary of the genetics of DN. Family-based linkage studies of DN, which test the co-segregation of DN with genetic markers across chromosomes, have mapped this phenotype to specific chromosomal regions, including loci on chromosomes 2p, 3q, 7p, 18q, and 22q. These signals suggest that highly penetrant Mendelian genes containing rare genetic variants with large effect sizes exist at these loci. GWASs, which test for associations with common variants between unrelated case and control subjects, have identified several additional loci that are associated with DN, including the identification of associations at *ELMO1*, *FRMD3*, *MYO16*/*IRS2*, and *AFF3*. In contrast to the loci identified through linkage studies, variants underlying these associations have small effects on disease risk

these loci, highlighting several potential genes including the angiotensin II type 1 receptor gene (*AGTR1*) on chromosome 3q and the carnosinase 1 (*CNDP1*) gene on chromosome 18q. Importantly, by virtue of the approach used to identify these loci, these signals suggest that highly penetrant Mendelian genes containing rare genetic variants with large effect sizes exist at these loci. Using a parallel approach that tests for associations with common variants between unrelated case and control subjects, genome-wide association studies (GWASs) have identified several additional loci that are associated with DN, including the identification of reproducible associations at the engulfment and cell motility 1 (*ELMO1*) and FERM domain containing 3 (*FRMD3*) loci in multiple studies and across various populations. In contrast to the loci identified through linkage studies, these associations have more modest effects on DN risk, an observation that is consistent with a complex pattern of inheritance in which variants at multiple loci likely contribute to increased susceptibility.

In this chapter, we review evidence supporting a genetic basis for DN, discuss efforts to identify the genes that underlie its risk, and present the major findings of this area to research to date.

Familial Clustering of DN

Evidence of familial clustering, or familial aggregation, supports the notion that genetic factors play a major role in the susceptibility of DN (Table 7.1) [4–11]. In the earliest investigation of familial clustering of DN, Seaquist et al. examined the risk of DN among 2 sets of families with type 1 diabetes (T1D): 11 with probands who were free of DN and 26 with probands who had undergone kidney transplantation due to DN [4].

Table 7.1 Summary of studies supporting the familial clustering of DN

Reference	Population	Number of families and index cases	Prevalence of DN in sibling/relatives	OR
Seaquist et al. [4]	Caucasian, T1D	37 sib pairs; w/ ESRD: $n = 26$	83%	24
		w/o DN: $n = 11$	17%	
Borch-Johnsen et al. [5]	Caucasian, T1D	49 sib pairs; w/ DN: $n = 20$	33%	4.9
		w/o DN: $n = 29$	10%	
Quinn et al. [6]	Caucasian, T1D	110 sib pairs; w/ DN: $n = 38$	72%	2.5
		w/o DN: $n = 72$	25%	
DCCT [7]	Caucasian, T1D	114 extended families; w/ DN: $n = 13$	61%	5.3
		w/o DN: $n = 101$	28%	
Pettitt et al. [8]	Pima Indians, T2D	316 extended families; Offspring w/ 0 proteinuric parents: $n = 247$	14%	Ref.
		Offspring w/ 1 proteinuric parents: $n = 240$	23%	1.8
		Offspring w/ 2 proteinuric parents: $n = 12$	46%	2.5
Freedman et al. [9]	African-American, T2D	97 extended families; w/ ESRD: $n = 52$	37%	8.1
		w/o ESRD: $n = 45$	7%	
Faronato et al. [10]	Caucasian, T2D	134 sib pairs; AER (+): $n = 56$	45%	3.9
		AER (−): $n = 78$	17%	
Canani et al. [11]	Caucasian, T2D	90 sib pairs; w/ DN: $n = 41$	53%	3.8
		w/o DN: $n = 49$	26%	

T1D type 1 diabetes, *T2D* type 2 diabetes, *DN* diabetic nephropathy, *ESRD* end-stage renal disease, *AER* albumin excretion rate, *Ref.* reference

Interestingly, they found that while only 17% of diabetic siblings of probands without DN had evidence of DN, defined as overt proteinuria, 83% of diabetic siblings of probands with DN went on to develop DN. The reported odds ratio for siblings of probands with overt proteinuria relative to siblings with normoalbuminuria in this study was 24.0, suggesting that the risk of DN to siblings of patients with DN was 24 times greater than that to siblings of patients without this complication. However, because of its small sample size and potential ascertainment bias of concordant siblings in this study, it's likely that the estimated relative odds of DN reported in this study is inflated. Nonetheless, increased risk of DN to family members of patients with DN has been confirmed in several studies since this initial report.

In a study of 49 T1D patients (20 with DN, defined as urinary albumin excretion greater than 300 mg/24 h, and 29 without DN) attending the Steno Diabetes Center in Denmark and having diabetic siblings, 33% of siblings to T1D patients with nephropathy had DN [5]. In contrast, DN was only observed in 10% of siblings to normoalbuminuric patients in this study. In a study of 110 families identified through Joslin Clinic patients, the cumulative risk of advanced DN (i.e., persistent proteinuria or end-stage renal disease (ESRD)) in siblings after 25 years of T1D was 72% if the proband had persistent proteinuria but only 25% if the proband did not: a difference of nearly 50% in the risk of DN to T1D siblings that is dependent on the proband's renal status [6]. Comparably, in families of 372 T1D subjects from the Diabetes Control and Complications Trial (DCCT), a 5.4-fold increased risk of DN was observed in relatives of DN-positive subjects compared to DN-negative subjects [7].

Familial clustering of DN has also been observed in families with type 2 diabetes (T2D) [8–11]. Pettitt et al. examined the risk of proteinuria among 316 Pima Indian families with diabetes in 2 generations. In this study, the risk of proteinuria among diabetic offspring with a parent with proteinuria was 1.8 times higher than that of offspring of diabetic parents without proteinuria [8]. The adjusted prevalence of proteinuria among individuals with one diabetic parent with proteinuria was 23% compared to only 14% among offspring with two diabetic parents with normoalbuminuria. The prevalence of proteinuria among offspring with two diabetic parents with proteinuria was even greater, with 46% of these individuals having this complication. In 52 multigenerational African-American families, Freedman et al. found that 37% of T2D-induced ESRD patients had either first-, second-, or third-degree relative with ESRD, compared to only 7% of T2D controls [9]. Diabetic individuals from families with a relative with ESRD were at an eightfold increased risk of developing ESRD. Studies by Faronato et al. and Canani et al. similarly demonstrated that T2D siblings of probands with DN from Caucasian families had three to four times the risk of developing micro- and macroalbuminuria compared to sibling of normoalbuminuric probands [10, 11].

Heritability of DN and DN-Related Traits

In addition to investigations of the familial aggregation of DN, several studies have estimated the heritability (h^2), or the proportion of total variation due to genetic effects, of DN and several DN-related traits (e.g., urinary albumin excretion rate

Table 7.2 Heritability (h^2) of DN and DN-related traits

Reference	Population	Number of families/ samples	DN-related traits	h^2
Forsblom et al. [12]	Caucasians, T2D	267 nuclear families	AER	0.30
Fogarty et al. [13]	Caucasians, T2D	96 extended families (630 individuals)	ACR	0.31
Langefeld et al. [14]	Caucasians, T2D	310 extended families (662 individuals)	ACR	0.46
			eGFR	0.75
Krolewski et al. [15]	Caucasians, T2D	63 extended families (426 individuals)	ACR	0.23
Placha et al. [16]	Caucasians, T2D	63 extended families (406 individuals)	eGFR	0.45
Sandholm et al. [17]	Caucasians, T1D	1925 T1D individuals	AER	0.38
Sandholm et al. [18]	Caucasians, T1D	1820–2843 T1D individuals	Microalbuminuria	0.03
			Combined DN	0.34
			Macroalbuminuria and ESRD	0.51
			ESRD	0.54

Combined DN (microalbuminuria, macroalbuminuria, and ESRD)
T1D type 1 diabetes, *T2D* type 2 diabetes, *DN* diabetic nephropathy, *ESRD* end-stage renal disease, *ACR* albumin-to-creatinine ratio, *eGFR* estimated glomerular filtration rate, *AER* albumin excretion rate

(AER) and estimated glomerular filtration rate (eGFR)) in families with diabetes (Table 7.2) [12–18].

In 96 large, multigenerational families that included 630 individuals with T2D and 639 individuals with normoglycemia enrolled in the Joslin Study on the Genetics of Type 2 Diabetes, Fogarty et al. estimated that 27% of the variance in albumin-to-creatinine ratio (ACR) was genetically determined among all family members, irrespective of their diabetes status [13]. In analyses restricted to diabetic individuals, this estimate rose slightly to 31%, and, supporting previous reports of familial clustering of AER among nondiabetic family members, h^2 was estimated to be 0.20 in nondiabetic individuals from this collection.

A subsequent analysis of the Joslin Study on the Genetics of Type 2 Diabetes collection restricted to families with a middle-age at onset of T2D reported similar estimates of heritability with ACR, ranging from 0.20 in all family members to 0.39 in relative without diabetes [15]. An important strength of the Joslin T2D family collection is that its members were ascertained for studies on the genetics of T2D, not kidney complications. As such, these estimates of heritability are unlikely to be biased due to an enrichment of DN cases. Reinforcing the estimates obtained from this collection, Forsblom et al. and Langefeld et al. reported similar heritability for AER in 267 nuclear T2D families from Finland ($h^2 = 0.30$) and for ACR in 310 T2D sibling pairs from the United States ($h^2 = 0.46$ in T2D members and $h^2 = 0.35$ in all family members) [12, 14].

To evaluate the possible mode of inheritance of ACR in families with T2D, we performed a formal quantitative segregation analysis of this trait in members of the

Joslin Study on the Genetics of Type 2 Diabetes collection [19]. In this analysis, evidence for the genetic effects on ACR was derived from its transmission between relatives across large pedigrees, an approach that provides substantial power in assessing this effect over studies limited to nuclear families. In models where the genetic effect was assessed separately in all members and in T2D members alone, the model that most completely described the control of ACR levels in these pedigrees combined the effects of at least one major locus (with a relatively common allele frequency between 0.25 and 0.40) with significant residual genetic variation that could be due to multiple other genetic factors. These results are consistent with a previous segregation analysis of overt nephropathy by Imperatore et al. that also supported the existence of a major DN gene with a common allele frequency in a collection of 715 nuclear Pima Indian families [20]. Additionally, Sandholm et al. reported that genetic variants explain 38% of the AER heritability in 1925 unrelated patients with T1D [17].

eGFR has also been shown to be a heritable trait in families with diabetes [14, 16]. The first study to investigate this estimated the heritability of eGFR to be 0.75 among Caucasian T2D sibling pairs and 0.69 in analyses that included all available family members [14]. Similarly, we found eGFR to be highly heritable in T2D subjects (h^2 ranging from 0.29 to 0.47) and all family members (h^2 ranging from 0.28 to 0.31) from the Joslin Study on the Genetics of Type 2 Diabetes collection [16]. To date, no formal segregation analysis of eGFR in T2D has been reported.

Most recently, Sandholm et al. estimated the heritability of several dichotomous DN phenotypes, including "combined" DN (defined as microalbuminuria, macroalbuminuria, or ESRD) and ESRD alone in 1820–2843 unrelated T1D patients in the Finnish Diabetic Nephropathy (FinnDiane) Study [18]. While heritability estimates varied widely across comparisons (h^2 ranging from 0.35 for combined DKD to 0.51 for advanced DN, defined as macroalbuminuria and ESRD), this study confirmed that DN is highly heritable among T1D patients. Importantly, as the estimated heritability for early DN (i.e., microalbuminuria) was only 0.03, this study suggests that genetic factors contribute most to the more advanced stages of DN.

Linkage-Based Efforts to Uncover the Genetic Basis of DN

Evidence of familial aggregation and the heritability of DN and its related traits provide compelling evidence that the increased risk of nephropathy in diabetes among affected family members is, as least in part, due to shared genes or set of genes among relatives. These studies have motivated research efforts over the past two decades to uncover the specific genes that contribute to this risk.

The earliest efforts to identify these genetic factors employed a classic gene mapping approach, termed family-based linkage analysis, that aims to identify shared chromosomal regions that co-segregated with DN in affected family members using a set of highly polymorphic variable number tandem repeats (VNTRs) or, more recently, single nucleotide polymorphism (SNP) markers [21]. Such analyses

produce a statistical estimate called the logarithm (base 10) of odds, or LOD score, that tests whether a genetic marker is concordant with a trait of interest and the extent to which genetic linkage exists between the two. In doing so, linkage analysis is able to aid in mapping traits to a particular chromosomal region. In general, significant evidence of linkage is considered once a LOD score exceeds 3.0.

In complex diseases, like DN, linkage analysis offers a model-free, or nonparametric, approach which is advantageous when the underlying genetic model is not known. Linkage-based approaches, however, are limited in both the magnitude of the genetic effects that they are able to detect and the resolution with which they are able to pinpoint susceptibility loci. Overall, linkage studies have been most successful in identifying major disease loci, i.e., those with effect sizes 2.0 or greater, and typically localizing evidence of linkage to regions spanning several megabase pair (Mb) in length and containing tens to hundreds of potential candidate disease genes.

Evidence of linkage has been reported across nearly all 22 autosomes (Table 7.3). Consistent linkage with DN and DN-related traits across multiple studies, however,

Table 7.3 Loci with significant evidence of linkage with DN and DN-related traits (MLS ≥ 3.0, $P < 1.0 \times 10^{-5}$)

Chr	Position (Mb)[a]	MLS/*p*-value	Phenotype	Population	Study design	Reference
1	11.68–36.22	3.81	eGFR, T2D	Caucasian and African-American	Extended families	Freedman et al. [22]
1	235.89–244.06	3.78	eGFR, T2D	Mexican-American	Sib pair	Schelling et al. [23]
2	44.85–66.75	4.31	eGFR, T2D	Caucasian and African-American	Extended families	Freedman et al. [22]
2	50.84–68.24	3.02	eGFR, T2D	Mexican-American	Sib pair	Schelling et al. [23]
2	195.29–213.29	4.1	eGFR, T2D relatives	Primarily Caucasian (94%)	Extended families	Placha et al. [16]
3	102.18–117.41	4.55	Early-onset ESRD, T2D	African-American	Sib pair	Bowden et al. [24][b]
3	139.19–167.24	3.1	DN, T1D	Caucasian	Sib pair	Moczulski et al. [25]
5	41.03–67.23	3.4	ACR, T2D	Caucasian	Extended families	Krolewski et al. [15]
7	6.03–26.03	4.0	eGFR, T2D, all relative-pairs	Primarily Caucasian (94%)	Extended families	Placha et al. [16]
7	82.79–96.06	6.0×10^{-5}	Proteinuria/ESRD, T2D	African-American	Sib pair primarily	Iyengar et al. [26]
7	151.57–154.57	4.23	eGFR, T2D	Mexican-American	Sib pair	Schelling et al. [23]
7	151.57–155.99	3.1	ACR, T2D	Primarily Caucasian (94%)	Extended families	Krolewski et al. [15]

(continued)

Table 7.3 (continued)

Chr	Position (Mb)[a]	MLS/*p*-value	Phenotype	Population	Study design	Reference
8	66.07–87.17	8.7×10^{-6}	eGFR, T2D	Mexican-American	Sib pair	Schelling et al. [23]
10	85.01–101.01	3.6	eGFR, T2D	Caucasian	Extended families	Placha et al. [16]
14	54.37–70.22	2.0×10^{-5}	Proteinuria/ESRD, T2D	American-Indian	Primarily Sib pair	Iyengar et al. [26]
16	76.04–87.94	3.56	Creatinine clearance, T2D	West African	Sib pair	Chen et al. [27]
18	58.82–73.11	3.72	ESRD, early-onset T2D	African-American	Sib pair	Bowden et al. [24][b]
18	70.80–75.10	6.1	Proteinuria, T2D	Turkish	Extended families	Vardarli et al. [28]
18	73.11–77.85	6.4×10^{-6}	eGFR, T2D	Mexican-American	Sib pair	Schelling et al. [23]
19	6.11	3.13	ESRD, late-onset T2D	African-American	Sib pair	Bowden et al. [24][b]
19	55.69–59.10	3.1	DN, T1D	Caucasian (95%)	Sib pair	Rogus et al. [29]
22	28.86–36.75	3.7	ACR, T2D	Caucasian (94%)	Extended families	Krolewski et al. [15]

MLS maximum LOD score, *T1D* type 1 diabetes, *T2D* type 2 diabetes, *DN* diabetic nephropathy, *ESRD* end-stage renal disease, *ACR* albumin-to-creatinine ratio, *eGFR* estimated glomerular filtration rate, *OSA* ordered subset analysis

[a]Approximate positions for reported LOD-1 intervals are provided in megabase pairs (Mb) relative to NCBI Build GRCh37/hg19. For studies not reporting LOD-1 intervals, the approximated position of the peak/flanking markers is provided if available

[b]Bowden et al. [24] also identified 1 significant (LOD = 3.59, chromosome 7p) and 2 suggestive loci (LOD = 2.94, chromosome 12 and LOD = 2.85, chromosome 16) in OSA among ESRD patients with long duration of DM; however these findings could potentially be spurious [30]

has been localized to only a modest subset of potential candidate loci, with signals on chromosomes 2p, 3q, 7q, and 18q emerging as the most promising candidates implicated to harbor major DN susceptibility genes (Fig. 7.1). In particular, linkage reported near the *AGTR1* gene, a component of the renin-angiotensin pathway, on chromosome 3q has been of particular interest. Moczulski et al. first reported evidence of linkage to this region (maximum LOD score (MLS) = 3.1 located only 15 kilobase pair (kb) downstream of *AGTR1*) in a linkage scan of 3 candidate regions involving 66 T1D discordant sib pairs from the Joslin Diabetes Center [25]. This signal was replicated by Osterholm et al. [31] and Bowden et al. [24] in genome-wide linkage scans of 83 Finnish T1D sibling pairs and 48 African-American families with T2D, respectively. Fine-mapping of this locus in T1D patients from Finland, Iceland, and the British Isles consequently identified a strong association in a noncoding region approximately 10 Mb centromeric of the original linkage peak [32]. Although the gene underlying this linkage signal is unclear, these efforts further reinforce the likelihood that this region harbors a gene (or genes) that

contributes to the risk of DN while also suggesting that this susceptibility locus is likely common to both T1D and T2D.

A second locus with consistent support of linkage to DN was identified on chromosome 18q in 18 extended Turkish families with 115 members with T2D [28]. Evidence of linkage to this same region was shown in both African-American T2D patients with ESRD [24] and with eGFR in 378 multiethnic families from the Family Investigation of Nephropathy and Diabetes (FIND) collection [23]. In the FIND study, the findings at this locus were primarily driven by Mexican-American families who comprised 52% of the families enrolled in this collection. Resequencing efforts in 135 T2D DN cases and 107 T2D non-DN controls later identified a significant association at a trinucleotide VNTR in exon 2 of *CNDP1* that is composed of five, six, or seven repeated leucine residues [33]. In this study, the five-leucine allele was present in only 59% of chromosomes in patients with DN compared to 88% in those without DN. Functional studies confirmed the protective effects of this allele by demonstrating its ability to inhibit the production of extracellular matrix components in cultured human podocytes exposed to high glucose. Similarly, transforming growth factor-beta (TGF-β) production was reduced in cultured mesangial cells exposed to high glucose. Additional support of this polymorphism's role in DN was provided by its association in 858 European-American subjects (294 ESRD patients with T2D, 258 T2D controls, and 306 healthy controls) [34].

Lastly, consistent significant evidence of linkage with DN phenotypes has also been observed at loci on chromosomes 7p and 22q [15, 16, 24, 35]. On chromosome 7p, significant linkage (MLS = 3.6) was first reported in an ordered subset analysis of African-American families with ESRD patients and a long duration of T2D [24]. In a study by Placha et al., a linkage scan for genes controlling variation in eGFR in 406 T2D and 428 nondiabetic members from 63 extended families in the Joslin Study on the Genetics of Type 2 Diabetes collection identified strong evidence for linkage at this same region (MLS = 4.0) [16]. Most recently, suggestive evidence for linkage on chromosome 7p (MLS = 2.81) has also been reported in an expanded linkage scan of the FIND collection that now includes 1235 multiethnic T2D families [35]. In the Joslin collection, a second scan for regions linked with variation in urinary albumin excretion found significant linkage on chromosome 22q (MLS = 3.7) [15]. Support for this region has also recently been confirmed in Mexican-American families from the FIND collection [35].

On chromosome 22q, the non-muscle myosin heavy chain 9 gene (*MYH9*), located less than 14 kb downstream of the apolipoprotein L1 (*APOL1*) gene, which has been shown to be a major gene for nondiabetic kidney disease among African-Americans [36–38] and is expressed in both glomerular podocytes and mesangial cells, represents a particularly interesting candidate gene [39, 40]. As first demonstrated by Freedman et al., *MYH9* SNPs appear to contribute to the risk of nephropathy in African-American T2D patients [41]. In this study, a comparison of 751 ESRD patients with clinically diagnosed T2D and 227 T2D controls identified significant associations at 3 *MYH9* SNPs (rs4821480, rs2032487, and rs4821481). In a subsequent study, these same variants trended toward an association with ESRD in 536 T2D cases and 467 T2D controls of European-American

ancestry [42]. These observations, however, were not confirmed in a recent study of T2D patients from the United Kingdom [43]. An important distinction between the study by McKnight et al. and the two previous reports is that the former examined these associations in T2D nephropathy patients with CKD; less than 100 of whom had ESRD. In consideration of this fact and the support garnered from multiple linkage studies of DN in T2D, continued investigation of the role of variants in the *MYH9* region in DN is warranted.

Mapping Genes for DN in the Genome-Wide Association Study (GWAS) Era

The completion of the Human Genome Project in 2001, an international scientific effort to sequence and map all 3.3 billion nucleotides that make up the entire human genome [44, 45], and the International HapMap Project's (www.hapmap.org) haplotype map of the block-like structure of human genome shortly thereafter [46], coupled with advances in affordable, high-throughput genotyping technology, ushered in a new era of disease gene mapping that, for the first time, allowed researchers to comprehensively and simultaneously interrogated hundreds of thousands to more than one million genetic markers across the entire genome in thousands of patients with unprecedented accuracy. These genome-wide association studies (GWASs) focus primarily on common SNPs, with a minor allele frequency greater than 5%, and have shifted gene mapping strategies from family-based linkage approaches to population-based studies primarily centered on unrelated case and control subjects. Since the first major GWAS published by the Wellcome Trust Case Control Consortium in 2007 [47], GWASs have proven to be extremely powerful in detecting disease loci that are associated with many complex human traits and diseases, including T1D, T2D, coronary heart disease, bipolar disorder, Crohn's disease, and rheumatoid arthritis [48]. To date, GWAS-based approaches have identified more than 50,000 unique SNP-trait associations [49].

Several GWASs for DN have been conducted [17, 18, 50–62]. The major findings from these studies are summarized in Fig. 7.1 and Table 7.4.

The first reported GWAS for DN was a low-density analysis of 56,648 SNPs in 188 Japanese patients with T2D (94 cases with either proteinuria or ESRD and 94 normoalbuminuric controls) [50]. From this discovery panel, 402 SNPs were selected for replication in a larger collection that included 466 T2D DN cases and 266 T2D controls. Using this two-stage approach, one SNP located in intron 24 of the solute carrier family 12 member 3 (*SLC12A3*) gene on chromosome 16 emerged as the most strongly DN-associated SNP in these collections (p-value = 8.7×10^{-5}). Further analysis identified a SNP in *SLC12A3*'s 23rd exon that results in an amino acid substitution (arginine to glutamine at codon 913) that was strongly associated with DN (p-value = 2.0×10^{-5}).

Table 7.4 Loci identified through GWASs of DN and DN-related traits ($p < 1.0 \times 10^{-5}$)

Chr	Pos.[a]	SNP	Nearest gene	*p*-value	Effect size (OR/ beta)	DN phenotype	Population	Study design	*N* samples	*N* Samples discovery cohort	*N* markers	Reference
2	100,460,654	rs7583877	*AFF3*	1.2×10^{-8}	1.29	ESRD vs. non-ESRD	Caucasian	Case-control	11,847	6652	2.4 million	Sandholm et al. [58]
2	129,027,961	rs13427836	*HS6ST1*	6.3×10^{-7}	0.19	ACR	Population-based	Quantitative	Up to 7787	Up to 5825	2.2 million	Teumer et al. [62]
2	213,168,768	rs7588550	*ERBB4*	2.1×10^{-7}	0.66	Prot./ESRD vs. normo.	Caucasian	Case-control	11,847	6231	2.4 million	Sandholm et al. [58]
4	87,529,078	rs61277444	*PTPN13*	1.9×10^{-6}	1.41	ESRD vs. non-ESRD	Caucasian	Case-control	12,540	5150	37 million	Sandholm et al. [18]
4	87,529,078	rs61277444	*PTPN13*	6.0×10^{-6}	1.42	ESRD vs. no DKD	Caucasian	Case-control	12,540	3406	37 million	Sandholm et al. [18]
6	154,947,408	rs955333	*SCAF8/ CNKSR3*	1.3×10^{-8}	0.73	Prot./ESRD vs. normo.	Transethnic	Case-control	13,736	6197	906,600	Iyengar et al. [61]
6	154,954,420	rs12523822	*SCAF8/ CNKSR3*	5.7×10^{-9}	0.57	Prot./ESRD vs. normo.	American-Indians	Case-control	2154	857	906,600	Iyengar et al. [61]
7	29,255,470	rs39059	*CHN2/ CPVL*	5.0×10^{-6}	1.39	Prot./ESRD vs. normo.	Caucasian	Case-control	1705	–	359,193; 2.4 million	Pezzolesi et al. [55, 56]
7	36,917,995	rs741301	*ELMO1*	8.0×10^{-6}	2.67	Prot./ESRD vs. normo.	Japanese	Case-control	920	188	81,315	Shimazaki et al. [51]
7	148,141,082	rs1989248	*CNTNAP2*	6.0×10^{-7}	1.26	Micro./prot./ ESRD or eGFR<45 vs. normo.	Caucasian	Case-control	12,540	3135	37 million	Sandholm et al. [18]

(continued)

Table 7.4 (continued)

Chr	Pos.[a]	SNP	Nearest gene	*p*-value	Effect size (OR/ beta)	DN phenotype	Population	Study design	*N* samples	*N* Samples discovery cohort	*N* markers	Reference
7	148,141,082	rs1989248	*CNTNAP2*	1.8×10^{-6}	1.29	ESRD vs. no DKD	Caucasian	Case-control	12,540	3406	37 million	Sandholm et al. [18]
9	86,164,176	rs10868025	*FRMD3*	5.0×10^{-7}	1.45	Prot./ESRD vs. normo.	Caucasian	Case-control	1705	–	359,193; 2.4 million	Pezzolesi et al. [55, 56]
10	83,291,690	rs72809865	*NRG3*	7.4×10^{-6}	1.17	Micro./prot./ ESRD vs normo.	Caucasian	Case-control	12,540	5150	37 million	Sandholm et al. [18]
10	97,284,081	rs1326934	*SORBS1*	5.7×10^{-7}	0.84	Prot./ESRD vs. normo.	Caucasian	Case-control	7801	1462	11.1 million	Germain et al. [60]
11	3,060,725	rs451041	*CARS*	3.1×10^{-6}	1.36	Prot./ESRD vs. normo.	Caucasian	Case-control	1705	–	359,193; 2.4 million	Pezzolesi et al. [55, 56]
11	88,008,251	rs649529	*RAB38*	5.8×10^{-7}	−0.14	ACR	Population-based	Quantitative	Up to 7787	Up to 5825	2.2 million	Teumer et al. [62]
13	110,252,160/ 110,252,608	rs1411766/ rs17412858	*MYO16/ IRS2*	1.8×10^{-6}	1.41	Prot./ESRD vs. normo.	Caucasian	Case-control	1705	–	359,193; 2.4 million	Pezzolesi et al. [55, 56]
15	94,141,833	rs12437854	*RGMA/ MCTP2*	2.0×10^{-9}	1.80	ESRD vs. non-ESRD	Caucasian	Case-control	11,847	6652	2.4 million	Sandholm et al. [58]
22	36,708,483	rs5750250	*MHY9*	7.7×10^{-8}	1.27	Prot./ESRD vs. normo.	African-American	Case-control	6108	3221	906,600	Iyengar et al. [61]
22	36,657,432	rs136161	*APOL1*	5.2×10^{-7}	1.36	Prot./ESRD vs. normo.	African-American	Case-control	6108	3221	906,600	Iyengar et al. [61]

Normo. normoalbuminuria, *Micro.* microalbuminuria, *Prot.* proteinuria, *ESRD* end-stage renal disease, *ACR* albumin-to-creatinine ratio, *eGFR* estimated glomerular filtration rate

[a]Positions for reported SNPs are relative to human reference sequence genome build GRCh37/hg19

In a second GWAS from the same investigators, Shimazaki et al. identified a strong association at rs741301 (p-value = 8.0×10^{-6}), located in intron 18 of the *ELMO1* gene on chromosome 7p [51]. Subsequent functional studies demonstrated increased expression of *ELMO1* in the presence of high glucose [51, 63]. Supporting its potential role in the pathogenesis of DN, ELMO1 has also been shown to contribute to the progression of chronic glomerular injury by promoting excess TGF-β, collagen type 1, fibronectin, and integin-linked kinase expression and dysregulation of renal extracellular matrix (ECM) metabolism.

Since the initial report by Shimazaki et al., other variants at the *ELMO1* locus have been shown to be associated with DN in multiple independent collections [64–66]. Confirmation of *ELMO1*'s potential role in the susceptibility of DN was first demonstrated in a study by Leak et al. that identified strong associations between multiple variants located in intron 13 of *ELMO1* and ESRD in two large African-American cohorts with T2D [66]. Variants located in intron 13 were also associated with overt proteinuria in a family-based study of Pima Indians with T2D [64]. Of note, while statistically significant, the associations observed in Pima Indians were in the opposite direction of those observed in African-Americans. Additionally, in a comprehensive investigation of variants across this locus using GWAS data from the Genetics of Kidneys in Diabetes (GoKinD) collections, we further established *ELMO1*'s role in conferring increased susceptibility to DN in T1D by demonstrating that *ELMO1* variants are also associated with its risk in Caucasian T1D patients [65]. The strongest associations in this study mapped to intron 16 of *ELMO1*.

Importantly, each of these studies is consistent with *ELMO1*'s role in DN and suggests that extensive allelic heterogeneity, contributed by the diverse ancestral genetic backgrounds of the different ethnic groups examined in each of these studies, exists across this locus. It is likely that rare polymorphisms in *ELMO1*, either the same variants or variants in strong or complete linkage disequilibrium, may be common to each ethnic group and merely tagged by the common variants identified in each study. Further investigation of rare SNPs at the *ELMO1* locus is likely necessary to fully understand the commonality of these associations and to elucidate the mechanism(s) underlying their role in DN.

Using a pooling approach, in which genomic DNA from pools of 105 Pima Indians with T2D and ESRD and 102 Pima Indians with T2D and either normoalbuminuria or microalbuminuria were examined, Hanson et al. identified strong associations at variants in the plasmacytoma variant translocation (*PVT1*) gene on chromosome 8 [53]. Subsequent fine-mapping of this locus revealed the strongest evidence for association at rs2648875 (p-value = 2.0×10^{-6}) located in intron 8 of *PVT1*. Confirmation of this association was later shown by Millis et al. in a subset of patients with ESRD from the GoKinD collections [67].

In a GWAS sponsored by the Juvenile Diabetes Research Foundation (JDRF), the National Institutes of Diabetes and Digestive and Kidney Disease (NIDDK), and the Centers for Disease Control and Prevention (CDC) in T1D participants of the GoKinD study [68], we identified 13 SNPs located in 4 genomic loci that were strongly associated with DN with p-values $<1 \times 10^{-5}$ [55, 56]. The strongest association occurred on chromosome 9q with rs10868025 (p-value = 5.0×10^{-7}), a SNP

located near the 5′-end of the *FRMD3* gene. Three additional genomic regions located on chromosomes 7p, 11p, and 13q were also associated with DN. On chromosome 7p, rs39059 localizes to the first intron of *CHN2* (beta chimerin) isoform 2 and upstream of an alternatively spliced *CPVL* (serine carboxypeptidase vitellogenic-like) transcript. On chromosome 11p, rs451041 is located in an intronic region of the *CARS* (cysteinyl-tRNA synthetase) gene. And on chromosome 13q, the region bounded by rs1411766/rs1742858 is located approximately 384 kilobase pair (kb) telomeric to the myosin heavy chain Myr 8 (*MYO16*) gene and 120 kb centromeric to the insulin receptor substrate 2 (*IRS2*) gene. Interestingly, significant evidence of linkage was recently identified at this same locus in studies of patients with nondiabetic ESRD and all-cause ESRD [69, 70]. The imputation of ungenotyped SNPs at each of these loci, using population haplotype data available from the HapMap Project and the MACH software program (www.sph.umich.edu/csg/abecasis/MACH), identified 11 additional SNPs that were highly correlated with the original associations, including three variants that were more strongly associated with DN than our lead genotyped SNPs at their respective loci (rs1888747 on chromosome 9q and rs39075 and rs39076 on chromosome 7p).

Importantly, three loci identified in this GWAS have been confirmed in multiple diverse collections of T1D and T2D patients [55, 71–75]. Evidence of replication of the associations at the 9q, 11p, and 13q loci with severe nephropathy was first observed among Caucasian participants with >19,000 person-years of follow-up from the DCCT/Epidemiology of Diabetes Interventions and Complications (EDIC) study [55]. Examination of SNPs at these loci in four independent cohorts of Japanese patients with T2D provided additional support of the association at chromosome 13q, suggesting that this region may harbor a susceptibility locus common in both TD and T2D nephropathy [71]. Further expanding support at this locus, we reported a significant association at rs1411766 in 646 normoalbuminuric controls and in 743 nephropathy patients of European ancestry from the Joslin Study of Genetics of Nephropathy in Type 2 Diabetes collection [72]. Meta-analysis of these data with those of the Japanese and GoKinD collections significantly improved the strength of the association (p-value = 9.7×10^{-9}).

Similarly, in addition to confirmation in the DCCT/EDIC study, associations at common variants on the 9q locus have also been confirmed in both the Joslin Study of Genetics of Nephropathy in Type 2 Diabetes collection and in African-Americans with ESRD due to T2D [73–75]. Importantly, Martini et al. later identified a potential homeodomain factor (HOMF) transcription factor binding site at the lead SNP (rs1888747) at the *FRMD3* locus that affects protein binding in glomerular extracts from C57 black 6 mice, providing evidence that this SNP likely has a functional role in DN susceptibility [76]. Thus, associations identified in the GoKinD collections on chromosomes 9q (near the *FRMD3* gene), 11p (near the *CARS* gene), and 13q (within an intergenic region between *MYO16* and *IRS2*) appear to be true susceptibility loci of kidney disease common to both T1D and T2D.

The first GWAS for DN in African-Americans individuals with and without T2D identified several novel regions with evidence of association with T2D-associated ESRD [57]. As part of their approach, McDonough et al. used comparisons of T2D-

ESRD cases and nondiabetic, non-nephropathy controls to identify 67 candidate SNPs that were then genotyped in T2D controls to discriminate between T2D-ESRD loci and T2D loci. In combined analyses of 1674 T2D ESRD cases and 1719 non-T2D controls, a total of five loci achieved p-values $<1.0 \times 10^{-5}$. Among these, rs9493454 at AU RNA binding protein/enoyl-CoA hydratase (*AUH*) on chromosome 9 and rs7735506 at ribosomal protein S12 (*RPS12*) on chromosome 6 were most strongly associated with DN in comparisons between T2D-ESRD cases and T2D non-DN controls.

In conjunction with a linkage analysis for diabetic renal failure and albuminuria, Igo et al. performed a "sparse" genome-wide association scan of DN and ACR in the FIND collection using ~5500 SNPs from their linkage panel [35]. In this study, the strongest association with DN was observed on chromosome 18 in the American-Indian subgroup (rs1241893; p-value $= 3.0 \times 10^{-5}$). On chromosome 11, associations with ACR were found at rs722317 in both European-American and Mexican-American samples (combined p-value of 7.3×10^{-5}). Recently, a complete transethnic GWAS that included >900,000 SNPs and more than 13,000 unrelated individuals of European-American, African-American, Mexican-American, or Indian-American ancestry was completed in this same collection [61]. In this analysis, genome-wide significance, i.e., a p-value $<5 \times 10^{-8}$, was observed in an ethnic group-specific analysis of Mexican-Americans at rs12523822 on chromosome 6q between the SR-like carboxyl-terminal domain-associated factor 8 (*SCAF8*) and connector enhancer of KSR family of scaffold protein (*CNKSR3*) genes. This same signal was also strongly associated with DN in this study's transethnic meta-analysis. Interestingly, strong associations were also observed on chromosome 22q near the *APOL1* and *MYH9* genes; however, after adjusting for the *APOL1* G1 and G2 variants that are strongly associated with nondiabetic nephropathy in African-Americans [37, 77, 78], these variants accounted for this association, likely due to the inclusion of unrecognized nondiabetic kidney disease among cases included in this analysis.

As part of the GEnetics of Nephropathy: an International Effort (GENIE) consortium, Sandholm et al. performed a meta-analysis of GWASs of T1D DN in 6691 individuals using 2.4 million SNPs [58]. Additional genotyping of the 41 top-ranked SNPs identified in this discovery cohort in 5873 additional individuals revealed genome-wide significant associations with ESRD at rs7583877 in the AF4/FMR2 family member 3 (*AFF3*) gene and at an intergenic SNP (rs12437854) on chromosome 15q between the RGM domain family, member A (*RGMA*) and multiple C2 domains, transmembrane 2 (*MCTP2*) genes (p-values $= 1.2 \times 10^{-8}$ and 2.0×10^{-9}, respectively). Although the biological relevance of these genes in DN are not well known, functional analyses performed by Sandholm et al. suggest that *AFF3* may play a role in transforming growth factor-β1 (TBF-β1)-induced fibrotic responses of epithelial cells and, thereby, influences renal tubule fibrosis, a pathological hallmark of severe DN.

As done for findings that emerged from the GoKinD GWAS, Maeda et al. also performed a replication study of the top associations reported in the GENIE study using 2300 Japanese patients with T2D; however, none of the signals derived from

the European T1D cohorts examined in GENIE were replicated in these Japanese patients [79]. Although a significant association of rs7588550 in *ERBB4*, a locus suggestively associated with DN in GENIE, was observed with DN, the effect direction was not consistent with that in the GENIE study (p-value = 0.0126 and odds ratio (OR) = 1.26 versus p-value = 2.1×10^{-7} and odds ratio (OR) = 0.66, relative to the G allele of rs7588550). This same trend for the association of rs7588550 with DN was also observed in an independent Japanese cohort (596 nephropathy cases and 311 controls). No evidence of association of the SNPs at the *AFF3* and *RGMA*/*MCTP2* loci was present in these Japanese cohorts.

As a follow-up to their initial GWAS, members of the GENIE consortium from the FinnDiane Study performed a sex-specific GWAS on the risk of ESRD in 3652 Finnish men and women with T1D separately [59]. Although no sex difference was found for the SNPs that were previously reported to be associated with ESRD in the GENIE study, interestingly, a novel variant on chromosome 2q (rs4972593) between the Sp3 transcription factor (*SP3*) and the cell division cycle associated 7 (*CDCA7*) genes was associated with ESRD in women (p-value = 3.0×10^{-8}) but not in men (p-value = 0.77). Replication efforts in samples from the Ireland-Warren 3-Genetics of Kidneys in Diabetes UK (UK-ROI) and GoKinD studies and an Italian cohort further supported this association (replication p-values, women = 0.02 and men = 0.90). No evidence of this association was seen among African-American, European-American, or Mexican-American women with T2D from the FIND study.

Most recently, this GWAS effort by the GENIE consortium was expanded to include both a more comprehensive set of genetic variants (~37 million SNPs) and a larger number of subjects (12,540 individuals); however, in this analysis, no variant reached genome-wide significance despite [18]. The top signals from this study were observed at SNPs near the contactin-associated protein-like 2 (*CNTNAP2*) gene (p-value = 6.0×10^{-7}) and in the protein tyrosine phosphatase, non-receptor type 13 (*PTPN13*) gene (p-value = 1.9×10^{-6}). Interestingly, in examining whether *SP3* and *CDCA7* exhibit sex-specific differential gene expression, Sandholm et al. found that *SP3* was among the top sex-specific differentially expressed genes in glomeruli, with significantly higher expression of *SP3* occurring in women than in men.

A multistage GWAS by Germain et al. identified the sorbin and SH3 domain containing 1 (*SORBS1*) gene as a new candidate gene for DN in patients with T1D [60]. Among 4 cohorts included in this analysis, including a discovery cohort made up of 683 DN cases and 779 non-DN controls and participants of the GoKinD, UK-ROI, and FinnDiane studies, rs13626934, located in an intronic region of *SORBS1*, achieved a p-value of 5.9×10^{-7} in a combined meta-analysis of these 4 cohorts. However, signal at this locus was not observed in the FinnDiane cohort, perhaps due to different patterns of allelic heterogeneity and linkage disequilibrium across *SORBS1* in the various European populations included in this analysis. In a meta-analysis excluding patients from FinnDiane, strong evidence of this association was observed among the remaining cohorts (p-value = 2.4×10^{-8}). Functionally, *SORBS1* has been shown to be differentially upregulated in glomeruli of rats with DN compared to rats without DN and to be expressed in tubules and glomeruli in human kidney samples from patients with T2D, supporting a potential role for this gene in DN [80].

GWASs for quantitative DN-related traits have also been reported [17, 62]. Although a strong association was identified by Sandholm et al. among 1925 T1D from the FinnDiane Study at the glycine receptor subunit α-3 (*GLRA3*) gene (p-value = 1.5×10^{-9}; beta = 0.21), this association was not confirmed in a meta-analysis of 7 independent cohorts. This cohort achieved nominal significance; however, this association was in the opposite direction to that in the discovery cohort (beta = −0.02), resulting in a nonsignificant combined p-value = 0.30 in the meta-analysis of the discovery and replication cohorts. This study also examined whether a previously reported association with ACR in the cubilin (*CUBN*) gene in nondiabetic patients [81] was associated with this trait in diabetic individuals but found no evidence of this association. Similarly, although Teumer et al. reported genome-wide significance at CUBN in their GWAS of ACR in more than 54,000 individuals, no evidence of association was seen when these analyses were restricted to individuals with diabetes [62]. Among >7000 diabetic patients, however, novel associations with ACR were observed at both the RAB38, member RAS oncogene family (*RAB38*, p-value = 5.8×10^{-7}), and heparan sulfate 6-O-sulfotransferase 1 (*HS6ST1*, p-value = 6.3×10^{-7}) genes. Combined evidence from both Rab38 knockout rats, which show increased urinary albumin concentrations relative to controls, and gene expression data in diabetic kidney disease patients, which show high *RAB38* expression in tubuli compared with control subjects, implicate *RAB38* as a gene involved in albuminuria in humans.

Lessons Learned from GWASs of DN

Over the last decade, a great deal of progress has been made in the identification of novel genetic factors for DN. Despite this success, however, much work remains. While GWASs have yielded several significant findings, few loci have reached genome-wide significance, and fewer have been shown to be consistently associated with DN. Nonetheless, given strong evidence of its heritability and the apparent polygenic nature of this complex disease, it is clear that additional genetic loci responsible for susceptibility to DN remain to be identified.

Evaluating the studies to date that have investigated the genetics of DN, it is clear that several obstacles have limited progress in this field. Recently, these challenges have been highlighted in a number of editorials and reviews on the state of this area of research [82–85]. From these commentaries, several common themes have emerged. Specifically, the studies that have been conducted thus far have largely been underpowered, have suffered from poor phenotype definitions that have included a mosaic of DN definitions, and have been limited in their focus by primarily surveying common genetic variants. To improve upon these studies, it is clear that strategies moving forward need to include (*i*) increased sample sizes to detect modest genetic effects, (*ii*) improved phenotyping to reduce heterogeneity of the DN phenotype, and (*iii*) expanded searches of additional classes of susceptibility variants by studying rarer variants.

The leading researchers investigating the genetic basis of DN are mindful of these needs and are addressing these as they continue to explore the genome for variation responsible for its susceptibility. A major effort in this regard is the JDRF's Diabetic Nephropathy Collaborative Research Initiative (DNCRI), which has brought together investigators from across the globe, including those from the Joslin Diabetes Center, the GENIE consortium, the DCCT/EDIC study, and the FinnDiane Study, and assembled nearly 30,000 samples from participants with T1D. This initiative is working closely with investigators from the surrogate markers for micro- and macrovascular hard endpoints for innovative diabetes tools (SUMMIT) consortium, a large-scale effort to identify genetic and nongenetic marker for DN in T2D, to combine resources and empower DN gene discovery.

In addition to performing the largest comparison of dichotomous DN phenotypes, two parallel subprojects from the DNCRI are focusing on identifying genetic variants that are associated with longitudinal changes in renal function decline and repeated measures of renal function. As has recently been formally assessed by Chan et al., the most fruitful approach to identifying variants for DN, particularly low-frequency contributors, is to focus on more homogeneous phenotypes of this disease [86]. The approaches being implemented in these subprojects, which center on identifying genetic variation that contributes to the risk of renal function decline in DN, thereby represent the most robust efforts thus far to identify genetic factors contributing to the predominant clinical feature of DN [87].

In studies of rare forms of nondiabetic kidney disease, including nephrotic syndrome and familial focal segmental glomerulosclerosis, several rare missense and nonsense mutations have been identified in genes that play a critical role in the structure and/or proper functioning of the glomerular filtration barrier, including nephrin (*NPHS1*), podocin (*NPHS2*), actinin alpha 4 (*ACTN4*), transient receptor potential cation channel subfamily C (*TRPC6*), and phospholipase C, epsilon 1 (*PLCE1*) in families with multiple affected members [88–92]. While such progress has not yet been seen in DN, the recent emergence of next-generation sequencing technology, which allows large genomic regions (e.g., all protein coding regions of the genome, also known as whole exome sequencing) or entire genomes (i.e., whole genome sequencing) to be sequenced rapidly and accurately, is beginning to facilitate more comprehensive interrogations of DN susceptibility variants. To date, only a single study has been reported using this technology [18]. Despite leveraging extreme phenotypes that included early-onset advanced DN cases and long-duration T1D controls, no exome-wide significant associations (p-value $<5 \times 10^{-7}$) were observed. Nonetheless, this study is the first of its kind and is a major step toward interrogating low-frequency and rare disease predisposing variation that contributes to the risk of DN.

Conclusions

For more than 20 years, evidence in favor of a genetic basis contributing to an increased risk of DN in T1D and T2D has formed the foundation for studies aimed at identifying the causal genes responsible for its development. During this period,

strategies used to map genes for DN have been driven by our understanding of variation across our genome and the technologies available to interrogate it. As both have evolved, so too have our approaches. From the studies reported to date, although several candidate loci and associated variants have been identified, it is clear that no genetic markers exist that can identify those at risk of developing DN.

To advance this area of research and further drive discovery of genes for DN, studies need to take advantage of advances in next-generation sequencing technology that enable researchers to affordably and comprehensively investigate genetic variation across the entire genome, including low-frequency and rare variants that are not well-captured by current GWAS platforms. In case-control study settings, however, tens to hundreds of thousands of individuals are needed to identify disease-associated low-frequency and rare variants. Because such variants will be present at much higher frequency in affected relatives, family-based studies, on the other hand, provide us best opportunity to detect these classes of variants and their associations with DN [93]. Depending on the structure of these families, a study investigating relatively few large, multigenerational pedigrees may prove more powerful than studies involving 10,000–100,000, or more, unrelated individuals.

As important, more focused genetic research is crucial to the advancement of research on DN and a key step toward early identification of those at risk of DN and implementing targeted interventions through precision medicine. Work done by our group and others over the past few years has helped to redefine the course of kidney disease in patients with diabetes [87, 94, 95]. These studies have established a new paradigm that now recognizes progressive renal function decline, and not albuminuria, as the predominant clinical feature of this disease. As the field moves forward, family-based approaches investigating patients with rapid progression of renal function decline should greatly facilitate efforts to identify variants in genes that have a major effect on the risk of DN.

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Chapter 8
Pathology of the Kidney in Diabetes

Behzad Najafian and Charles E. Alpers

Introduction

In a classical paper published in 1936, Kimmelstiel and Wilson for the first time described mesangial expansion and nodular glomerulosclerosis in diabetic kidney disease (DKD) [1]. We have since learned much more about DKD lesions, although natural history of progression of these lesions is better known in type 1 diabetes, while majority of patients with DKD suffer from type 2 diabetes, calling for further studies in the latter population. The pathology of DKD is also more homogeneous in type 1 diabetes, and there is some controversy if all kidney lesions observed in type 2 are attributable to diabetes or they may be related to concurrent conditions such as aging, hypertension, and atherosclerosis which are commonly present in type 2 diabetic patients. For these reasons, we will initially discuss pathology of DKD in type 1 diabetic patients and then provide comparisons with type 2 diabetes.

DKD in Type 1 Diabetic Patients

The most characteristic features of DKD occur in the glomeruli and include thickening of glomerular basement membrane (GBM), accumulation of mesangial matrix, and arteriolar hyalinosis, which typically occurs in both afferent and efferent arterioles. Evolution of these changes has a well-defined sequence. Using morphometric methods, thickening of GBM is identifiable within 2 years of onset of diabetes. There is a direct and linear relationship between thickness of GBM and duration of

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diabetes [2]. In early stages of DKD, when the patients are normoalbuminuric, there is substantial overlap between GBM thickness in persons with diabetes and nondiabetic individuals which in part may be related to inter-individual variability in the rate of progression of basement membrane thickening or in baseline values of GBM thickness, which is typically unknown [3]. In a study of identical twins who were discordant for type 1 diabetes, all diabetic siblings had thicker GBM and greater mesangial expansion, estimated by the fraction of the volume of glomerulus occupied by mesangium [Vv(Mes/glom)] compared with their nondiabetic siblings. Of note, some of the values for the diabetic subjects were within the "normal range" [4]. Thus, assuming that identical twins had similar GBM thickness and Vv(Mes/glom) values at the onset of diabetes, without a knowledge about the baseline values, these changes would have not been appreciated in diabetic siblings. Age and gender should be taken into account when interpreting GBM thickness values. Ramage et al. showed that GBM thickness in nondiabetic children increases with age, from an average of about 190 nm at 1 year to 300 nm at 11 years, with a reduced rate of increase after the age of 11 years [5], and no difference between males and females in pediatric population. In adulthood, GBM becomes thicker in nondiabetic males averaging about 370 nm in nondiabetic males and 325 nm in females with slight increase in thickness observed up to the fourth decade of life and some decline afterward [6]. In comparison, in normoalbuminuric young type 1 diabetic patients with an average age of 17 years and 8 years of diabetes, the average GBM thickness was 428 nm with a direct relationship with diabetes duration which was not affected by gender [7]. In an older group of type 1 diabetic patients with an average age of about 38 years and about 25 years of diabetes, the average GBM thickness ranged from 465 nm in normoalbuminuric to 700 nm in macroalbuminuric patients [3], where only rare microalbuminuric and virtually no macroalbuminuric patients showed GBM thickness values within the normal range. When examined by transmission electron microscopy, GBM is composed of three distinct components, namely, the lamina rara externa (immediately underneath the foot processes), lamina densa (in the middle and more electron dense), and lamina rara interna (subendothelial). Thickening of GBM in DKD is primarily due to expansion of lamina densa and occurs in a diffuse and uniform fashion. However, especially in advanced DKD, rare glomerular capillaries with thin GBM can be seen. This phenomenon is hypothesized to result from new capillary formation. The hallmark of DKD is accumulation of extracellular matrix, either in the form of thickening of basement membranes or accumulation of mesangial matrix (Fig. 8.1) [8, 9]. This accumulation is related to an imbalance between synthesis, controlled by transcription and translation, and degradation of matrix components, regulated by the interplay between matrix metalloproteinases and their inhibitors [10]. GBM thickening is associated with increased densities of α_3 and α_4 chains of type IV collagen, as hyperglycemia increases production of these molecules by podocytes [11–13].

The earliest lesion of DKD which is appreciable by light microscopy, especially by periodic acid-Schiff stain, is mesangial expansion. Using morphometry, increased Vv(Mes/glom) can be detected as early as 4–5 years after the onset of diabetes [14]. In contrast to thickening of GBM which is more or less linear with increasing dia-

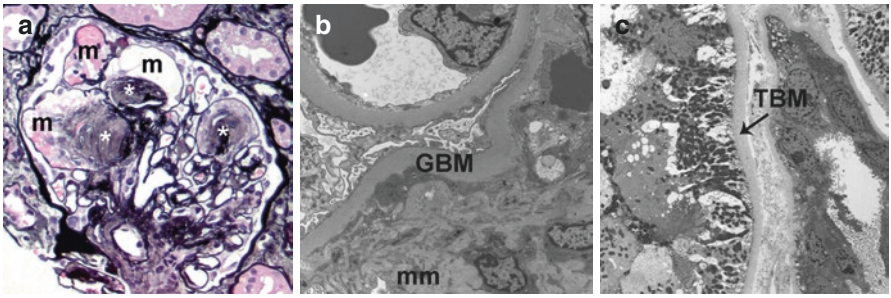

Fig. 8.1 Classical biopsy findings in DKD. (**a**) A glomerulus with nodular glomerulosclerosis or Kimmelstiel-Wilson nodules (*asterisks*) and mesangial expansion due predominantly to increased mesangial matrix and microaneurysm formation (m); Jones methenamine silver stain. (**b**) Thickening of glomerular basement membrane (GBM) and increased mesangial matrix (mm); transmission electron microscopy. (**c**) Thickening of tubular basement membranes (TBM); transmission electron microscopy

betes duration, progression of mesangial expansion is slower in the first few years after the onset of diabetes and becomes faster with increased duration of diabetes [15]. Expansion of mesangium in DKD is due primarily to increased mesangial matrix [7]. Even in the earlier stages when Vv(Mes/glom) is still within the normal range, the fraction of mesangium which is matrix [Vv(MM/glom)], as opposed to mesangial cells, is increased compared to nondiabetic subjects [15]. As mesangium expands, it protrudes into peripheral capillary walls within the subendothelial space, the so-called mesangial interposition. This leads to reduced filtration surface area. Thus, an inverse relationship exists between Vv(Mes/glom) and peripheral GBM filtration surface density [Sv(PGBM/glom)] (Fig. 8.2) [8, 16]. On the other hand, this reduction in Sv(PGBM/glom) is at least partially compensated by increased glomerular volume, preserving the total filtration surface area. Mesangial expansion can be diffuse or nodular. Fraying of the mesangial matrix leads to unfolding of the GBM, conjoining of adjacent capillary loops, and formation of microaneurysms or nodular glomerulosclerosis (so-called Kimmelstiel-Wilson nodules). The accumulated mesangial matrix in the nodules is hypocellular and may show a distinctive lamellated appearance which is best appreciated by Jones methenamine silver stain. The nodules are often surrounded by patent glomerular capillaries or microaneurysms. The microaneurysms may become sclerotic, creating large scarred nodules. Although nodular lesions typically occur in advanced DKD and at least 15 years after the onset of T1D [17, 18], occasional nodular lesions can be seen in earlier stages of DKD when the overall mesangial expansion is mild and diffuse. Therefore, in contrast to the classification proposed by Tervaert et al., the presence of nodular glomerulosclerosis does not always indicate severe DKD. It is noteworthy that nodular glomerulosclerosis is not pathognomonic to DKD and can also be seen in other conditions, perhaps again as a consequence of mesangiolysis, such as light chain deposition disease, immune complex processes, idiopathic nodular glomerulosclerosis, and chronic thrombotic microangiopathy [19].

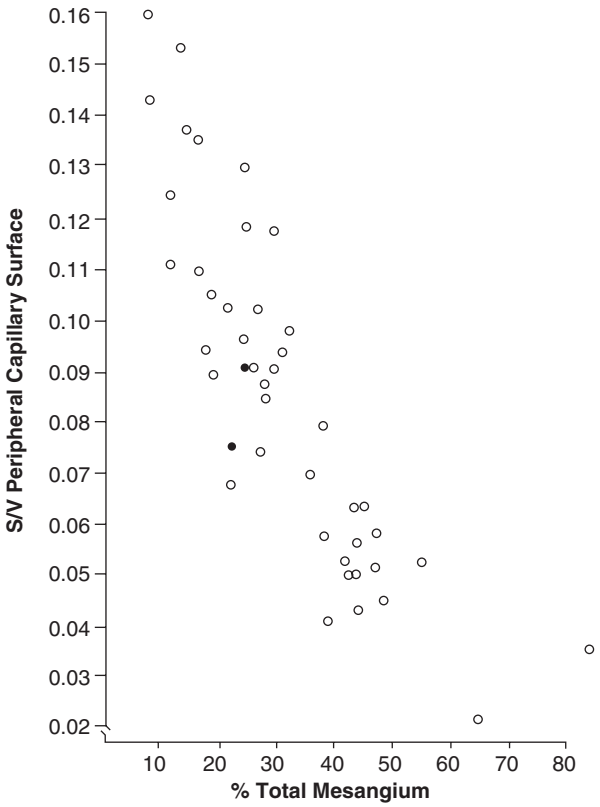

Fig. 8.2 Relationship of percentage total mesangium and S/V of the peripheral capillary surface. $r = -0.86$, $P < 0.0005$. (Figure reproduced from Ref. [8] with permission of the publisher)

Another group of characteristic lesions of DKD result from accumulation of hyaline and are referred to as exudative lesions. These include arteriolar hyalinosis, fibrin caps, and capsular drops (Fig. 8.3). Concomitant hyalinosis of afferent and efferent arterioles is almost specific to DKD and can be seen within 3–5 years after the onset of diabetes [20]. Hyalinosis starts in the subendothelial space but can expand to replace the entire media of arterioles. Some glomeruli may show multiple efferent arterioles at the vascular pole [21]. Fibrin cap, a misnomer which would more appropriately be called "hyaline cap," refers to accumulation of hyaline in glomerular capillary subendothelial spaces. Accumulation of hyaline under parietal epithelial cell lining of Bowman's capsule is called "capsular drop."

While DKD is primarily defined by accumulation of extracellular matrix and its exudative lesions, there is a body of evidence that podocyte injury plays a crucial role in progression of the diseases and kidney prognosis in diabetic patients. About 1/3 of diabetic patients with normal urine albumin excretion rate show increased nephrin excretion in the urine, indicative of early podocyte injury even before the onset of microalbuminuria [22]. Similarly, foot process effacement, commonly regarded as an evidence of podocyte injury, is detectable in normoalbuminuric

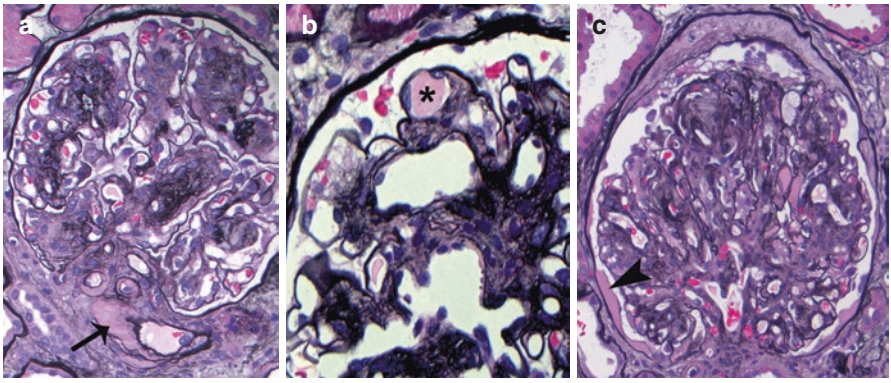

Fig. 8.3 Exudative lesions of DKD. (**a**) Arteriolar hyalinosis (*arrow*). (**b**) Fibrin cap in a glomerular capillary (*asterisk*). (**c**) Capsular drop (*arrowhead*). Jones methenamine silver stain

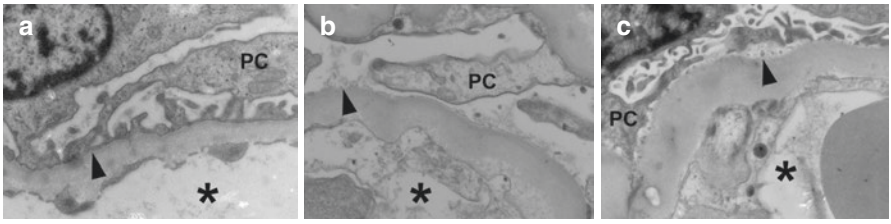

Fig. 8.4 Podocyte (PC)-GBM interfaces (*arrowheads*) are classified into areas with intact foot processes (**a**), areas with no foot process coverage (**b**), and areas with a mixture of intact and detached foot processes (**c**). *Capillary lumen. (Figure reproduced from Ref. [24] with permission of the publisher)

diabetic patients [23] and becomes more severe as albuminuria increases [24]. Various mechanisms are proposed for podocyte injury in diabetes, including reduced expression of $\alpha_3\beta_1$ integrin [25], apoptosis, glucose-induced oxidative stress, and autophagy [26, 27]. Electron microscopy studies show evidence of detachment of podocytes from GBM in normoalbuminuric patients. Similar to foot process effacement, detachment becomes more severe as albuminuria increases (Fig. 8.4) [24]. Podocyte loss and reduced density of podocytes in the glomeruli lead to secondary focal and segmental glomerulosclerosis (FSGS). Notably, FSGS is a relatively late finding in type 1 diabetic patients, when patients are commonly macroalbuminuric. There is a distinct predilection for FSGS lesions to occur at the glomerulotubular junction. A serial section study showed that over half of the FSGS lesions occur at or adjacent to the glomerular tubular outlet, consistent with tip lesion [28, 29]. Thus, it is important to realize that tip lesion is not limited to a subset of primary FSGS and can be seen in various proteinuric conditions, including DKD. A combination of increased shear stress to podocytes at the tubular pole of the glomerular tuft [30] and injury to tubular epithelial cells secondary to the tubulotoxic effect of proteinuria might be involved in predilection of FSGS to this region in conditions with heavy proteinuria [31]. Bowman's capsule thickening and duplication is a common

finding at the FSGS site, perhaps reflecting direction of part of the glomerular ultrafiltrate through into Bowman's capsule, leading to dissection of the capsular basement membrane. This dissection can extend into the glomerulotubular junction, leading to stricture and occlusion of the glomerular tubular outlet and eventually creation of atubular glomeruli (Fig. 8.5), or extend into the proximal tubule [28, 29].

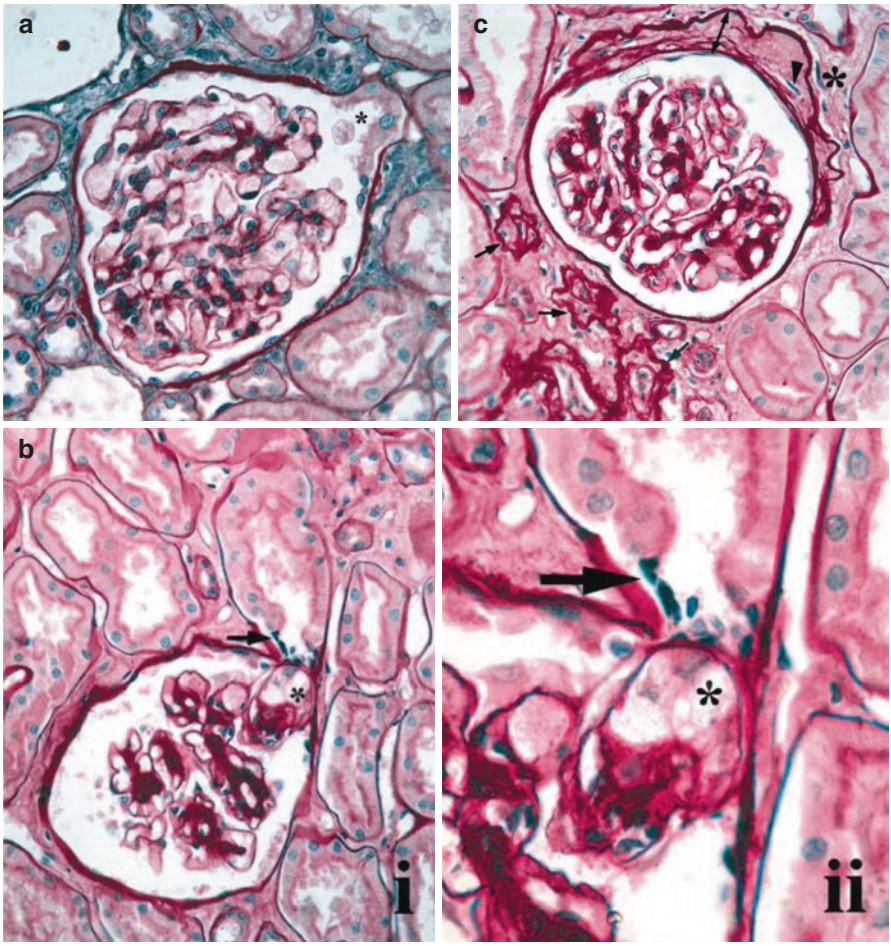

Fig. 8.5 (**a**) A glomerulus attached to a normal tubule (NT). * Glomerulotubular junction. (**b**) (i) A glomerulus attached to a short atrophic tubule (SAT), with a tip lesion at glomerulotubular junction. PAS-stained; magnification, ×630. (ii) A higher-magnification view of the tip lesion, allowing better appreciation of a dilated loop (*), with foam cells within the tip lesion and flat epithelial cells (*arrow*) covering the very beginning of the proximal tubule. (**c**) An atubular glomerulus (AG). The glomerular tuft is indistinguishable from other glomeruli. Bowman's capsule is markedly thickened and wrinkled at a site opposite to the vascular pole, where a tubular connection is expected. ↔, reduplicated Bowman's capsule; arrowhead, a spindle-shape cell within the reduplicated Bowman's capsule; arrow, atrophic tubules adjacent to the atubular glomerulus; * periglomerular fibrosis. PAS-stained; magnification, ×630. (Figure reproduced from Ref. [29] with permission of the publisher)

Thickening of tubular basement membranes (TBM) parallels GBM thickening and is an early finding in DKD (Fig. 8.1) [4, 9]. TBM thickening related to diabetes is diffuse and homogeneous with a different appearance from the nonspecific TBM thickening in atrophic tubules where basements become irregular, corrugated, and duplicated and frequently associated with deposition of cellular debris. Supportive of this difference, TBM width in diabetic patients correlates strongly with GBM width and Vv(Mes/glom) but only weakly with the volume fraction of renal cortex that is interstitium [Vv(Int/cortex)] [9]. Moreover, tubulointerstitial fibrosis follows glomerulopathy in T1D patients. In fact, as a result of tubular hypertrophy, Vv(Int/cortex) initially reduces [32]. Expansion of cortical interstitium is initially due primarily to an increase in the cellular component, while increased interstitial fibrillar collagen deposition occurs relatively late, when GFR decline is already present [32].

Using immunofluorescence microscopy, GBM and TBM commonly show modest linear staining with IgG (polytypical) and albumin in diabetic patients. This finding is related to diabetes, regardless of the presence or absence of DKD. The exact cause of this phenomenon remains unclear, although alterations in chemical properties of extracellular matrix, immunoglobulins, or both might be involved. A recent study suggested an association between the intensity of IgG staining and renal outcomes, but this finding requires further validation, especially given the absence of a proper way to precisely standardize fluorescent intensity of IgG staining [33].

Structural-Functional Relationships of DKD in T1D

The natural history of DKD in T1D patients is characterized by an initial long period of normal or high GFR and normoalbuminuria, during which the disease has a slow progression rate. This initial period is followed by a more rapid pace of increasing albuminuria and GFR loss [34]. The structural-functional relationship models of DKD follow a similar course. Initially, when the patients are normoalbuminuric, classical DKD glomerular structural parameters, including GBM width, Vv(Mes/glom), and Sv(PGBM/glom), may be within the normal range. As progression of DKD leads to microalbuminuria and macroalbuminuria, GBM thickness and Vv(Mes/glom) increase, and Sv(PGBM/glom) reduces. These parameters show considerably less overlap with normal values in microalbuminuric patients and almost no overlap in macroalbuminuric patients [3]. Persistent microalbuminuria is associated with progression of the lesions and increased risk for developing macroalbuminuria [3]. Vv(Mes/glom) and GBM width directly and Sv(PGBM/glom) inversely correlate with urine albumin excretion rate (AER) from normoalbuminuria to macroalbuminuria (Fig. 8.6). Importantly, increased GBM width can predict progression of DKD in T1D patients from normoalbuminuria to microalbuminuria or even to macroalbuminuria and ESRD [35]. In a longitudinal study, none of the normoalbuminuric patients with long-standing T1D and normal GBM width progressed to proteinuria or ESRD after an average follow-up of 11 years [35]. Vv(Mes/glom), fractional volume of mesangial matrix per glomerulus [Vv(MM/glom)], and

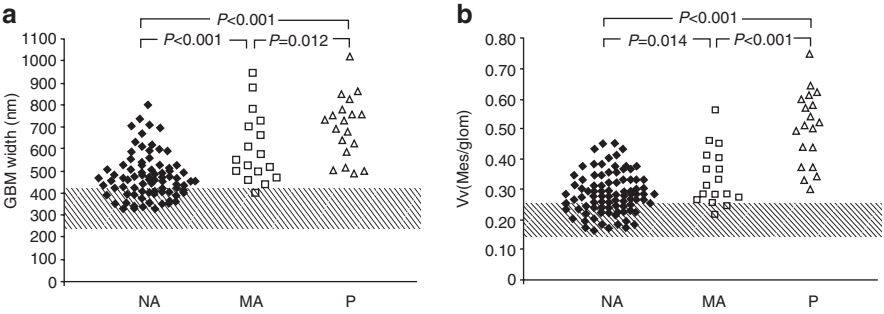

Fig. 8.6 (**a**) GBM width in 88 normoalbuminuric (NA), 17 microalbuminuric (MA), and 19 proteinuric (P) patients with type 1 diabetes. The hatched area represents the mean ± 2 SD in a group of 76 age-matched normal control subjects. All groups are different from control subjects. (**b**) Vv(Mes/glom) in 88 normoalbuminuric (NA), 17 microalbuminuric (MA), and 19 proteinuric (P) patients with type 1 diabetes. The hatched area represents the mean ± 2 SD in a group of 76 age-matched normal control subjects. All groups are different from control subjects. (Figure reproduced from Ref. [3] with permission of the publisher)

GBM width are inversely and Sv(PGBM/glom) is directly related to GFR (Fig. 8.7) [3]. In fact, there is a direct correlation between the total peripheral capillary filtration surface area and GFR from hyperfiltration to renal insufficiency.

T1D patients are fairly homogeneous in regard to DKD structural-functional relationship models, and such models have been shown to be robust [36]. Current models can better predict AER than GFR. About 70% of AER and only about 20–30% of GFR variances are explainable by structural-functional relationship models developed by multiple regression analysis based on glomerular lesions alone. However, models developed by piecewise linear regression analysis can explain much larger fraction of AER and GFR variances, approaching prediction of over 80% of AER and over 65% of GFR variances. Piecewise linear regression analysis examines if the relationships can be explained by two regression lines of different slopes, intersecting at a breakpoint. Thus, the improved predictability of the piecewise linear regression analysis models mirrors the natural history of DKD with an initial slow progression prior to a breakpoint and fast progression thereafter. Importantly, the breakpoints found in two separate studies one based on a small cohort and another based on a larger cohort of T1D patients were both in the microalbuminuric and normal GFR ranges [28, 36], suggesting that the shift from a slow to a fast progression phase occurs relatively early and during the initial clinically silent phase. In addition, these results indicate that glomerular lesions alone can explain a major proportion of AER and GFR variance in T1D patients. In fact, predictability of these models showed relatively minor improvements by adding Vv(Int/cortex) and glomerulotubular junction abnormalities as other predictor variables [28]. In contrast to these results, some studies have suggested that GFR decline in DKD is primarily driven by interstitial fibrosis, rather than diabetic glomerulopathy [37, 38].

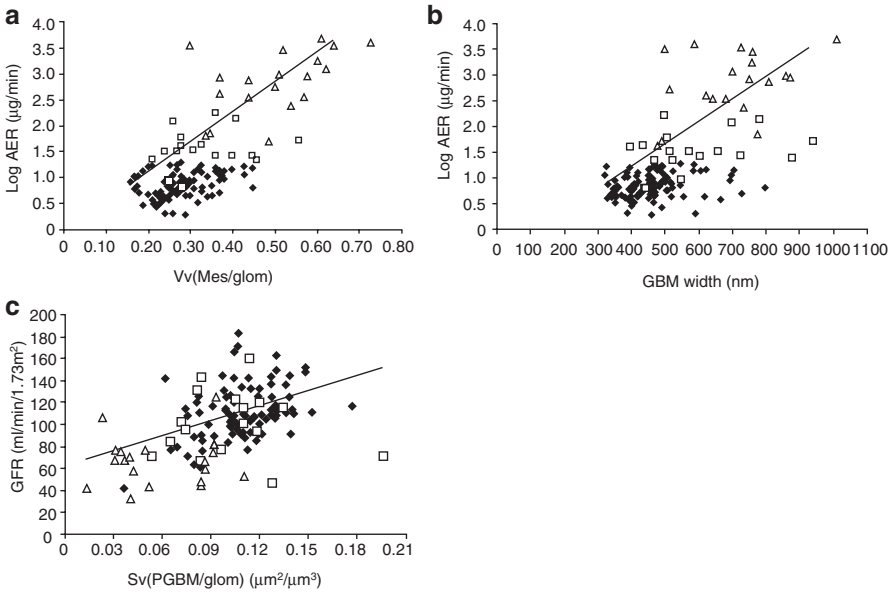

Fig. 8.7 (**a**) Correlation between Vv(Mes/glom) and AER in 124 patients with type 1 diabetes. ♦, Normoalbuminuric patients; ■, microalbuminuric patients; △, proteinuric patients. $r = 0.75$, $P < 0.001$. (**b**) Correlation between GBM width and AER in 124 patients with type 1 diabetes. ◇, Normoalbuminuric patients; ■, microalbuminuric patients; △, proteinuric patients. $r = 0.63$, $P < 0.001$. (**c**) Correlation between Sv(PGBM/glom) and GFR in 125 patients with type 1 diabetes. ◇, Normoalbuminuric patients; ■, microalbuminuric patients; △, proteinuric patients. $r = 0.48$, $P < 0.001$. (Figure reproduced from Ref. [3] with permission of the publisher)

However, as pointed out earlier, increased Vv(Int/cortex) in DKD in T1D patients is seen in later stages when diabetic glomerulopathy becomes more advanced. Moreover, appreciation of contribution of glomerular lesions in GFR loss requires careful measurement of glomerular structural parameters using morphometric techniques. The importance of vascular lesions in advancement of chronic injury in DKD should not be underestimated. An autopsy study showed that the sclerotic glomeruli in T1D patients are more often clustered in the plane vertical to the renal capsule, indicative of the importance of vascular lesions and chronic ischemia in glomerulosclerosis [39].

There is a large body of evidence about podocyte injury and progression of DKD. Foot process width, a parameter that is commonly regarded a sign of podocyte injury, was directly correlated with AER and inversely with GFR across a wide range of albuminuria [24]. Another study showed that the density of podocytes per glomerular volume [Nv(Podo/glom)] was inversely related to AER in normotensive proteinuric T1D patients [40]. Such association was not found between AER and total number of podocytes per glomerulus, perhaps signifying the importance of imbalance between the number of podocytes and glomerular volume as suggested in other glomerulopathies. Of note, relationship between

AER and Nv(Podo/glom) was not seen in microalbuminuric T1D patients. Moreover, another study showed that podocyte structural parameters did not predict progression to proteinuria or ESRD during long-term follow-up in normoalbuminuric T1D patients [36], suggesting that podocyte injury may play a more critical role in progression of DKD in later stages of the disease or, alternatively, the role of podocyte injury may become evident only after a certain fraction of podocytes are lost [41].

The endothelial cells develop structural changes in DKD. There is a reduction in glomerular endothelial fenestration in normoalbuminuric patients which persists in micro- and macroalbuminuria [24]. Although endothelial glycocalyx cannot be observed using routing electron microscopy techniques, it has been shown that DKD is associated with increased heparanase activity that leads to reduced endothelial glycocalyx, a change that can contribute into albuminuria and infiltration of macrophages into the kidney [42].

DKD in Type 2 Diabetic Patients

The frequency of DKD among clinical biopsies, regardless of the status of diabetes, has progressively increased over the last three decades in the USA, currently approaching ~20% [43, 44]. This is while an autopsy study showed that ~19% of diabetic patients with obvious DKD lesions did not present with clinical manifestations of DKD, suggesting that DKD may be underdiagnosed based on indication biopsies [45]. Although type 2 diabetes is by far the most common etiology of ESRD, there are more studies available describing the natural history of DKD lesions in type 1 compared to type 2 diabetes. In general, similarities between DKD lesions in T1D and T2D patients are substantial. Classical glomerular lesions of DKD, including GBM thickening, mesangial expansion, and reduced glomerular filtration surface area, similar to type 1 diabetes, are present and progress with diabetes duration [46]. Studies performed on adults who develop type 2 diabetes later in life when hypertension and atherosclerotic vascular lesions are already present suggest that pathologic findings are more heterogeneous in T2D compared to type 1 diabetic patients [9, 47–49]. Fioretto et al. identified three different patterns or categories of lesions in kidney biopsies from microalbuminuric and macroalbuminuric Northern Italian T2D patients: category I with almost normal biopsies (35% of microalbuminuric and 10% of proteinuric patients) (Fig. 8.8), category II with classical lesions of DKD similar to T1D (30% of microalbuminuric and 55% of proteinuric patients), and category III with disproportionately advanced tubulointerstitial fibrosis, arteriolar hyalinosis, arteriosclerosis, or global glomerulosclerosis, despite minor diabetic glomerulopathy [9]. Of note, these categories correlated with some clinical phenotypes. Thus, the presence of classical DKD lesions (category II) was associated with longer duration of diabetes, poorer glycemic control, faster GFR

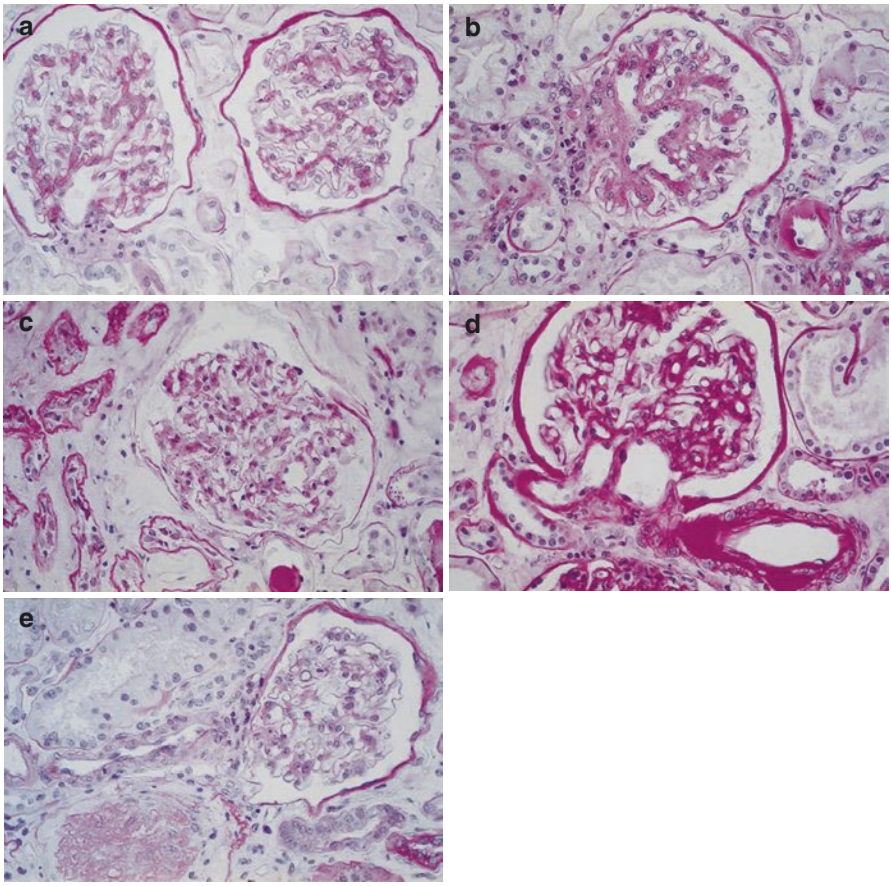

Fig. 8.8 (**a**) Glomeruli from a patient in category C I. Glomerular structure is near normal with minimal mesangial expansion (PAS). (**b**) Glomerulus from a patient in category C II, with well-established diabetic nephropathology. Diffuse mesangial expansion, advanced arteriolar hyalinosis, and mild interstitial fibrosis are present (PAS). (**c**) Glomerulus from a patient in category C III (a), with near-normal glomerular structure and tubular basement membrane thickening, tubular atrophy, and severe interstitial fibrosis (PAS). (**d**) Glomerulus from a patient in category C III (b), with mild mesangial expansion and severe arteriolar hyalinosis, affecting both afferent and efferent glomerular arterioles (PAS). (**e**) Glomeruli from a patient in category C III (c). Glomerular structure is near normal in one glomerulus, while the adjacent shows global glomerular sclerosis (PAS). (Figure reproduced from Ref. [18] with permission of the publisher)

decline, and retinopathy [50, 51]. In contrast, retinopathy was rare in patients exhibiting category I or II on biopsies [52]. In contrast, biopsy studies performed in Pima Indians who generally develop type 2 diabetes at a younger age suggest that the relationships between albuminuria and DKD structural changes are more similar to those seen in type 1 diabetic patients [53], reflecting more homogeneity in DKD lesions in these younger type 2 diabetic patients.

Structural-Functional Relationships in T2D Patients

There is substantial similarity in structural-functional relationships of DKD between type 1 and type 2 diabetes, albeit these relationships may be less precise in type 2 diabetes, which may be at least partly related to the heterogeneity of lesions in older type 2 diabetic patients. A study on 47 Caucasian adults (average age about 60 years) with type 2 diabetes and proteinuria showed direct relationships between Vv(Mes/glom), Vv(MM/glom), and GBM width and proteinuria and inverse relationships between Vv(Mes/glom), Vv(MM/glom), Vv(Int/cortex), and GFR [54]. A longitudinal study in Japanese type 2 diabetic patients found GBM width and Vv(Mes/glom) as predictors of progression of albuminuria after 6 years of follow-up [55]. Another longitudinal study performed on Northern Italian type 2 diabetic patients showed that GBM width and Vv(Mes/glom) predicted GFR decline after follow-up for 4 years [50]. Importantly, even in patients who were normoalbuminuric at the baseline, these lesions predicted GFR loss at follow-up. More recently, a study performed on a large cohort of Pima Indians with type 2 diabetes suggested that both glomerular and tubulointerstitial lesions were significant contributors into GFR loss. On one hand, glomerular parameters, including Vv(Mes/glom), percentage of global glomerular sclerosis, nonpodocyte (mesangial and endothelial) cell number per glomerulus, GBM width, mean glomerular volume, and podocyte foot process width; lower Sv(PGBM/glom); and fewer endothelial fenestrations were each associated with GFR decline after adjustment for main clinical parameters [56]. Moreover, a composite glomerulopathy index, reflecting the combined effects of the statistically significant morphometric variables listed above, was strongly associated with GFR loss. On the other hand, when GFR slope was modeled as a threshold, only Vv(Int/cortex) was associated with the slope. Importantly, these relationships between biopsy structural parameters and GFR loss were even present when the baseline GFR was normal or elevated, suggesting that the deteriorating impact of these lesions on renal function starts at very early stages of DKD when the disease is clinically silent.

Podocyte injury starts early in type 2 diabetic patients. Normoalbuminuric patients with type 2 diabetes show increased urine nephrin and/or podocin mRNA compared to nondiabetic persons [57]. Injured podocytes can detach from the GBM and fall into the urine. In fact, microalbuminuric and proteinuric type 2 diabetic patients show increased shedding of podocytes into the urine (podocyturia) [58]. Since podocytes do not regenerate efficiently, podocyte loss is generally regarded as a cumulative insult to the glomerulus and in time leads to podocyte depletion in the glomeruli. The number of podocytes in a glomerulus can be assessed either in relative (to glomerular volume), i.e., podocyte number density per glomerular volume, or absolute (i.e., podocyte number per glomerulus) terms. It has been shown that both number and number density of podocytes per glomerulus are reduced in microalbuminuric and macroalbuminuric type 2 diabetic patients [53, 59, 60]. Podocyte loss increases with diabetes duration, and as expected this is associated with

increased AER [46, 59]. Once podocyte loss is severe enough, it ensues into segmental and eventually global glomerulosclerosis. Importantly, Meyer et al. found that podocyte number per glomerulus in microalbuminuric Pima Indian persons with type 2 diabetes was not only the strongest predictor of AER increase but also predicted progression to overt nephropathy [61].

Nondiabetic Renal Disease in Diabetic Patients

Currently, indication biopsies in diabetic patients are performed if the clinical history raises suspicion for a nondiabetic renal disease (NDRD), including [62] nephrotic-range proteinuria or kidney failure in the absence of diabetic retinopathy, with diabetes duration less than 5 years or with normal GFR, reduced GFR with diabetes duration less than 5 years, unexplained microscopic hematuria or acute kidney injury, or rapidly worsening kidney function in patients with previously stable kidney function. Therefore, it is not surprising to find a high incidence of NDRD in clinical biopsies from diabetic patients [63–68]. Given the prevalence of diabetes, up to 25% of all clinical renal biopsies are done in diabetic patients [69]. The prevalence of NDRD in biopsies from diabetic patients is variable in the literature and ranges from 10% to 85% [70–73]. However, the incidence of NDRD in research or protocol biopsies is remarkably lower than in clinical biopsies [74]. The likelihood of finding NDRD in indication biopsies from diabetic patients is affected by the criteria and biopsy threshold used, as well as ethnic and geographic factors. In a study performed at Columbia University, of all indication biopsies from diabetic patients, 37% had DKD alone, 36% had NDRD alone, and 27% had DKD plus NDRD [69]. In the NDRD alone group, the most common diagnosis was FSGS (22%), followed by hypertensive nephrosclerosis, acute tubular injury, IgA nephropathy, membranous nephropathy, and pauci-immune glomerulonephritis. In the DKD plus NDRD group, however, acute tubular injury was the most common finding (43%), followed by hypertensive nephrosclerosis, FSGS, and IgA nephropathy. Diabetes duration ≥12 years was found to be the best predictor of DKD alone.

It is noteworthy that some lesions listed among the most commonly reported forms of NDRD in biopsies from diabetic patients, e.g., focal and segmental glomerulosclerosis and hypertensive nephrosclerosis, can reflect processes secondary to DKD, rather than independent concurrent diseases. There is no general consensus on how to report such lesions. Therefore, studies on NDRD made based on extracting data from pathology reports may well be affected by reporting routines of one center vs. another. Another example would be the presence of interstitial eosinophilic aggregates that are commonly regarded as an allergic reaction to presumptive drugs. However, Dai et al. showed that this finding, which can be seen in about 40% of indication biopsies from diabetic patients, does not correlate with a clinical history of drug allergy or the number of medicines used by patients and instead it correlates with the severity of chronic injury in renal parenchyma [75].

DKD Classification of Pathologic Lesions

Classification of pathologic lesions facilitates uniform reporting of biopsy findings and reproducibility of data generated from biopsy studies. Tervaert et al. [76] proposed a pathologic classification for DKD mainly based on the glomerular lesions. The classification consists of four progressive classes, including GBM thickening (class I), mesangial expansion (class II which is divided into classes IIa if mild and IIb if severe), presence of Kimmelstiel-Wilson nodules (class III), and extensive global glomerulosclerosis (class IV). Vascular and tubulointerstitial lesions are included in a separate scoring system. An et al. in a large study performed on type 2 diabetic patients showed that the severity of glomerular and interstitial lesions inversely impacts renal prognosis [77]. Another study showed that progression of glomerular, tubulointerstitial, and vascular lesions evaluated by this classification was associated with poor renal prognosis [33]. On the other hand, some studies have challenged the prognostic significance of glomerular lesions according to this classification [78, 79]. Whether this classification has any predictive value in early stages of DKD, when treatments are more likely to affect outcomes, remains to be validated. In an effort to study the net cumulative effect of various DKD lesions on renal prognosis, Hoshino et al. proposed a D-score calculated by summing the scores of all components in Tervaert classification which led to improvement in prediction of renal outcome, with a D-score ≤ 14 predicting excellent outcomes [80]. However, it should be noted that all studies confirming a prognostic value for this classification so far basically have reported that more severe glomerular or tubulointerstitial lesions portend worse outcomes, which is not surprising and does not help identifying patients at greater risk of progression at earlier stages. In addition, some other important aspects of DKD with predictive value for renal dysfunction, such as heterogeneity of patterns of renal injury in T2D [70], and some other morphologic features with predictive value for renal dysfunction, such as podocyte loss [53], glomerulotubular junction abnormalities [28], or endothelial fenestration [56], are not included in this classification.

Are DKD Lesions Reversible?

It has been shown that kidney lesions developed in diabetic murine models are reversible following normoglycemia. Islet transplantation in STZ-induced diabetic rats normalizes blood glucose and leads to reversal of diabetic kidney lesions in 2 months [81]. BTBR ob/ob diabetic mice, a model of type 2 diabetes with kidney lesions mimicking those seen in human DKD, show reversal of diabetic lesions after 6 weeks of leptin replacement-associated normoglycemia [82]. However, as explained earlier in this chapter, human DKD lesions in contrast to murine models gradually develop in a long time. Similarly, long-term normoglycemia is required for human DKD lesions to improve or reverse. Fioretto et al. showed that after

10 years of normoglycemia following pancreas transplantation, marked reversal of diabetic glomerulopathy lesions can be seen in type 1 diabetic patients with a diabetes duration of approximately 20 years, while 5 years of normoglycemia after pancreas transplantation was not enough to lead to appreciable changes [83]. Most strikingly, Kimmelstiel-Wilson nodules had completely disappeared in the 10-year biopsies. GBM and TBM width, Vv(Mes/glom), and Vv(MM/glom) were all reduced at 10 years compared with the baseline and 5-year values, and these parameters in some patients returned to the normal at 10-year biopsies (Fig. 8.9) [9]. Reversal of diabetic glomerulopathy was also associated with improvement of tubulointerstitial lesions and reduction in total cortical interstitial collagen [28].

Given the limited regeneration capacity of podocytes and the role of podocyte loss in progression of DKD lesions (see above), it is important to find out if podocyte regeneration is needed for reversal of DKD. Animal models have suggested that progenitor cells on Bowman's capsule may be involved in replacing lost podocytes in the glomerular tuft [82, 84–87]. Pichaiwong et al. showed that leptin treatment of BTBR ob/ob mice not only led to reversal of renal diabetes lesions but also was associated with podocyte regeneration in the glomeruli [82]. Evidence as to whether or not that is the case in reversal of DKD lesions in humans is scanty. In one study that addresses this issue, Andeen et al. showed that early DKD in clinical biopsies was associated with increased number of parietal cells with a podocyte phenotype (Fig. 8.10), indicative of the potential for podocyte restoration [19].

The effect of pharmaceutical intervention to reverse DKD or reduce its progression has also been explored in limited studies. Five years of RAAS blockade by losartan or enalapril in normotensive normoalbuminuric type 1 diabetic patients did not prevent progression of DKD lesions but reduced progression of retinopathy [88]. On the other hand, 6 years of treatment with losartan slowed progression of mesangial expansion in microalbuminuric Pima Indian patients with type 2 diabetes [89].

Animal Models of DKD

Animal models have been widely used to explore the pathogenesis of DKD [90, 91]; however, in general, they do not faithfully replicate human DKD. A detailed discussion of models of DKD in several animal species is presented in Chapter 13 of this book. Most murine models show only the earliest features of human DKD, and an ideal model is yet to be developed. In response to this need, the nephropathy subcommittee of the Animal Models of Diabetic Complications Consortium (AMDCC) sets validation criteria for rodent models of DKD based on the clinical and pathological features of human DKD [92], including [1] >50% decrease in renal function, [2] >10-fold increase in albuminuria, and [3] pathological features including advanced mesangial matrix expansion with or without nodules, thickening of the GBM, arteriolar hyalinosis, and tubulointerstitial fibrosis.

Common rodent models used for type 1 diabetes include streptozotocin (STZ)-induced diabetic mice, Akita mice, OVE26 FVB mice, and nonobese diabetic

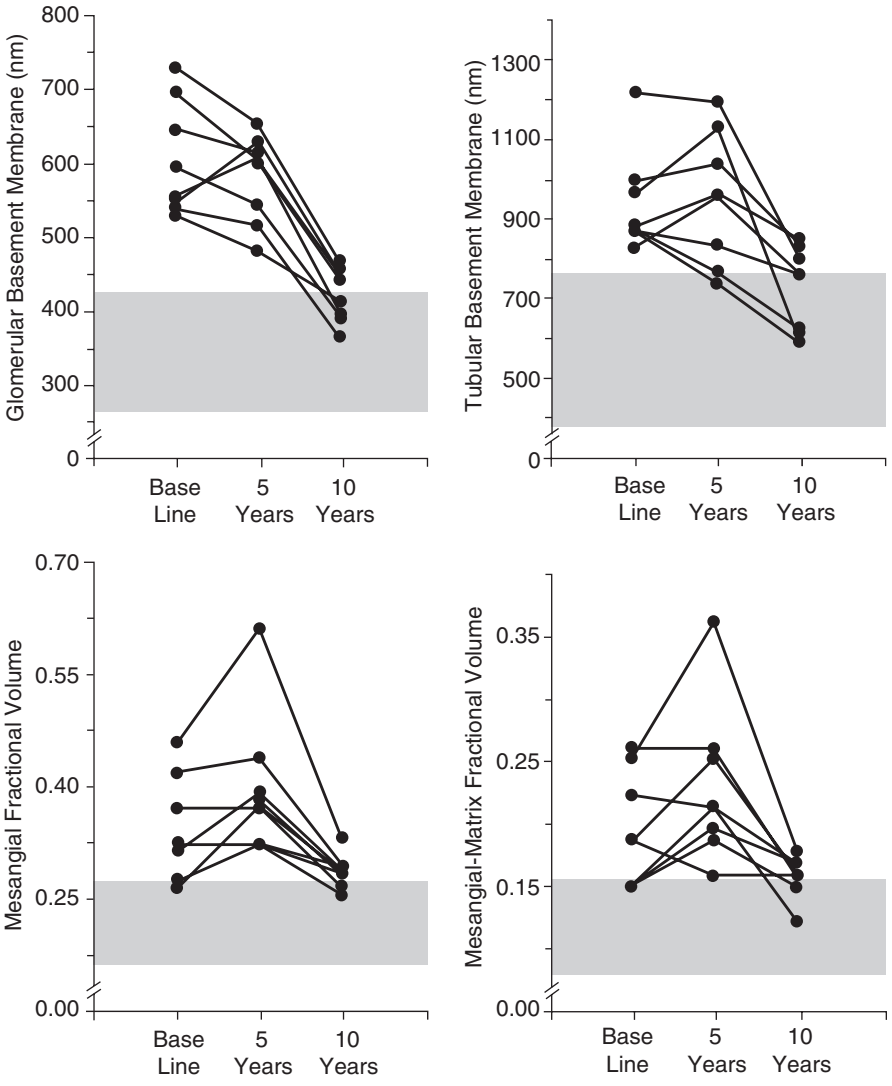

Fig. 8.9 Thickness of the glomerular basement membrane, thickness of the tubular basement membrane, mesangial fractional volume, and mesangial-matrix fractional volume at baseline and 5 and 10 years after pancreas transplantation. The mesangial fractional volume is the proportion of the glomerulus occupied by the mesangium; the mesangial-matrix fractional volume is the proportion of the glomerulus occupied by mesangial matrix. The shaded areas represent the normal ranges obtained in the 66 age- and sex-matched normal controls (means ±2 SD). Data for individual patients are connected by lines. (Figure reproduced from Ref. [9] with permission of the publisher)

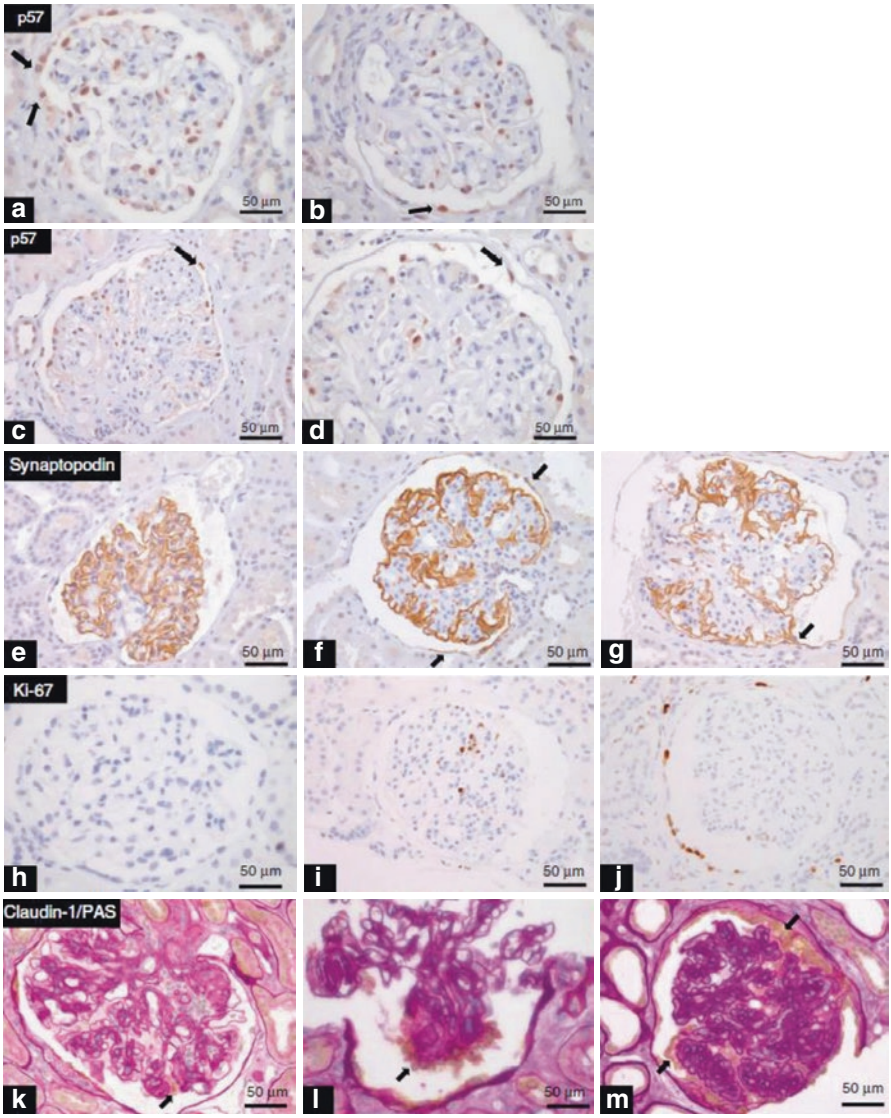

Fig. 8.10 Immunophenotypic alterations in podocyte and parietal epithelial cells in diabetic nephropathy (DN). (**a**–**d**) Cells marking as podocytes were present in parietal epithelial cell locations and significantly increased in histologically early DN (**a**, **b**), with a nonsignificant increase in advanced DN (**c**, **d**) compared with controls (original magnification ×400). (**e**–**g**) Synaptopodin highlighted a significantly increasing percentage of staining of cells lining Bowman's capsules from controls (**e**) to early (**f**) to advanced DN, including areas of segmental adhesions (original magnification ×400). (**h**–**j**) Ki-67-expressing cells were identified on the glomerular tuft and Bowman's capsule in morphologically early (**i**) and advanced (**j**) DN but only rarely in controls (**h**) (original magnification ×400). (**k**–**m**) Claudin-1/PAS revealed claudin-1-positive cells in areas of increased mesangial matrix in early DN (**k**), in areas of "capping" of segmentally sclerotic regions (**l**), and having a variable glomerular distribution in advanced DN (**m**) (original magnification ×400). (Figure reproduced from Ref. [19] with permission of the publisher)

(NOD) mice. STZ is a chemical toxin for pancreatic β cells. Therefore, injection of sufficient dose of STZ can make virtually any pre-existing model diabetic, although it should be noted that susceptibility to develop diabetic DKD varies among different strains [92]. For example, C57BL/6J mice in general are resistant to the development of kidney injury, including DKD [92]. It should be noted that STZ, especially if used at high doses (150–200 mg/kg), is nephrotoxic [93]. Multiple injections of low doses can avoid this problem to some degree [94]. Diabetic C57BL/6J mice develop mesangial expansion and some thickening of GBM, but not nodular glomerulosclerosis or tubulointerstitial fibrosis [92]. STZ-induced diabetes has been tried on other mouse strains such as DBA/2, CD1, and 129/Sv and also in rats [95]. Multiple genetic models for type 1 diabetes have also been developed. Akita mice have an *Ins2+/C96Y* mutation (a single nucleotide substitution in the *Ins2* gene) [96], which leads to abnormal folding of the insulin protein with subsequent toxic injury to pancreatic β cells and development of diabetes. It has been shown that the genetic background of Akita mutation mice affects the severity of albuminuria and histological changes. Although *Ins2+/C96Y* mutation causes comparable hyperglycemia in C57BL/6, DBA/2, and 129/SvEv mice, the DBA/2-*Ins2+/C96Y* mice develop more severe albuminuria, but C57BL/6 and 129/SvEv mice develop more prominent increase in mesangial matrix [97]. However, Akita mice, regardless of the background strain, do not develop advanced DKD lesions, such as mesangiolysis, nodular glomerulosclerosis, or tubulointerstitial fibrosis. Thus, these mice can be considered for modeling early to moderate DKD [95]. Moreover, C57BL/6-*Ins2+/C96Y* mice develop diffuse granular mesangial IgA deposits starting at 20 weeks of age, which is a confounding factor for analysis of the contribution of diabetes to the mesangial injury that may develop [98]. Another model of type 1 diabetes is the OVE26 FVB mice with transgenic overexpression of calmodulin in pancreatic β cells with subsequent deficiency in insulin production within the first week of life [99]. This model develops progressive albuminuria, starting by 2 months of age. GFR increases from 2 to 3 months of age, followed by a subsequent decline from 5 to 9 months, with increased systolic and diastolic blood pressures. Diabetic OVE26 FVB mice develop glomerulomegaly, GBM thickening, podocyte loss, mesangial matrix increase, nodular glomerulosclerosis, and tubulointerstitial fibrosis [100]. Therefore, this model exhibits some of the features of advanced DKD in humans. The nonobese diabetic (NOD) mouse, which develops type 1 diabetes through autoimmune destruction of islet cells, is similar to humans [101, 102]. However, this model faces some disadvantages to others including the complex genetic background required for development of disease, the inconsistent timeline for onset of hyperglycemia, and the development of autoimmunity including deposition of immune complexes in glomeruli [103]. Perhaps for the same reasons, the extent of diabetic kidney injury in NOD mice has not been well characterized.

The most common type 2 diabetes murine models include db/db mice, KK-Ay mice, T2DN/Mcwi mice, eNOS−/− *db/db* mice, *OVE26–TTrhRen* double trans-

genic mice, BTBR *ob/*ob mice, Zucker diabetic fatty (ZDF) rats, Wistar fatty rats, Otsuka Long-Evans Tokushima fatty (OLETF) rats, and Goto-Kakizaki (GK) rats.

db/db mice have a deletion mutation in the leptin receptor (*LepRdb/db*) which causes abnormal splicing and results in a defective receptor for the adipocyte-derived hormone leptin [104]. Defected leptin signaling leads to abnormal hypothalamic responses, ensuing in hyperphagia, obesity, hyperlipidemia, hyperinsulinemia, insulin resistance, and diabetes, which is more severe in male mice than in females. Male *db/db* mice become hyperglycemic at 6–10 weeks of age, followed by moderate to severe albuminuria at 8–25 weeks of age. Renal function declines at 15–18 weeks. db/db mice develop GBM thickening, podocyte loss, and moderate mesangial matrix expansion, but not mesangiolysis, nodular glomerulosclerosis, or severe tubulointerstitial fibrosis [26, 105]. KK mice develop mild insulin resistance and obesity, which is more severe in male animals [106, 107]. KK mice develop mild increase in mesangial matrix and GBM thickening. However, STZ-induced diabetic KK/H1J mice show more severe mesangial matrix expansion with nodular glomerulosclerosis and arteriolar hyalinosis [108]. The KK-*Ay* mouse was developed by transferring the yellow obese gene (*Ay* allele) into the KK mouse, which then becomes severely obese, hyperglycemic, and albuminuric. The kidneys of these mice show diffuse and moderate to severe mesangial matrix expansion with mesangial cell proliferation, segmental glomerulosclerosis, nodular glomerulosclerosis, and podocyte loss [109, 110]. The Zucker fatty (ZF) rat has a homozygous missense mutation (*fatty, fa*) in the leptin receptor gene (*Lepr*), resulting in obesity without diabetes. Zucker diabetic fatty (ZDF) rats are derived from the ZF strain. These rats are obese and develop progressive insulin resistance and diabetes [111, 112]. They are not hypertensive and show an initial increase in GFR which later on declines to normal level. Pathological changes include glomerulosclerosis, tubulointerstitial fibrosis, and inflammation [113]. The Wistar fatty (WF) rat is a congenic strain of the Wistar Kyoto (WKY) rat with a *fa/fa* homozygous missense mutation in the *Lepr* gene, resulting in obesity, hyperinsulinemia, and hyperlipidemia [114, 115]. Diabetes in the WF rats is milder than in the ZDF rats. However, the WF rats develop GBM thickening, foot process effacement, mesangial expansion, and tubulointerstitial inflammation. The Otsuka Long-Evans Tokushima fatty (OLETF) rat is a robust model of type 2 diabetes. Almost all male OLETF rats develop diabetes by 25 weeks of age [116]. These rats develop albuminuria, proteinuria, and elevated GFR. Long-lasting diabetes in the OLETF rats is associated with glomerulomegaly, increased mesangial matrix, GBM thickening, nodular glomerulosclerosis, and tubulointerstitial fibrosis [117]. The Goto-Kakizaki (GK) rat is a nonobese model of type 2 diabetes, developed from a colony of Wistar rats through selection of rats with hyperglycemia [118]. The GK rats demonstrate impaired glucose tolerance test as early as 2 weeks of age, due to hypoplasia of pancreatic islet cells and insulin resistance [119]. GK rats develop type 2 diabetes by 12 weeks of age. However,

they are relatively resistant to develop DKD [120], although some levels of GBM thickening and mild to moderate mesangial expansion have been reported in this model [121]. T2DN/Mcwi mice which are developed from a cross between GK and fawn-hooded hypertensive (FHH) rats [122] develop diabetes and progressive proteinuria, focal glomerulosclerosis, severe mesangial matrix expansion, and GBM thickening and later on nodular glomerulosclerosis and arteriolar hyalinosis [123].

Additional genetic stressors have been incorporated into some of the genetic models of DKD to accelerate progression of the lesions. The full knockout of endothelial nitric oxide synthase (eNOS) on the *db/db* C57BL/KsJ background results in eNOS−/− *db/db* mice which are hypertensive and develop marked albuminuria and reduced GFR with aging, extensive mesangial matrix expansion with nodules, mesangiolysis, increased GBM thickness, arteriolar hyalinosis, and tubulointerstitial fibrosis [124, 125]. Chronic activation of the renin-angiotensin system (RAS) in hyperreninemic transgenic (*TTRhRen*) mice is another approach to accelerate kidney lesions [126]. STZ injection to *TTRhRen* transgenic mice results in albuminuria and kidney lesions. *OVE26–TTrhRen* double transgenic mice develop very prominent albuminuria with glomerulosclerosis and interstitial fibrosis [126]. Combining the black and tan, brachyuric (BTBR) mouse strain with natural insulin resistance, with the *ob/ob* leptin mutation, results in BTBR ob/ob mice [127, 128]. These mice develop hyperglycemia and albuminuria with prominent mesangial matrix expansion, focal nodular glomerulosclerosis, mild GBM thickening and arteriolar hyalinosis and podocyte loss. Importantly, many of these lesions, including podocyte loss, can be reversed by administration of leptin [82]. However, the phenotype reported by some other labs has been milder than what was originally described, perhaps reflecting the impact of environmental factors on DKD in this model [129].

Characteristics of some of the discussed models are tabulated in Table 8.1. In summary, most currently studied mouse models of diabetes show early morphological changes of human DKD, such as mesangial matrix expansion and, in some cases, podocyte loss, including *db/db* and Akita mice. There are few models that exhibit features of both morphologically early and late DKD; of these, the eNOS−/− *db/db* mice, OVE26 FVB mice (a type 1 diabetes model), and BTBR *ob/*ob mice (modeling type 2 diabetes and obesity) appear to be the most robust. The BTBR *ob/*ob mouse model is particularly noteworthy for the relative rapidity in which lesions develop, making it well suited for studies of new therapeutics. Despite the plethora of diabetic mouse models, all models available to date possess important limitations in their practicality and/or fidelity in recapitulating all of the features of human disease. The tubulointerstitial and vascular lesions of DKD have been particularly challenging to model in the mouse. Designing better models of DKD that will allow identification of underlying mechanisms remains an important research objective, which in turn will facilitate testing of therapeutic interventions that can ameliorate or even reverse the structural alterations of DKD.

Table 8.1 Renal functional and pathologic characteristics of various murine models of DKD

Model	Strain	Characteristic								
		Hypertension	GFR decline	Albuminuria	Mesangial expansion	Glomerulosclerosis	Arterial hyalinosis	GBM thickening	IFTA	References
Type 1 diabetes										
STZ	C57BL/6J	–	–	–	+	–	–	+	–	[108, 130]
	DBA/2J	–	–	++	+	+	+	++	–	[108, 130]
Akita (*Ins2+/C96Y*)	C57BL/6J	+	–	+	+	–	NR	NR	–	[97]
	129/SvEv	+	–	++	+	–	NR	NR	–	[97]
	FVB/NJ	+	–	++	++	–	NR	–	–	[131]
OVE26	FVB/NJ	+	–	+++	+++	++	+	++	+	[132, 133]
OVE26—TTRhRen	FVB/NJ	+++	+	+++	+++	NR	–	NR	++	[126]
Type 2 diabetes										
ob/ob	BTBR	–	–	++	+++	++	+	+	+	[82, 128]
	FVB/NJ	–	NR	++	++	+	+	++	+	[134, 135]
	C57BL/KsJ	–	–	+++	++	–	+	+	–	[136]
eNOS−/− *db/db*	C57BL/KsJ	++	++	+++	+++	++	+	++	+	[124]
KK	KK/H1J	NR	–	++	+++	++	+	++	–	[108]
	KK-A(y)/Ta	–	–	+++	+++	++	–	+	–	[110]
ZDF	ZDF/Gmi™	–	–	++	++	+	NR	NR	+	[112, 137]
WFR	fa/fa	NR	+	+++	+	–	NR	+	+	[115]
OLETF	OLETF	+	–	++	++	+	NR	+	+	[117, 138]
GK	T2DN/Mcwi	+	–	++	+++	+	+	+	+	[123]

Modified from Azushima et al. [139]

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Part III
The Glomerulus

Chapter 9
The Mesangial Cell in Diabetic Nephropathy

Tri Q. Nguyen and Roel Goldschmeding

Introduction

The glomerular capillary tuft consists of three distinct cell types: fenestrated endothelial cells that are situated between the capillary lumen and the glomerular basement membrane (GBM), podocytes that cover the outermost layer of the GBM, and mesangial cells that lie between the capillary loops. Surrounded by their matrix, mesangial cells form the central stalk of the glomerulus and are part of a functional and structural unit that interacts closely with endothelial cells and podocytes.

Traditionally, it has been assumed that mesangial cells play the major role in the pathogenesis of diabetic glomerulopathy, considering that both diffuse mesangial expansion and nodular mesangial expansion, the latter also known as Kimmelstiel-Wilson nodules, are the structural hallmarks of experimental and human DN (Fig. 9.1). However, recent data show that also dysregulated glomerular endothelial cells and podocytes, as well as bone marrow-derived mesangial progenitor cells and inflammatory cells, significantly contribute to the development of diabetic glomerulosclerosis [1]. The roles of endothelial dysfunction, podocytes, and inflammatory processes in the pathogenesis of DN are discussed in Chaps. 10, 11, and 12 of this book. In this chapter, we will review the role of mesangial cells, and their interplay with other glomerular cells, in the pathophysiology of DN.

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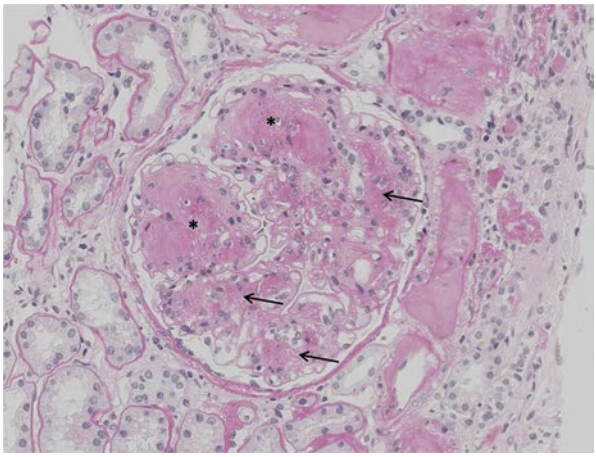

Fig. 9.1 Mesangial expansion is the structural hallmark of DN. Glomerulus in a kidney biopsy from a patient with DN showing both diffuse mesangial expansion (*arrows*) and nodular mesangial expansion, also known as Kimmelstiel-Wilson nodules (*asterisks*)

Development and Origin of the Mesangial Cell

The development of the glomerulus occurs in several stages, starting with the condensation of the renal vesicle, after which the comma-shaped body is formed, followed by the S-shaped body and a capillary loop stage, and finally the mature glomerulus [2]. At the S-shaped stage, glomerular endothelial progenitor cells, probably derived from angioblasts, migrate into a vascular cleft and start to secrete platelet-derived growth factor-B (PDGF-B), which promotes the recruitment of mesangial cells that express PDGF receptor-β (PDGF-Rβ) [3, 4]. Mesangial cells thus invade the developing glomerulus and form a branching stalk of cells protruding into the single capillary loop, thereby splitting it into a capillary network with multiple interconnecting capillaries [5]. Genetically modified mice lacking either PDGF-B or PDGF-Rβ mice fail to develop mesangial cells, and their glomeruli appear as a single balloon-like capillary loop consisting of only endothelial cells, podocytes, and basement membrane material [6, 7]. Interestingly, this exact glomerular phenotype is also observed in mice lacking the G domain of laminin α5, which is thought to be essential for the adhesion of mesangial cells to the GBM during nephrogenesis [8].

Mesangial cells originate from a subpopulation of undifferentiated metanephric mesenchymal cells [9]. Since mesangial cells do not express specific markers, their exact origin is still a matter of debate. Staining for α-smooth muscle actin (α-SMA) in fetal kidneys suggested that mesangial cells originate from mesenchymal cells found in the lower cleft of the S-shaped body and then migrate to the periphery of the capillary tuft [10]. A comparable study using immunohistochemistry for PDGF-Rβ, desmin, and α-SMA identified a common origin of mesangial cells and smooth muscle cells in afferent and efferent arterioles, suggesting that mesangial cells are co-recruited from arteriolar smooth muscle cell progenitors [3]. The origin of mesangial cells has also been studied extensively in the rat anti-Thy1.1 model,

which is characterized by acute mesangial damage and mesangiolysis, followed by repopulation of lost mesangial cells. These studies demonstrated that also progenitor cells in the juxtaglomerular apparatus [11] and bone marrow-derived cells [12] can be recruited to replace injured mesangial cells.

Function of the Mesangial Cell

Mesangial cells play an important role in the maintenance of mesangial matrix homeostasis by generating and controlling the turnover of extracellular matrix [13]. The extracellular matrix consists of numerous protein components, including collagen type IV, laminin, fibronectin, and heparan sulfate proteoglycans, that do not only provide structural support for the mesangium but are also involved in matrix-cell signaling related to mechanical stretch and in binding of growth factors [13, 14]. By their contractile properties, mesangial cells are also able to regulate glomerular capillary wall tension, intraglomerular capillary flow, and filtration surface area, in response to various vasoactive hormones and growth factors [13]. For example, mesangial cells express a number of ion channels that regulate cell volume, contractility, and cell proliferation in response to angiotensin II, endothelin, bradykinin, and epidermal growth factor [15].

Mesangial cells are crucial for maintenance of the structural architecture and integrity of the glomerular capillary tuft. As mentioned above, mesangial cells play a key role in the formation of the capillary tuft during glomerulogenesis. Also in mature glomeruli, mesangial cells provide a mechanical function to preserve the integrity of the capillary tuft. For example, loss of mesangial cells and mesangiolysis in glomerular diseases like thrombotic microangiopathy and DN often result in dilation of glomerular capillary loops and ultimately formation of microaneurysms.

In addition, mesangial cells have phagocytic properties that enable them to remove macromolecules and immune complexes that accumulate in the mesangium. The uptake and degradation of these molecules by mesangial cells can be both receptor-independent and receptor-dependent [13]. By phagocytosis, mesangial cells also have a function in the clearance of apoptotic cell bodies [16, 17].

Mesangial Cell Response to Diabetic Injury

Expansion of the mesangium, both diffuse and nodular, is the hallmark of diabetic nephropathy. In addition, numerous in vitro and in vivo studies have shown adverse responses of mesangial cells to various injuries related to metabolic and hemodynamic effects of the diabetic condition, including high glucose and angiotensin II. These observations underline the pivotal role that mesangial cells play in the development of diabetic glomerulopathy.

Effect of Glucose and Insulin Dysregulation on Mesangial Cells

High extracellular glucose induces glucose uptake in mesangial cells via increased expression of the insulin-dependent glucose transporter GLUT1 [18]. Elevated glucose levels in the mesangial cell lead to activation of metabolic pathways that result in reactive oxidative stress, which in turn activates a number of signaling pathways that lead to enhanced production of extracellular matrix (ECM) proteins. The stimulation of ECM protein synthesis can occur via protein kinase C activation, extracellular signal-related (ERK) pathways, and production of transforming growth factor (TGF)-β1, its downstream mediator connective tissue growth factor (CTGF) and gremlin [19–21]. Part of these changes might be mediated by HIF-1α, since diabetic mice have a significant increase of HIF-1α in the nucleus of mesangial cells, and high glucose enhances the expression of HIF-1α and its target genes known to be involved in the development of DN in cultured mesangial cells [22].

Interestingly, increased uptake by mesangial cells of extracellular glucose alone cannot activate all pathways described above. For example, either overexpression of GLUT1 in mesangial cells or increased exposure of cultured mesangial cells to high extracellular glucose is sufficient to enhance fibronectin production, but does by itself not increase TGF-β1 expression [23]. This suggests that also other diabetic factors and possibly also the interaction of mesangial cells with other cells might be important for activation of pathways that together lead to development of DN. In this respect, the effect of advanced glycation end products (AGEs) on mesangial cells might be important, since mesangial cells express the receptors for AGEs and uptake of AGEs leads to oxidative stress and ECM production [24].

High glucose concentration can also lead to apoptosis of mesangial cells and mesangiolysis [25]. Interestingly, observations in human biopsies with advanced DN suggest that the characteristic Kimmelstiel-Wilson nodules may develop in areas of and secondary to mesangiolysis. Although not documented in the literature, a sequence has been postulated of mesangiolysis followed by formation of microaneurysms, which subsequently undergo thrombosis and organization with excessive layered and nodular matrix accumulation (Fig. 9.2). It is still unclear what determines whether a mesangial cell responds to high glucose by proliferation and increased production of mesangial matrix or undergoes apoptosis and mesangiolysis takes place. Theoretically, this might relate to the acuteness and severity of the stress peaks that might or might not allow for timely adaptation of the cell to its altered microenvironment, including exposure to inflammatory cytokines [26].

Transient receptor potential channels (TRPC) are present in a wide variety of cells, including mesangial cells. Upon activation, these nonselective cation channels increase the intracellular calcium concentration. Interestingly, chronic high glucose exposure leads to downregulation of TRPC1 and TRPC6 in mesangial cells, resulting in impaired calcium influx. This leads to decreased mesangial cell contractility and might account for the disturbed glomerular hemodynamics seen in DN [27].

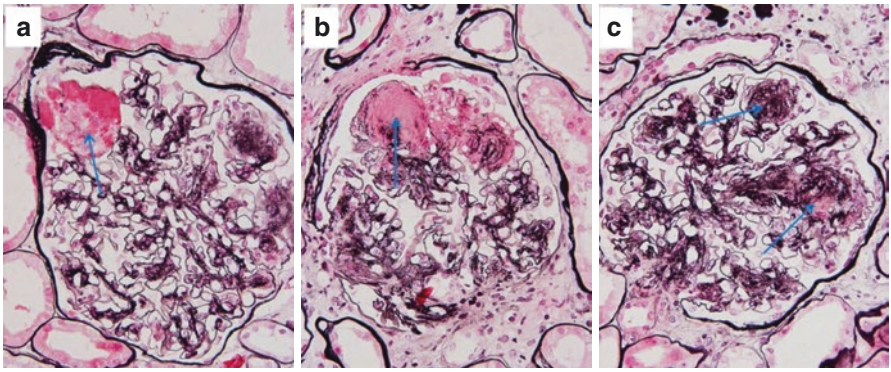

Fig. 9.2 Nodular mesangial expansion develops in areas of mesangiolysis. Three glomeruli in a single kidney biopsy from a patient with advanced and progressive DN showing different stages of the presumed sequence of events ultimately leading to the formation of nodular mesangial expansion (Kimmelstiel-Wilson nodules): mesangiolysis (**a**), thrombosis (**b**), and organization and layered extracellular matrix deposition (**c**). (Photos courtesy of prof. Jan J. Weening, MD PhD)

Hyperglycemia also results in upregulation of insulin-like growth factor (IGF)-I and insulin receptors on mesangial cells in diabetic mice [28]. Moreover, insulin deficiency itself also results in increased expression of IGF-1 and the IGF-1 receptor in mesangial cells of diabetic rats, which is accompanied by increased proliferation, and synthesis of fibronectin and collagen IV [29]. Remarkably, also elevation of insulin promotes the proliferation of mesangial cells, stimulates the production of IGF-1 and TGF-β1, and upregulates the expression of the angiotensin II type 1 receptor in mesangial cells [30]. These data suggest that high glucose, insulin deficiency and hyperinsulinemia all have profound effects on the diabetic phenotype of mesangial cells.

Effect of Mechanical Stress and Hypertension on Mesangial Cells

Hemodynamic factors relevant to the pathogenesis of diabetic nephropathy include systemic hypertension, intraglomerular hypertension, and vasoactive hormones, such as angiotensin II. It is known that in the early phase of diabetes, intraglomerular hypertension causes stretch of mesangial cells. This mechanical stretch results in mesangial cell hypertrophy, proliferation, increased production of ECM proteins, and altered ECM metabolism, leading to mesangial expansion [31]. Of note, the contribution of angiotensin II to the development of diabetic nephropathy might also involve non-hemodynamic mechanisms, since binding to angiotensin II type 1 receptors on mesangial cells induces pathways that are directly involved in accumulation of ECM components and in induction of profibrotic growth factors [32].

The effect of hypertension on mesangial cells in diabetes might also relate to altered expression of the bradykinin 2 receptor. This receptor forms a complex with angiotensin-converting enzyme and plays a role in the cross talk between the renin-angiotensin system and the kinin-kallikrein system, and it has been shown that decreased expression of the bradykinin 2 receptor in mesangial cells is associated with increased expression of genes involved in DN, overt albuminuria, and mesangial expansion [33].

Effect of Dyslipidemia on Mesangial Cells

Dyslipidemia is a frequent complication of DN and is also directly involved in the development and progression of DN. For example, both mesangial cells and podocytes express receptors for triglyceride-rich lipoproteins (TGRLs). Via the production of inflammatory cytokines and growth factors, including TGF-β1, TGRLs can activate inflammatory pathways in mesangial cells that result in the generation of reactive oxygen species, leading to accumulation of ECM proteins. In addition, oxidized LDL can bind to scavenger receptors on mesangial cells, activation of which results in increased chemokine production and ECM synthesis [34].

Effect of Growth Factors on Mesangial Cells

A variety of growth factors that are dysregulated in DN also have a direct effect on the profibrotic phenotype of mesangial cells, and as stated above, many of these can be produced by mesangial cells themselves in response to high glucose and other diabetic injuries. For example, TGF-β1 induces α-SMA expression and transformation of mesangial cells into myofibroblast-like cells, and both TGF-β1 and CTGF induce mesangial cell migration and upregulation of fibronectin expression [35]. Interestingly, the growth factor bone morphogenetic protein (BMP)-4 has been implicated in activation of mesangial cells and accumulation of extracellular matrix in the mesangium in DN [36], while the expression of its family member BMP-7 is decreased in DN. Moreover BMP-7 therapy has been shown to preserve kidney function and glomerular morphology in diabetic rats [37]. Other growth factors that have been implicated in activation of mesangial cells in DN include PDGF, hepatocyte growth factor, and IGF-I [38–40].

Interestingly, the effect of growth factors on mesangial cells in DN can be context-dependent. This is illustrated by the seemingly paradoxical observation that acute exposure to epidermal growth factor (EGF) resulted in decreased collagen production by mesangial cells in vitro [41], while also chronic in vivo inhibition of the EGF receptor slowed progression of DN in mice [42].

Targeting of Mesangial Cells

Several systemic approaches that result in reducing mesangial cell proliferation and decreased mesangial cell expansion have been applied successfully in experimental models. These include the use of antisense oligodeoxynucleotides (ODN) against proliferating cell nuclear antigen and Ki-67 or E2F-decoy ODN [43] Although not entirely specific for the mesangial cell, systemic therapies that are directed against growth factors including PDGF, TGF-β1, and CTGF have resulted in pronounced inhibition of mesangial cell proliferation and ECM expansion in DN [44, 45]. Because of its pivotal role in DN, it would be desirable to design therapies that even more specifically target the mesangial cell for treatment or prevention of DN. One option might be the administration of size-specific nanoparticles that accumulate in the mesangium [46]. Unfortunately, it will be challenging to devise truly mesangial cell-specific drug delivery because of the lack of specific cell surface markers.

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Chapter 10
The Glomerular Endothelium in Diabetic Nephropathy: Role of Heparanase

Johan van der Vlag and Baranca Buijsers

Glomerular endothelial cells are highly specialized cells covering the inner side of glomerular capillaries. Glomerular endothelial cells are highly fenestrated, and under normal conditions the fenestrated area constitutes up to 50% of the entire endothelial surface, which facilitates the passage of water and small solutes [1]. The maintenance and formation of the fenestrae depends on vascular endothelial growth factor (VEGF) that is produced by podocytes [2].

The non-diaphragmed fenestrae of the glomerular endothelium traverse the cytoplasm and have a diameter of 50–80 nm [3]. Although the size of the fenestrae seems relatively large in relation to the size of circulating proteins, such as albumin with a diameter of 3.6 nm, it has been clearly shown that the glomerular endothelial glycocalyx contributes to the glomerular filtration barrier function. Removal of non-covalently bound components of the endothelial glycocalyx by infusion of hypertonic sodium chloride into the renal artery has been shown to cause a 12-fold increase in the fractional clearance of albumin without detectable changes in endothelial morphology [4].

Dysfunction of the (glomerular) endothelium is associated with the development and progression of diabetic vascular complications, including diabetic retinopathy and diabetic nephropathy [5, 6]. There are many factors and mechanisms that determine endothelial health and function, such as nitric oxide (NO), which is produced by the endothelial cells via endothelial nitric oxide synthase (eNOS) and which is crucial for endothelial health, integrity, and function [5, 7]. Importantly, eNOS has an anti-inflammatory effect, which is relevant for diabetic nephropathy, since diabetic nephropathy can be considered as an inflammatory glomerular disease [8]. It is therefore not surprising that a decreased NO production and bioavailability are observed in diabetes, thereby contributing to endothelial dysfunction (Fig. 10.2) [5].

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The glomerular endothelium cells are covered by, and fenestrae are filled with, a carbohydrate-rich layer, the glycocalyx, which represents a barrier that restricts passage of proteins from blood to urine [2]. The endothelial glycocalyx is a gel-like structure that is composed of a network of cell membrane-bound glycoproteins and proteoglycans, such as syndecans, glypicans, perlecan, and versican. The proteoglycans form the structural and functional backbone of the glycocalyx. In particular, the glycosaminoglycan (GAG) side chains of the proteoglycans contribute to the glomerular charge barrier function due to the overall highly negative charge of GAGs, whereas in addition GAGs can bind soluble molecules such as albumin, orosomucoid, and lumican and many other factors [1, 3, 9].

Heparan sulfate (HS) is one of the main sulfated GAGs in the glomerular endothelial glycocalyx. In addition to the contribution of HS to the glomerular filtration barrier function, HS is also a key player in cellular and tissue homeostasis, due to its capacity to selectively bind growth factors, chemokines, and many other factors. Heparanase is the only known mammalian endoglycosidase that is capable of degrading heparan sulfate. Chondroitin sulfate (CS) is another sulfated GAG that is typically present in the glycocalyx at a ratio of 1:4 compared to HS, although this ratio is variable depending on, for example, endothelial cell activation status. The major non-sulfated GAG in the glycocalyx is hyaluronan (HA). HA is a very efficient water binder, 1 gram of HA is able to bind 1000 gram of water, and therefore HA is responsible for the gel-like structure of the glycocalyx, whereas HA plays an important role in structural maintenance of vascular integrity [10]. Degradation of hyaluronan by hyaluronidase causes both a reduced glycocalyx thickness and an increased passage of albumin across the endothelium, illustrating the importance of an intact glycocalyx in barrier function [10, 11].

Due to the many functions and complexity of the glycocalyx, any disruption or damage to the glycocalyx contributes toward various vascular pathologies, including diabetic nephropathy [9, 12]. For instance, upon endothelial activation through inflammation, both the glycocalyx thickness and composition are changed, which is mainly due to action of the heparan sulfate-degrading enzyme heparanase [10]. In animal models for diabetes, a threefold increase in permeability of the negatively charged albumin can be observed, which is correlated to a reduced endothelial glycocalyx thickness and a reduced presence of glycocalyx proteins, such as versican and decorin [3, 13]. In contrast to the increased permeability for albumin, the permeability for Ficoll, with a similar size to albumin, but neutral in charge, is not affected in these animal models for diabetes. Therefore, it can be concluded that the charge barrier function, rather than the size barrier function is affected in these animal models for diabetes. Therefore, it may be suggested that endothelial damage, in particular a compromised glycocalyx, seems to precede damage to the podocyte/glomerular basement membrane [3].

Heterogeneity of Renal Endothelial Cells

Endothelial cells from renal arteries, arterioles, capillaries, venules, veins, and glomerular capillaries are phenotypically distinct, which may include differences in glycocalyx composition. The renal endothelium has several functions, such as

oxygen/nutrient delivery and/or charge-selective filtration, whereas the endothelial cells in different renal compartments perform different functions to maintain kidney homeostasis [14–16]. Endothelial cells derived from different renal compartments display varying chemokine receptor expression patterns, thereby mediating renal compartment-specific immune cell recruitment under inflammatory conditions [14]. Regarding reported changes in the renal endothelial glycocalyx in diabetic nephropathy, the majority of published research has focused on the glomerular endothelium. Changes in glycocalyx, in, for example, peritubular capillaries and other renal vasculature, and their corresponding effects on endothelial function have to our knowledge not been adequately addressed yet. It can be hypothesized that a better understanding of glycocalyx composition and function in different renal vascular beds may contribute to a better understanding of endothelial cell dysfunction in progressive kidney injury, including diabetic nephropathy.

Glomerular Endothelial Abnormalities in Diabetic Patients

Morphological abnormalities of the glomerular endothelium in diabetic patients have been reported in several studies. For instance, in patients with type 1 diabetes, a reduction in the amount of fenestrated glomerular endothelium was observed, ranging from 41% in controls to 32% in normo- and microalbuminuric patients and to 25% in macroalbuminuric patients [17]. The reduced glomerular endothelial cell fenestration was related to typical histological diabetic nephropathy lesions and a reduced renal function.

A similar decrease of glomerular endothelial fenestration was reported for type 2 diabetes patients [18]. Moreover, this latter study revealed that endothelial damage and podocyte damage occur simultaneously, which seems in contradiction with the idea of some researchers that in diabetic nephropathy podocyte injury is the primary event and endothelial damage occurs as a secondary effect. Nevertheless, aforementioned findings support the concept that the glomerular filtration barrier is only functional when all layers are intact [18, 19].

Heparan Sulfate as Part of the Extracellular Matrix

The glycocalyx in fact is part of the extracellular matrix (ECM). Constituents of the glycocalyx are, among others, the heparan sulfate proteoglycans (HSPGs) consisting of the polysaccharide HS linked to a proteoglycan core protein, such as syndecans and glypicans, the polysaccharide hyaluronan as well as other glycosylated proteins, and proteins that bind to these polysaccharides and glycosylated proteins [10]. HSPGs are involved in cell-cell and cell-ECM interaction via their HS chains. Furthermore, HS domains mediate binding of specific proteins, which is essential for the organization of cell surface protein-receptor interactions and for the creation of chemotactic gradients of growth factors and chemokines. For example, HS mediates the interaction of fibroblast growth factor-2 (FGF-2), vascular endothelial

growth factor (VEGF), and heparin-binding epidermal growth factor-like growth factor (HB-EGF) in conjunction with their corresponding receptors [20, 21], thereby underscoring a crucial role for HSPGs in physiological and pathological processes in the kidney.

Biosynthesis and Regulation of Heparan Sulfate

HS biosynthesis occurs in the Golgi apparatus via a complex multistep process, which is characterized by chain initiation, polymerization, and several modifications, such as N-deacetylation and O-sulfation [22–24]. All these different modifications give rise to an enormous structural diversity of HS, which dictates binding and modulation of growth factors, complement factors, chemokines, cytokines, selectins, enzymes, and other proteins [22, 24]. HS is degraded by heparanase, a β(1–4)-endoglucuronidase that cleaves HS via hydrolysis [25]. The recognized cleavage site is a tetrasaccharide that is accommodated within the heparanase-binding cleft. Heparanase is encoded by the HPSE1 gene, which is located on chromosome 4 at 4q21.3. Alternative splicing leads to expression of two mRNA transcripts that have the same open reading frame and encode the same 543 amino acid polypeptides, i.e., HPSE1a and HPSE1b. Heparanase is synthesized as a 68 kDa pre-proheparanase, which is targeted to the endoplasmic reticulum, via its signal peptide, and after cleavage of the signal peptide, the 65 kDa proheparanase is formed. The proheparanase is then targeted to the Golgi apparatus where it will be secreted in vesicles (Fig. 10.1) [21, 22, 26].

Heparanase is regulated in multiple ways. Heparanase activity is negatively regulated by heparanase 2, which lacks enzymatic activity and thus is an inactive heparanase variant, although the exact mechanism needs to be identified [27]. Furthermore, heparanase expression is regulated by methylation of the HPSE1 promotor and by the actions of cellular tumor antigen p53, inducing constitutively silencing of the gene. Additionally, members of the SP1 and ETS transcription factor families can induce HPSE1 expression. Moreover, HPSE1 expression can be induced by inflammatory mediators such as TNFα and IL-1β via their downstream transcription factor NF-κβ, as well as factors involved in cell growth like transcription factor early growth response protein 1 (EGR1). Factors involved in diabetic conditions also induce glomerular heparanase expression, such as components of the renin-angiotensin-aldosterone system (RAAS), advanced glycation end products, high glucose, reactive oxygen species (ROS), and endothelin-1 (Fig. 10.2) [21]. On the other hand, cellular quiescence induced by nitric oxide that is produced by eNOS and anti-inflammatory intracellular signaling through the vitamin D receptor suppress heparanase expression. The regulation of heparanase expression through vitamin D signaling was demonstrated by an increased heparanase expression and the development of proteinuria in knockout mice incapable of converting vitamin D into its active form and by reduction of heparanase expression and proteinuria after treatment with the active form of vitamin D (Fig. 10.2) [21, 28].

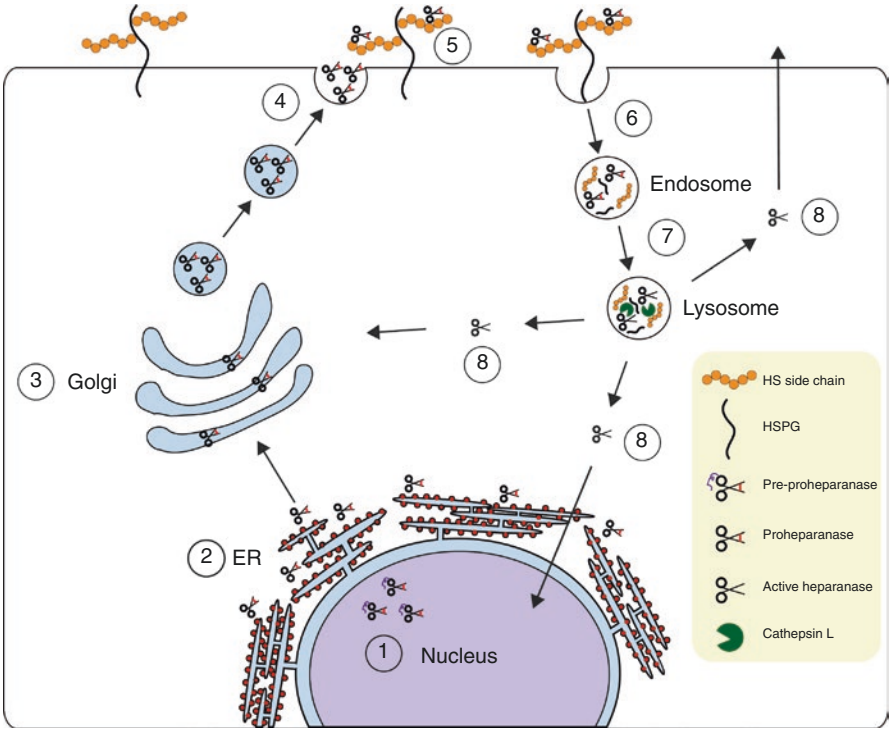

Fig. 10.1 Schematic overview of heparanase biosynthesis and trafficking. (1) Heparanase is synthesized in the nucleus as pre-proheparanase and subsequently trafficks to the endoplasmic reticulum (ER). (2) In the ER the signal peptide of pre-proheparanase is cleaved off resulting in proheparanase. (3) Proheparanase trafficks to the Golgi apparatus, where proheparanase is packaged into vesicles. (4) Subsequently, proheparanase is secreted into the extracellular matrix. (5) Once proheparanase is located in the extracellular matrix, proheparanase can bind to cell-associated HSPGs (in particular to syndecan). (6) Binding of proheparanase to HSPGs then causes internalization of the complex consisting of HSPGs and proheparanase by endocytosis. (7) As endosomal maturation takes place, the endosomes will become more acidic and thus convert into lysosomes, which will activate cathepsin L. Cathepsin L will cleave out an internal linker domain of proheparanase thereby processing proheparanase into the active heparanase heterodimer. (8) Upon activation, heparanase can be transported back to the Golgi apparatus, where heparanase will cause further remodeling of the intracellular HS structures. Furthermore, heparanase can be transported to the nucleus, where it is involved in the process of chromatin remodeling, probably by affecting histone acetyltransferase activity. Finally, heparanase can be transported back to the cell surface where it will be secreted into the ECM and degrade HS of the glycocalyx

Heparan Sulfate in Charge-Selective Filtration

It has been thought for decades that negatively charged HS in the glomerular basement membrane (GBM) was essential for the charge-selective permeability of the glomerular filtration barrier (GFB). HS in the GBM appears to be decreased in many glomerular diseases, like diabetic nephropathy. This decreased HS expression

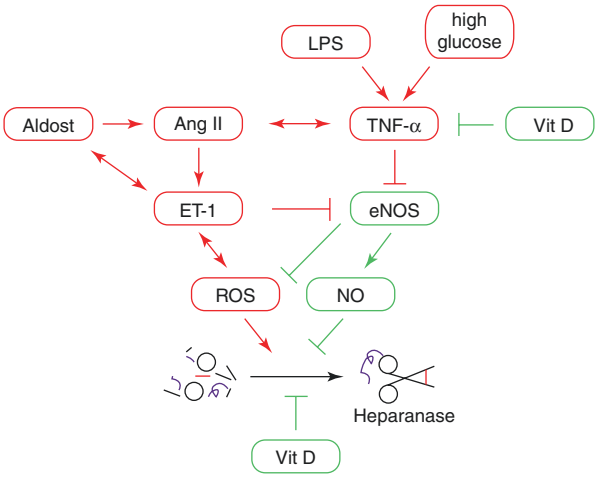

Fig. 10.2 Factors involved in the regulation of heparanase expression. In inflammatory conditions (such as in the presence of LPS) or under conditions of high glucose, TNF-α is induced. TNF-α will subsequently reduce eNOS expression, while it induces angiotensin II expression. Angiotensin II induces endothelin-1 expression, which induces aldosterone expression; creating a positive feedback loop as aldosterone further increases angiotensin II expression. Furthermore, endothelin-1 stimulated ROS production and reduces eNOS expression. eNOS reduces heparanase expression via reducing the ROS production and increasing NO production. Finally, vitamin D reduces heparanase expression either directly or indirectly via reduction of TNF-α expression. The abbreviations used are standing for LPS (lipopolysaccharide), Vit D (vitamin D), TNF-α (tumor necrosis factor-α), Aldost (Aldosterone), Ang II (angiotensin II), ET-1 (endothelin-1), ROS (reactive oxygen species), eNOS (endothelial nitric oxide synthase), and NO (nitric oxide). The factors that reduce heparanase expression are depicted in green, while the factors that increase heparanase expression are shown in red

is inversely correlated with the level of urinary protein excretion [29]. However, the primary role of HS in the GBM in charge-selective filtration was questioned by studies that applied several genetically engineered mouse models with disturbed HS(PG) expression in the GBM. Mice lacking the most abundantly expressed core protein agrin, or both agrin and perlecan, lack the majority of anionic sites in the GBM, but did not develop proteinuria or glomerular abnormalities [30, 31]. Furthermore, mice deficient for the cell surface HSPG syndecan-1 or endothelial NDST-1 also did not show albuminuria [32]. Mice lacking the essential HS-polymerizing enzyme EXT1 in their podocytes clearly showed glomerular ultrastructural abnormalities like foot process effacement, but only mild albuminuria was observed [33]. Finally, overexpression of heparanase in mice displayed a fivefold reduction of GAG-associated anionic sites in the GBM, but no severe albuminuria or ultrastructural abnormalities were observed [34]. Taken together, several mouse models targeting HS expression in the GBM do not display proteinuria, whereas a reduced HS expression in the capillary filter is associated with proteinuria in proteinuric patients. The primary role of HS, in the GBM, in determining charge-selective filtration can be rejected based on aforementioned studies. However,

limitations of the aforementioned studies were that targeting of HS in the GBM was performed under non-pathological conditions, which still warrants a possible role of reduced HS in the GBM under pathological conditions. Importantly, aforementioned studies shifted focus to the glomerular endothelial glycocalyx as the primary barrier in charge selectivity of the capillary filter under normal conditions [2].

Tight Control of Heparanase Activity Under Healthy Conditions

Heparanase facilitates HS turnover and recycling, thereby also contributing to remodeling of HS within the glycocalyx that may be important for disruption of barrier function and/or inflammatory processes. Because heparanase can disrupt the ECM and cell surface signaling processes, it is of great importance that heparanase activity is tightly controlled under healthy conditions. It has, for instance, been shown that heparanase can modulate HS within the glycocalyx, thereby facilitating binding of chemokines and growth factors and leukocytes [35]. Furthermore, heparanase is involved in autophagy, a cellular defense mechanism that generates metabolic precursors and ATP, clears cell debris and misfolded proteins, and is important for cell survival under stressful conditions. Autophagy deficiencies in podocytes lead to accelerated diabetes-induced podocytopathy in mice with streptozotocin-induced diabetes mellitus. Both HS and heparanase can influence autophagy. HS can constitutively inhibit autophagy, while heparanase positively stimulates the autophagy process through a nonenzymatic mechanism [21, 36].

The enzymatic activity of heparanase relies on an acidic environment. Raising the lysosomal pH by administration of substances such as chloroquine and bafilomycin A1 can therefore block the heparanase enzymatic activity. Under normal conditions, heparanase acts typically intracellular. Secreted proheparanase is quickly bound and taken up by the cells, mediated by the low-density lipoprotein receptor-related proteins and mannose-6 phosphate receptors, after which it is transferred to late endosomes and lysosomes [21]. Cathepsin L subsequently cleaves proheparanase into the mature active enzyme (Fig. 10.1) [37]. The proteoglycan syndecan-1 seems to be critical for proheparanase processing after its internalization [38].

Upon heparanase activation in the endosome, heparanase can be taken up by the Golgi system again, although its enzymatic activity will be relatively low due to the neutral pH in the Golgi (Fig. 10.1). Furthermore, heparanase can be transported from the endosome to the nucleus where it may facilitate chromatin remodeling, thereby increasing transcriptional activity of specific genes, which is mechanistically still poorly understood (Fig. 10.1). HS in the nucleus may inhibit histone acetylation, whereas heparanase can relieve this HS-mediated inhibition. Therefore, loss of nuclear heparanase is associated with tumor progression and tumor cell dedifferentiation [39, 40].

Upon activation, heparanase can also be transported from the endosome back to the cell surface where it can degrade HS in the ECM including the glycocalyx of

endothelial cells and podocytes (Fig. 10.1). The degradation of HS in the ECM and glycocalyx impairs barrier function of the glomerular capillary filter and causes release and modulation of the HS-bound chemokines and growth factors and generates potentially bioactive HS fragments. Under physiological healthy conditions transport of activated heparanase from endosome back to the cell surface does not occur often, except in placental trophoblasts and blood-borne immune cells due to the requirement of heparanase activation for physiological tissue remodeling and cell invasion. However, under inflammatory conditions, as in diabetes mellitus, extracellular heparanase activity is increased [21, 41].

Glomerular Endothelium in Kidney Disease: Role of Heparanase

Dysfunction of the endothelium due to, for instance, disturbance of metabolism of the endothelium by hyperglycemia in diabetic nephropathy is defined by impaired endothelium-dependent vasodilation and endothelial activation. This impaired vasodilation and endothelial activation is associated with an environment that promotes initiation and complications of atherogenesis due to its proinflammatory, proliferative, and procoagulatory effects [6]. The observed vasodilation is mainly due to the key role the endothelium plays in vascular homeostasis and damage to the endothelium will therefore disturb the balance between vasoconstriction and vasodilation, thereby initiating a cascade of events promoting or exacerbating atherosclerosis [6].

The endothelial glycocalyx exerts a key function in many physiological processes, including vascular permeability, attenuation of blood cell-vessel wall interactions, mechanotransduction, signaling, and vascular protection. Glycocalyx damage may disturb these physiological processes, potentially causing several vascular pathologies, such as development of proteinuria and inflammation, including diabetic nephropathy [2, 9, 12].

Increased glomerular heparanase activity has been demonstrated in human proteinuric glomerular diseases, including diabetic nephropathy [42]. In general, both podocytes and glomerular endothelial cells show increased heparanase expression associated with proteinuric glomerular disease, whereas heparanase levels in tubular cells are typically high also under healthy conditions [22, 43]. Increased expression of glomerular heparanase corresponds to loss of glomerular HS in rats, while the onset of proteinuria and loss of glomerular HS can be prevented by administration of a neutralizing antibody against heparanase or the heparanase inhibitor PI-88, thereby directly linking the development of proteinuria to loss of HS as mediated by heparanase [27, 44].

Heparanase Is Crucial for the Pathogenesis of Diabetes Mellitus and Diabetic Nephropathy

Heparanase has been implicated in the pathogenesis of various inflammatory kidney diseases, in acute kidney injury caused by sepsis [45], and has a potential role in the development of diabetes and diabetes complications. Increased extracellular heparanase activity is associated with pancreatic β-cell failure. Islet-specific autoreactive T cells can produce heparanase that promotes the migration of leukocytes going from the pancreatic blood vessels into the islets, which causes an immune response while simultaneously depleting islet β-cells of the intracellular HS necessary for cell survival [46]. Moreover, heparanase is overexpressed in the pancreas under obese conditions, causing polarization of islet-infiltrating macrophages toward a damaging inflammatory M1 phenotype, leading to pancreatic β-cell failure. On the other hand, heparanase can be associated with immunomodulatory protective effect, as it stimulates regulatory T helper type 2 cells production in some nondiabetes mouse models. This indicates that heparanase-mediated effects are model-dependent and that our understanding of heparanase is incomplete [21].

A ground breaking study demonstrated that heparanase is crucial for the development of experimental diabetic nephropathy. In contrast to wild-type mice, heparanase-deficient mice are completely resistant to streptozotocin-induced diabetic nephropathy [47]. Heparanase-deficient mice failed to develop proteinuria, and their urinary albumin excretion rate was normal, while a fivefold increase in urinary excretion rate was observed in the wild-type mice after streptozotocin-induced diabetic nephropathy. The role of heparanase under pathological conditions is supported by the fact that SST0001, a heparanase inhibitor, lowers albuminuria in type 1 and type 2 diabetic mice [47].

The diabetic milieu is one of the strongest inducers of heparanase expression. Patients with type 1 or type 2 diabetes show both a reduction in systemic glycocalyx volume and initially microalbuminuria that is associated with urinary heparanase levels [22]. High glucose levels alter the biosynthesis of sulfated GAG domains, in particular that of HS, indicating a possible role of hyperglycemia in systemic glycocalyx reduction. Furthermore, both hyperglycemia and glycated serum proteins contribute to heparanase upregulation in certain cell types, among others, endothelial cells and podocytes, whereas other factors such as ROS, aldosterone, and angiotensin II are amplifying factors as well [22, 48, 49].

An endothelial peptide, involved in the progression of diabetic nephropathy, called endothelin-1, activates podocytes to release heparanase. Furthermore, the endothelin-1-induced heparanase expression in podocytes causes an increase of transendothelial albumin passage. Moreover, the diabetes-induced upregulation of glomerular heparanase expression and corresponding reduction in HS expression, endothelial glycocalyx thickness, and development of proteinuria, which was

observed in wild-type mice, can be prevented by podocyte-specific deletion of the endothelin receptor [50].

Besides directly affecting endothelial glycocalyx, and thus barrier function, heparanase-mediated effects on inflammation provide an additional mechanism that contributes to the development of diabetic nephrology. Patients with diabetic nephropathy show increased levels of monocyte chemoattractant protein-1 (MCP-1) in their renal tissue and urine, thereby suggesting that macrophages have a pathogenic role in the development of proteinuria and glomerular damage and the progression of renal disease in humans [51]. This association between renal macrophages and pathological lesions in human diabetic nephropathy was indeed confirmed. The lysosomal cysteine protease cathepsin L, which facilitates processing and activation of enzymes, such as proheparanase in the extracellular matrix, can be secreted by macrophages [44, 52]. Furthermore, it was shown that macrophages are more prone to activation by, for instance, LPS or INF-γ, after they have been pretreated with heparanase, resulting in, among others, an increased TNF-α production (Fig. 10.3) [42, 53]. The mechanism behind this sensitization of macrophages, and also other cell types, by heparanase is only poorly understood, but it has been suggested that toll-like receptors (TLRs) 2 and 4 mediate cellular hyperactivation by binding of heparanase-generated HS fragments [54, 55]. Additionally, increased cellular activation can be due to heparanase-mediated shaving of cells, which may improve the accessibility of cytokine receptors for their specific ligands. A recent study even showed that heparanase-mediated sensitization is not limited to macrophages, because heparanase-mediated hypersensitivity for insulin was shown in breast cancer cells [56], thereby postulating the possibility of sensitization of other cell types involved in diabetic nephropathy such as glomerular endothelial cells but also podocytes.

Pathological events mediated by podocytes are also related to cleavage of cytoskeleton-associated proteins such as dynamin and synaptopodin by cathepsin L, which leads to impairment of the actin cytoskeleton and effacement of the podocyte foot process, for example, by cathepsin L-mediated cleavage of synaptopodin [57]. Besides macrophages, also podocytes in response to injury can secrete cathepsin L into the extracellular space. It has been shown that cathepsin L expression is increased in many glomerular diseases, like in diabetic nephropathy, and treatment of this increased cathepsin L expression with a cathepsin L inhibitor results in reduction of proteinuria [52, 58]. Cathepsin L-deficient mice with streptozotocin-induced diabetes do not lose their renal function and do not develop albuminuria, mesangial matrix expansion, tubulointerstitial fibrosis, podocyte injury, or renal macrophage influx, which is most likely caused by the inability of cathepsin L-deficient mice to activate heparanase [52]. Furthermore, MCP-1 inhibition results in decreased albuminuria and is associated with a shift in tissue macrophage phenotype toward alternatively activated M2 macrophages, resulting in a reduced glomerular cathepsin L and heparanase expression and restoration of the glomerular glycocalyx barrier function [21].

One of the hallmarks of glomerular lesions in diabetic nephropathy is destabilization of glomerular capillaries and corresponding mesangiolysis and glomerular

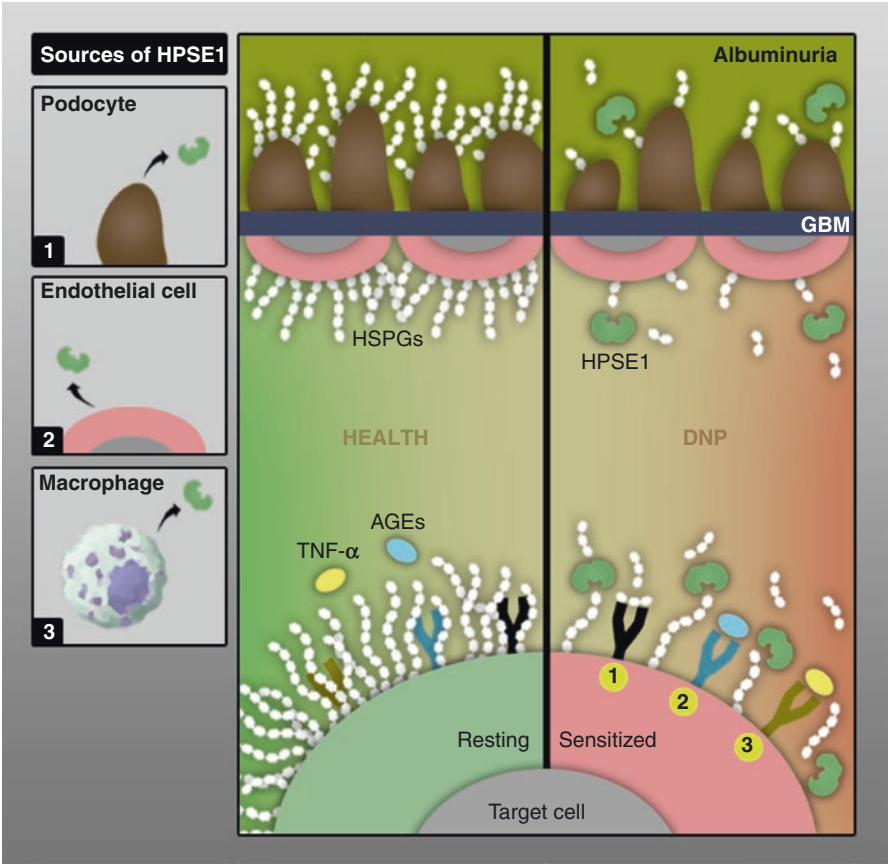

Fig. 10.3 The interplay between glomerular and immune cells leading to increased glomerular heparanase activity and albuminuria. During the development of diabetic nephropathy (DNP), glomerular heparanase activity is increased, thereby degrading heparan sulfate (HS) in the glomerular filtration barrier, which leads to proteinuria. Heparanase may be derived from immune cells such as macrophages and glomerular cells, such as podocytes and glomerular endothelial cells (left panel). The local inflammatory cytokine milieu acts on both immune and glomerular cells, thereby further enhancing heparanase and cathepsin L expression (not shown), which is required to activate pro-heparanase. Exciting recent data show that active heparanase can sensitize cells by degrading HS at the cell surface, as depicted in the lower part of the figure. Heparanase-generated HS then binds to TLR2 and TLR4 (depicted by 1), thereby increasing cellular activation. Additionally, heparanase-mediated shaving of cells may improve the accessibility of cytokine receptors (depicted by 2 and 3) for their specific ligands, which also leads to increased cellular activation. Overall, the increased glomerular heparanase activity shaves the glycocalyx from both endothelial cells and podocytes, thereby facilitating albuminuria

hypertrophy [59]. Induction of increased HS turnover and O-sulfation by increased activity of extracellular heparanase results in enlarged angiogenic growth factor binding. In case of sustained heparanase activity, heparanase in the ECM is released together with its bound growth factors, thereby facilitating angiogenesis and vessel

destabilization [60]. Moreover, angiogenesis is boosted by upregulated intracellular heparanase expression via direct enhancement of cellular VEGF production [61]. Finally, immunocytes are recruited due to the action of heparanase, which constitutes another important role in the angiogenesis process [21].

It can be concluded that heparanase activity, most likely in relation to reduction in HS expression levels, plays a crucial role in pathogenesis of proteinuria in experimental diabetic nephropathy at several levels.

Heparanase as a Pharmacological Target

Intracellular heparanase expression and activity plays a key role in cell survival and communication and should therefore not be targeted for therapy, except in cases where cells have to be killed like in cancer. As outlined, extracellular heparanase activation can cause inflammation, vessel destabilization, and fibrosis. A promising treatment strategy in kidney disease would thus be targeting of the extracellular heparanase activity. Some compounds that are aiming to inhibit heparanase expression or reduce heparanase activation are being developed and are already being tested for their therapeutic benefit. However, those compounds are thus far mainly used and developed in the context of cancer therapies and might target both intracellular and extracellular heparanase.

One approach is the development of drugs that compete with natural HS substrate by binding to the HS substrate-binding domain of heparanase, and this class of drugs are called HS mimetics [21, 62, 63]. One drawback of HS mimetics is that due to the structural resemblance to natural HS, these compounds can bind to many other HS-binding proteins, increasing the possibility of off-target effects. There are hundreds of proteins known to possess the capacity to interact with HS, together called the heparan sulfate interactome, or heparanome. This interactome includes proteins that are involved in various cellular and biological processes such as cell attachment, migration, invasion and differentiation, morphogenesis, organogenesis, blood coagulation, lipid metabolism, inflammation, and responses to injury [64]. For example, HS mimetics can modulate HS-mediated interactions between thrombin, antithrombin III, and protein C inhibitor, thereby influencing coagulation [64]. Furthermore, HS mimetics can be taken up by cells and modify the intracellular regulatory function of heparanase, as outlined. Another downside of HS mimetics is their probability to provoke an inflammatory response since HS can serve as ligand for TLR-2 and TLR-4 on macrophages and epithelial cells [54].

In addition to HS mimetics, such as SST0001 [62], synthetic HS tetrasaccharides containing unsubstituted glucosamine residues, like GP545, are under development. These synthetic HS structures are resistant to heparanase activity and can therefore be applied as a heparanase inhibitor [65]. Furthermore, multiple oligosaccharides that are derived from marine algae are currently tested for their possible ability to modify HS-heparanase interactions. Sulfated polysaccharides that resemble glycosaminoglycans are present in different algae species in the marine environment. One

compound that has been tested thus far is λ-carraheptaose, which is a highly sulfated polysaccharide derived from red algae. The λ-carraheptaose seems to act simultaneously as competitive inhibitor of heparanase, and thus as HS-mimetic, and as inhibitor of FGF-2 signaling. Another compound, a fucosylated form of chondroitin sulfate, which is extracted from marine echinoderm, was shown to reduce heparanase expression in the glomerulus thereby protecting rats from streptozotocin-induced diabetic nephropathy [21].

Administration of HS, heparin, low-molecular-weight heparin or the heparinoid danaparoid all are, in potential, able to reduce albuminuria in patients with diabetes mellitus, but these compounds have some off-target effects. One example is sulodexide, which is a highly purified mixture consisting of 80% of low-molecular-weight heparin and of 20% of dermatan sulfate [66], for which there are some conflicting studies. Sulodexide was shown to be effective in restoring the glycocalyx thickness and showed a trend toward normalization of systemic albumin clearance in a study of type 2 diabetes mellitus patients, but in two other studies, no such effect was observed in type 2 diabetes mellitus and diabetic nephropathy patients [21]. These contradicting findings might be explained by the use of sulodexide derived from different animal sources, and therefore the presence of different biological active structures in different preparations. Due to the lack of insight into the specific structures within different sulodexide preparations that are responsible for heparanase inhibition and subsequent anti-proteinuric effects, wrong conclusions may have been drawn from aforementioned studies.

Another strategy to inhibit heparanase-mediated HS breakdown is by directly blocking the HS-binding site on heparanase. There are three potential HS-binding domains in heparanase identified that could serve as a target for heparanase inhibition. A peptide directed against Lys158-Asp171 domain of heparanase physically interacts with HS and heparin and inhibits heparanase activity [67]. Moreover, a polyclonal antibody and two monoclonal antibodies raised against this region may provide a new class of drugs leading to a reduced heparanase activity [67, 68]. This new class of drugs, yet to be tested in models of kidney disease, might become an appealing treatment option for patients with diabetic nephropathy or other heparanase-mediated nephropathies.

Some drugs that are currently used in renal medicine have been shown to suppress glomerular heparanase expression and activity, such as angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), and vitamin D [28, 49]. These drugs have been associated with reduced albuminuria in clinical trials, which might be explained by their ability to reduce heparanase activity. Endothelin-A (ETa)-receptor blockade is currently under exploration in randomized clinical trials for its renoprotective potential, since it reduces albuminuria clinically. Selective ETa-receptor blockade facilitates preferential ETb-receptor stimulation by endothelin-1 and increased nitric oxide production by the endothelium. Moreover, ETa-receptor blockade causes suppression of heparanase activity associated with restoration of the glomerular glycocalyx, restoration of the barrier function, and reduction of albuminuria [21].

Several drugs currently tested or used for treatment of renal dysfunction target pathways that reduce heparanase activity both in immunocytes and podocytes and

might therefore be adequate to resolve residual albuminuria treatment. Clinical studies that have therapeutically targeted monocytes by blocking their chemokine receptor CCR2 (also known as CD192) or by blocking the CCR2 ligand, MCP-1, support the idea that albuminuria can be reduced by targeting immunocyte activation as both approaches led to reduced albuminuria in patients with diabetic nephropathy [69, 70]. The obtained reduction in albuminuria due to blockage of the CCR2 ligand was associated with a reduced cathepsin L release by tissue macrophages [21]. As outlined, cathepsin L is important in heparanase activation, and reduction of cathepsin L expression/activity was therefore further associated with reduced heparanase activity and restoration of the glycocalyx filtration barrier. Due to its key role in heparanase activation, cathepsin L could also be considered a potential therapeutic target. Furthermore, cathepsin L is shown to be required for the development of albuminuria and diabetic nephropathy [52]. Only several non-specific cathepsin L inhibitors have been tested and showed reduction of proteinuria in experimental models of anti-glomerular basement membrane glomerulonephritis [21, 71, 72]. To date, more specific cathepsin L inhibitors have been developed; however, their therapeutic effects in glomerular diseases have not yet been tested.

Another promising treatment strategy includes the use of heparanase 2, which is an inactive heparanase variant as it shares 44% identity and 59% similarity with heparanase but lacks enzymatic activity [27]. It has been shown that heparanase 2 inhibits heparanase activity [27, 73]. The mode of action of heparanase 2 may rely on its higher affinity to HS compared to heparanase, thereby blocking the binding of heparanase to HS. As heparanase cannot bind to HS on the cell surface, it fails to get internalized and will therefore remain inactive. In addition, heparanase 2 may physically interact with heparanase, thereby preventing the cleavage of HS chains. Furthermore, heparanase 2 is not likely to activate macrophages, which is problem in case of HS mimetics aiming to inhibit heparanase activity. However, the potential of heparanase 2 as an inhibitor of heparanase activity in glomerular diseases remains to be elucidated in experimental models.

Besides application of heparanase as a target for therapy, heparanase activity might also serve as a suitable biomarker for risk stratification and treatment titration as urinary heparanase excretion is increased in patients with diabetes, especially in case of albuminuria, whereas no urinary heparanase activity is present in healthy individuals.

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Chapter 11
The Podocyte in Diabetic Nephropathy: Recent Advances

Gavin I. Welsh and Richard J. Coward

Introduction

Diabetic nephropathy (DN) is the leading cause of kidney failure, accounting for approximately a third of patients in the UK and half of patients in the USA entering end-stage renal failure and requiring dialysis or kidney transplantation. It is increasing rapidly due to the global epidemic of type 2 diabetes. In most cases DN is characterized by progressive albuminuria due to damage to the glomerular filtration barrier of the kidney. Initially this begins with the loss of small amounts of urinary albumin in the range of 30–300 mg/L (microalbuminuria), but as the kidney damage progresses, this develops into macroalbuminuria (>300 mg/L). Diabetic patients with albuminuria have a greatly increased chance of dying due to cardiovascular events such as stroke and heart attacks [1]. It is now clear that the glomerular podocyte is a key target cell in the prevention of albuminuria. Landmark genetic and biological studies over the last decade point compellingly to the podocyte as a critical cell in maintaining glomerular filtration barrier function and an important early target cell in DN [1, 2]. There are now over 50 human genetic mutations associated with albuminuria, all of which code for proteins found in the podocyte [3]. Podocyte cell injury plays a pivotal role in the pathogenesis of diabetic nephropathy. The characteristic podocyte response to injury or cell stress is actin cytoskeleton reorganization, which typically leads to foot process effacement, resulting in proteinuria. This is followed by irreversible podocyte depletion which coincides with the progression of glomerular disease, as these cells do not have the ability to proliferate and regenerate. Numerous human and experimental studies have demonstrated that the podocyte number or density was diminished during DN. Reduced podocyte number is also a predictor of DN progression [4–7].

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The glomerulus is the filtration unit of the kidney and is composed of a bundle of capillaries, which are highly permeable to water, and yet can selectively allow passage of solutes while retaining larger macromolecules. This selectivity is achieved through the action of the glomerular filtration barrier. The glomerular filtration barrier consists of the glomerular basement membrane, glomerular endothelial cells and glomerular epithelial cells or podocytes. Podocytes are terminally differentiated epithelial cells that are critical in preventing protein passage across the filtration barrier. Podocytes have branching and interdigitating processes, and filtration takes place through slits between these processes. The slit diaphragm, a critical component of the filtration barrier, is an ultra-thin zipper-like structure that bridges the gap between interdigitating podocyte foot processes. The slit diaphragm is a cell junction and signalling complex essential for regulating podocyte cytoskeletal dynamics. Podocytes have a remarkably elaborate and highly specialized cell biology and morphology which are essential for maintaining glomerular function and integrity in healthy kidneys [2]. The podocyte is critically important in preventing albuminuria. Damage to or loss of podocytes is linked to the development of albuminuria and occurs early in the progression of diabetic nephropathy [4, 6, 8, 9]. Although good progress has been made in recent years, the mechanisms underlying podocyte injury have not yet been fully identified. Therefore, understanding the critical factors and signalling pathways that control this cell in the setting of diabetes is highly desirable.

Damage/changes to the specialized cell biology of the podocyte have been reported to be due to podocyte hypertrophy, endoplasmic reticulum stress, autophagy, epithelial-to-mesenchymal transition (EMT), detachment and apoptosis. Historically this was thought to be due to the diabetic milieu especially high glucose. However, we have shown that diminished insulin signalling to the podocyte is also detrimental to glomerular function [10–12]. We have recently described the role of the podocyte in the pathogenesis of DN [13]. In this chapter, we will discuss new insights into the mechanisms underlying podocyte injury in the progression of DN (displayed in Fig. 11.1) which may point to novel therapeutic targets to develop important renoprotective treatments for DN.

Diabetic Environment and Insulin Sensitivity

Hyperglycaemia has been demonstrated to be a key factor underlying podocyte injury [14], and podocytes have been shown to be the direct target of circulating hormones, lipids and adipokines whose levels are altered in diabetes [13]. For example, free fatty acids (FFA) are elevated in insulin-resistant states, are involved in the pathogenesis of diabetic nephropathy and induce insulin resistance in human podocytes [15]. Interestingly insulin resistance has emerged as a key driver of impaired glomerular function and the development of renal complications. Insulin resistance plays a major role in the pathogenesis of both type 1 and type 2 diabetes [16, 17] being associated with albuminuria and nephropathy [18, 19]. Insulin

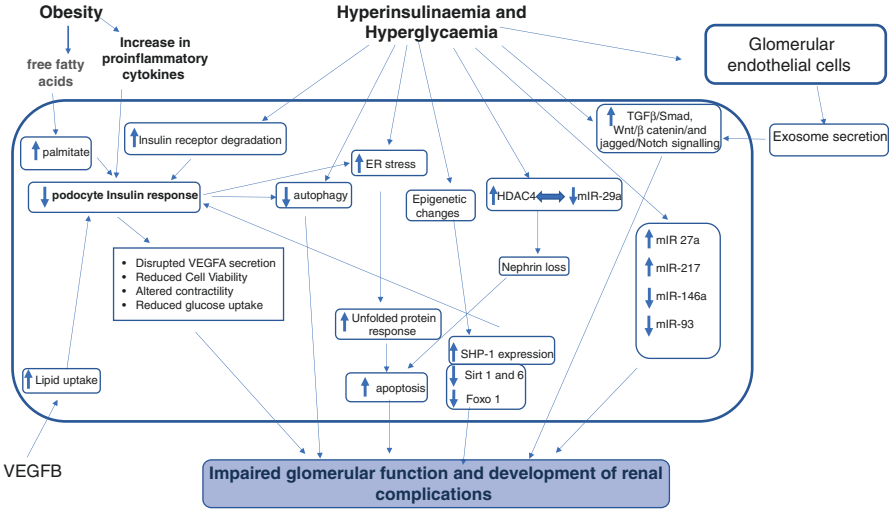

Fig. 11.1 Schematic overview of pathophysiological mechanisms of podocyte damage in the pathogenesis of diabetic nephropathy. VEGF vascular endothelial growth factor, ER endoplasmic reticulum, HDAC histone deacetylase, SHP-1 protein tyrosine phosphatase type 1, mIR micro-RNA

resistance is also associated with the development of albuminuria in nondiabetic individuals [20]. Furthermore, renal disease is also common among people with severe forms of genetic insulin resistance [21]. We have shown that the podocyte is a direct target for insulin action and that loss of this signalling leads to a diabetic nephropathy like phenotype importantly in the absence of hyperglycaemia [10]. Recently we have shown that degradation of the insulin receptor, caused by high levels of insulin, drives early podocyte insulin resistance and that both the IR and nephrin are required for full insulin sensitivity of this cell [22]. This could be highly relevant for the development of nephropathy in type 2 diabetic patients who are commonly hyperinsulinaemic in the early phases of their disease. In both type 1 and type 2 diabetes, glomerular insulin signalling is lost early in the development of kidney disease [23] suggesting that targeting, and enhancing, this pathway, in these settings, could be beneficial.

Podocyte EMT

A number of phenotypic and morphological changes are seen in the injured podocyte which are described as an epithelial-to-mesenchymal transition. These result from hyperglycaemia induced changes to several podocyte signalling pathways such as upregulation of the TGFβ/Smad, Wnt/β catenin/ and jagged/Notch pathways which have been described in detail in a recent review article [24]. Interestingly it has been

demonstrated that high glucose induces glomerular endothelial cells to secrete exosomes that are internalized by podocytes causing podocyte EMT possibly via TGF-β1 in the exosomes and activation of podocyte Wnt/β-catenin signalling [25].

Podocyte Endoplasmic Reticular (ER) Stress and Autophagy

In a diabetic environment cellular metabolic overload results in increased cellular oxidative stress and ER-stress which leads to the activation of unfolded protein response (UPR) [26]. UPR is a positive cellular response that in its early phase either refolds accumulated unfolded proteins or degrades unfolded protein by the ubiquitin-proteasome pathway. This is probably extremely important for the podocyte as it a terminally differentiated cell with minimal capacity to replicate, so maintaining its cellular function under stress is crucial. Misfolded proteins are detected as a result of ER membrane stress which, in turn, activates several signalling events and triggers a compensatory response to prevent further accumulation of misfolded protein. However, when the unfolded protein and cellular damage exceed a threshold, chronic and unresolved stress results in a change from an adaptive to pro-apoptotic responses [26].

There is now evidence that glucose/oxidative stress-mediated ER stress plays a role in chronic vascular complications in DN [27]. Hyperglycaemia or increased glycation of proteins has been shown to mediate apoptosis partly through increases in ER stress in cultured murine podocytes [28, 29]. Activation of the UPR has also been observed in mouse glomerular mesangial cells exposed to glucose and glucosamine [30], and in kidneys from diabetic rats administered streptozotocin for 16 weeks [31]. Furthermore, microarray analysis of human biopsies from patients with established DN showed that UPR genes were upregulated proportionally to the severity of diabetic renal lesions [32].

Recently a link between podocyte insulin sensitivity and ER stress has been demonstrated. Madhusudhan et al. have elegantly shown that under diabetic conditions ER adaptive mechanisms are impaired in the podocyte and that this is exacerbated when the cell is rendered more insulin resistant by partially knocking down its insulin receptor in a podocyte-specific manner. Studying human and murine DN, they have shown that nephropathy was associated with alterations in the UPR with impairment of the nuclear translocation of XBP-1. Genetic ablation of the transcription factor XBP-1 or activation of ATF6 (downstream of XBP-1) in the podocyte of diabetic mice aggravates DN. Of interest, mice with genetically impaired podocyte insulin signalling exhibited impaired UPR (XBP-1 activation) that was associated with more severe diabetic kidney disease when compared with diabetic controls [33].

Autophagy, regulated by the mammalian target of rapamycin complex 1 (mTORC1) is, with the UPR, also essential to maintain cellular homeostasis and in the context of ER stress contributes towards the elimination of toxic and damaged cellular components [34]. Genetic loss of mTORC1 in podocytes or administration of rapamycin (a mTORC1 inhibitor) resulting in activation of autophagy [35] has

been shown to prevent progressive DN [36, 37]. In contrast, mTORC1 activation in podocytes, resulting in inhibition of autophagy, leads to accelerated DN [38]. Loss of insulin sensitivity in cultured podocytes results in suppression of autophagy and addition of rapamycin in these cells attenuates insulin resistance [39]. In the future, understanding how to manipulate podocyte ER stress and autophagic pathways may prove fruitful in developing novel therapies for DN.

Podocyte Vascular Endothelial Growth Factors

In the past decade, it has become clear that several vascular endothelial growth factors are produced by the podocyte and are altered in diabetes. A key factor produced by the podocyte, which signals to the endothelium, is vascular endothelial growth factor A (VEGFA). Podocyte produced VEGFA is crucial for glomerular function both during development [40] and also in maturity [41]. It is also clear that its production needs to be tightly regulated as either too much or too little is detrimental [40].

Recent studies have shown a connection between insulin resistance and the subsequent production of VEGFA in podocytes [42]. This finding is likely to be important in the setting of DN with many elegant studies using transgenic mice highlighting the importance of podocyte VEGFA levels in the progression of this condition [43]. A new aspect of VEGFA signalling in the glomerulus is potential crosstalk between VEGFA secreted from podocytes and the GECs glycocalyx in the setting of diabetes. There is clear evidence that the GECs glycocalyx is lost both systemically and within the diabetic glomerulus and that this contributes to both cardiovascular and renal complications [44]. Mechanistically there are a number of pathways which led to loss of the glomerular glycocalyx including hyperglycaemia [45] and reactive oxygen species (ROS) [46].

During the early phases of diabetes, an increase in VEGFA causes glycocalyx shedding from the GECs. Furthermore, the inhibitory isoform of VEGFA, called VEGF-$A_{165b,}$ also plays a role in maintaining the GECs glycocalyx in diabetes. Oltean et al. [47] have shown that in diabetic patients with progressive nephropathy, the renal expression of VEGF-A_{165b} is lost. They went on to develop several murine models of DN and have shown that genetic overexpression or pharmacological administration of VEGF-A_{165b} to the mouse, acting through VEGF receptor 2 in the GECs, restores damaged glomerular endothelial glycocalyx and improves renal function. VEGF-A_{165b} also improved the permeability of isolated human diabetic glomeruli suggesting the response is conserved across murine and human species [47].

Very recently another member of the VEGF family, VEGF-B, has been implicated in the development of diabetic nephropathy through causing increased podocyte lipid uptake and subsequently inducing insulin resistance in this cell type. This is believed to be through VEGF-B engaging with the VEGFR1 (Flt1) and Neuropilin-1 (NRP1) in glomerular endothelial cells and upregulation of fatty acid transporter protein 3 (FATP3) and FATP4 which facilitates the passage of free fatty acids through the filtration barrier and into the podocyte. Elegant studies by Falkevall

et al. [48] in which VEGF-B was systemically genetically or pharmacologically inhibited in diabetic and high-fat fed mice have revealed that podocyte insulin sensitivity is increased and that mice are subsequently protected from DN. Furthermore, they show that in patients with diabetic nephropathy that their glomerular VEGF-B levels are increased, and this closely correlates with their degree of albuminuria. Genetic overexpression of VEGF-B in the podocyte mirrors these findings when the mice are fed a high-fat diet. Going forward it will be interesting to understand the precise mechanism through which lipids are taken up by podocytes when VEGF-B is increased in the glomerulus, the precise cellular origin of VEGF-B and if there are any VEGF-B receptors in the podocyte that facilitate lipid uptake. These could reveal novel therapeutic targets to prevent the progression of DN in the future.

Epigenetics

There is increasing evidence that epigenetic modifications, resulting from prolonged exposure to hyperglycaemia, play an important role in podocyte injury and diabetic nephropathy. These modifications include methylation of cytosine residues of the DNA and acetylation and methylation of lysine residues of the histone proteins which are the principal component of chromatin. These changes have been shown to continue even after the normalization of glucose levels explaining in part why diabetic complications persist in patients even after hyperglycaemia is controlled [49]. One of the first reported demonstrations of these epigenetic modifications in podocytes involved the adapter protein $P66^{Shc}$ which mediates receptor tyrosine kinase signalling and oxidative stress-induced apoptosis [50]. This protein has been strongly implicated in the pathogenesis of diabetic nephropathy as $p66^{Shc}$-deficient mice are protected against this condition [51]. High glucose was shown in podocytes to induce hypomethylation and hyperacetylation of the $p66^{Shc}$ promoter resulting in high levels of $p66^{Shc}$ expression leading to mitochondrial $p66^{Shc}$ translocation, ROS generation and oxidative stress [50]. Subsequently, hyperglycaemia has also been shown to result in increased histone H3 lysine acetylation and histone H3 lysine methylation of the promotor of the tyrosine phosphatase SHP1 resulting in high expression of this protein and inhibition of insulin signalling pathways even after glycaemic control had been achieved [52, 53]. Furthermore, elevated circulating lipids, which are known to cause podocyte insulin resistance, have been shown to alter histone modifications of the FOXO1 promotor in podocytes, an effect again which is sustained after lipid levels have returned to normal [15, 54]. FOXO1 is a key regulator of gluconeogenic genes, and overexpression has been reported to ameliorate podocyte injury in diabetic animal models and high glucose-treated podocytes by decreasing apoptosis and promoting mitophagy, via regulation of the PTEN-induced PINK1/Parkin-dependent signalling and inhibiting epithelial-mesenchymal transition [55–58].

Histone acetylation, which is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDAC), plays a crucial role in the regulation of gene

expression [49]. The histone deacetylases are a family of enzymes (HDACS 1–11 and Sirts 1–7), several of which have been implicated in podocyte injury and the pathogenesis of diabetic nephropathy [59, 60]. Sirt 1 and 6 levels and activity are downregulated in high-glucose and AGE-BSA-treated podocytes and in diabetic animal models [59, 61–63]. Sirt1 deficiency has been shown to exacerbate podocyte injury in both diabetic and glomerular disease models [59, 60]. Sirt1, via crosstalk with AMPK, plays a key role in the regulation of insulin action in podocyte [61]. Furthermore, downregulation of Sirt1 in podocytes has been shown to result in epigenetic modulation and subsequent upregulation of the tight junction protein Claudin 1. Upregulation of Claudin 1 activates the β-catenin/Snail pathway leading to downregulation of the slit diaphragm protein podocin and the actin-binding protein Synaptopodin resulting in podocyte foot process effacement [59]. Interestingly the effects of hyperglycaemia on the downregulation of both Sirt1 and AMPK could be ameliorated by treatment of the podocytes with metformin, a commonly used diabetes treatment, suggesting a potential mechanism by which this drug improves the insulin sensitivity of podocytes and prevents diabetes-related complications [64]. Sirt6 has been shown to protect against podocyte injury by reducing inflammation, blocking apoptosis, maintaining the actin cytoskeleton and promoting autophagy through epigenetic regulation of Notch signalling [63]. Therefore, both Sirt1 and 6 are required for the maintenance of a healthy podocyte phenotype and downregulation of these proteins as seen in diabetes leads to podocyte damage.

HDAC proteins have been shown to also have non-epigenetic roles in regulating the progression of podocyte injury in diabetes by deacetylating several nonhistone proteins. Sirt1 deacetylates the transcription factors FOXO4, NFκB and STAT 3, all of which play a role in diabetic nephropathy through increased expression of their target genes, and increased acetylation of these proteins has been observed in diabetic animal models and human diabetic kidneys [65]. Sirt1 also has a role in the maintenance of the podocyte actin cytoskeleton integrity by regulating the acetylation of the actin-binding and polymerizing protein cortactin [60]. HDAC4 expression is upregulated in streptozotocin-induced diabetic rats, kidneys from diabetic patients and in podocytes treated with high glucose, advanced glycation end products or TGF-β. HDAC4 regulates podocyte autophagy via deacetylation of the transcription factor STAT1 [66]. HDAC4 has also been shown to deacetylate nephrin, and high glucose leads to decreased nephrin acetylation leading to increased nephrin loss and podocyte apoptosis which is ameliorated by HDAC4 knockdown in podocytes [67]. This control of nephrin acetylation and loss is part of a complex pathway involving the reciprocal regulation of HDAC4 and the micro RNA(mIR)-29a [67]. In contrast to HDAC4, the levels of mIR-29a are reduced in high glucose-treated podocytes and in diabetic animals. HDAC4 reduces the expression of mIR-29a via decreased acetylation of its proximal promoter. In high glucose-treated podocytes, knockdown of HDAC4 leads to increased acetylation of the mIR-29a promoter, upregulated levels of mIR-29, increased nephrin acetylation and reduced podocyte apoptosis. Importantly HDAC4 is a target for mIR-29a and thus mIR-29a can reciprocally regulate the expression of this protein. In diabetic animals overexpressing mIR-29a nephrin levels are restored, HDAC signalling is reduced and

podocyte viability and renal function are improved. Knockdown of mIR-29a leads to increased HDAC4 activity, podocyte apoptosis and renal damage. Therefore, a tight choreography of this reciprocal pathway is important for the health of the podocyte, and the above results suggest that this is deleteriously altered in diabetic nephropathy [67].

Interestingly, several other podocyte mIRs have been implicated recently in the pathogenesis of diabetic nephropathy. For example, the expression of mIR-27a is stimulated by high glucose in cultured podocytes and is upregulated in renal biopsy samples from patients with diabetic nephropathy. Increased mIR-27a expression in podocytes leads to decreased expression of PPARγ and subsequent activation of β-catenin signalling resulting in increased podocyte EMT and apoptosis [68]. Increases in the levels of miR-217 are also seen in high glucose-treated podocytes, and this has been linked to podocyte injury and insulin resistance via regulation of PTEN-mediated autophagy signalling [69]. Conversely loss of mIR-146a in podocytes, which is seen in both glomeruli from diabetic patients and animal models, leads to increased susceptibility to diabetes induced damage via upregulation of ErbB4 and Notch1 [70]. Levels of mIR-93 are also downregulated in the kidneys of experimental animal models of diabetes. mIR-93 through modulation of its target Msk2, a histone kinase and its target H3S10 plays a critical role in chromatin reorganization in podocytes. Importantly inducible expression of mIR-93 specifically in podocytes led to major improvements in key features of diabetic nephropathy in diabetic db/db mice including much reduced mesangial matrix expansion, and increased synaptopodin, and nephrin levels [71].

Finally, long noncoding RNAs have also been shown to play a role in the podocyte in the development of diabetic nephropathy. Long noncoding RNA (lncRNA) taurine-upregulated 1 (Tug1) was shown to regulate mitochondrial bioenergetics in podocytes by epigenetic targeting of expression of the transcription factor PPARγ coactivator 1α (PGC-1α). Tug1 expression is reduced in db/db mouse model of diabetes and overexpression of TUG1 in these animals led to key improvements in biochemical and histological features associated with diabetic nephropathy including rescued expression of PGC-1α and its downstream targets and improvements in podocyte mitochondrial bioenergetics [72].

Podocyte Targeted Treatment of Diabetic Nephropathy

There is evidence that strategies that enhance cellular insulin-sensitivity, including metformin and peroxisome proliferator-activated receptor gamma (PPARγ) agonists, such as rosiglitazone, are beneficial in preventing kidney damage in models of diabetic nephropathy in both type 1 and type 2 diseases, as well as other nondiabetic chronic kidney diseases [64, 73]. It is possible that these drugs are exerting part, or all, of their beneficial effects by directly enhancing insulin sensitivity of the podocyte. For example, we have shown that rosiglitazone directly augments insulin signalling in human immortalized podocytes in vitro [74]. Resveratrol,

a non-flavonoid polyphenol, has been shown to have beneficial effects in the treatment of diabetic kidney disease and has recently been shown in a diabetic mouse model to protect podocytes against apoptosis by stimulating autophagy [75]. Furthermore, astragaloside IV, a traditional Chinese herbal remedy, has been reported to prevent the progression of diabetic nephropathy in streptozotocin-induced diabetic mice by attenuating ER stress and promoting autophagy in podocytes [76]. Therefore, understanding the mechanisms behind podocyte damage during diabetes is an important step in treating this condition and directly targeting the podocyte may be beneficial in kidney disease states especially diabetic nephropathy.

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Chapter 12
Inflammatory Processes in Diabetic Glomeruli

Daphne H. T. IJpelaar

Introduction

Diabetic nephropathy was traditionally thought to be the result of hyperglycaemia and haemodynamic factors (e.g. hyperfiltration); however, in the last years more evidence has been gathered that indicate a causative role for inflammatory factors in development of diabetic renal damage. This chapter describes these inflammatory molecules and inflammatory cells and provides a possible mechanism involved in inflammatory glomerular damage in diabetic nephropathy.

Inflammatory Molecules

From the 1990s onwards, human studies have shown that inflammatory molecules are upregulated in serum of diabetic patients with diabetic nephropathy compared to patients without renal involvement [1, 2]. In addition, experimental models of diabetic nephropathy suggest that several inflammation-related molecules are involved in the development of diabetic nephropathy or progression of renal damage (Table 12.1) [3].

A number of pro-inflammatory cytokines are upregulated in diabetic nephropathy, such as IL-1, IL-6, IL-18 and TNFα [3]. The expression and effect of these cytokines depend on the cell type, context and kinetics of diabetic stimuli such as hyperglycaemia and AGEs (advanced glycation end products). These molecules are mainly expressed by inflammatory cells but also by intrinsic renal cells. IL-1, for

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Table 12.1 Pro-inflammatory molecules in diabetic nephropathy per glomerular cell type

Group of molecules	Molecule	Glomerular cell type		
		Mesangial cell	Endothelial cell	Podocyte
Cytokines	IL-1	x [4]	x	x [5]
	IL-6	x [6]		
	TNFα	x	x	x
Chemokines	MCP-1	x [7]	x [8]	x
Adhesion molecules	VCAM-1	x [9]		
	ICAM-1	x	x	
	P/E-Selectin	x		
Transcription factor	NF-kB	x	x	x [10]

x means: the molecule is present in this cell type
Abbreviations: *IL-1* interleukin-1, *IL-6* interleukin-6, *TNFα* tumour necrosis factor alpha, *MCP-1* monocyte chemoattractant protein 1, *VCAM-1* vascular cell adhesion molecule 1, *ICAM-1* intercellular adhesion molecule 1, *NF-kB* nuclear factor kappa-light-chain-enhancer of activated B cells

example, is expressed by glomerular endothelial and epithelial cells, and by mesangial cells after stimulation with AGEs, and is thought to have a role in macrophage attraction by upregulation of adhesion molecules in endothelial but also in mesangial cells [11]. Furthermore, IL-1 induces prostaglandin synthesis in mesangial cells and thus changes glomerular haemodynamics [12].

IL-6 mRNA is upregulated in glomerular cells in diabetic nephropathy and in infiltrating cells [6]. More specifically, IL-6 mRNA levels are increased in mesangial cells and podocytes, and its level was associated with severity of diabetic nephropathy and GBM thickness [13]. Because the renal and urinary concentration of IL-6 protein is correlated with the degree of urinary albumin excretion [14, 15], IL6 might have a causal role in diabetic nephropathy and albuminuria. Il-18 is constitutively expressed in tubular epithelial cells and upregulated particularly in the interstitium in diabetic nephropathy and is discussed in greater detail in Chaps. 13 and 15 of this book. TNFα can be induced by all resident cells as well as infiltrating monocytes. It is cytotoxic to renal cells by stimulation of, amongst others, reactive oxygen species and apoptosis [16, 17]. Furthermore, it not only changes haemodynamics through an imbalance between vasodilatory and vasoconstrictive mediators but also increases endothelial permeability [17].

Next to the stimulation of locally produced cytokines, several chemokines and adhesion molecules are upregulated in resident renal cells. The production is induced by the earlier described cytokines, but also glucose or AGEs themselves are potent stimulators of chemokine production. Chemokines are involved in chemoattraction of inflammatory cells such as monocytes and macrophages, to sites of inflammation. The best defined chemokine in diabetic nephropathy is monocyte chemoattractant protein-1 (MCP-1, also called CC chemokine ligand 2 or CCL2). MCP-1 is systemically induced in several cell types including endothelial and epithelial cells and in the glomerulus also broadly expressed in mesangial [7] and endothelial cells [9]. Its expression is stimulated by AGEs, glucose, stretching of mesangial cells and angiotensin II, amongst others [18]. The exact role of MCP-1 in glomerular inflammation in diabetic nephropathy

will be discussed in the next paragraph. Increased expression of adhesion molecules such as ICAM-1 (intercellular adhesion molecule 1) and selectins enhances the homing of the inflammatory cells. In agreement with this finding, macrophage infiltration in MCP-1 and ICAM-1 knockout mice was reduced in diabetic nephropathy [19, 20].

Finally, transcription factors such as NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) are involved in upregulation of several intracellular processes. NF-kB is present in all cells in an inactive state, and it is activated by stimuli such as cytokines, oxygen radicals, activation of the renin-angiotensin system and in tubular cells by proteinuria [10]. It is able to bind to the promotor region of several genes involved in diabetic nephropathy, e.g. TGFβ (transforming growth factor beta), MCP-1 and ICAM-1 [21], leading to upregulated expression of these molecules. In conclusion, inflammatory molecules are locally upregulated in glomerular resident cells, and inflammation is probably enhanced by chemoattraction of systemic inflammatory cells.

Inflammatory Cells

Increased levels of MCP-1 in renal tissue as well as in urine of patients with diabetic nephropathy suggest that influx of macrophages might have a pathogenic role in the development of diabetic renal damage [22]. In addition, the level of urinary MCP-1 is linked to the level of urinary albumin excretion [23]. In experimental models of type 1 and type 2 diabetes mellitus, macrophages are prominently present in glomeruli when nephropathy develops. Several intervention studies in these models showed that amelioration of renal damage and albuminuria coincides with a decrease in the number of glomerular macrophages [8]. Furthermore, in CD11b diphtheria toxin receptor transgenic mice, a mouse model in which administration of diphtheria toxin specifically leads to depletion of monocytes/macrophages; depletion of macrophages resulted in prevention of renal damage, again suggesting a causal role of macrophages in the pathogenesis of diabetic nephropathy [24]. Studies in MCP-1 knockout mice, or after inhibition of MCP-1 or blockage of its receptor C-C chemokine receptor type 2 (CCR2), all show that MCP-1 and the presence of macrophages are essentially involved in the induction of the inflammatory reaction in diabetic nephropathy [19, 25–27].

The question remains whether the number of inflammatory cells or their phenotypes are related to severity of diabetic renal damage. The number of infiltrating renal macrophages is inversely correlated with outcome in several renal diseases [28]. Since macrophages that display a pro-inflammatory and pro-fibrotic cytokine profile are also capable of secreting cytokines which are beneficial in kidney repair and remodelling, their activation and exact expression profile are very important. One of the main functions of macrophages is phagocytosis, the major mechanism to remove pathogens or cell debris. We recently studied the mechanism of MCP-1 inhibition in apo E knockout mice, made diabetic with streptozotocin, and showed that MCP-1 inhibition did not change the total number of glomerular macrophages

but was linked to a switch in tissue macrophage phenotype to less inflammatory macrophages, as depicted by an increase of CD206 positive macrophages [29]. Stimulation of isolated renal macrophages after in vivo MCP-1 inhibition showed less IL-1 and more IL-10 expression compared to isolated renal macrophages without MCP-1 inhibition. These changes in ex vivo phenotype were accompanied by restoration of the glomerular glycocalyx in vivo, probably as a result of a reduced presence of activated heparanase and thus less degradation of heparan sulphates (see Chap. 10 of this book). As a result, these mice showed less urinary albumin excretion. In addition, other interventions such as inhibition of the endothelin A receptor or substitution of vitamin D also shifted the phenotype of macrophages towards a less inflammatory one [30, 31].

In patients with diabetic nephropathy, only a few studies described the presence or phenotype of glomerular inflammatory cells. Already in 1993, macrophages were found by immunohistochemistry in glomeruli of renal biopsies of patients with non-insulin-dependent diabetic nephropathy [32]. In addition, Nguyen et al. [33] showed that macrophages are present in glomeruli and interstitium in renal biopsies of patients with type 1 and 2 diabetes mellitus. In that study the number of glomerular macrophages was correlated with baseline creatinine levels, but not with decline in renal function over time. Recent observations revealed the presence of macrophages in all pathological classes of diabetic nephropathy (classes I through IV, pathological classification of DN as described in Chap. 8 of this book) in autopsy tissue of patients with type 2 diabetic nephropathy. Here, the number of glomerular CD163-positive cells, as a marker of less inflammatory macrophages, was positively associated with class of diabetic nephropathy, interstitial fibrosis, tubular atrophy and glomerulosclerosis [34]. These findings indicate that macrophages infiltrate in all stages of diabetic damage, but phenotype might change during ongoing damaging.

In conclusion, in particular in experimental studies of diabetic nephropathy, evidence has been gathered that inflammatory cells play an important role in development of diabetic nephropathy and that these cells can change function during this process.

Mechanism of Inflammation and Glomerular Damage in Diabetic Nephropathy

The influence of inflammatory cells on glomerular diabetic damage depends on four processes:

(a) Pro-inflammatory molecules and chemoattraction (Fig. 12.1)
(b) Adhesion and transendothelial migration (Fig. 12.2)
(c) Activation or differentiation (Fig. 12.3)
(d) Intraglomerular actions (Fig. 12.3)

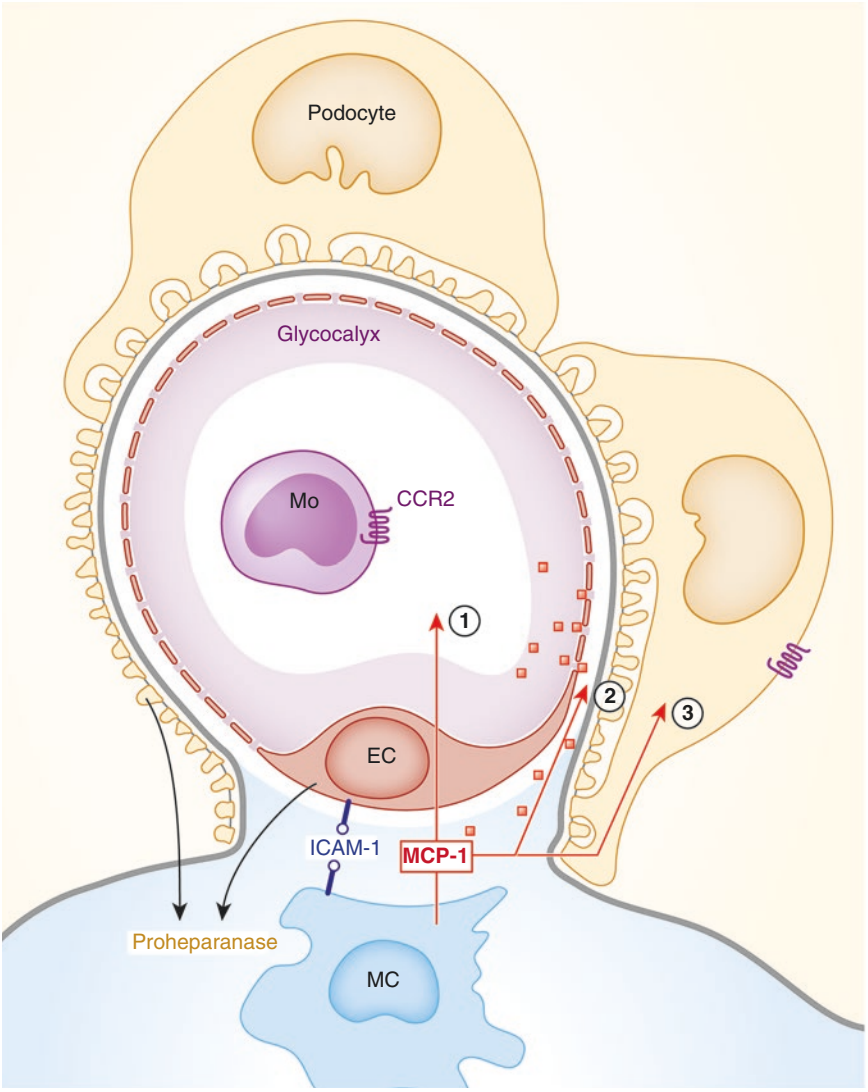

Fig. 12.1 Pro-inflammatory molecules and chemoattraction. MCP-1 is induced in diabetic glomeruli. Here three effects of MCP-1 are shown. (1) It is released into the bloodstream and activates mobilization of monocytes from the bone marrow into the blood in a CCR2-dependent manner. (2) A chemokine gradient is formed along the glycocalyx, needed for homing and migration. (3) MCP-1 can bind its CCR2 receptor on podocytes. In addition, several other molecules are induced within the glomerulus in diabetic milieu, e.g. ICAM-1 and proheparanase. EC endothelial cell, MC mesangial cell, Mo monocyte

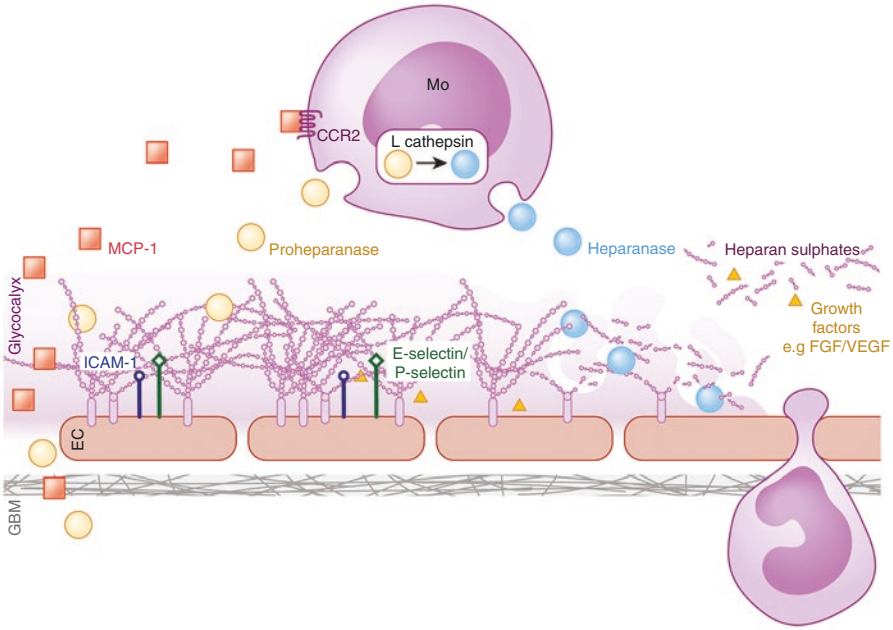

Fig. 12.2 Adhesion and transendothelial migration. First rolling and adhesion of monocytes to the endothelial layer are enabled by multiple receptors such as E-selectin and ICAM-1. Bound monocytes activate proheparanase by intracellular L-cathepsin. Heparanase degrades heparan sulphates of the glycocalyx, leading to a disrupted glycocalyx. This is needed for migration of monocytes/macrophages into the glomerulus. This also causes leakage of serum proteins such as albumin across the glomerular filtration barrier. Disruption of heparan sulphate also releases growth factors such as vascular endothelial growth factor (VEGF) and FGF, known to be involved in angiogenesis. GBM glomerular basement membrane, EC endothelial cell, Mo monocyte

Pro-inflammatory Molecules and Chemoattraction

The production and secretion of pro-inflammatory molecules in a diabetic milieu have been described in several glomerular cell types, including mesangial cells, endothelial cells and podocytes [35]. One of the most important chemokines that is induced in diabetic milieu is MCP-1. It is produced by several glomerular cell types such as endothelial cells, podocytes and mesangial cells, mainly in response to pro-inflammatory molecules such as IL-1 and TNFα [7]. Once induced, MCP-1 forms a chemokine gradient enabling directed migration of cells expressing the appropriate chemokine receptor, chemokine receptor type 2 (CCR2 or CD192). The CCR2 receptor on monocytes thus enables them to attach and migrate along the increasing chemokine gradient created by extracellular heparan sulphates present. Interestingly,

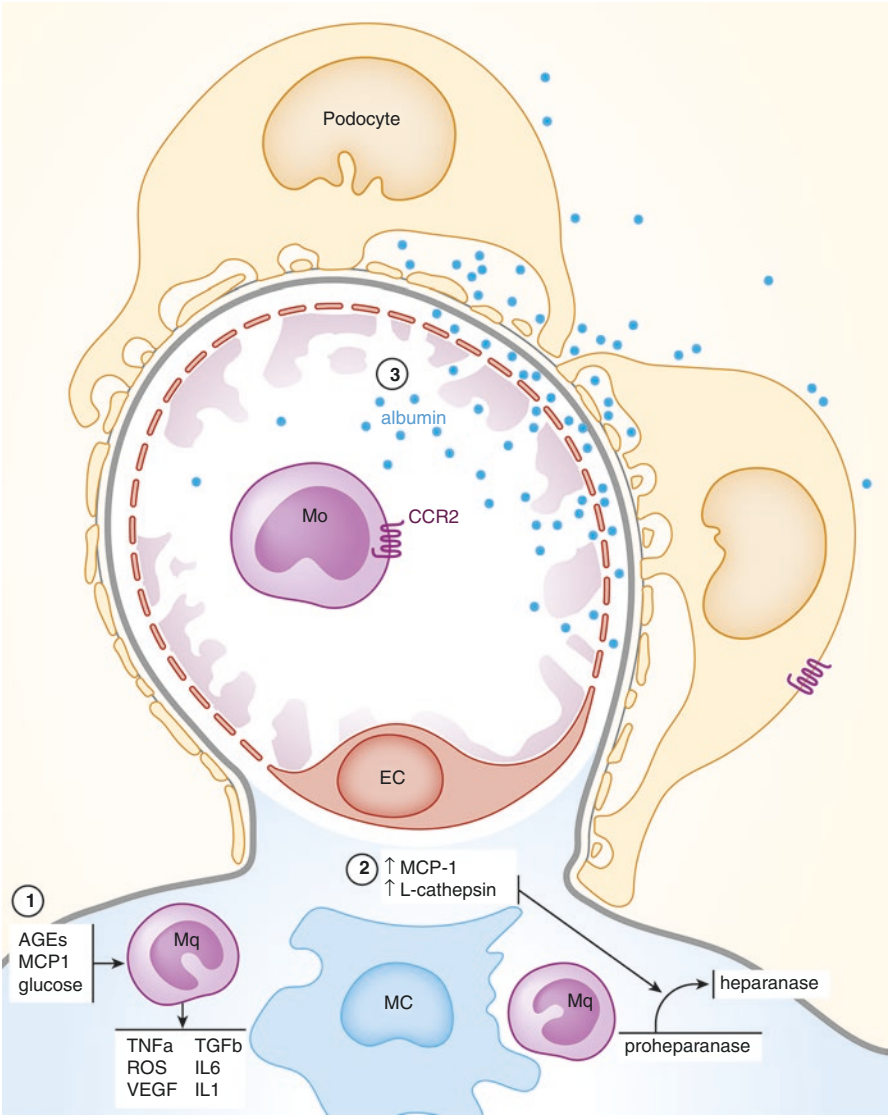

Fig. 12.3 Intraglomerular effects of macrophages. (1) Intraglomerular macrophages are activated by chemokines (such as TNFα), AGEs and high glucose and subsequently release several molecules: TNFα, ROS, VEGF, TGFβ, IL-6 and IL-1, amongst others. (2) Macrophages further stimulate macrophage recruitment by induction of MCP-1 and activation of proheparanase by L-cathepsin. (3) Prolonged inflammation results in a chronically interrupted endothelial glycocalyx and in podocyte damage, causing albuminuria. ROS reactive oxygen species, EC endothelial cell, Mq macrophage, Mo monocyte, MC mesangial cell

binding to heparan sulphates in the extracellular matrix is also essential for several other chemokines to exert proper function. For example, fibroblast growth factor 2 (FGF2) is more active when bound to heparan sulphates compared to the unbound growth factor. Heparan sulphates are also the main compound in the endothelial surface glycocalyx, facilitating direct physical interaction between endothelial and inflammatory cells upon modification.

There are three effects of MCP-1 release upon the inflammatory response in diabetic nephropathy (Fig. 12.1). First, after its expression in diabetic glomeruli, mainly in mesangial cells and podocytes [8], MCP-1 is released into the bloodstream facilitating mobilization of monocytes or its precursors from the bone marrow. This process is CCR-2 dependent, as CCR2 knockout mice show monocyte retention in the bone marrow after stimulation [36]. Secondly, within the glomerulus, a gradient of MCP-1 and other molecules such as ICAM-1 is formed along the glycocalyx. ICAM-1 enhances leukocyte rolling and attachment of monocytes to the endothelial layer. Finally, MCP-1 can also bind the CCR2 receptor on podocytes that might eventually lead to reduced presence of slit diaphragm proteins such as nephrin and to foot process effacement [37]. The protein expression of CCR2 receptor on podocytes is enhanced in diabetic nephropathy.

Adhesion and Transendothelial Migration

Adhesion of inflammatory cells to the endothelial layer is enabled by binding of leukocyte receptors to endothelial cells [38]. For example, P-selectin is involved in rolling of leukocytes on the activated endothelium. In addition, the adhesion is even more stimulated by the chemokines, presented on endothelial cells, such as ICAM-1 (Fig. 12.2).

Once monocytes are bound to the endothelial cells, inflammatory cells need to migrate across the endothelial layer. For this purpose, the glycocalyx needs to be degraded locally by heparanase. Under physiologic conditions heparanase is intracellularly expressed in immunocytes such as monocytes. However, activation of the monocyte induces release of proheparanase and its activator L-cathepsin [39]. Apart from immunocytes, heparanase can also be released by other cells, such as endothelial cells and podocytes, as a reaction on inflammatory mediators such as TNFα, IL-1 and the transcription factors NF-kB and EGR1 but also by the renin-angiotensin-aldosterone system, reactive oxygen species, endothelin-1 and glucose itself. Heparanase is produced as a 68 kDa pre-proheparanase and processed by the Golgi apparatus into a 65 kDa proheparanase. Activation of heparanase, which under physiological conditions predominantly functions intracellularly in its active or inactive form, is tightly regulated. Secreted proheparanase is internalized and activated by L-cathepsin into heparanase. Intracellular heparanase is involved in modification of sulphating heparan sulphates that results in continuous adaption of extracellular heparan sulphate expression to environmental changes such as inflammation. However, release and prolonged expression of extracellular heparanase will

lead to degradation of the heparan sulphate within the extracellular matrix and glycocalyx, resulting in an interrupted chemokine gradient. Finally, this enables leukocyte transmigration.

Urinary albumin excretion is one of the first signs of diabetic nephropathy and is likely linked to a disrupted glycocalyx [39]. Presence of active heparanase is linked to albuminuria in several models of glomerulonephritis as well as of diabetes mellitus. In agreement, heparanase-deficient diabetic mice are protected from glomerular macrophage influx and albuminuria [40, 41].

Release of heparan sulphates with its bound growth factors may exert paracrine effects. FGF2 and vascular endothelial growth factor (VEGF) are pro-fibrotic and angiogenic growth factors, which are known to be involved in angiogenesis and fibrosis in diabetic nephropathy as well [38, 39].

In summary, degradation of the endothelial glycocalyx by heparanase will thus lead to influx of monocytes, albuminuria and vessel destabilization/angiogenesis. The various roles of heparanase in the pathogenesis of DN are discussed in greater detail in Chap. 10 of this book.

Activation or Differentiation

Macrophages require activation to function properly. In immune complex-mediated glomerular disease, macrophage activation is probably the result of the interaction with T-cells or the reaction to phagocyted immune complexes. However, the mechanism of activation in chronic diseases like diabetes mellitus is less clear. Chemokines, such as MCP-1 (in vitro activities are summarized in [7]) and interferon gamma (IFNɣ), themselves may activate macrophages. Furthermore, other diabetic factors can directly activate macrophages. Goldberg et al. [42] found that AGEs and high glucose induce TNFα expression in macrophages (Fig. 12.3). In addition, modified albumin and free fatty acids can also induce the expression of cytokines in these macrophages [43, 44].

Next to activation of recruited macrophages, also resident macrophages might be activated and could play a role in diabetic damage. Studies have mainly focused on the role of interstitial resident macrophages in immune complex-mediated inflammation [45]; however, the role of (glomerular) resident macrophages in diabetic nephropathy is not exactly known.

Intraglomerular Actions

Infiltrated and activated macrophages release lysosomal enzymes (including L-cathepsin), reactive oxygen species, TGF−β, VEGF and cytokines such as TNFα, IL-1 and IFNɣ, leading to cell damage and fibrosis (Fig. 12.3). TNFα has been shown to be detrimental in the pathogenesis of glomerular damage.

Macrophage-specific deletion of TNFα resulted in a reduction of basal glomerular levels of TNFα and a blocked increase in TNFα after induction of diabetic nephropathy, leading to decreased renal damage [24]. The above described molecules cause damage to all glomerular cell types, leading to upregulation of pro-inflammatory and fibrotic molecules.

Activated macrophages also stimulate the release of MCP-1 and thus start a vicious circle of inflammation. This vicious circle is enhanced by the activation of heparanase by macrophages by extracellular release of L-cathepsin. Increased heparanase will lead to a positive feedback loop of increased inflammation, increased influx of inflammatory cells and the subsequent inflammatory response.

Apart from enhanced degradation of proteoglycans by heparanase, L-cathepsin might also damage the proteoglycans expressed by the podocyte, leading to degradation of podocyte-specific proteins such as dynamin and synaptopodin, which are essential for the arborized podocyte structure and restriction of albumin passage across the glomerular filtration barrier [46].

In conclusion, diabetic milieu leads to upregulation of pro-inflammatory molecules and attraction of inflammatory cells, mainly macrophages, thereby degrading the glycocalyx. This results in albumin passage across the glomerular filtration barrier. Furthermore, infiltrated and activated macrophages produce cytokines, leading to glomerular damage. The timing of infiltration and expression profile of macrophages is detrimental in the resulting damaging or repair effect. Persistent activation of macrophages leads to progressive diabetic nephropathy.

The awareness that inflammation is essentially involved in development of diabetic nephropathy offers prospects for new treatment modalities. In recent years, promising clinical trials are performed with MCP-1 inhibitors or CCR2 blockers, suggesting that MCP-1 and subsequently attractions and activation of inflammatory cells are crucially involved in development of diabetic nephropathy [8, 47].

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Part IV
The Tubulointerstitium

Chapter 13
Proteinuria and Tubulotoxicity

Norberto Perico, Ariela Benigni, and Giuseppe Remuzzi

Introduction

Tubulointerstitial injury is common to all chronic progressive renal diseases, irrespective of the initial trigger or site of injury. Once viewed as an inconsequential corollary to pathologic events enveloping glomeruli, tubulointerstitial disease is now recognized as an indispensable and prominent participant in the progression of renal disease [1]. Many, if not most, forms of progressive, non-cystic renal diseases are glomerular in origin, and yet, it is the intensity of accompanying evolving injury of the tubulointerstitial compartment, rather than the extent of glomerular changes, that predicts overall decline in renal function [1].

Although historically proteinuria has been considered as simply a surrogate marker of the severity of underlying glomerular damage, clinical and experimental data reported during more than two decades of intensive investigation indicate that proteinuria is an independent risk factor and plays an important role in the pathogenesis of the progression of renal disease [2, 3]. In 1932 Alfred Chanutin and Eugene Ferris [4] observed that removing the three quarter of the total renal mass in the rat led to a slowly progressive deterioration in the function of the remaining nephrons, with progressive azotemia and glomerulosclerosis. The glomerular lesions of the remnant kidneys were associated with abnormal glomerular permeability and proteinuria. At that time proteinuria was considered a marker of the extent

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of the glomerular damage, despite the fact that Franz Volhard and Theodor Fahr in 1914 [5] and Wilhelm von Mollendorff and Philip Stohr in 1924 [6] already found that renal damage was related to exuberant protein excretion in the urine. In 1954 Jean Oliver [7] recognized protein droplets in the cytoplasm of tubular cells. They suggested that such findings were possibly the results of an impairment on the process of reabsorption of plasma proteins normally carried out by renal tubule and proposed that proteinuria could lead to nephron structural and functional damage.

Nowadays it is well known that glomerular ultrafiltration of excessive amounts of plasma proteins and protein-associated factors incites tubulointerstitial damage and further promotes the effects of glomerular disease on the tubular compartment. The noxious substances in the proteinuric ultrafiltrate may set off tubular epithelial injury with tubular apoptosis, secondary generation of inflammatory mediators, and peritubular inflammation [8]. The mechanisms whereby increased urinary protein concentration leads to nephrotoxic injury are multifactorial and involve complex interaction between numerous pathways of cellular damage.

Tubular Cell Apoptosis and Tubuloglomerular Disconnection

In progressive kidney diseases, a significant link has been shown between the degree of proteinuria and tubular atrophy, which is the most severe consequence of proximal tubule cell apoptosis. The knowledge that proteinuria is a stimulus for tubular apoptosis was first derived by several in vitro and in vivo experimental studies. In cultured human proximal tubule cells, albumin caused apoptosis via caspase-9-mediated mitochondrial pathway characterized by upregulation of the proapoptotic Bcl-2 protein Bax, translocation of cytochrome c from mitochondria to cytosol, and alteration in the mitochondrial membrane potential [9]. This pathway was confirmed in rat proximal tubule cells by inhibition with Bcl-2 transfection and was found to be mediated by PKC-delta, a subfamily of PKC serine/threonine protein kinases [10]. Mechanistically, PKC-delta could favor apoptosis at multiple levels, including the activation of apoptotic genes, phosphorylation of caspases, interaction with apoptotic regulators, remodeling of cell membranes, or interference with mitochondrial function, including endoplasmic reticulum (ER)-mitochondria cross talk during ER stress-induced apoptosis [10].

Renal proximal tubule cells have the remarkable capacity to reabsorb large quantities of albumin through megalin and cubilin receptor-mediated endocytosis [11]. Megalin was proposed to act as the sensor that determines to what extent the cells will be protected from or injured by albumin, depending on albumin concentration [12]. It has been shown that megalin interacts with survival protein kinase B (PKB/Akt), crucial for the phosphorylation of Bad, the Bcl-2-associated death promoter. The exposure of cultured proximal tubule cells to low concentrations of albumin led to activation of PKB and phosphorylation of the Bad protein, inhibiting an apoptotic response. Conversely, albumin at high concentrations more typical of proteinuric

diseases decreased the expression of megalin on the plasma membrane of proximal tubule cells that was associated with the reduction of PKB activity and Bad phosphorylation, favoring apoptosis [12] (Fig. 13.1) . Instead of a direct interaction between megalin and PKB/Akt, a more recent study indicated the possibility of an indirect association mediated by the adaptor protein disabled 2 (Dab2) [13]. Indeed, it was shown that Dab2, which binds to the third NPXY motif of megalin, interacted with PKB/Akt via proline-rich domain and that the expression of both proteins was downregulated in association with albumin-induced apoptosis in human proximal tubule cells, thereby supporting a link between albumin endocytosis and apoptotic cell injury [13].

Proximal tubule cell apoptosis was reported to contribute to tubular atrophy and glomerular-tubule disconnection in response to proteinuria in the rat model of passive Heymann nephritis, to the extent that angiotensin-converting enzyme (ACE) inhibitors that reduced proteinuria prevented both tubular atrophy and disconnection [14] (Fig. 13.2) (Table 13.1). Apoptotic cells expressing both proximal and

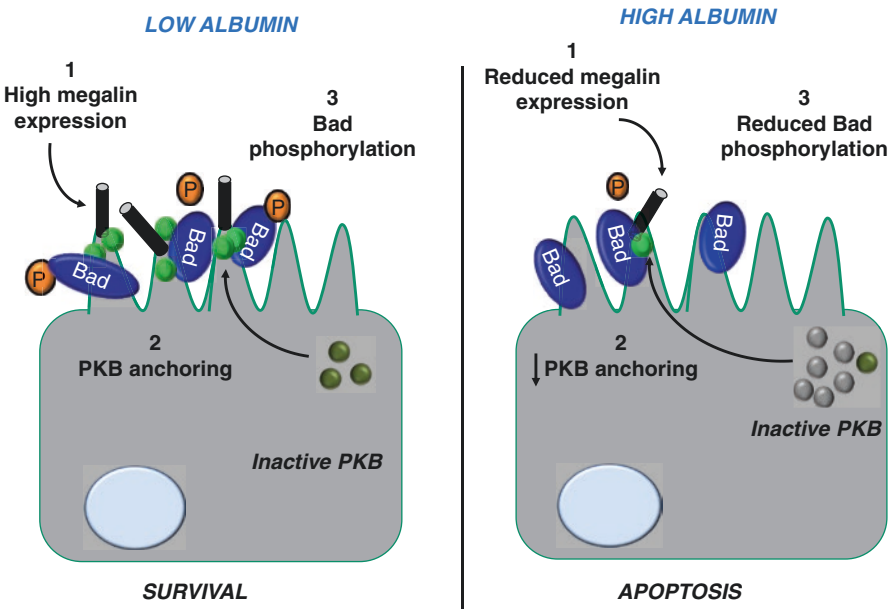

Fig. 13.1 Megalin acts as a sensor which determines if proximal tubule cells are protected or injured by albumin. Exposure of proximal tubule cells to low albumin concentrations activates protein kinase B, which promotes the phosphorylation of Bad protein, protecting against apoptosis. Conversely, albumin at high concentrations more typical of proteinuric diseases decreases the expression of megalin on the plasma membrane of proximal tubule cells, thereby reducing protein kinase B activity. The drop in protein kinase B activity ultimately leads to a reduction in Bad phosphorylation and consequently induces apoptosis in proximal tubule cells. Abbreviations: Bad Bcl2-associated death promoter, PKB protein kinase B. (Adapted from Ref. [12])

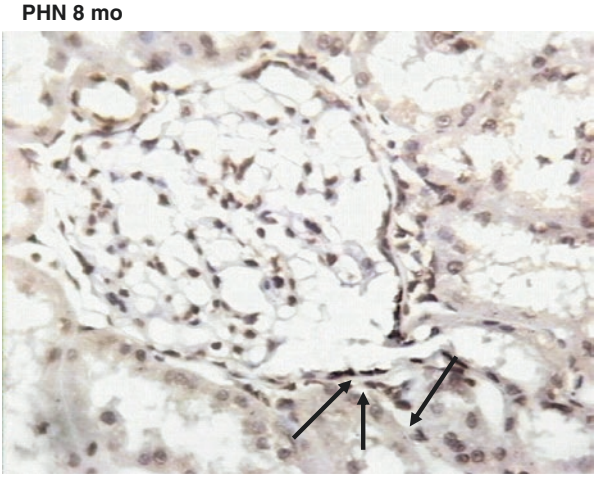

Fig. 13.2 Apoptosis and glomerular-tubular disconnection in rats with passive Heymann nephritis. In kidneys of passive Heymann nephritis rats at 8 months, atrophic tubules invariably show signs of apoptosis, as indicated by strong terminal dUTP nick-end labeling staining (*arrow*), which precedes and may favor disconnection. Abbreviations: PHN passive Heymann nephritis. (Adapted from Ref. [14])

Table 13.1 Proteinuria parallels glomerular-tubular disconnection in rats with passive Heymann nephritis

	Control	PHN	
	4 mo	4 mo	8 mo
Proteinuria *mg/day*	32 ± 6	663 ± 5*	742 ± 35*
Atubular glomeruli %	0.5	9*	27°

PHN Passive Heymann nephritis. (From Ref. [14]). *P < 0.05 vs control; °P < 0.05 vs PHN 4 mo

distal tubular phenotype were detected in biopsy specimens from patients with primary focal segmental glomerulosclerosis [15]. A strong positive correlation was found between proteinuria and the number of apoptotic cells [15].

Protein-Bound Lipids in Tubule Cell Apoptosis

Which of the filtered factors during proteinuric disease play a predominant role as activator of tubule cells has been a matter of debate for many years. It has been argued that fatty acids bound to albumin may contribute considerably to tubulointerstitial injury in proteinuric disease. Fatty acids carried by filtered albumin have been proposed as the chief instigators of tubulointerstitial damage in the model of

protein-overload proteinuria based on more severe macrophage infiltration and tubular apoptosis in rats injected with the fatty acid-carrying albumin, with respect to those injected with fatty acid-depleted albumin [16].

In cultured proximal tubule cells, albumin repletion with fatty acids and its association with linoleic acid induced more apoptosis than the exposure to defatted albumin alone [17]. Furthermore, another study showed that non-delipidated albumin or albumin conjugated with palmitate, but not fatty acid-free albumin, altered both tubule mitochondrial viability and membrane potential and caused cytochrome c release [18]. In concert with the decline of mitochondrial parameters, fatty acid overload led to a redox imbalance which deactivated the antioxidant protein peroxiredoxin 2 and caused a peroxide-mediated apoptosis through the redox-sensitive pJNK/caspase-3 pathway. These data were taken to suggest that attempts at lowering circulating fatty acid levels may be important in both preserving redox balance and limiting tubule cell damage [18]. A novel biochemical mechanism has been proposed linking lipotoxicity to tubule apoptosis in proteinuric conditions [19]. The study focused on the Na^+/H^+ exchanger NHE1, a regulator for proximal tubule cell survival through interaction with the membrane phosphoinositide phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2], which initiated formation of a signaling complex that culminated in Akt activation and opposition to apoptotic stress. Starting from the concept that diseased glomeruli with impaired permselectivity allow filtration and proximal tubule reabsorption of nonesterified fatty acids (NEFA) bound to albumin, it was shown that an accumulation of metabolites of NEFA, long-chain acyl-CoA (LC-CoA) could stimulate lipoapoptosis by competing with the structurally similar PI(4,5)P2 for NHE1 binding, thus interrupting PI(4,5)P2 prosurvival activity [19]. Taking advantage of the $eNOS^{-/-}$db/db mice as a model of progressive, albuminuric kidney disease with features of diabetic nephropathy, it has been shown that in the kidneys of these mice, NEFA and LC-CoA contents were increased when compared with littermate controls [19]. Proximal tubule apoptosis, as determined by TUNEL-positive cells and enhanced caspase-2 activation, was documented along with decreased NHE1 activity in the renal cortex [19].

Tubular Cell Activation and Damage

Receptor-mediated endocytosis of excessive proteins at the apical pole of the proximal tubule cells is associated with phenotypic changes characteristic of an activated state. Insights into specific mechanisms linking protein uptake to cell activation have come from in vitro studies using polarized proximal tubule cells to assess the effect of apical exposure to proteins. Collectively, they show that protein overload induces a proinflammatory phenotype [20–23].

Plasma proteins (delipidated or lipid-enriched albumin, IgG, and transferrin) upregulated gene expression and production of vasoactive, inflammatory, and

fibrogenic mediators, as the vasoconstrictor peptide endothelin-1, chemokines such as monocyte chemoattractant protein-1 (MCP-1), regulated upon activation, normal T cell expressed and secreted (RANTES), interleukin-8 (IL-8) and fractalkine, and the profibrogenic cytokine TGF-β [8, 20–24] (Fig. 13.3). Moreover, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2, as well as membrane surface expression of the αvβ5 integrin [25], were also highly increased in vitro upon stimulation by plasma proteins. Intracellular signaling pathways activated by protein overload in proximal tubule cells included, among others, the transcription factor NF-kB, extracellular signal-regulated kinase and Janus kinase/signal transducer, and activator of transcription (JAK/STAT) pathways, requiring reactive oxygen as a second messenger [20, 26–29]. Exposure of proximal tubule cells to high

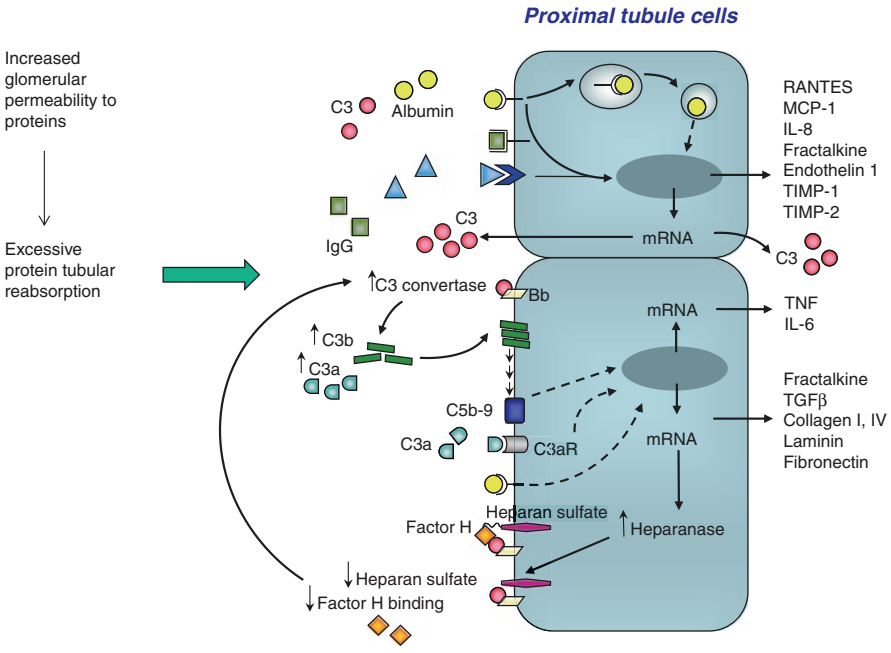

Fig. 13.3 Proximal tubular cell phenotypic changes induced by proteins. Protein overload of proximal tubular cells as a consequence of increased glomerular permeability to proteins activates intracellular signals that cause increased production of inflammatory and vasoactive mediators and growth factors, eventually released into the interstitium where they promote fibrosis. Abbreviations: IgG immunoglobulin G, IL-6 interleukin-6, IL-8 interleukin-8, MCP-1 monocyte chemoattractant protein-1, RANTES regulated upon activation, normal T cell expressed and secreted, TIMP-1 tissue inhibitor of metalloproteinases-1, TIMP-2 tissue inhibitor of metalloproteinases-2, TGF-ß transforming growth factor-β, TNF tumor necrosis factor

albumin doses also activated the renin-angiotensin system via a protein kinase C (PKC)-NADPH oxidase-dependent pathway [30].

Extrapolation from such in vitro data to the human nephropathies may be difficult considering the conflicting data observed with different proteins in different cell systems [31], as well as the reported changes in the expression of several genes of unknown function [32]. However, transcriptome analysis of renal proximal tubule epithelial cells isolated by laser capture microdissection from kidney biopsies of patients with proteinuric nephropathies identified 168 differentially regulated genes in comparison with proximal tubule cells from non-proteinuric controls, revealing differential expression of a number of genes encoding for signal transduction, apoptotic, and inflammatory proteins [33]. An additional study identified 231 "albumin-regulated genes" differentially expressed by human kidney tubule epithelial cells exposed to albumin [34]. These findings were translated to human disease by studying mRNA levels of these genes in the tubulointerstitial compartment of kidney biopsies from patients with IgA nephropathy using microarrays. The expression of a 11-transcript subset which included genes for growth factors, collagen 1 alpha 1, and proteins implicated in fibrotic and apoptotic pathways was related to the degree of proteinuria. The 11-mRNA subset was also sufficient to distinguish biopsies of subjects with IgA nephropathy from control biopsies. A recent study used multiplex-tandem PCR analysis of laser capture microdissected proximal tubule epithelial cells isolated from kidney biopsies of patients with different renal diseases to identify and quantitate "real-time" gene transcription profiles that describe signaling pathway associated with renal fibrosis [35]. A total of nine genes that discriminated disease samples from controls were identified. Among them, C3 and kidney injury molecule (KIM-1) expression levels were remarkably elevated in proximal tubule cells from patients with the heaviest proteinuria.

Evidence implicates megalin as a central element of the signaling pathway linking protein reabsorption and gene regulation in proximal tubule cells [36] (Fig. 13.4). Megalin is subjected to regulated intra-membrane proteolysis (RIP), an evolutionarily conserved process linking receptor function with transcriptional regulation. Through RIP, megalin is subjected to PKC-regulated, metalloprotease-mediated ectodomain shedding, producing a membrane-associated C-terminal fragment that in turn forms the substrate for the γ-secretase which releases the C-terminal, cytosolic domain. The latter translocates to the nucleus where it interacts with other proteins to regulate expression of specific genes. This function may explain the phenotypic change of proximal tubules in proteinuric kidney disease. Evidence that megalin contributes to the early activation of proximal tubule cells during nonselective proteinuria was derived from experiments in megalin-knockout/NEP25 mice treated with the immunotoxin LMB2, a model for nephrotic syndrome, focal segmental glomerulosclerosis, and tubulointerstitial injury. Megalin-deficient proximal tubule cells reabsorbed less proteins and expressed less tubule cell injury markers, such as MCP-1 and heme-oxygenase 1 [37].

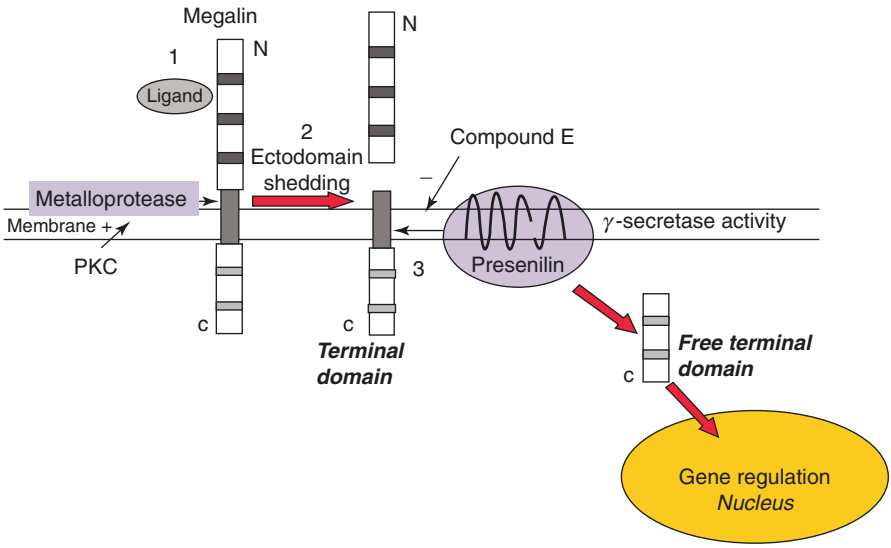

Fig. 13.4 Megalin as central element of signaling pathway linking protein reabsorption and gene regulation in proximal tubule cells. Megalin is subjected to regulated intra-membrane proteolysis, an evolutionarily conserved process linking receptor function with transcriptional regulation. In particular, metalloprotease activity, activated by ligand binding (1), and regulated by protein kinase C, results in ectodomain shedding (2) of megalin. Ectodomain shedding produces a membrane-associated C-terminal fragment which, in turn, becomes the substrate for γ-secretase activity acting in the membrane and releasing the free C-terminal domain into the cytosol (3). The latter translocates to the nucleus where it acts as a transcriptional regulator of specific genes. Presenilin is the active component of the γ-secretase protein complex and is specifically inhibited by Compound E. Abbreviations: PKC protein kinase C. (Adapted from Ref. [36])

Ultrafiltered Growth Factors Activate Tubular Phenotypic Changes

The proximal tubule bears receptors for ultrafiltered proteins, such as growth factors [8]. Usually these molecules are present in high-molecular-weight precursor forms or bound to specific binding proteins which regulate their biological activity. They can be found in nephrotic tubular fluid. In experimental proteinuria in rats, there is translocation of insulin-like growth factor I (IGF-I) from plasma into tubular fluid, primarily as the 50-kDa complex [38]. Similarly, hepatocyte growth factor (HGF) is present in early proximal tubular fluid from rats with streptozotocin-induced diabetic nephropathy [39]. Under physiologic conditions the high molecular weight of TGF-β complexes prevents glomerular ultrafiltration of this pluripotent growth factor. However, in proteinuric glomerular diseases, TGF-β is found in early proximal tubular fluid, and at least a portion is bioactive [39]. The remainder is likely activated during downstream tubular flow by acidification of tubular fluid and, perhaps, by the increasing urea concentrations and by the presence of enzymes such as PAI-1. The concentration of TGF-β in glomerular ultrafiltrate from rats with diabetic

nephropathy is approximately 30 pM, which is one to two orders of magnitude greater than required for documented biological responses [39]. There are several responses by tubular cells to these growth factors that collectively can be described as activation or as a moderate change toward a cell phenotype resembling cell injury. This includes a moderate increase in collagen type I and IV production in response to IGF-1 [38]. HGF modestly increases the expression of fibronectin in tubular cells [40]. HGF has also unique effects in proximal tubular cells. It actually completely blocks the expression of collagen α1III (Col3A1) [40], which is consistent with an anti-fibrogenic role. TGF-β also increases the transcription of genes encoding Col3A1 and collagen α_2I (Col1A2) as well as fibronectin in proximal tubular cells. Thus, ultrafiltered growth factors induce moderately increased expression of extracellular matrix proteins in tubular cells which most likely contributes to interstitial fibrosis. Increased tubular proliferation has been also observed following the induction of albuminuria of glomerular origin possibly suggesting that multiple growth factors and mitogens may be recruited in the context of an adaptive response to minimize the loss of filtered proteins [41]. How this could be balanced with the potential toxicity of excess and prolonged over-reabsorption of abnormally filtered proteins and protein-associated factors is not clear.

Role of Complement Activation

Among specific components of proteinuria, serum-derived complement factors can be highly harmful especially upon activation in the proximal tubule [42]. C3 is an essential factor of both the classical and alternative pathways of complement activation that lead to the formation of C5b-9 membrane attack complex. Renal tubular epithelial cells appear most susceptible to luminal attack by C5b-9 because of the relative lack of membrane-bound complement regulatory proteins such as membrane cofactor protein (CD46), decay-accelerating factor, or CD55 and CD59 on the apical surface [43], as opposed to other cell types such as endothelium or circulating cells ordinarily exposed to constant challenge from complement. In vitro, proximal tubular cells exposed to human serum activate complement via the alternative pathway leading to fixation of the C5b-9 membrane attack complex on cell surface [44]. These events were followed by marked cytoskeleton changes with disruption of the network of actin stress fibers, formation of blebs, and cytolysis. Increased production of superoxide anion and hydrogen peroxide and synthesis of proinflammatory cytokines such as IL-6 and TNF-α were also observed [45].

In proteinuric experimental models, C3 and ultrafiltered proteins co-localized to proximal tubule cells engaged in high protein uptake since stages that preceded inflammatory cell accumulation in the renal interstitium [46]. Treatment with an ACE inhibitor by limiting proteinuria effectively reduced both tubular accumulation of plasma proteins and C3 and interstitial inflammation [47]. In the mouse model of protein-overload proteinuria, the results of experiments with C3-deficient kidneys transplanted into wild-type mice or vice versa suggested that ultrafiltered C3

contributed more to tubulointerstitial injury induced by protein overload than locally synthesized C3 [46]. C3 and other complement proteins were also found in proximal tubules in renal biopsy from proteinuric patients [42, 48].

Studies on the mechanisms of complement activation and deposition of C5b-9 on tubule cells focused on properdin as a key factor in the initiation of alternative pathway during proteinuria. A strong staining for properdin was observed on the luminal surface of the tubules in kidney biopsies from patients with proteinuric diseases, suggesting that during proteinuria filtered properdin may bind to proximal tubule cells and act as a focal point for complement alternative pathway activation [49]. That properdin may be an important determinant in intratubular complement activation was supported by the detection of properdin in the urine of proteinuric patients associated with increased urinary levels of SC5b-9, the soluble form of the effector phase of complement activation [50]. Properdinuria was also associated with worsening of renal function indicating a role for properdin in proteinuria-mediated renal injury [50]. Tubular heparan sulfate was identified as the ligand for properdin during proteinuria acting as a docking platform for alternative pathway activation via properdin [51]. There is also evidence that factor H, one of the most important fluid phases as well as surface bound regulators of the alternative pathway, also binds to tubular heparan sulfate, although to a different epitope than properdin [52]. This observation might be of importance for future treatment prospects to prevent proteinuria-induced alternative pathway activation and tubular injury in proteinuric renal disease. Such an approach would be expected to be the most effective if combined with antiproteinuric therapy and possibly if administered to patients with forms of nephrotic syndrome which are resistant to steroids or other therapies. Indeed a previous in vitro study showed that protein load on proximal tubule cells reduced both heparan sulfate density and the binding of factor H to tubule cells, thereby enhancing the complement activation potential of proximal tubule cells [53].

Inflammasome Activation in Tubule Cells

The inflammasome-forming NOD-like receptor (NLR) genes are key regulators of the innate immune response, which integrate various danger signals into caspase-1-activating platforms leading to the processing and secretion of the proinflammatory cytokines IL-1β and IL-18. Emerging evidence suggests an important role for NLRP3, the best understood inflammasome, and IL-1β/IL-18 in the pathogenesis of acute and chronic inflammation and tissue remodeling in the kidney [54, 55]. In vitro and in vivo studies are available showing that proteinuria could cause inflammasome activation in the proximal tubules [56, 57]. Actually, albumin-bound free fatty acids triggered inflammasome activation through mitochondrial reactive oxygen species production in cultured proximal tubule epithelial cells [56]. In human kidney biopsies, the expression of inflammasome-related proteins such as caspase-1, IL-1β, and IL-8 in renal tubules correlated with the magnitude of proteinuria regardless of the underlying disease [57]. This association was further supported by

in vitro data showing that in a rat tubule cell line, bovine serum albumin (BSA) induced caspase-1 activation and maturation of IL-1β and IL-8 in a time- and dose-dependent manner. A significant overlap of NLRP3 protein expression with the ER marker calreticulin was detected, suggesting that ER stress induced by albumin could play an important role in the activation of inflammasome [57].

Autophagy and Tubular Injury

Autophagy is an intracellular catabolic process whereby cytoplasmic components are sequestered into autophagosomes and delivered to lysosomes for degradation in order to sustain cellular metabolism. Under pathological or stress conditions such as hypoxia or ischemia/reperfusion, autophagy is activated and may act as an adaptive and protective mechanism for cellular survival [58]. In the kidney autophagy is essential for the homeostasis and physiological function of podocytes and renal proximal tubular cells [59]. There is evidence that urinary protein overload in cultured proximal tubule cells activated the autophagic pathway [60]. Cell pretreatment with rapamycin, an inhibitor of the mammalian target of rapamycin pathway, was used to further promote autophagosome formation. This manipulation indeed increased autophagy activation and reduced the apoptotic response to excess urinary protein exposure. Conversely, chloroquine that blocks autophagic degradation had opposite effects. Similar data were obtained in vivo in proximal tubule cells of rats with BSA-induced proteinuria [60]. Moreover, mice with autophagy deficiency in proximal tubule cells due to lack of autophagy-related gene *Atg5* were highly susceptible to the induction of tubulointerstitial lesions by protein overload. Altogether the data from in vitro and in vivo models suggest that autophagy may play an important role in protecting renal proximal tubule cells from harmful proteinuria as it does from other stresses including ischemia/reperfusion and nephrotoxic drugs.

Immune Interstitial Response to Tubular Injury

The interstitium of normal kidneys contains numerous resident monocytic myelocytes [61] which express dendritic cell (DC) markers and can indeed present antigens [61]. DC has recently been described to form an immune sentinel network through the entire kidney, where they probe the environment in search of antigens [62]. An inflammatory environment converts the tolerogenic status of resident DC into an immunogenic one, favoring recruitment of T cells. It is known that cross-presentation by DC is a major mechanism for the immune surveillance of tissue against foreign antigens [63]. In this process professional antigen-presenting cells, such as DC, acquire proteins from other tissue cells through endocytic mechanisms, especially phagocytosis or micropinocytosis. The internalized antigen can then be processed and presented on MHC class I molecules to the extracellular environment

[64]. The outcome of cross-presentation with regard to immunity depends on the expression of immunostimulatory signals after the uptake of the antigen [63].

Until recently, the role of resident DC that accumulates in the renal parenchyma of non-immune-mediated proteinuric nephropathies remained poorly understood. Recent studies, however, have provided new insights into the activation of DC in the setting of proteinuria. Administration of ovalbumin – which is freely filtered by the glomerulus – to normal mice leads to concentration of the protein principally in proximal tubules and to its transfer to DC in the kidney and renal lymph nodes [65]. Here, ovalbumin is presented to $CD8^+$ T cells, thereby inducing proliferation of these cells. The importance of kidney DC activation to renal injury has been recently demonstrated by the fact that in transgenic NOH mice (which selectively express the antigen ovalbumin and hen egg lysozyme in podocytes), DC depletion resolved established periglomerular mononuclear infiltrates [66]. In vitro experiments have also shown that exposure of rat proximal tubule cells to excess autologous albumin, as in the case of proteinuric nephropathies, results in the formation of the N-terminal 24-residue fragment of albumin ($ALB_{1–24}$) [67]. This peptide is taken up by DC, where it is further processed by proteasomes into antigen peptides. These peptides were shown to have the binding motif for MHC class I and to be capable of activating $CD8^+$ T cells. Moreover, in vivo, in the proteinuric rat model of renal mass ablation, accumulation of DC in the renal parenchyma peaked 1 week after surgery and decreased thereafter, concomitant with their appearance in the renal draining lymph nodes. DC from renal lymph nodes loaded with the albumin peptide $ALB_{1–24}$ activated syngeneic $CD8^+$ T cells in primary culture [67]. Thus, inflammatory stimuli released from damaged tubules after protein overload may represent danger signals that, in the presence of albumin peptides, alter DC to promote local immunity via $CD8^+$ T cells that are activated in regional lymph nodes and recruited in the renal interstitium. These findings provide a link among proteinuria, protein tubular uptake, immune response directed against self-proteins, and progressive tubulointerstitial injury.

Tubule MicroRNAs and Interstitial Fibrosis

Recent studies link fibrosis to changes in microRNAs (miRNAs) [68–70], a class of short (21–24 nucleotides) noncoding RNAs that regulate gene expression through posttranslational and epigenetic mechanisms thereby affecting several cellular processes, from development to disease conditions [68]. A number of miRNAs have been shown to be relevant to fibrotic processes in diabetic nephropathy, including miR-29 and miR-200 families, miR-192, and miR-21 [70–73]. These miRNAs are regulated by TGF-β in renal cells, and normalization of their expression ameliorated fibrosis in in vitro and in vivo models of diabetes [72]. More recently, miR-184 has been shown to be a downstream effector of albuminuria driving renal fibrosis in rats with diabetic nephropathy [74]. Indeed, in Zucker diabetic fatty (ZDF) rats, miR-184 showed the strongest differential upregulation compared to lean rats (18-fold).

Tubular localization of miR-184 was associated with reduced expression of lipid phosphate phosphatase 3 (LPP3) and collagen accumulation. Transfection of NRK-52E cells with miR-184 mimics reduced LPP3, promoting a profibrotic phenotype. Albumin was a major trigger for miR-184 expression. Interestingly, anti-miR-184 counteracted albumin-induced LPP3 downregulation and overexpression of plasminogen activator inhibitor-1. In ZDF rats, ACE inhibitor treatment limited albuminuria and reduced miR-184, with tubular LPP3 preservation and tubulointerstitial fibrosis amelioration. Albumin-induced miR-184 expression in tubule cells was epigenetically regulated through DNA demethylation and histone lysine acetylation and was accompanied by binding to NF-kB p65 subunit to miR-184 promoter. These findings suggest that miR-184 may act as a downstream effector of albuminuria through LPP3 to promote tubulointerstitial fibrosis (Fig. 13.5) and offer the rationale to investigate whether targeting miR-184 in association with albuminuria-lowering drugs might be a new strategy to achieve fully antifibrotic effect, at least in diabetic nephropathy [74].

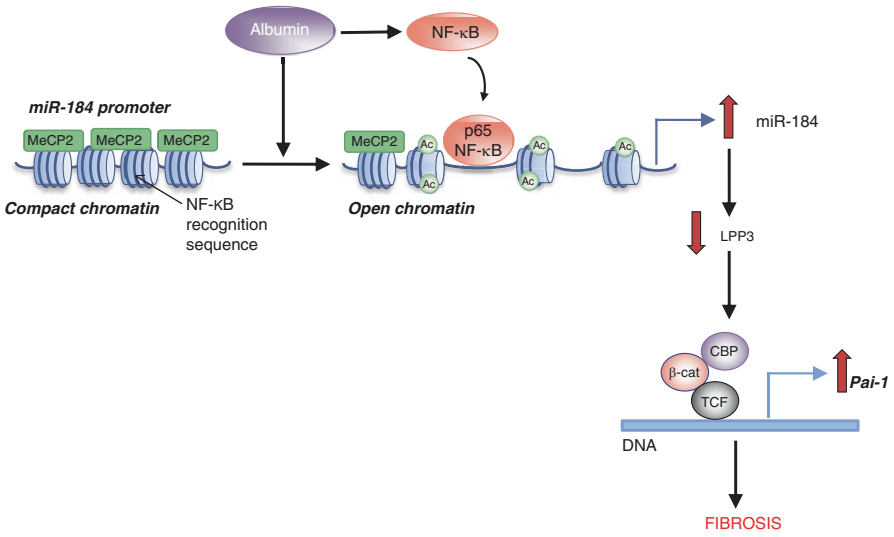

Fig. 13.5 Albumin overload promotes renal fibrosis via epigenetic regulation of microRNA-184. Hypothetical pathway through which albumin overload promotes renal fibrosis via epigenetic regulation of miR-184. Albumin reduces binding of MeCP2 to miR-184 and fosters histone lysine acetylation, favoring accessibility of NF-κB-p65 to its recognition sequence on the miRNA promoter. This results in miR-184 upregulation and repression of the downstream target LPP3, which in turn upregulates Pai-1 transcription through the β-catenin–TCF signaling pathway in a cAMP response element binding protein (CREB) binding protein (CBP)-dependent fashion. Abbreviations: Ac acetylation, β-Cat β-catenin, CBP CREB binding protein, CREB cAMP response element binding protein, LPP3 Lipid phosphate phosphatase 3, MeCP2 methylcytosine-binding protein 2, miR-184 microRNA-184, PAI-1 plasminogen activator inhibitor-1, TCF T cell factor. (From Ref. [74], under the Creative Commons Attribution 4.0 International License at http://creativecommons.org/licences/by/4.0)

Conclusions

The knowledge of mechanisms and mediators of interstitial inflammation and fibrosis in chronic proteinuric nephropathies, such as diabetic nephropathy, has been expanded with potential targets for pharmacological interventions, including the modulation of tubule cell responses to albumin-bound lipids, apoptosis, complement activation, and possibly regulation of inflammasome, autophagy, and miRNA expression. Independent of heterogeneous pathophysiology underlying glomerular dysfunction, the abnormal passage of plasma proteins across the filtering barrier contributes to propagate injury to the tubule which promotes vicious cycles of tubular epithelial degeneration and glomerular scarring and would be even more detrimental in patients with critically low number of nephrons [75]. The increasing recognition of proteinuria as a major risk factor for progression of chronic kidney disease in diabetic patients calls for continuous investigation of these mechanisms in animal models of proteinuric diseases, for new testing of agents to be used in combination with current antiproteinuric treatments, eventually potentiating their renoprotective effects.

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Chapter 14
Tubuloglomerular Communication in Diabetic Nephropathy

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Introduction

Diabetic nephropathy (DN) is the main cause for the initiation of dialysis, and preventing its onset and progression is an important clinical challenge. The lesions seen in diabetic nephropathy are primarily located in the glomerulus, and its onset is marked by the appearance of albuminuria. Hyperfiltration, in which there is excessive blood flow into the glomerulus, and other hemodynamic abnormalities as well as inflammatory changes are keys to its development. Considering that diabetes is a disorder of glucose and energy metabolism, it is likely that metabolic abnormalities in the kidney are involved in its pathology. Our laboratory has recently shown that proximal tubular abnormalities of the genes that encode sirtuins, which are key molecules in glucose and energy metabolism, are important in the onset of diabetes, as are abnormalities of the metabolism of nicotinic acid [1]. We have demonstrated that these metabolic derangements trigger the glomerular lesions. Moreover, recent large-scale clinical trials revealed that glomerular hyperfiltration and following the decline in renal function are ameliorated by the mitigation of diabetic overactivation of sodium-coupled glucose cotransporter 2 (SGLT2) inhibitors leading to the inhibition of decline in renal function in DN [2, 3]. This phenomenon is a proof of the regulatory role of proximal tubular dysfunction in glomerular filtration and glomerular damages through so-called tubuloglomerular feedback. These findings represent the tubular dysfunction, especially in proximal tubules as the prodrome of diabetic kidney disease. In this chapter, we discuss the associations between sirtuin genes, abnormalities of nicotinamide adenine dinucleotide (NAD^+) metabolism in diabetic proximal tubules, and glomerular dysfunction, or "tubuloglomerular communication" in DN.

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Proximal Tubular Sirtuin 1 and Glomerular Damages

Diabetes, Sirtuin, and NAD⁺ Metabolism

Sirtuins (hereafter Sirt) are mammalian homologues of the *Sir2* gene for a NAD⁺-dependent deacetylase identified in yeasts and nematodes. In addition to its action as a deacetylase, *Sir2* gene is also known to be responsible for longevity. Transfer of the *Sir2* gene into yeasts and nematodes is known to extend individual lifespans [4]. A number of different sirtuin isoforms are known in mammals, from *Sirt1* to *Sirt7*, of which *Sirt1* has been the best studied; its expression is induced by calorie restriction and has been demonstrated to enhance longevity in mammals. *Sirt1* regulates the expression of proteins involved in lifespan and stress resistance, many of which are encoded by genes associated with glucose, lipid metabolism, and energy metabolism. It has been reported, for example, that proteins such as uncoupling protein-1 (UCP-1), peroxisome proliferator-activated receptor α (PPARα), peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC1α), and PPARγ are deacetylated by Sirt1, enhancing their activity [5]. The functions of Sirt1 are thus intimately involved in the pathology of diabetes and insulin resistance. For example, systemically Sirt1-deficient mice and hepatocyte-specific knockout mice exhibit insulin resistance [6, 7]. Adipose tissue-specific Sirt1 knockout mice display increased obesity in response to a high-fat diet [8]. Mice with systemic overexpression of Sirt1, on the other hand, exhibit diminished insulin resistance [9], while the development of obesity in response to a high-fat diet is suppressed in mice with adipose tissue-specific overexpression of Sirt1 [8]. These data suggest that Sirt1 enables the effective systemic energy utilization and promotes insulin sensitivity.

As described above, Sirt1 is a deacetylase that requires NAD⁺ as its coenzyme. The intracellular NAD⁺ level is thus a key to its enzymatic activity. NAD⁺ is also an important coenzyme for oxidation-reduction reactions in addition to those catalyzed by Sirt1, and its maintenance is essential to obtain the energy. There are two NAD⁺ synthesis systems, one of which is a *de novo* pathway for its synthesis from tryptophan, an essential dietary amino acid, and the other of which is a salvage pathway for its resynthesis from nicotinamide (NAM), with the latter being important *in vivo*. The rate-limiting step in this salvage pathway is the synthesis of nicotinamide mononucleotide (NMN) from NAM and 5′-phosphoribosyl-1-pyrophosphate by nicotinamide phosphoribosyltransferase (NAMPT) (Fig. 14.1) [9]. On the other hand, NAM is catabolized by the methylation enzyme nicotinamide N methyltransferase (NNMT) that is expressed mainly in the kidney and liver. NAM is converted into methylnicotinamide (MNA) which is further broken down into the final metabolite N-methyl-4-pyridone-3-carboxamide or N-methyl-2-pyridone-3-carboxamide (2PY, 4PY) by the enzyme aldehyde oxidase (AOX) (Fig. 14.2).

There are two isoforms of mammalian NAMPT, i.e., intracellular nicotinamide phosphoribosyltransferase (iNAMPT) and extracellular NAMPT (eNAMPT). The activity of iNAMPT has been shown to contribute to energy metabolism [10]. For example, overexpression of iNAMPT in the liver suppresses the development of

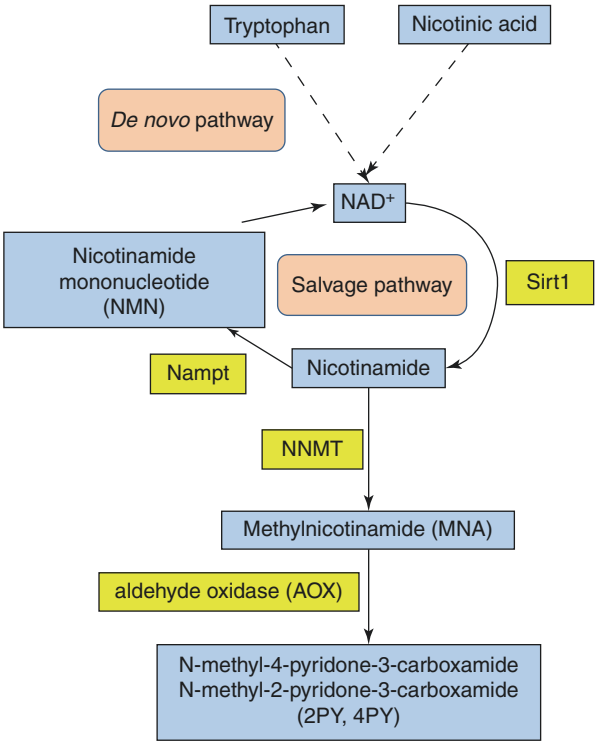

Fig. 14.1 NAD+ metabolism. Abbreviations: NMN, nicotinamide mononucleotide; NAMPT, intracellular nicotinamide phosphoribosyltransferase; NNMT, nicotinamide n-methyltransferase

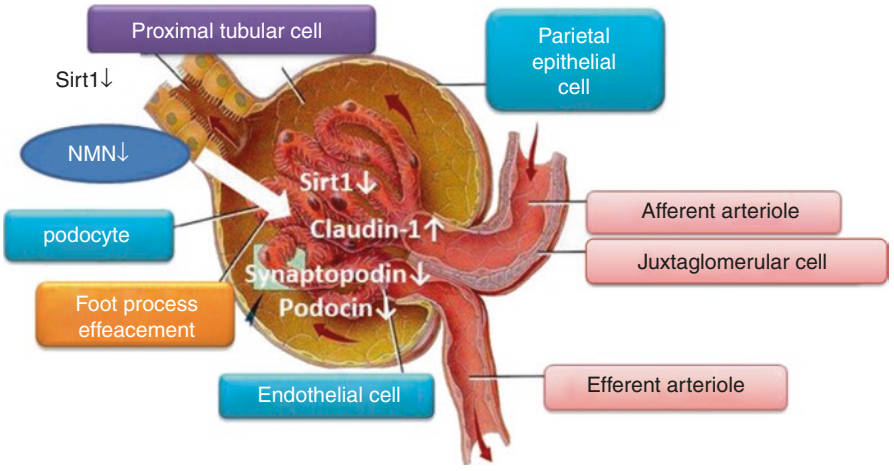

Fig. 14.2 Tubuloglomerular communication. Downregulation of Sirt1 decreases the excretion of NMN from the proximal tubules, which is uptaken by podocytes upstream of and close to the proximal tubules. The reduced uptake levels of NMN decrease Sirt1 expression in podocytes, after which ectopic overexpression of claudin-1 in podocytes disrupts the slit membrane structure by reduced expression of synaptopodin and podocin. These molecular changes lead to podocyte foot process effacement. These molecular events, propagating from proximal tubular cells to podocytes, initiate albuminuria in diabetic nephropathy. NMN, nicotinamide mononucleotide; iNAMPT, intracellular nicotinamide phosphorybosyltransferase

fatty liver [11]. Systemic administration of an iNAMPT expression vector normalizes impaired glucose tolerance [12]. Caloric restriction both increases the expression of iNAMPT in muscle and elevates the mitochondria count [13]. It has been suggested that these effects may be caused by the activation of Sirt1 as a result of the enhanced supply of NAD+ due to the activation of iNAMPT. The activation of Sirt1 also activates iNAMPT via increase in NAM levels, inducing the activation of the salvage pathway. Conversely, Sirt1 activation at the same time suppresses the activity of the clock gene, suppressing iNAMPT expression and inhibiting the excessive activation of Sirt1/iNAMPT pathway [14, 15]. More and more attention is now being focused on NAD/iNAMPT/Sirt1 as new players and their involvement in energy metabolism, which has been named the "NAD World" by Imai et al. of Washington University [16]. It was recently demonstrated that the levels of iNAMPT and NAD^+ are downregulated by high-fat stress in the liver and many other organs, and in the systemic administration of NMN, the iNAMPT product has been shown to improve impaired glucose tolerance induced by a high-fat diet, suggesting the importance of this hypothesis *in vivo* [17].

Proximal Tubule Sirt1 and Abnormal NAD^+ Metabolism in Diabetes

As regards the role of Sirt1 in renal tubular cells, a study has shown that Sirt1 promotes the nuclear translocation of Foxo3a and enhances the expression of catalases downstream of Foxo3a [18]. This action in the kidneys has been investigated using proximal tubule cells, suggesting the importance of the action of Sirt1 in this site. The proximal tubule is the site of extremely active resorption), consuming large amounts of energy. This suggests that Sirt1 may be important since Sirt1 is a key molecule in energy metabolism. Our lab investigated its significance by using *Sirt1* proximal tubule-specific gene-engineered mice. Proximal tubule-specific Sirt1-overexpresing mice were produced by using the sodium/phosphate transporter NptII, which is expressed specifically in the proximal tubule [19]. Proximal tubule-specific Sirt1-knockout mice were produced by mating floxed *Sirt1* mice with two types of proximal tubule-specific Cre mice: kidney androgen-regulated protein (KAP)-Cre mice and gamma GTP-Cre mice. The main pathological model of diabetes used was the type 1 diabetes model of diabetic nephropathy generated by streptozotocin (STZ) administration. First, temporal changes in Sirt1 expression in diabetic nephropathy were examined. Sirt1 expression in the proximal tubule in wild-type (WT) mice had already been downregulated by 8 weeks of STZ administration, and by week 24 it had further diminished. In the glomerulus, however, Sirt1 downregulation was first observed from around week 24. At week 8, although blood glucose was already elevated, albuminuria, representing glomerular lesions, had not yet started to appear, and Sirt1 expression in the proximal tubule was downregulated from this point. Molecular

changes in the proximal tubule thus occurred in advance of glomerular lesions. In the TG mice, Sirt1 expression in the proximal tubule was still preserved at week 24, and tests for albuminuria at this point revealed that even though Sirt1 was overexpressed only in the proximal tubule, the albuminuria seen in the WT mice was ameliorated in the TG mice. Electron microscopy also showed that the effacement of the podocyte foot processes seen in diabetic nephropathy had been mitigated. A microarray study of the gene profile expressed in the glomerulus revealed the change in the expression of claudin-1, the protein that constitutes tight junctions, in WT and TG mice. In diabetes, claudin-1 is strongly expressed in the glomerular podocytes and parietal epithelial cells (PECs) in Bowman's capsule, and this expression was suppressed in TG mice. Laser microdissection showed that in WT mice, Sirt1 was downregulated by STZ in both the proximal tubule and PECs, whereas claudin-1 was upregulated in PECs. In TG mice, Sirt1 was overexpressed in the proximal tubule even after STZ administration, and although the gene engineering was confined to the proximal tubule, the downregulation of Sirt1 by STZ administration was also restored in PECs. The elevated claudin-1 expression in PECs seen in WT mice was not observed in TG mice. The opposite findings were observed in proximal tubule-specific Sirt1-knockout mice.

In the next step, the function of claudin-1 in podocytes was investigated. When a claudin-1 expression vector was transferred into cultured podocytes, the expression of podocin and synaptopodin, proteins that suppress the albumin permeability of podocytes, was downregulated. We then injected Sendai virus-coated claudin-1 gene intravenously into mouse tails to carry out claudin-1 gene transfer into podocytes. Claudin-1 gene transfer in podocytes of normal mice caused increased albuminuria, and electron microscopy revealed the effacement of foot processes.

Epigenetic Gene Regulatory Mechanism Mediated by Sirt1

Gene transcription is regulated not only by canonical transcription factor-mediated mechanism but also by promoter modification by such as methylation or acetylation named epigenetic mechanism. The detailed molecular mechanism for the claudin-1 expression was examined by using human renal epithelial cell line, HRE cells. It was found that high glucose (HG) condition upregulated and the overexpression of Sirt1 downregulated claudin-1 expression, respectively. Although claudin-1 was previously shown to be regulated by protein kinase C (PKC) or PKA [20], the change in claudin-1 expression neither by HG nor Sirt1 was not changed by PKA or PKC inhibitor. Previous study using mouse renal inner medullary collecting duct cell revealed that Sirt1 regulates histone H3K9 methylation repressing the transcription of epithelial sodium channel alpha-subunit (alpha-ENaC) [21]. Computer search revealed that both mouse and human claudin-1 promoter contain CpG regions, consensus gene sequence for methylation modification. Methylation-specific PCR analysis was performed, which revealed that HG increased and Sirt1

overexpression decreased methylation of claudin-1 gene. These data indicated that the promoter methylation suppressed claudin-1 gene expression by Sirt1-mediated epigenetic gene regulation. Chromatin immunoprecipitation assay showed that Sirt1 induced histone H3K9 deacetylation and the silencing of DNA methyltransferase 1 (Dnmt1) abrogated Sirt1-mediated claudin-1 gene methylation. In conclusion, Sirt1 activation epigenetically suppressed claudin-1 expression by histone H3K9 deacetylation and by Dnmt1-mediated CpG methylation in podocytes or parietal epithelial cells. Diabetic condition downregulated Sirt1 in podocytes, which reduced claudin-1 gene methylation and increased claudin-1 gene expression (Fig. 14.2).

Tubuloglomerular Communication and Its Mediators

The abovementioned data show that downregulation of Sirt1 in the proximal tubule and in the podocytes resulted in upregulation of claudin-1. The analysis of proximal tubule-specific genetically recombinant mice also showed that upregulation of Sirt1 in the proximal tubule conserved the expression of Sirt1 in the glomerulus and suppressed the expression of claudin-1, ameliorating the development of albuminuria. This suggested that some factor released by the proximal tubule may mediate the regulation of Sirt1 expression in the glomerulus. We have termed the relationship in the opposite direction between the proximal tubule and the glomerulus, the "tubuloglomerular communication" (Fig. 14.2). To identify the mediator in this communication, conditioned medium (CM) experiments using a cell culture containing proximal tubule cells and podocytes were performed. After the proximal tubule cells had been cultured for 24 h, this CM was added to podocytes, and the changes in the latter were investigated. We found that when the CM from proximal tubular cells cultured at HG concentrations was added to podocytes, Sirt1 expression in the podocytes was downregulated and claudin-1 expression was upregulated. This suggested that the CM from proximal tubular cells cultured under HG contained some kind of humoral mediator. One of the candidates turned out to be nicotinamide mononucleotide (NMN), an intermediate product of NAD^+ metabolism (Fig. 14.1), whose production is downregulated when Sirt1 expression is downregulated. It has previously been reported that NMN secreted by perivascular adipose tissue brings about changes in the character of vascular smooth muscle cells [22]. The measurement of the concentration of NMN in CM revealed that it decreased in CM from proximal tubular cells cultured under HG conditions and that adding NMN to this CM restored Sirt1 expression and downregulated claudin-1 expression. Next, NMN was converted to a fluorescent substance with acetophenone and formic acid, and fluorescence staining of proximal tubular cells and podocytes was carried out. This revealed that intracellular NMN was downregulated in proximal tubular cells cultured under HG conditions. We also found that in diabetic mice with

STZ, the level of NMN in renal tissue was downregulated, whereas in TG mice the level was restored to normal. Finally, we injected fluorescent-labeled NMN into the renal artery to investigate the transfer of NMN into the kidneys in WT mice. After 1 h, NMN concentrated mainly in the tubule, followed by accumulation in the glomerulus after 2 h and 4 h. On the basis of these results, we thus demonstrated a single chain of events starting with abnormal NAD^+ metabolism in the tubules and ending with the onset of albuminuria (Fig. 14.2). Similar events are not detected in the 5/6 renal ablation model, suggesting that hyperglycemic stimulation is important in the pathological process. Consistently, in our study, essential parts of the results in STZ-induced diabetic mice were reproduced not only in obese-type diabetic *db/db* mice but also in the *db/db* mice crossed with proximal tubule-specific Sirt1 transgenic mice. Moreover, the phenotypes observed in diabetic mice were reproduced in tissue culture systems using proximal tubular cell line, HK-2 cell, and primary podocytes exposed to high glucose. The phenotypes in STZ-treated mice in this study are primarily due to diabetic condition, but not to STZ-induced nephrotoxicity.

Proximal Tubular Activation of SGLT2 and the Derangement of Tubuloglomerular Feedback

Tubuloglomerular Feedback

In the early stage of diabetes, renal tubular sodium reabsorption increases because of the activation of a couple of sodium absorptive transporters or channels. These changes in tubules affect the glomerular filtration through the mechanism named "tubuloglomerular feedback." Tubuloglomerular feedback is a feedback response to an increase or decrease in GFR. An increase in GFR causes an increase in Na and Cl ions in the glomerular filtrate in renal tubules. This is detected by macula densa cells of the distal renal tubule located in the juxtaglomerular apparatus, leading to the secretion of vasoconstrictors (e.g., adenosine and ATP) from macula densa cells, contraction of the afferent arteries, and a decrease in GFR. This is a mechanism to reduce further increase in GFR ([23], Fig. 14.3). At the early stages of the onset of diabetes, even without any changes in glomerulus, the increase in the sodium reabsorption in renal tubules decreases Cl delivery to the distal tubule), tubuloglomerular feedback, and afferent artery expansion and increases GFR, which represents hyperfiltration. In hyperfiltration in diabetes, an increase in albuminuria is often observed due to the rise in glomerular hydrostatic pressure. It also causes hypertrophy and hyperplasia of glomerular and tubular cells, leading to an increase in the kidney weight. Because tubular hypertrophy and renal tubular hyperplasia cause renal tubular injury and, in diabetes, urinary concentrations of tubular markers, NAG often increases.

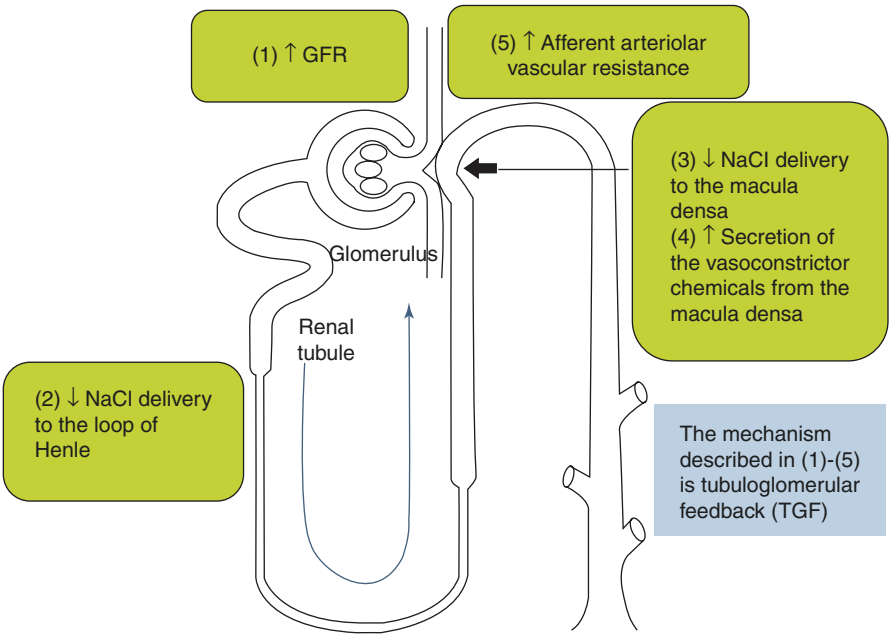

Fig. 14.3 Tubuloglomerular feedback. An increase in GFR is reflected in NaCl reabsorption in the renal tubules, which in turn is detected by the macula densa of the juxtaglomerular apparatus and reduces changes in GFR via vasoactive substances

Hyper-Activation of SGLT2

One of the culprits for the increased sodium reabsorption in diabetic milieu is the hyper-activation of sodium-coupled glucose cotransporter (SGLT). SGLT is one of the transporters that mediate glucose reabsorption and cellular glucose entry, expressed on the apical site of proximal tubular cells (PTs) [24]. Although SGLT is comprised of two isoforms, SGLT1 and SGLT2, only SGLT2 plays a dominant role in glucose transport in PTs [25], and SGLT2 inhibitors were recently made available for clinical use as glucose-lowering reagents. SGLT2 activity is supposed to be increased in diabetic conditions [24]. The backgrounds of this hypothesis are the phenomenon of the increased upregulation of urinary threshold of glucose reabsorption. It has been witnessed for a long time that while in normal subjects it is detected when blood glucose level is above 180 mg/dl, in diabetes, urinary glucose reabsorption increases as blood glucose level increases, leading to urinary glucose not detected until blood glucose level is higher than normal levels [26]. This blood glucose level at which urinary glucose excretion is detected is termed as renal threshold for glucose excretion (RT_G). Increased tubular reabsorption in the context of diabetes has been observed using a rat model of diabetes [27]. RT_G levels of approximately 415 mg/dL were reported, and glucosuria was not evident until blood glucose levels were above 400 mg/dL [28]. RT_G is often reported to be

approximately 180 to 200 mg/dL in healthy individuals [29], whereas, in patients with type 2 diabetes, RT_G is elevated [29, 30]. Many patients demonstrate elevated values above the normal range, with values ranging from 112 to 240 mg/dL. This hyper-activation leads not only to the increase in glucose reabsorption but also to the increase in Na reabsorption that is coupled with glucose entry through SGLT2. The mechanism for this hyper-activation of SGLT2 is not fully elucidated although we have some data from *in vitro* experiment where high glucose condition stimulates the increase in expression of SGLT2 in cultured PT cells through the activation of transcription factor, hepatic nuclear factor-1α (HNF-1α) (our unpublished observation). Therefore, we hypothesized that high glucose in serum or renal interstitium initiates the SGLT2 upregulation, leading to the increase in Na resorption, the inactivation of TGF mechanism, the induction of hyperfiltration, and finally glomerular damages. These mechanistic sequences represent the link between tubular functional abnormality and glomerular damages, another example of tubuloglomerular communication in DN.

The Effects on Glomerular Filtration by the Inhibition of SGLT2 in DN

This mechanistic link theoretically leads to the assumption that the inhibition of SGLT2 would restore TGF and ameliorate the dilation of afferent arteriole of glomerulus. Therefore, if diabetic patents are suffering from hyperfiltration, SGLT2 inhibitor would ameliorate this by reducing renal blood flow (RBF) and GFR and by raising renal vascular resistance. This hypothesis was clearly demonstrated by the experiments performed to diabetic patients. Cherney D et al. reported the results of inulin clearance and para-aminohippuric acid (PAH) clearance measured in diabetic patients with or without 2-week treatment with SGLT2 inhibitor, empagliflozin [31]. In this experiment, empagliflozin reduces inulin clearance and PAH clearance, which indicated the SGLT2 inhibitor reduced both GFR and RBF. It also increased renal vascular resistance. These data indicated SGLT2 inhibitor ameliorates hyperfiltration and that SGLT2 inhibitor has a potency to mitigate albuminuria and renal damages in DN. Some data obtained in clinical trial of several SGLT2 inhibitors showed that SGLT2 inhibitors reduced GFR and ameliorated albuminuria for a short period of time [32, 33]. And finally, recently the renoprotective effects of SGLT2 inhibitors have been demonstrated in two large-scale clinical trials, the EMPA-REG OUTCOME study using empagliflozin [2] and the CANVAS Program using canagliflozin [3]. In the EMPA-REG OUTCOME study, empagliflozin has a protective effect against the progression of kidney disease in type 2 diabetic patients. Patients with type 2 diabetes and estimated GFR of at least 30 ml/min received either empagliflozin or placebo once daily. It was demonstrated that the number of empagliflozin-treated patients whose nephropathy was worse was lower compared with placebo. Also, the number of empagliflozin-treated patients who doubled their serum creatinine level was lower compared with placebo, and the initiation of renal

replacement therapy was significantly lower in the empagliflozin group. The CANVAS Program integrated data from two trials involving a total of 10,142 participants with type 2 diabetes and high cardiovascular risk. Participants in each trial were randomly assigned to receive canagliflozin or placebo. The primary outcome was a composite of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke. The rate of the primary outcome was lower with canagliflozin than with placebo. The results also showed a possible benefit of canagliflozin with respect to the progression of albuminuria and the composite outcome of a sustained 40% reduction in the estimated glomerular filtration rate, the need for renal replacement therapy, or death from renal causes. Both trials are discussed in greater detail in Chap. 29 of this book. The outcomes of these trials endorse the significant role of tubuloglomerular communication in DN.

Conclusions

Previous studies, including our own, suggested that Sirt1 in the kidney exerts a protective effect against the development of diabetic nephropathy by suppressing the expression of claudin-1 in glomerulus. These results show that the renal tubuloglomerular communication is implicated in the development of diabetic nephropathy and that NMN may play an important role as a mediator. This NMN-mediated renal tubuloglomerular communication is a safeguard against the development of diabetic nephropathy, and the breakdown of the glomerular barrier function that develops from the disruption of tubular NAD^+ metabolism is important in the very early stages. The significance of this communication mechanism is also demonstrated by the renoprotective effects by SGLT2 inhibitors blocking the hyper-activation of SGLT2 in PTs leading to the amelioration of glomerular hyperfiltration in DN. The concept of tubuloglomerular communication will provide a novel strategy against DN.

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Chapter 15
Mechanisms of Interstitial Fibrosis in Diabetic Nephropathy

Ivonne Loeffler and Gunter Wolf

Introduction

The tubulointerstitium in the normal kidney consists of vascular components; some cells, including fibroblasts and lymphocytes; and the extracellular matrix (ECM) [1]. The ECM components, which are produced by interstitial fibroblasts and tubular epithelial cells, include the tubular basement membrane and a few collagen and reticular fibers [1]. In the early diabetic nephropathy (DN), the interstitium is expanded, and tubulointerstitial fibrosis and tubular atrophy follow glomerular changes [1, 2].

Tubulointerstitial fibrosis is characterized by myofibroblast accumulation, excessive deposition of extracellular matrix (ECM), and the destruction of renal tubules [3, 4]. The pathophysiology of interstitial fibrosis is divided into four overlapping phases: (1) cellular activation and injury phase, (2) the fibrogenic signaling phase, (3) the fibrogenic phase, and (4) the destructive phase [5]. In the initial priming phase, injury to the tubular cells within the kidney results in the formation of local inflammation [6]. Tubular, perivascular, and mononuclear cells are activated, begin to populate in the interstitium, and release proinflammatory and injurious molecules. The purpose of this inflammation is to repair the tissue damage, respectively; fibrosis is thought to result from wound healing processes that fail to terminate [6]. While much is known about the contribution of various molecules and signaling pathways to this "repair" process, little is known about what eventually goes wrong [6]. The second phase is characterized by the production of fibrosis-promoting factors, and in the third phase, the ECM production increases as well as matrix degradation decreases [5]. The presence of inflammatory cells (T cells, monocytes/macrophages, fibrocytes) and the production of profibrotic cytokines induce the activation and

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recruitment of matrix-producing cells [6]. Although interstitial fibroblasts, which take on the phenotypic appearance of activated myofibroblasts, are the major source of the expanded ECM, vascular pericytes, fibrocytes, and transdifferentiated endothelial and/or tubular cells may also contribute [6, 7]. The excessive accumulation of ECM results in the fourth phase, in that the number of intact nephrons progressively declines resulting in a continuous reduction in glomerular filtration [5]. Fibronectin and collagen IV are major ECM proteins that serve as a scaffold for the deposition of other proteins, such as collagen type I and III [8]. Furthermore, the adhesive glycoprotein fibronectin, which is initially produced, is thought to function as a fibroblast chemoattractant to amplify the fibrotic response [9].

The pathophysiology of DN is enormously complex, and there are a number of independent and overlapping cellular and molecular pathways leading to renal interstitial fibrosis. Initiated by several diabetes-induced factors, renal cells undergo pathological changes and secrete profibrotic signaling molecules, with excessive matrix production and interstitial fibrosis as the final consequence. This chapter summarizes current understanding of the mechanisms in fibrogenesis.

Cell Types Involved in Interstitial Fibrosis

In DN myofibroblasts appear in the interstitium, similarly to those appearing in tubulointerstitial fibrosis resulting from other disorders [1]. Although the origin of the matrix-producing myofibroblasts in the fibrotic kidney is the focus of several recent reviews and debates [10–15], it is a crucial advance in our understanding of renal fibrosis that multiple cell types are responsible for the accumulation and remodeling of ECM [16]. Limited by technical condition for in vivo research, the exact percentage of the individual renal cell types on the fibrogenesis in DN is still not clear. Nevertheless, this appears to be of minor importance, because the interstitial fibrosis is more a global process in which, via cross talk, different cell types in the interstitium contribute in different degrees and at different stages of disease to the fibrosis. At least six distinct cell types with mesenchymal phenotypes can be detected in the kidney (Fig. 15.1), but due to the relative lack of specific markers, it can be challenging to distinguish the one cell type from the other [17].

Activated Myofibroblasts

In its initial stage, interstitial fibrosis shows an enhanced occurrence of myofibroblasts, which possess unique contractile properties and were originally identified as the cells being responsible for wound contraction [1, 17]. Myofibroblasts represent an activated population of resident fibroblast, and this myofibroblastic activation is illustrated by α-SMA expression [17, 18]. It is thought that the role of the expression of α-SMA is that it causes fibrotic tissue contraction similar to that in wound

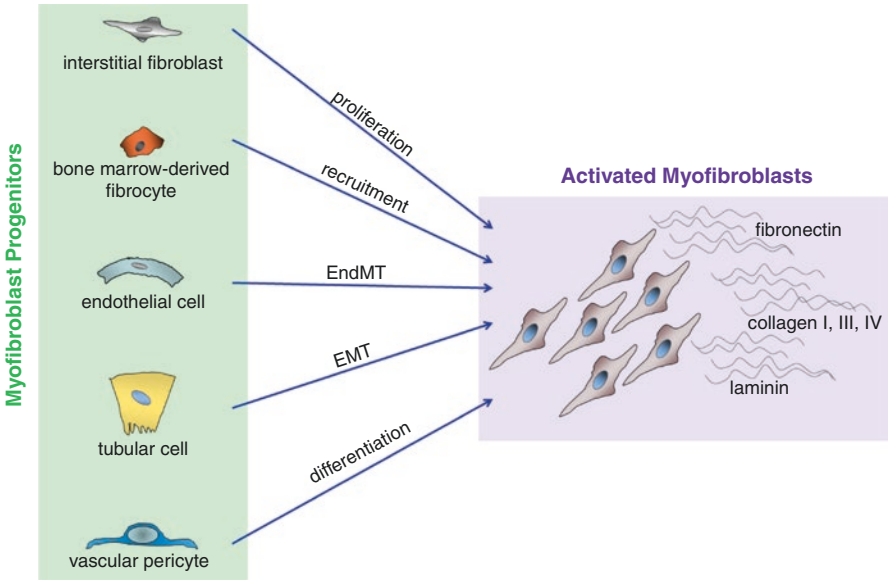

Fig. 15.1 Multiple origins of activated myofibroblasts in tubulointerstitial fibrosis. Once stimulated by diabetes-related factors, progenitor cells (interstitial fibroblasts, fibrocytes, endothelial cells, tubular cells, and pericytes) contribute to the population of activated myofibroblasts via different processes/mechanisms, namely, proliferation, recruitment, endothelial-to-mesenchymal transition (EndMT), epithelial-to-mesenchymal transition (EMT), and differentiation. Activated myofibroblasts promote fibrogenesis by production of excessive amount of matrix proteins, such as fibronectin; collagen types I, III, and IV; and laminin

contraction and/or that the cells may acquire chemoattractant activity by the expression of α-SMA [1]. De novo expression of α-SMA is not the only change during the activation to a myofibroblast but also the increased proliferative activity and matrix synthesis capacity [17]. Moreover, it has been described that depending on the stage of experimental fibrosis, among renal myofibroblasts a high degree of variability due to different α-SMA expression exists, suggesting that interstitial (myo)fibroblasts are a heterogeneous population of cells in the kidney [17, 19]. Various stimuli have been found to induce fibroblast activation [17]. The transformation from a quiescent to an activated population of fibroblasts can be initiated by four distinct mechanisms: (i) in response to autocrine or paracrine growth factor production; (ii) by direct cell-cell contact to inflammatory cells; (iii) by ECM-integrin interaction; and (iv) following exposure to environmental stimuli, such as those which occur under diabetic conditions (e.g., high glucose, advanced glycation end products (AGEs), and oxidative stress) [9]. Investigation of the molecular mechanism underlying the irreversible activation of myofibroblasts revealed nonreversible epigenetic modifications, such as methylation, as the molecular cause for the maintenance of the activated state [20]. Hypermethylation and the subsequent silencing of 12 genes have been identified, which sustain and perpetuate the activated state of interstitial myofibroblasts [20].

However, interstitial myofibroblasts have multiple origins: fibroblasts, pericytes, perivascular cells, and tubular and endothelial cells have been shown to contribute, by different mechanisms, to the population of activated and matrix-producing myofibroblasts (Fig. 15.1) [21].

Resident Interstitial Fibroblasts

Fibroblasts are mesenchymal cells and characterized by light microscopy as elongated, spindle- or stellate-shaped cells with rather pale cytoplasm and oval (or round) nuclei [19]. Interstitial fibroblasts in the normal kidney have an endocrine role – they are the major producers of erythropoietin (EPO) [22]. Thus, although the normal renal cortex contains relatively few fibroblasts, the presence of normal interstitial fibroblasts is essential for homoeostasis and protection against anemia [22]. Conversely, in fibrogenesis, activated fibroblasts reduce or even lose their ability to produce EPO, which is the major cause of renal anemia [19]. However, in healthy kidneys, cortical fibroblasts have also key roles in mediating intercellular communication with neighboring/infiltrating cells and ECM and maintenance of renal tissue architecture [9]. They have an endocytic and antigen-presenting capacity, facilitating their role in mediating inflammatory processes, and they are able to respond to a variety of autocrine and paracrine factors released by cells infiltrating the renal tissue or by resident renal cells [9]. For example, it has been demonstrated that human cortical fibroblasts modulate proximal tubule cell growth, and conversely proximal tubule cells modulate the biological behavior of cortical fibroblasts in the human kidney through paracrine mechanisms (*see also point 2.4*) [9, 23]. The embryonic origin of renal fibroblasts is still largely unknown, but several possible sources exist: they might arise (i) from the metanephric mesenchyme itself along with tubulogenesis; (ii) from the uninduced intermediate mesenchyme in the embryonic kidney in which the developing structures are embedded; and (iii) from different structures, e.g., the neural crest [19, 22]. Whatever the source of the normal resident fibroblasts is, their number increases in disease by proliferation when stimulated with cytokines (Fig. 15.1) [22, 24]. Although during the progression of DN many renal cells are initially growth arrested in the G1-phase of the cell cycle and undergo cellular hypertrophy, the interstitial fibroblasts contribute to the overall renal growth with proliferation [25]. Whereas, in the resting, quiescent state, interstitial fibroblasts express CD73 (also known as ecto-5′-nucleotidase) in their plasma membrane as well as platelet-derived growth factor receptor β (PDGFRβ) and fibroblast-specific protein 1 (FSP1), during the activation process the cells transform into myofibroblasts by expressing α-SMA, which initiates and sustains the process of fibrosis (Fig. 15.1) [1, 24].

Bone Marrow-Derived Cells (Fibrocytes)

Fibrocytes, circulating monocytes of bone marrow origin, are another potential source for myofibroblasts in the diseased kidney (Fig. 15.1) [22, 24]. Fibrocytes display the characteristics of both fibroblasts and hematopoietic cells, as they are spindle-shaped and have the ability to produce type I collagen as well as express the hematopoietic cell marker CD45 [24].

The collagen-producing bone marrow-derived cells have been shown to contribute to tissue fibrosis in models of renal fibrosis: In response to kidney injury, fibrocytes mobilize and infiltrate into renal parenchyma and participate in fibrogenesis [18, 22, 26]. The role of circulating fibrocytes as contributors to myofibroblast formation remains controversial as well [19]. The controversy about their contribution to fibrosis becomes clear, as the differences in the experimental findings are obvious: for example, Lin and colleagues [27] published that less than 0.1% of all myofibroblasts are bone marrow-derived cells, whereas LeBleu et al. [28] found that 35% are fibrocytes derived from bone marrow. Of particular note is that nearly all data in the research regarding the mechanisms of interstitial fibrosis were generated using the model of unilateral ureteral obstruction (UUO), because this model recapitulates various key features of fibrotic responses together with tubular damage and is therefore the most intensively used model to study fibrosis in the kidney [29]. Although the exact contribution of fibrocytes to fibrogenesis in DN remains unclear, the role of bone marrow cells in tissue repair is widely accepted, and as fibrosis, in general, is the end result of uncontrolled tissue repair and wound healing processes, it is reasonable that fibrocytes have a role also in fibrogenesis when they accumulate excessively at the injured site [29].

Tubular Cells

There is complex cross talk between fibroblasts, ECM proteins, and proximal tubular cells, and early changes and proximal tubular injury in diabetes affect these interactions and contribute to tubulointerstitial fibrosis [30]. As mentioned above, there is evidence for reciprocal paracrine activation of proximal tubular cells and fibroblasts: proximal tubular cells release fibrogenic signals to cortical fibroblasts, and vice versa renal fibroblasts can modulate proximal tubule cell growth and transport [30]. These interactions are modified by the tubular basement membrane components laminin and collagen type IV in the tubulointerstitium [30]. A characteristic of DN is enlarged kidneys with initial hyperplasia that is followed in time by hypertrophy [31]. Previously, it has been already foreseen that part of the association between renal growth and fibrogenesis could be the fact that similar networks of cytokines and growth factors that induce cellular hypertrophy can also stimulate

ECM synthesis and deposition [32]. At that time, it has been shown that the tubuloepithelial hypertrophy is a precursor of the later irreversible changes in the tubulointerstitial architecture leading to tubular atrophy and interstitial fibrosis [7, 25]. Furthermore, it has been proposed that diabetic hypertrophy is a transitional phase to senescence, which is described as a process that limited the proliferation of cells [31]. The senescence of tubular cells is mediated by increased p21 expression and associated with early stage of DN [31, 33]. Besides the increased p21, the potential role of cell cycle arrest in the development of interstitial fibrosis in DN has been further underlined by markedly upregulation of other cell cycle regulatory proteins, such as p16 and p27 [31, 34, 35]. It is accepted that proximal tubular epithelial cell arrest in G2/M is involved in fibrogenesis [36], as G2/M-arrested proximal epithelial cells increase expression of collagen and acquire a profibrotic phenotype by increased expression of cytokines (TGF-β and CTGF) responsible for enhancing proliferation and collagen production of fibroblasts [20, 31].

Tubular atrophy/loss as well as interstitial fibrosis can also occur by EMT (epithelial-to-mesenchymal transition), a mechanism in which proximal tubular epithelial cells transdifferentiate to acquire myofibroblast phenotypes (Fig. 15.1) [37]. Once activated by key regulators (e.g., Snail and TGF-β), the tubular cells start the EMT program that involves cytoskeletal reorganization and de novo acquisition of classic mesenchymal markers [10, 20, 38]. Of note, EMT induces a variety of intermediate cell phenotypes, not all of which complete their transition to fibroblasts [39].

Furthermore, injured tubular epithelial cells display dramatic metabolic rearrangements, such as a profound suppression of fatty acid oxidation, which highly impacts on the regeneration capacity and fibrogenesis [20]. Renal proximal tubular epithelial cells are the most energy-demanding and therefore metabolically active cells in the body [20, 40]. The ATP that they use is mostly produced in their mitochondrial and peroxisomal compartments, by the oxidation of fatty acids, because it generates more ATP than does oxidation of glucose [40, 41]. In DN, an altered renal lipid metabolism and renal lipid accumulation have been described [42]. Recently it has been demonstrated that the downregulation of key enzymes and regulators of fatty acid oxidation as well as the increased lipid accumulation in tubule epithelial cells is directly linked to tubulointerstitial fibrosis [41].

The proximal tubule in the early diabetic kidney is exposed to high glucose levels, glycated proteins, and ultrafiltered albumin and responds, inter alia, with both hyperreabsorption of glucose and reabsorption of albumin [30, 43, 44]. Glucose entry into proximal tubular cells is insulin-independent and the Na^+-glucose cotransporter SGLT2, which is directly localized in the brush border membrane of the early and later sections of proximal tubules, is responsible for all glucose reabsorption in the early proximal tubule [30]. After reabsorption across the luminal membrane by SGLT2, the glucose transporter GLUT2 mediates the glucose transport across the basolateral membrane [30]. Hyperglycemia enhances the reabsorption of glucose in the proximal tubule by upregulation of SGLT2 and GLUT2 expression [30]. The reabsorption via SGLT2 seems to represent the major cause for the glomerular hyperfiltration after hemodynamic changes, such as activation of renin-angiotensin-

aldosterone system (RAAS) [45]. It has been shown that blockade of RAAS does not completely ameliorate the hyperfiltration in diabetic renal disease, which suggests a contribution of tubular factors on hyperfiltration [30, 45]. The increase in proximal tubular reabsorption leads to deactivation of the tubuloglomerular feedback mechanism and enhanced glomerular filtration rate [45]. Multiple selective inhibitors of SGLT2 are currently in clinical trials that show promising renoprotective effects in diabetes and significantly ameliorate hyperfiltration, inflammation, oxidative stress, and albuminuria [45]. Normally, albumin and other ultrafiltered proteins are filtered through the glomerulus and reabsorbed via endocytosis in the tubular system (the proximal convoluted tubule reabsorbs 71%, the loop of Henle and distal tubule 23%, and collecting duct 3% of the glomerular filtered albumin), and only traces of protein are found in the urine under physiological conditions [46]. Several receptors for the receptor-mediated endocytosis of albumin have been identified, including megalin and cubilin [47]. Dysfunction of albumin reabsorption in the proximal tubules, due to inhibition of megalin- and cubilin-mediated endocytosis, explains the albuminuria in early-stage diabetes [46, 47]. Several data suggest that albumin itself initiate a series of events that leads to fibrosis [47]. The tubulotoxicity of albuminuria causes fibrosis as albumin induces the expression of a number of inflammatory and fibrogenic mediators, leading to infiltration of inflammatory cells into interstitium [47]. A further mechanism of the pathophysiologic effect of albuminuria on tubular cells is the albumin-induced activation of local angiotensin II (ANG II) production and upregulation of the receptor of transforming growth factor (TGFBR), contributing to the profibrotic effects of transforming growth factor (TGF) on proximal tubular cells [48].

Also for the pathogenesis of DN, another hypothesis of tubulointerstitial injury, based on polyuria that is associated with poor glycemic control, is proposed [43]. It has been demonstrated that hyperglycemia-induced polyuria with increased tubular fluid pressure in the nephron is quite obviously the cause of tubular dilatations in collecting ducts [43]. Several data indicate that, at least in earlier stages, the tubular expression of proinflammatory and profibrogenic molecules in experimental DN occurs mainly in nephron segments that have undergone mechanical dilatation due to increased fluid pressure [43]. Therefore, it is believed that also osmotic polyuria plays important contributory roles in the induction and progression of tubulointerstitial fibrogenesis in diabetic nephropathy [43].

Endothelial Cells

Progressive renal disease is characterized in part by progressive loss of peritubular capillaries, associated with increased apoptosis of endothelial cells, and it correlates with the development of tubulointerstitial fibrosis [37, 49]. The biological functions of endothelium, an interior covering of blood vessels, are numerous and in DN vascular endothelial dysfunction has been shown, which results in oxidative stress and upregulation of proinflammatory mediators [50]. Oxidative stress is a central process in the pathophysiology of endothelial dysfunction: in endothelial cells, reactive

oxygen species (ROS) can be generated and lead to the production of oxygen peroxide and subsequent modifications of the cellular phenotype [51]. Endothelial functional alterations promote tissue inflammation by upregulation of cytokine stimuli, which are able to induce the expression of distinct patterns of adhesion molecules on the luminal surface, thereby promoting the recruitment of circulating leukocytes [51].

Although the endothelium receives direct oxygen supply from red blood cells, global or regional hemodynamic disturbances may be responsible for endothelial hypoxia and activate the endothelial cells with subsequent adhesion of leukocytes to the endothelium [51]. Current evidence underlines this renal endothelial hypoxia, resulting from capillary rarefaction and vasoconstriction, as the actor of an important profibrotic vicious circle in progression of renal fibrosis [51].

A potential hypothesis to describe an alternative mechanism for myofibroblast accumulating in the renal cortex has been the capacity of altered endothelial cells to acquire functional and structural characteristics of mesenchymal cells, a mechanism which is called EndMT (endothelial-to-mesenchymal transition) (Fig. 15.1) [52]. Endothelial cells in different mouse models of chronic kidney disease, including DN, have shown to contribute to the emergence of fibroblasts during kidney fibrosis – approximately 30 to 50% of fibroblasts, detected in the mouse models of renal fibrosis, coexpressed the endothelial marker CD31 and markers of fibroblasts/myofibroblasts such as fibroblast-specific protein 1 (FSP-1) and α-smooth muscle actin (α-SMA) [53].

Vascular Pericytes

The simple definition of a pericyte is "a cell of the connective tissue about capillaries or other small blood vessels," but especially in the healthy renal interstitium, such a definition could easily categorize most cells as pericytes [19]. Pericytes are a subset of stromal cells that partially cover capillary walls, thereby stabilizing endothelium [18]. As mentioned before, in the literature, controversial opinions about the origin of myofibroblasts are discussed: some studies have disputed the contribution of epithelial and/or endothelial cells in the emergence of myofibroblasts and fibrosis, and a suggestion that bone marrow contributes to the total myofibroblast population has also been put forward, but other studies favor the idea that vascular pericytes may serve as precursors to myofibroblasts in fibrosis (Fig. 15.1) [14, 28, 54]. Independent research groups have identified perivascular fibroblasts and pericytes, derived from FoxD1-positive metanephric mesenchymal cells, as the major contributors to the myofibroblast population in the model of kidney fibrosis [27, 55]. Following kidney injury, pericytes are detached from the endothelium, undergo migration and proliferation, and finally differentiate into myofibroblasts, resulting not only in destabilization of microvasculature but also in interstitial fibrosis [18, 27, 55].

Inflammatory Cells

Inflammation appears to be the critical pathway in development of DN, and the tubulointerstitial fibrosis is typically conditioned by the infiltration of inflammatory cells including dendritic cells, lymphocytes, macrophages, and mast cells [39]. Inflammation is initiated with the entry of neutrophils, which take up cell debris and phagocytose apoptotic bodies as well as release inflammatory and profibrotic cytokines after degranulation [24]. Then, macrophages accumulate in the diabetic kidney interstitium, which are well documented to contribute to inflammation and fibrosis [31]. Macrophages are the major drivers of inflammation and fibrosis in kidney disease because of their capacity to synthesize and secrete several different molecules, such as growth factors, enzymes, and matrix proteins, which promote and sustain the fibrogenic process [20]. Interestingly, whereas T-cell deficiency is associated with reduced fibrosis in nondiabetic kidney diseases, in DN the major mechanism of the inflammatory sequence, the macrophage infiltration, was not affected by the absence of lymphocytes [24]. How or whether the various inflammatory response processes can affect disease-specific responses and subsequent tubulointerstitial injury from fibrosis remains unclear [24]. Dendritic cells originate from the same bone marrow myeloid progenitor cells as macrophages, and they abundantly accumulate in normal kidney interstitium [24]. Although the significance of dendritic cells in diabetic kidney fibrosis remains unclear, it has been shown that in proteinuric diseases, renal dendritic cells capture and carry filtered antigens to T cells, leading to the production of proinflammatory cytokines [24]. Although the role of mast cells in renal fibrogenesis remains controversial, in DN the number of mast cells in the interstitium is significantly increased and correlates with interstitial fibrosis [24]. It has been shown that they contribute directly to ECM accumulation and also influence fibroblast activity in DN [56].

However, the inflammatory cells and their produced cytokines play a crucial role in the sequential process of diabetic kidney fibrogenesis, including in each type of fibroblast activation process [24].

Mediators Involved in Interstitial Fibrosis

Several molecular factors are well known to mediate fibrogenesis in DN in a stage-dependent manner. Uncontrolled hyperglycemia induces the production of various profibrotic molecules with TGF-β as the major player in the induction and progression of tubulointerstitial fibrosis (Fig. 15.2). The other glucose-induced factors mediate their profibrotic action in cooperation with but also independent from TGF-β. The production and deposition of extracellular matrix proteins in the interstitium is then the final outcome of this complex interplay of mediators.

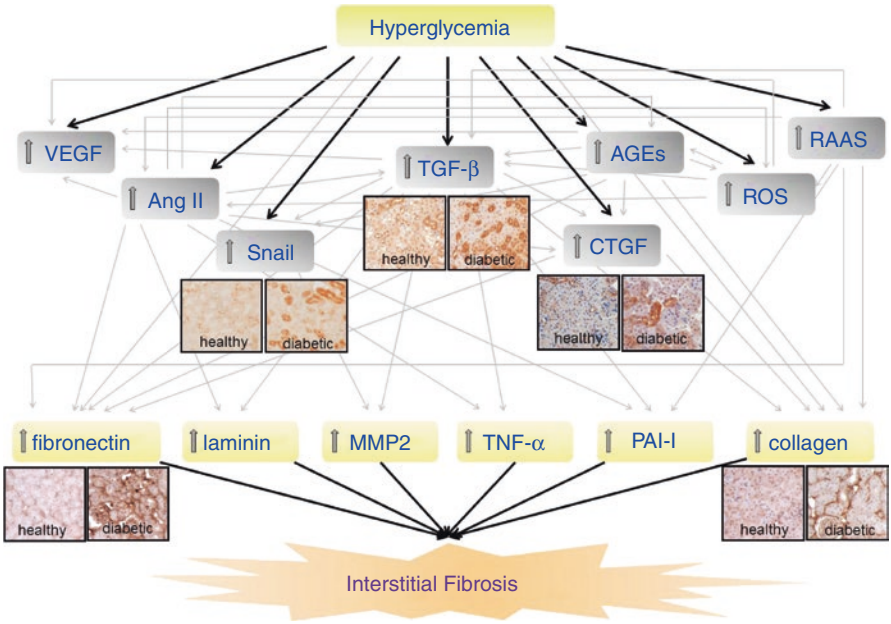

Fig. 15.2 Summary of molecular factors mediating fibrogenesis in DN. Induced by uncontrolled hyperglycemia, various profibrotic molecules are produced and signaling pathways are activated, which are involved in the induction and progression of tubulointerstitial fibrosis. Among all these factors, TGF-β is the major contributor to fibrogenesis, as its effect is multifactorial. The final outcome of the induction of the shown molecular factors is the excessive production and deposition of fibronectin, collagens, and laminin. For more details see text. The black-rimmed images below TGF-β, snail, CTGF, fibronectin, and collagen represent unpublished immunohistochemical stainings from our group. Compared to healthy kidneys, the kidney sections from mice with diabetic nephropathy show significantly increased expression of TGF-β, Snail, and CTGF that leads to accumulation of fibronectin and collagen in the interstitium. AGEs, advanced glycation end products; Ang II, angiotensin II; CTGF, connective tissue growth factor; MMP2, matrix metalloproteinase-2; PAI-I, plasminogen activator inhibitor-1; RAAS, renin-angiotensin aldosterone system; ROS, reactive oxygen species; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor

Glucose and AGEs

DN develops partly because of changes in glucose metabolism with hyperglycemia and partly because of a genetic predisposition. Hyperglycemia is pivotal for the initiation of the pathological process [2]. Glycemia and glycated proteins induce interstitial fibrogenesis in DN through mechanisms) that are identical to those thought to cause tubular atrophy and progressive interstitial fibrosis in virtually every other renal disease leading to complete renal failure [43]. Hyperglycemia shows direct effects on renal cells that leads to increased matrix production and thickening of basement membranes [57]. For example, exposure of human renal proximal tubular cells to high glucose increases the amount of collagen IV and fibronectin in the culture supernatant and decreases the pathways which are

responsible for their degradation [7]. Furthermore, hyperglycemia facilitates reactive oxygen species (ROS) generation in various renal cells, including tubular cells, and the high glucose-induced proliferation and activation of renal fibroblasts are mediated by ROS signaling [58].

A key morphological change associated with sustained hyperglycemia in the diabetic kidney is the accumulation of glycogen, known as Armanni-Ebstein lesions [59]. Glycogen is a complex branched polymer of glucose that is not found in healthy kidneys due to its high metabolic turnover, but accumulated in the diabetic kidney, primarily localized in the thick ascending limb of Henle and distal tubules [59]. That the diabetes-induced glycogen accumulation in the tubular cells is associated with fibrosis was confirmed since a new antifibrotic agent called FT011 attenuated fibrosis in experimental nephropathy by reduction of the renal glycogen concentration and the progression of DN [59].

Among the irreversible changes that occur in DN as a result of hyperglycemia is the formation of advanced glycation end products (AGEs) through a nonenzymatic and irreversible reactions between sugars and the free amino groups on proteins, lipids, and nucleic acids (Maillard reaction) [60, 61]. These chemically heterogeneous compounds are known to have a wide range of cellular and tissue effects implicated in the development and progression of diabetic pathology [60]. Besides others, AGEs) are able to influence the balance between synthesis and degradation of ECM components in variety of ways, leading to the accumulation of ECM proteins and interstitial fibrosis [60]. On the one hand, the expression of extracellular proteins such as fibronectin and types I and IV collagen is increased by AGE) in a dose- and time-dependent manner, and on the other hand, AGE reduces the expression and activity of degradative matrix metalloproteinases [60]. The effects of AGE can be both direct (through receptors of AGE (RAGE)) and indirect, via generation of ROS [60]. Furthermore, AGEs promote the release of proinflammatory cytokines and expression of profibrotic factors, such as transforming growth factor β1 (TGF-β1) and connective tissue growth factor (CTGF), mainly by interacting with, e.g., monocytes/macrophages and glomerular endothelial cells [61]. In addition to these cells, RAGE has been also identified on the surface of tubular cells in kidneys, where an activation leads to increased expression of TGF-β1, which is responsible for development of activated myofibroblasts [62]. Tubular RAGE is significantly upregulated in kidney biopsies of patients with DN [42]. The model of UUO (chronic unilateral ureteral obstruction), which is characterized by proliferation of interstitial fibroblasts, has shown that RAGE, the receptor of AGEs), promotes interstitial fibrosis via Snail activation and tubular cell cycle arrest at G2/M [63].

TGF-β, CTGF, and IGF

Transforming growth factor-beta (TGF-β), a potent profibrotic cytokine, and its type II receptor are increased in type 1 and 2 diabetes [64, 65]. The TGF-β-mediated effects influence the pathology of different renal cells, including tubular

and endothelial cells, which subsequently leads to inflammation and tubulointerstitial fibrosis [37, 64]. Whereas) in the early stages of diabetic kidney disease, TGF-β is stimulated by hyperglycemia, in later stages persistent production of TGF-β may be due to stimulation by glycated proteins (e.g., AGEs); the influence of growth factors, e.g., angiotensin II (ANG II); and TGF-β auto-induction [37, 64, 66].

It is obvious that TGF-β is the key cytokine inducing the production of different extracellular matrix (ECM) proteins in renal cells, but TGF-β is also known to inhibit matrix degradation by induction of plasminogen activator inhibitor-1 (PAI-1), resulting in ECM accumulation and tubulointerstitial fibrosis [9, 67].

Furthermore, there are several tubular and endothelial pathological events that are mediated by TGF-β1 during the progression of interstitial fibrosis, such as EMT (epithelial-to-mesenchymal transition)- and EndMT (endothelial-to-mesenchymal transition)-like changes and epithelial and endothelial cell apoptosis [37, 49]. In various experimental models and human DN, the expression of TGF-β has been associated with apoptotic tubular epithelial cells, causing tubular atrophy, and also the loss of glomerular and peritubular capillaries is associated with increased apoptosis) of endothelial cells, which is directly induced by TGF-β [49]. However the contribution of TGF-β to fibrosis is multifactorial: TGF-β not only induces matrix production or the transition of tubular and endothelial cells but also stimulates proliferation of interstitial fibroblasts [25].

It is well known that activation of TGF-β/Smad signaling plays a central role in pathogenesis of tubulointerstitial fibrosis [68]. Recent investigations of the mechanism underlying the initial interaction of the TGF-β receptor (TGFBR) with Smads show that Kindlin-2 physically interacts with both TGFBRI and Smad3, amplifying the TGF-β-driven fibrogenesis in proximal tubular cells and in the kidney [68, 69].

Connective tissue growth factor (CTGF) is another prosclerotic cytokine and has also been shown to be involved in both the early and later stages of DN, where it is strongly induced [67]. CTGF, originally identified as a growth factor secreted by vascular endothelial cells in culture, is an important downstream mediator of the profibrotic effects of TGF-β, and TGF-β- and Smad-responsive elements in the CTGF promotor have been identified [8]. Downstream of a cascade of events induced by hyperglycemia, CTGF and TGF-β1 work in a coordinated manner to promote increased expression of extracellular matrix proteins (collagen types I, III, IV and fibronectin) [67]. CTGF) has also TGF-β-independent effects to enhance renal fibrosis: it has been shown that renal tubular expression of CTGF correlates with interstitial fibrosis in DN and that specific downregulation of CTGF attenuates nephropathy in mouse models of type 1 and 2 DN [31].

The insulin-like growth factor (IGF-I) is a potent mitogenic polypeptide, and the local renal IGF-I system is upregulated in key diabetic kidney tissues and in renal fibroblasts under hyperglycemic conditions [70, 71]. IGF-I increases the DNA and protein synthesis in tubular cells and promotes the glucose-induced extracellular matrix (ECM) accumulation in tubular cells and fibroblasts in cooperation with CTGF) [70, 71].

Snail and Twist

Snail is a transcription factor that can specifically bind to E-box motifs because of its zinc finger domain [72]. Snail can be activated by the TGF-β/Smad3 pathway but also by the noncanonical TGF-β pathways as well as in a TGF-β-independent manner [72]. In renal biopsies of patients with DN as well as in kidneys of type 2 diabetic mice, the level of Snail was upregulated, which was associated with enhanced EMT (epithelial-to-mesenchymal transition)-like changes and tubulointerstitial fibrosis [73,] [73].

Snail controls major biological processes responsible for renal fibrogenesis, including mesenchymal reprogramming of tubular epithelial cells, shutdown of fatty acid metabolism, cell cycle arrest, and inflammation of the microenvironment surrounding tubular epithelial cells [72]. Conversely, established fibrosis and EMT can be ameliorated/reversed in vivo by inhibition or depletion of Snail [39, 74, 75].

Besides Snail, the basic helix–loop–helix transcription factor Twist, well known as an essential factor in embryonic development, is the other main regulator of the tubular EMT program [76, 77]. New insights into mechanisms of EMT contribution to renal fibrosis have been provided: After injury of the epithelia, TGF-β promotes Snail and Twist expression, which activate the EMT program in epithelial cells, and then these epithelial cells undergo an incomplete/partial EMT, remain the basement membrane attached and arrested in the tubules, and promote fibrosis in a non-cell-autonomous manner [75, 78].

ANG II, ROS, and VEGF

Uncontrolled hyperglycemia and mechanical stress (hypertonus and hyperfiltration) lead to an activation of the renin-angiotensin aldosterone system (RAAS) in cells of the glomerulus as well as in tubular cells [79, 80]. Activated RAAS plays an important role in the progression of DN, and the inhibition of RAAS, a standard therapy in patients with DN since several years, shows anti-inflammatory and antifibrotic processes [64, 81]. In fact, the hormone angiotensin II (ANG II) itself induces in renal cells many cytokines, chemokines, and growth factors and can induce simultaneously a variety of effects on cells such as contractility, inflammation, differentiation, proliferation, apoptosis, or extracellular matrix gene activation, which are capable of interacting with each other, and according to the specific environmental, hormonal or homoeostatic conditions within the kidney can lead to the development of fibrosis [52, 64]. The mechanisms by which ANG II promotes renal fibrosis remain incompletely understood, but it is known that ANG II stimulates reactive oxygen species (ROS) and TGF-β signaling (upregulation of TGF-β and its receptors) and activates the EGF receptor (EGFR) [37, 48, 82, 83]. It has been proposed that a vicious cycle may exist: ANG II-ROS-Src-EGFR signaling induces TGF-β activation, which may in turn further increase ROS production and Src kinase

activity, thereby further enhancing TGF-β-dependent fibrogenesis [83]. The profibrotic action of ANG II and ROS is strengthened by the stimulating effects of AGEs, as both ANG II and ROS promote the synthesis of AGEs [60, 64, 79]. In addition, it is seen that glucose-induced ANG II increases tubular reabsorption of ultrafiltered proteins and that the upregulation of SGLT2 expression and glucose reabsorption in diabetes has been linked to activation of ANG II AT1 receptors leading to tubular inflammation and fibrosis [30, 84]. At last, the proliferative effects of ANG II on some renal cells, e.g., fibroblasts and distal tubular cells, should also not be underestimated [25].

The homodimeric glycoprotein VEGF (vascular endothelial growth factor) is an endothelial-specific growth factor that promotes endothelial cell proliferation, differentiation, and survival and furthermore a potent inducer of vascular permeability and dilation [85]. In the kidney VEGF and its receptors are predominantly expressed by podocytes and tubular cells but also in the collecting duct, and these expressions are increased in patients with type 1 and 2 diabetes and also in experimental animals [85, 86]. Not only hypoxia, as the main stimulus of VEGF, but also several other factors, such as hyperglycemia, TGF-β1, IGF-I, ANG II, AGEs, and ROS, also have the potential to upregulate VEGF expression [85]. A functional role of VEGF in the pathophysiology of DN is most likely, because inhibition of VEGF has beneficial effects on diabetes-induced functional and structural alterations, such as hyperfiltration, albuminuria, and glomerular hypertrophy [85]. Besides its well-studied function as regulator of capillary formation and function of endothelial cells, VEGF has also nonangiogenic properties particularly in cells of nonendothelial lineage [86].

HGF and BMP-7 as Antifibrotic Factors

Several antifibrotic and renoprotective agents have been shown to partially alleviate TGF-β-induced fibrosis and include bone morphogenic protein 7 (BMP-7) and hepatocyte growth factor (HGF) [87]. The renal expression of HGF, a multifunctional polypeptide playing an important role in kidney development, is limited to mesenchyme-derived cells, including mesangial cells, endothelial cells, interstitial fibroblasts, and macrophages [88]. For example, HGF has therapeutic effects to suppress TGF-β-induced tubulointerstitial fibrosis in hyperglycemia-linked nephropathy [89].

Identified as an osteogenic factor and homodimeric member of the TGF-β superfamily, BMP-7 is primarily expressed in kidney tubules and glomeruli and plays an important role in kidney development and the regulation of nephrogenesis [87, 90]. In human and experimental DN, the renal cortical expression of BMP-7 is progressively decreased, and it has been shown that the inhibition of BMP-7 signaling is mediated by the upregulation of CTGF by TGF-β in vitro and in vivo [91, 92]. BMP-7 presents a novel therapy for DN, because in experimental BMP-7 treatment leads to (i) reversed TGF-β-induced EMT, (ii) inhibited renal fibrosis, and (iii) reversed proteinuria to normal in a dose-dependent manner [87, 91, 93, 94].

Key Mechanisms Leading to Interstitial Fibrosis

The sections above discussed the mechanisms of fibrosis regarding the involved cells and the diabetes-induced factors that activate or mediate these pathologic changes, but further mechanisms are known, which are not directly restricted to a specific cell type or mediator. Figure 15.3 shows a summary of the key mechanisms leading to interstitial fibrosis.

EMT and EndMT

The matrix-producing myofibroblasts in the kidney, which contribute to progression of fibrosis in DN by facilitating deposition of interstitial ECM, can be derived from various cellular sources (Fig. 15.1). Tubular and endothelial cells are involved in two mechanisms, called epithelial-to-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndMT), in that the cells undergo biochemical changes, which lead to a transdifferentiation to activated myofibroblasts. EMT of tubular cells is a highly regulated process, which is defined by four

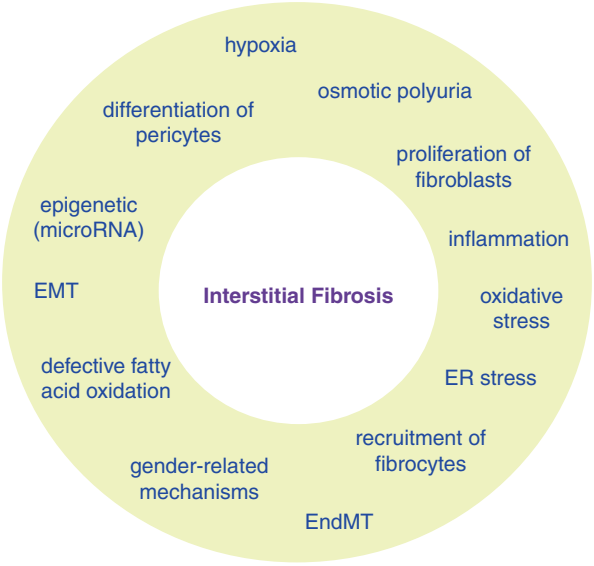

Fig. 15.3 Key mechanisms involved in interstitial fibrosis in DN. The figure summarizes the mechanisms that are known to play important roles in the interstitial fibrogenesis in DN. This various mechanisms reflect the sum of the numerous processes that renal cells undergo (see also Section "Cell Types Involved in Interstitial Fibrosis" and Fig. 15.1) in response to the factors of the diabetic milieu (see also Section "Mediators Involved in Interstitial Fibrosis" and Fig. 15.2). EMT, epithelial-to-mesenchymal transition; EndMT, endothelial-to-mesenchymal transition; ER, endoplasmic reticulum

key steps: (1) loss of epithelial cell adhesion (e.g., E-cadherin adhesion complex), (2) de novo α-SMA expression and actin reorganization, (3) disruption of tubular basement, and (4) enhanced cell migration and invasion of the interstitium [9, 95, 96]. EndMT is considered to be a unique form of EMT, as endothelial cells are a specialized type of epithelia, and during the process the cells lose their endothelial (e.g., CD31) and not epithelial cell markers, which is the only difference between this two mechanisms [24, 53]. Several factors are known to play an important role in the induction of EMT and EndMT: Snail, TGF-β, CTGF, and AGEs but also chronic hypoxia in the tubulointerstitium and metalloproteinases have been shown to stimulate EMT in models of renal disease [3, 10, 53, 74, 87, 97–99]. The existence and significance of EMT as well as EndMT in DN are still a subject of controversial debates, but the in vitro evidence for EMT/EndMT induced by different diabetic factors is compelling, and many previous studies have suggested that both EMT and EndMT occur in diabetes and play a vital role in the progression of tubulointerstitial fibrosis [10, 73, 98, 100, 101]. Although the true frequency of tubular or endothelial cells that complete the transition and become fibroblasts in DN is unknown and likely depends on the persistence of inflammation or epigenetic control of fibrotic gene regulation, the relevance of EMT/EndMT for progression of renal fibrosis is highlighted by studies in which administration of BMP-7, HGF, metformin, paricalcitol, and others all inhibit EMT/EndMT and ameliorate fibrogenesis and preserve kidney function [24, 39, 93, 102, 103].

Hypoxia and Oxidative Stress

The fibrotic tubulointerstitium is a hypoxic environment, and the hypoxia results from microvascular rarefaction made worse by matrix expansion throughout the interstitium, requiring the oxygen gradient to diffuse greater distances from vasoconstricted or attenuated microvessels to adjacent ischemic tubules [39]. Chronic hypoxia is multifactorial and a potent regulator of gene expression for a broad spectrum of molecules and in DN an important factor aggravating tubulointerstitial fibrosis, partly by the induction of factors such as TGF-β, CTGF, and VEGF [64, 104–106]. This induction of growth factors and cytokines is mediated by hypoxia-inducible factor-1 (HIF-1), and ANG II can further increase this important transcription factor [64].

ANG II, together with hyperglycemia, also increases the generation of intracellular reactive oxygen species (ROS) in renal tubular epithelial cells, which mediate many negative biological effects, including oxidation of proteins and damage to DNA [31]. Oxidative stress in the form of increased mitochondrial ROS formation plays an important role in the pathogenesis of renal fibrosis [58]. Various biochemical pathways are stimulated through the increased generation of ROS mainly AGE formation, TGF-b, and VEGF [60, 64, 79]. Furthermore, the findings that antioxidants block glucose-induced increase in fibronectin and collagen type IV gene

expression and attenuate hyperglycemia-induced apoptosis in human tubular cells strongly indicate that oxidative stress is essential for renal fibrogenesis [107].

Inflammation

Inflammation appears to be the critical pathway for the development and progression of DN [24]. Several studies have demonstrated that cytokines, chemokines, growth factors, adhesion molecules, nuclear factors, and immune cells as monocytes, lymphocytes, and macrophages are all involved in diabetes pathogenesis and of course play an important role in diabetes complications [108]. The inflammation in diabetic kidneys is characterized by increased synthesis of proinflammatory cytokines (e.g., tumor necrosis factor (TNF-α) and the interleukins IL-1, IL-6, and IL-18) and enhanced tubular expression of chemoattractant cytokines (e.g., monocyte chemoattractant protein 1 (MCP-1) and RANTES) [64, 109]. Several components of the diabetic milieu, as hyperglycemia, AGEs, RAAS, and oxidative stress, can activate the inflammatory process in the kidneys, which results in the infiltration of the organ by monocytes, neutrophils, and lymphocytes, which secrete injurious molecules, such as proinflammatory cytokines and reactive oxygen species [64, 108, 109]. This leukocyte activity amplifies the inflammatory response and promotes cell injury and the development of fibrosis. Better understanding of the inflammatory response in diabetic kidneys is expected to identify novel anti-inflammatory strategies for the potential treatment of human DN [108]. For example, anti-inflammatory drugs, such as inhibitors of cyclooxygenase (COX), MCP-1, und TNF-α, show clear renoprotective effects and have a great potential in management of DN [110]. As the Smad family plays a very important role in inflammation and fibrosis in renal disease, Smad family could be also a therapeutic option for DN patients [108].

Altered Fatty Acid Metabolism and Lipotoxicity

Sustained hyperglycemia in diabetes promotes fatty acid synthesis and triglyceride accumulation in nonadipose tissues, a process termed lipotoxicity [42]. The effect of lipid accumulation in the kidney is well described in animal models of DN, and also in kidney biopsies of patients with DN, they found extensive accumulation of intracellular lipid droplets in tubular epithelial cells [42, 111, 112]. Lipid droplets are round membrane-coated organelles in which lipids are stored as a central core of potentially toxic triglycerides and cholesterol esters [42]. Proximal tubular epithelial cells have high levels of baseline energy consumption and rely on fatty acids as their energy source, whereat fatty acid uptake, oxidation, and synthesis are tightly balanced [41, 113]. The tubule epithelial lipid accumulation in kidneys of patients with diagnosed DN is associated with dysregulation of genes involved in both

triglyceride metabolism and cholesterol metabolism [42]. It is believed that the increased fatty acid uptake, the elevated active synthesis of triglycerides, and the decreased expression of master regulators of fatty acid oxidation contribute equally to lipid deposition in diabetic kidney as altered expression of receptors and transporters regulating cholesterol influx/efflux [20, 41, 42, 113]. The mechanism of lipotoxicity in patients with DN could be divided in generic cellular stress (altered mitochondrial energy production, ER stress, ROS production, and TGF-β release) and cell-specific stress (e.g., in tubular cells: CD36-mediated cellular stress and altered channel and transporter function) [111]. Particularly, a key role in kidney fibrosis has been described for the decreased fatty acid oxidation in renal tubular epithelial cells [41]. Experiments have shown on the one hand that the inhibition of fatty acid oxidation leads to a fibrotic phenotype of tubular cells and on the other hand that restoring fatty acid metabolism protected mice from tubulointerstitial fibrosis [41].

Epigenetic and Gender

Not all patients with diabetes mellitus or even with microalbuminuria progress to overt proteinuria and nephropathy and also sex differences in the genesis and progression of DN do exist. Whereas the molecular reasons for gender-related differences in the development of renal fibrosis remain largely unclear, it is likely that the risk for onset of DN is determined by genetic and epigenetic factors.

Hyperglycemia leads to different posttranslational modifications (histone acetylation and microRNAs) and DNA methylations, which are involved in pathogenesis and progression of DN [110]. MicroRNAs (miRNAs) are highly conserved small non-coding RNAs and posttranscriptional regulators of gene expression involved in numerous biologic processes [31, 74]. It is currently estimated that miRNAs regulate the expression of at least 60% of all protein-coding genes, and alterations in miRNA expression profiles have been observed in numerous pathological processes, including DN [114]. Among the multiple mechanisms by which different miRNAs cause renal damage under diabetic conditions, several miRNAs have been identified, which modulate the TGF-β-induced fibrosis in models of kidney fibrosis and DN [4, 110]. Some of the known miRNAs show profibrotic functions, but some of them show antifibrotic functions [114]. It has been demonstrated that TGF-β/Smad signaling promotes renal fibrosis either by inhibiting microRNAs (e.g., miR-29 and miR-200) or by inducing its expression (e.g., miR-21, miR-382, miR-192, miR-216a, and miR-377) [4, 110, 114, 115]. Other miRNAs have been shown to modulate the activity of Snail: e.g., it has been reported that plasma miR-130b downregulation is associated with increased Snail expression and increased tubulointerstitial fibrosis in DN. MiR-130b inhibition could induce Snail signaling activation and enhance EMT in vitro and in vivo [74]. However, a better identification of genetic or epigenetic factors could help to define patients with increased risk for development of DN, which need a more intensive monitoring and therapy.

The relationship between sex hormone levels and DN is unknown, and moreover the existing data regarding the sex differences in the incidence and progression of DN are inconclusive. Whereas some studies report that male sex is a risk factor and that the rate of progression of DN is greater in men compared with age-matched women, others indicate no sex difference or even increased risk in women [116]. Furthermore, the time point of the onset and the duration of diabetes, the puberty, as well as the menopause seem to play an important role for the differences between the sexes [116–118]. Accumulating evidence suggests that diabetes is a state of an imbalance in sex hormone levels in both women and men. It remains unclear whether the testosterone level in diabetic men is decreased or in diabetic women increases or vice versa or how it is with estrogen level [117]. This is similar to data to sex hormone levels in experimental models for diabetes: the studies were performed either only with male or only with female animals, respectively, and the levels of sex hormones not determined [117]. However, experimental evidence suggests that both estrogens and androgens play an important role in the pathophysiology of DN, but the precise mechanisms by which sex hormones contribute to the pathophysiology of diabetic renal disease are poorly understood [117].

MORG1 and Collagen Type VIII

By the latest findings of research to pathogenesis of tubulointerstitial fibrosis in DN, two proteins move into focus of scientific interest: MORG1 and collagen type VIII.

Mitogen-activated protein kinase organizer 1 (MORG1), also known as WDR83, is a member of the WD-40 domain protein family and was first isolated as a binding partner of an extracellular signal-regulated kinase (ERK) pathway scaffold protein [119]. Besides its function as a scaffold protein of the MAPK pathway, MORG1 plays also a central role in the HIF (hypoxia-inducible factor) signaling, where MORG1 acts as a scaffold of prolyl hydroxylase 3 (PHD3) [105, 120]. HIFs are the master transcription factors that enhance gene expression and regulate adaptive responses against tissue hypoxia. It has been shown that MORG1 activates/stabilizes PHD3 and assists in the regulation and degradation of protein and that suppression of MORG1, in turn, superinduced the HIF-mediated reporter gene activity in vitro as well as increased the basal HIF-α protein stability or rather decreased the HIF-α degradation in vivo. Very recent data indicate that MORG1 plays a role in the pathogenesis of DN by its function as a scaffold of PHD3 [73]. In a mouse model for type 2 DN, it was demonstrated that the suppression of MORG1 leads to an amelioration of DN by reduction of EMT-like changes and decreased tubulointerstitial fibrosis [73]. This resulted most likely by the preferred activation and stabilization of HIF-2 with subsequently increased expression of erythropoietin, which is a transcription target of HIF-2 and known to have potential renoprotective effects [105].

For collagen type VIII (gene: COL8), a nonfibrillar short-chain collagen and structural component of many extracellular matrices [121], an increased expression

in glomerular as well as tubular compartments of renal biopsies from patients with DN has been shown [122]. A direct relationship between the elevated COL8 expression and the hyperglycemia and induction of TGF-β, which is present in DN, has been demonstrated through cell culture experiments in that it has been shown that both, high glucose and TGF-β, can induce the expression of COL8 in mesangial and tubular cells [123]. A renoprotective effect showed the knockout of COL8 (COL8-KO) in diabetic mice [100, 123]. Compared with the diabetic wildtype mice, the diabetic COL8-KO mice showed significantly ameliorated albuminuria resulted from reduced glomerular changes (e.g., mesangial expansion), the first clinical sign for DN [123]. Further investigations to the role of COL8 in tubulointerstitial fibrosis of DN confirmed that the knockout of COL8 is renoprotective and therefore significantly reduced fibrosis in the tubular compartment was found. In addition, in contrast to diabetic wildtype animals, no signs of EMT-like changes were detected when COL8 was absent [100].

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Part V
Microvascular Involvement

Chapter 16
Microvascular Damage and Hemodynamic Alterations in Diabetic Nephropathy

Eliane F. E. Wenstedt and Liffert Vogt

Introduction

Generally, the vascular complications of diabetes are separated into macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (neuropathy, retinopathy, and nephropathy). Microvascular problems involve the microcirculation, which is often defined morphologically and considered to consist of vessels <150 μm, including arterioles, capillaries, and venules. However, a more feasible definition is based on functional aspects of arterial vessels and includes all arterial vessels that respond to increasing pressure by a myogenic reduction in lumen diameter, as well as the capillaries and venules [1]. These microvessels have an important role in the transportation of oxygen and nutrients to tissue cells, and, therefore, their adequate perfusion is essential for tissue and organ function [2, 3]. In diabetic nephropathy, damage of the microcirculation occurs. In this chapter, the manifestations, causative mechanisms, and specific treatment of microvascular damage in diabetic nephropathy will consecutively be discussed.

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Manifestations of Microvascular Damage in Diabetic Nephropathy

Renal Microcirculation and Hemodynamic Alterations

The renal microcirculation involves the interlobar, arcuate, and interlobular arteries and smaller microvessels like arterioles, capillaries, and venules. Two capillary beds are involved in the renal microcirculation, as it includes both the glomerular capillary bed and the peritubular capillary bed (Fig. 16.1). The first capillary bed emerges from the afferent arteriole and leads into the efferent arteriole, which merges into the peritubular capillary bed. Damage of the renal microcirculation has a central role in the origination and progression of diabetic nephropathy. However, judging the state of the microcirculation is not straightforward. An intact *macro*circulation, which can be estimated relatively easy through measurements like blood pressure, heart frequency, and duplex ultrasound (for the renal artery), does not automatically translate into a good functioning microcirculation [4]. Assessment of the microcirculation is more complicated and is not yet common practice in the clinical setting.

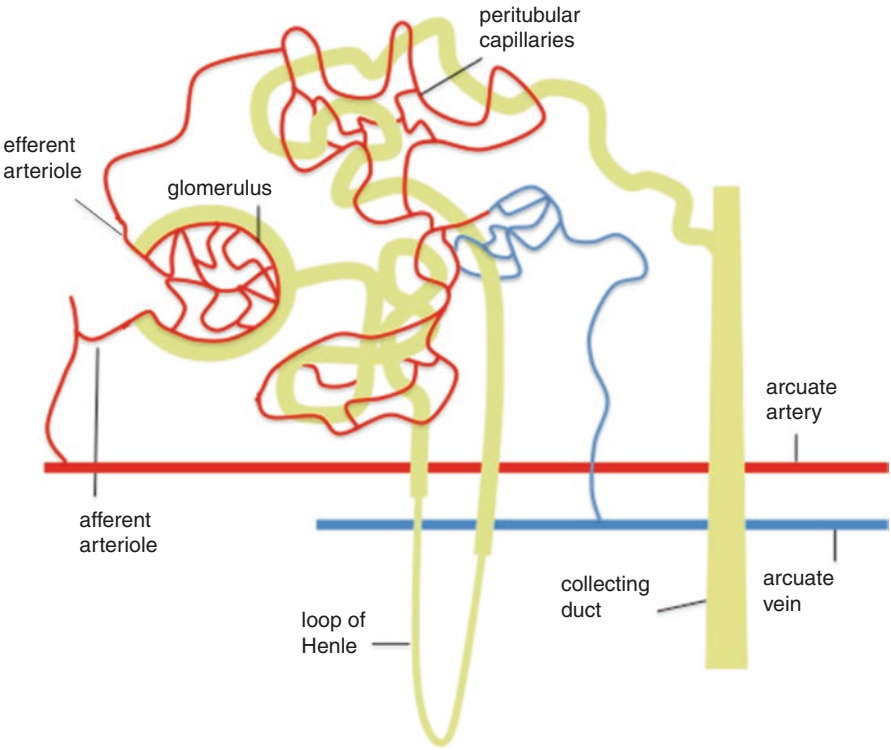

Fig. 16.1 The microcirculation of the kidney. The microcirculation of the kidney with the afferent arteriole, the glomerular capillary bed, the efferent arteriole, and the peritubular capillary bed

With the help of handheld microscopical devices, the microcirculation can be assessed visually, of which one of the most modern examples is sidestream dark field (SDF) imaging [5]. However, with regard to the renal microcirculation, most devices require direct access to the kidney, complicating clinical use [6]. Therefore, for clinical practice, it is necessary to use other estimates. In general, kidney function is represented by an estimation of the GFR, and kidney damage is assessed by the presence and the degree of albuminuria. Albuminuria is thought to represent damage to glomerular capillaries – the endothelium in particular – and is considered to be a hallmark of microvascular lesions in diabetic nephropathy, as the more damaged the glomeruli are, the more albumin is able to pass. Also, albuminuria may represent more widespread vascular damage, as suggested by the "steno hypothesis" [7]. This hypothesis proposes that disturbance of heparan sulfate metabolism can account as a common cause for albuminuria and other complications that occur in diabetes, both microvascular and macrovascular. Heparan sulfates comprise an important anionic component of the glomerular endothelium and the glomerular filtration barrier, and its associated enzymes are vulnerable to hyperglycemia. Reduced negativity of the glomerular barrier as a consequence of loss of the negatively charged heparan sulfates means an easier transfer for proteins with a negative charge, leading to albuminuria. Besides vascular damage, albuminuria may reflect the presence of hyperfiltration and increased intraglomerular pressure as a consequence of impaired regulation of the tone of the afferent arteriole. The glomerular damage in diabetic nephropathy furthermore involves thickening of the glomerular basement membrane, mesangial expansion, and podocyte injury, which is more extensively discussed in Chapter 9.2. Both the eGFR and the degree of albuminuria are significant predictors of end-stage renal disease and mortality and can best be combined for the most accurate prognosis [8–10]. The predictive value of these measurements in diabetics does not differ from that in nondiabetics [11] although it was also proposed that the GFR cannot be accurately estimated in patients with diabetes when compared to direct measurements with iohexol [12]. Albuminuria may therefore be the most representable measurement to assess impairment of the microcirculation in diabetes.

Rarefaction and Altered Blood Flow

Regardless of their cause, acute as well as chronic kidney diseases are characterized by an altered renal microcirculation, worsening with progress of the disease [13]. In nondiabetic animal models, renal ischemic injury induces renal microvascular rarefaction (a reduction of vessel number per volume of tissue) leading to albuminuria [14]. Also in diabetic animals, renal microvascular disease, among which rarefaction, precedes and likely promotes the decline in kidney function [15]. In both diabetic and nondiabetic humans, kidney function is negatively correlated with renal microcirculatory rarefaction, as assessed through biopsies [16]. Figure 16.2 shows clear microvascular rarefaction in a human diabetic kidney compared to a healthy control. Furthermore, besides anatomical changes like rarefaction, hemodynamic

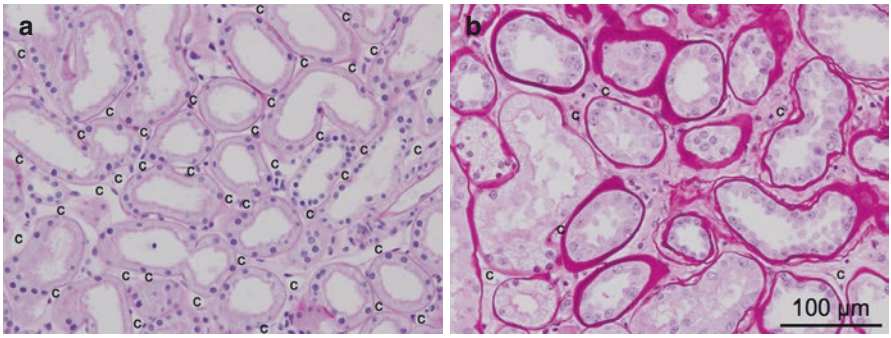

Fig. 16.2 Microvascular rarefaction in a diabetic kidney. Kidney tissue stained with PASD. (**a**) Normal human kidney. (**b**) Kidney of a patient with diabetic nephropathy. The peritubular capillaries are marked with a C. (Image with permission of J. Aten, PhD, Department of Pathology, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, The Netherlands)

changes take place in diabetic kidney disease. Diabetic patients with albuminuria demonstrate lower peritubular capillary flow than their normoalbuminuric counterparts [17]. But also in diabetic patients without albuminuria and without decline in kidney function, peritubular capillary flow is decreased compared to healthy controls [18]. Drug-induced increase of peritubular capillary flow is associated with better renal function [19]. A decrease in peritubular capillary flow negatively affects microcirculatory function, as it precedes and probably contributes to structural alterations of the microcirculation [20]. An intact microcirculation is important for the oxygenation of the surrounding tissue, and the microcirculatory alterations that have been described, involving both rarefaction and reduced flow velocity, induce hypoxia. Hypoxia leads to inflammation and induces tubulointerstitial fibrosis and glomerulosclerosis, which enhances microcirculatory dysfunction even more (Fig. 16.3).

Hyperfiltration

As briefly mentioned above, besides rarefaction of the tubular microvasculature, typical early abnormalities of diabetes comprise an increased GFR, or glomerular hyperfiltration, and intrarenal hypertension. Generally, hyperfiltration is believed to be the consequence of vasodilation of the afferent arteriole (by a disturbed myogenic response through hyperglycemia) and of vasoconstriction of the efferent arteriole (owing to activation of the RAS) (Fig. 16.4), as discussed in Chapter 11.3 [21]. There is evidence that the presence of hyperfiltration may eventually lead to worse renal outcomes in terms of a more rapid progression of GFR decline over time [22] and increased albuminuria [23–25], although there are also studies that do not show this association [26–28]. A complicating factor is that hyperfiltration is not uniformly defined and that methods for GFR evaluation differ between studies.

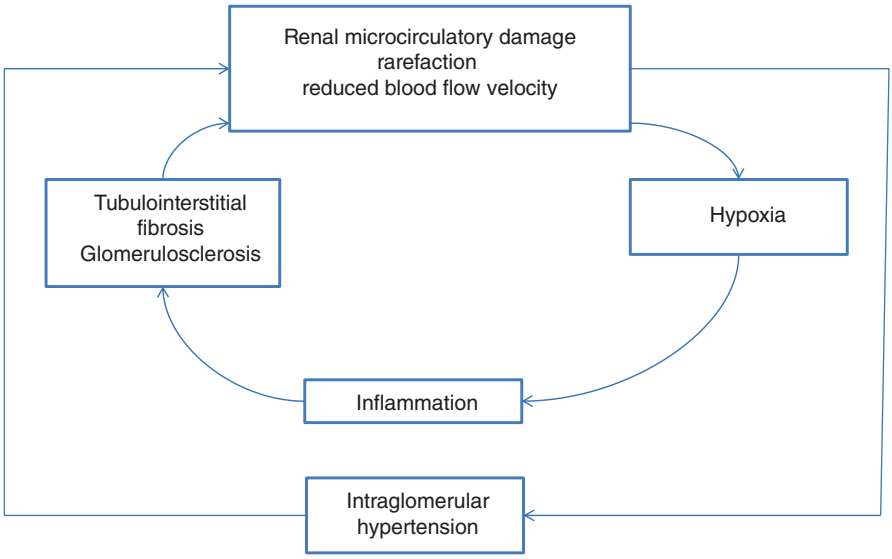

Fig. 16.3 The cascades involved in renal microvascular damage in diabetic nephropathy. Microvascular damage is centrally involved in diabetic nephropathy and is both a cause and a consequence of renal damage. (Reprinted with permission from Elsevier: Ref. [6])

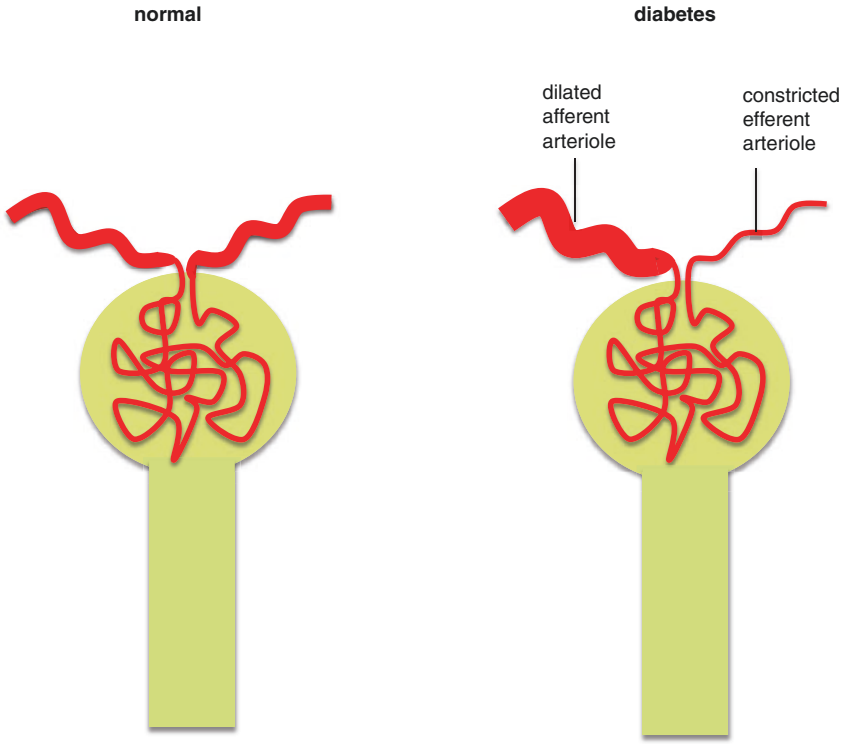

Fig. 16.4 Glomerular hyperfiltration. Vasodilation of the afferent arteriole and vasoconstriction of the efferent arteriole result in glomerular hyperfiltration

The microvascular alterations in the glomerulus – among which increased thickness of the glomerular basement membrane – and other changes like mesangial expansion and podocyte injury affect glomerular filtration capacity and may ultimately lead to loss of functional nephrons. Both glomerular hyperfiltration and its eventual consequences (i.e., reduced number of functional nephrons) negatively affect the kidney's ability to increase renal blood flow. In addition, thickening of both afferent and efferent arterioles as a result of hyalinosis, which comprises depositions of collagen, fibrinogen, and other extracellular matrix components in the vessel wall, may further increase renal vascular and decrease renal flow reserve. This is supported by studies showing that the increase in renal blood flow following dilatation with different hyperemic agents is lower in patients with diabetes compared to studies that examined nondiabetic patients [29]. Renal hemodynamic alterations appear to be similar in DM1 and DM2 [30].

Intrarenal Renin-Angiotensin System

An essential mediator in renal hemodynamics is the renin-angiotensin system (RAS). Evidence with regard to the systemic RAS in diabetic subjects is not univocal, as studies have described overactivation [31], underactivation [32], and normal activation [33] of this system. Either way, the effects of RAS inhibitors such as ACE inhibitors and ARBs with regard to renal and cardiovascular outcomes in diabetic nephropathy have been well established [34–36]. However, complete insight concerning the underlying mechanisms is still lacking. The fact that not all diabetic subjects show an overactivated RAS might suggest that RAS inhibitors induce their effect through other mechanisms than the systemic RAS [37]. Apart from the systemic RAS, the existence of a local RAS has been proven in various tissues, including the kidney, called the intrarenal RAS. This is a local autocrine and paracrine system involving both angiotensin-dependent and angiotensin-independent pathways. In both nondiabetic [38, 39] and diabetic nephropathy [40–42], the intrarenal RAS is upregulated. Possibly, RAS inhibitors exert their beneficial effect by influencing this upregulation [43]. In subjects with diabetic nephropathy, the intrarenal RAS is more upregulated than in subjects with nondiabetic nephropathy [44]. Overactivation of the intrarenal RAS can occur by the conventional ACE-dependent mechanisms but also by an ACE-independent pathway mediated by chymases, which are enzymes that cleave peptide bonds in proteins. It is suggested that increased chymase activity in diabetes is responsible for overactivation of the intrarenal RAS [45–47]. Another relatively newly identified regulator of the RAS system is ACE2, an enzyme that catalyzes the conversion of angiotensin I to angiotensin 1–9 and the conversion of angiotensin II to the vasodilatory angiotensin 1–7. Its distribution is more restricted than ACE, as it is found mainly in the heart and in the kidney. In the proximal tubule of the diabetic kidney, ACE2 is increased, possibly as a response to the increased activation of the intrarenal RAS [48, 49]. ACE2 is shown

to mediate hyperfiltration in diabetic mice [50]. Although the role of ACE2 in diabetic nephropathy is not yet completely unraveled, ACE2 inhibition worsens glomerular injury, pointing to a protective compensatory ACE2 increase in diabetic nephropathy [51]. Another consequence of activation of the intrarenal RAS is that it induces oxidative stress, which serves as one of the causative mechanisms in diabetic nephropathy and will be discussed under the next heading [52]. Also, the intrarenal RAS influences renal hemodynamics and the renal microcirculation. Increased intrarenal RAS activation is thought to predominantly constrict efferent arterioles, although increasing evidence also shows constriction of the afferent arterioles. Independent of hemodynamic changes, intrarenal RAS activation directly damages the glomerular basal membrane and podocytes [52]. Also, the autoregulatory capacity of the kidney is altered in the presence of an activated intrarenal RAS [53, 54].

Autoregulation

Autoregulation comprises the kidney's ability to regulate its own blood flow, which is defined by the capacity to keep renal blood flow and glomerular filtration rate constant despite variations in blood pressure. The two principal mechanisms that regulate renal blood flow are the myogenic response and tubuloglomerular feedback. The myogenic response regulates renal blood flow via stretch receptors in renal afferent arterioles that sense arterial pressure and cause arteriolar vasoconstriction in response to an increase in blood pressure. Tubuloglomerular feedback refers to the mechanism unique to the kidney, whereby changes in sodium chloride delivery in the distal tubule cause alterations in afferent arteriolar tone. If distal sodium chloride concentration is increased, constriction of afferent arterioles will cause tubular delivery to decrease. Next to the myogenic response and tubuloglomerular feedback, other mechanisms influence renal autoregulation, including the sympathetic nervous system, angiotensin II, and prostaglandins, among others. In different experimental models of diabetes, renal autoregulation is impaired already at an early stage and contributes to the development of diabetic nephropathy [55–57]. Whether impairment of renal autoregulation facilitates the development of diabetic nephropathy in humans is unknown. However, a previous study comparing the effects of acute blood pressure changes on glomerular filtration rate in type 1 diabetic patients with and without microalbuminuria showed that clonidine induced a similar blood pressure reduction in both groups but decreased glomerular filtration rate only in patients with microalbuminuria, suggesting impaired renal autoregulation in patients with diabetic nephropathy [58]. The capacity to regulate glomerular filtration rate after acute blood pressure lowering was associated with the duration of diabetes [59]. Moreover, the use of blood pressure-lowering medication in patients with diabetes, particularly diuretics and calcium-antagonists, may further affect renal autoregulation, contributing to an already compromised autoregulatory capacity [60, 61].

Microcirculation of Other Organs in Diabetic Nephropathy

The "diabetic triopathy" [62] comprises the three common microvascular complications of diabetes, which are nephropathy, retinopathy, and neuropathy. Although these three complications do not always occur together, they are clearly related. Since retinopathy commonly precedes nephropathy, virtually all patients with diabetic nephropathy display some stage of retinopathy [63]. Conversely, approximately one-third of the patients with retinopathy do not have microalbuminuria. Estimations greatly differ between studies and will be further discussed in Chapter 11.4. Damage of the renal microcirculation associates with damage of the retinal microcirculation, as retinal arteriolar narrowing is correlated with albuminuria [64]. The extent of albuminuria may predict the occurrence of retinopathy [65]. The relation between nephropathy and neuropathy is somewhat less robust, but still one-third of microalbuminuric patients and half of macroalbuminuric patients also have neuropathy [63]. The presence of neuropathy is associated with nephropathy lesions in diabetics [66]. The cumulative burden of the nephropathy, retinopathy, and neuropathy significantly increases the risk of cardiovascular disease [67]. Notably, apart from this "diabetic triopathy," the microcirculation of other organ systems is affected as well. Albuminuria is associated with cerebral anatomic alterations and disturbances in cognitive function, whereas a decline in GFR is not or less so [68, 69]. This might mean that not the decline in kidney function but the extent of microvascular damage (as represented by albuminuria) links kidney disease to cognitive function. Independent of albuminuria, the presence of cerebral microvascular disease predicts renal failure in patients with DM2 [70]. Furthermore, not only diabetes as disease itself is associated with dermal microvascular rarefaction [71], but also diabetic kidney damage – as measured by albuminuria – is associated with dermal microvascular rarefaction [72]. Another known feature in diabetics is microvascular dysfunction of the heart [73]. In diabetic nephropathy, coronary microvascular dysfunction is associated with the presence of albuminuria [74]. Lastly, diabetic nephropathy is associated with a high fracture risk [75]. Bone microstructure is altered in diabetics, particularly when microvascular complications are present [76]. This has led to the hypothesis that bone disease is just another microvascular complication of diabetes [77]. The coherency between diabetic nephropathy and microvascular damage in various other organ systems suggest that a common systemic microvascular process is present in diabetic nephropathy.

Endothelial Dysfunction and the Endothelial Glycocalyx in Diabetic Nephropathy

The Endothelium

The vascular endothelium is of major importance with regard to vascular function, wielding a wide range of functions ranging from regulating vascular tone to modifying permeability for nutrients, inflammatory cells, and other molecules. Therefore,

endothelial alterations have substantial impact on kidney biology. In diabetic nephropathy, the role of endothelial dysfunction is widely acknowledged, generally meaning that the endothelial function has changed in a way that bears negative consequences for organ function. In Chapter 9.2, the endothelial alterations in diabetic nephropathy are discussed more extensively. In humans, endothelial function cannot be measured directly. Thus, indirect estimates have to be used, including assessment of the degree of vasodilation and plasma levels of endothelium-derived mediators like nitric oxide, endothelin, and von Willebrand factor [78]. Nitric oxide is a major regulator of the endothelial vascular tone and is one of the most important vasodilating agents. In the endothelium, endothelial nitric oxide synthase (eNOS) is primarily responsible for the generation of nitric oxide. There is ample evidence that in diabetic nephropathy, eNOS is decreased [79]. One example is that diabetic eNOS knockout mice develop advanced diabetic nephropathy [80]. In early diabetes however, an upregulation of eNOS in the renal microvasculature might be responsible for intrarenal vasodilation [81]. There is a clear association between endothelial dysfunction and albuminuria, in both diabetic [82, 83] and nondiabetic subjects [84]. The endothelial glycocalyx might provide a link between these phenomena [85].

The Endothelial Glycocalyx

The importance of the endothelial glycocalyx is increasingly being recognized in diabetic nephropathy, and more and more research focuses on this miniscule layer that resides on the luminal side of the blood vessel endothelium. The glycocalyx ranges from 0.5 μm in capillaries to 4.5 μm in larger arteries and mostly comprises glycosaminoglycans, which are large negatively charged polysaccharides [86]. Both patients with type I diabetes [87] and type II diabetes [88] are known to have an impaired glycocalyx. The most abundant glycosaminoglycan of the glycocalyx is heparan sulfate. Increased activity of glycocalyx-degrading enzymes such as heparanase in diabetic nephropathy has been acknowledged for quite some time [89]. Endothelin-1 triggers podocytes to release heparanase [90]. Heparanase-mediated disruption of the glomerular glycocalyx is associated with albuminuria [90], and the degree of albuminuria reflects endothelial glycocalyx status [87]. The relationship between a damaged glycocalyx and albuminuria appears to be causal. Chemical destruction of the glycocalyx leads to albuminuria [91, 92], while conversely, restoration of the glycocalyx reduces albuminuria [85, 93].

Salt

Albuminuria is also associated with salt sensitivity [94], which refers to the extent of the individual blood pressure response after salt (sodium chloride) loading. Individuals are called salt-sensitive when they demonstrate a blood pressure increase after salt load, whereas the individuals that do not show an increase are deemed salt-resistant. Diabetic patients are thought to be prone for salt sensitivity [95]. Evidently, a salt-mediated rise of blood pressure bears negative consequences with regard to

kidney function and kidney damage. However, even independent of blood pressure, salt can damage the microcirculation and the blood vessel wall, thereby possibly contributing to albuminuria. High salt intake is associated with a reduction of vessel number per volume of tissue, called rarefaction, whereas reduction of salt intake increases vessel density [96–100]. In addition, salt induces endothelial dysfunction [101]. Both the endothelial glycocalyx and the vascular endothelium itself stiffen in response to salt excess [102, 103]. This stiffening impairs the effects of shear stress on the vascular wall, interfering with the pathways that normally lead to nitric oxide production and consequent vasodilatation. This touches upon the central role that the eNOS/nitric oxide pathway has in endothelial dysfunction and diabetic nephropathy [104, 105]. Monocyte chemotactic protein-1 (MCP-1) is another factor that is affected by salt and which can contribute to albuminuria. Whereas salt increases MCP-1 [106], inhibition of MCP-1 restores the glycocalyx [93] and reduces albuminuria [107]. In short, in diabetic nephropathy, high salt intake (which is quite common in a normal Western diet) is yet another factor that can impair the microcirculation.

Causative Mechanisms of Microvascular Damage in Diabetic Nephropathy

Hypertension

Hypertension has a higher prevalence in diabetic subjects than in nondiabetic subjects [108]. Also, the prevalence of hypertension is higher in diabetics with macroalbuminuria than in diabetics with normoalbuminuria [109]. One of the fundamental features of hypertension is rarefaction, since it is consistently found in models of both experimental [98, 110–113] and clinical [114–117] hypertension. The fact that rarefaction is also present in the very early phases of hypertension or borderline hypertension might suggest it is a cause rather than a consequence of increased blood pressure [118, 119]. Rarefaction is thought to contribute to the development of hypertension by increasing peripheral vascular resistance. Also, rarefaction negatively influences tissue oxygen concentration, impairing the metabolic activity of the tissue [73] (Fig. 16.3). Hypertension is a major driving force for vascular remodeling, contributing to renal abnormalities [120]. Microcirculatory alterations are predictive for a decrease of kidney function in hypertensives [121]. Also in diabetic nephropathy, renal rarefaction is present and is associated with worse renal outcome [14–16].

Renal microvascular disease leads to hemodynamic maladjustments that induce tubulointerstitial fibrosis and increased intraglomerular pressure [18]. Systemic hypertension itself contributes to glomerular hyperfiltration and intraglomerular hypertension as well. Increased intraglomerular pressure will lead to glomerular capillary dropout, resulting in rarefaction. The presence of rarefaction further increases intraglomerular pressure, ensuing a vicious cycle that worsens kidney

damage even more [13] (Fig. 16.3). Importantly, also in normotensive diabetic subjects, structural alterations of the microcirculation are present [122]. In fact, vascular remodeling is even greater in normotensive diabetic subjects than in hypertensive nondiabetic subjects [123]. Therefore, other factors than high blood pressure contributing to microvascular damage are present in diabetic nephropathy.

Hyperglycemia

Hyperglycemia can cause microvascular damage and hemodynamic alterations as well. Besides hypertension, it is another cause of hyperfiltration as it impairs the tubuloglomerular feedback mechanism [124]. Other factors among which obesity and insulin resistance may be contributing too [125]. Furthermore, hyperglycemia can cause direct damage to the endothelial glycocalyx [126, 127], and hyperglycemia itself as well as the metabolic consequences of glucose dysregulation can lead to endothelial cell dysfunction [79]. Hyperglycemia is associated with decreased nitric oxide, providing a link between diabetes and endothelial dysfunction [105] (Fig. 16.5). First, glucose itself impairs endothelial function. Glucose concentrations similar to the concentrations in diabetic patients are associated with a lower nitric oxide response of vascular endothelial cells [128]. Second, mechanisms

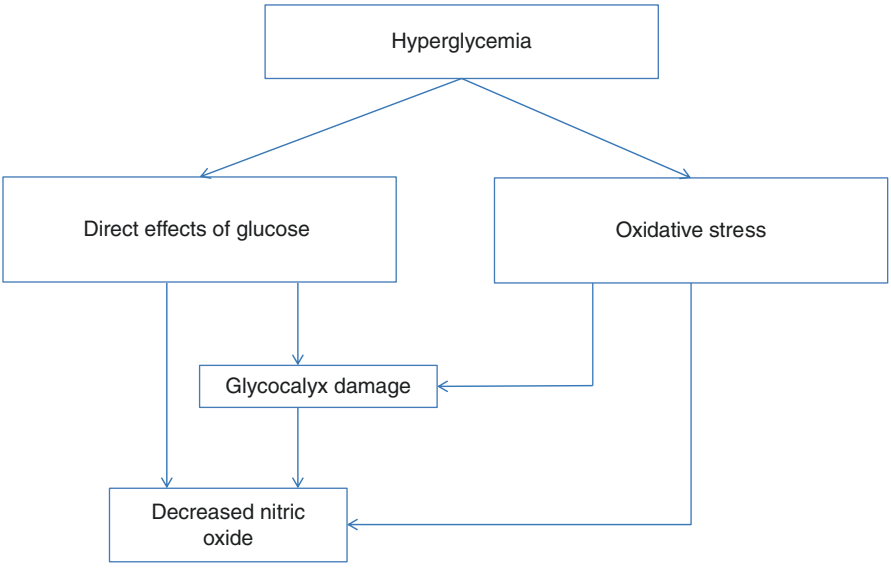

Fig. 16.5 Hyperglycemia and impaired nitric oxide availability. Impaired nitric oxide availability has a central role in the endothelial dysfunction that occurs with diabetic nephropathy. It is caused by directs effects of glucose, damage of the glycocalyx, and oxidative stress

related to oxidative stress decrease nitric oxide availability. There are four main molecular mechanisms involved in hyperglycemic damage of the vasculature [129]:

- Overproduction of superoxide by the mitochondrial electron transport chain increased polyol pathway flux.
- Increased hexosamine pathway flux.
- Activation of protein kinase C (PKC) isoforms.
- Increased advanced glycation end-product (AGE) formation.

Oxidative stress may be the linking mechanism as it can act as a triggering mechanism for each pathway [129–131]. By interfering in one of the last steps of glycolysis, namely, inhibiting the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), excess superoxide increases the pathways more upstream in the reaction, involving the polyol, hexosamine, protein kinase C, and AGE pathways (Fig. 16.6). Although this hypothesis serves as a very attractive unifying explanation in diabetic nephropathy, clinical trials that seized on this mechanism by administration of antioxidants were not successful [132, 133]. Newer theories focus on superoxide production by the mitochondria, which is supposed to be elevated in early diabetic nephropathy and reduced in a later phase of diabetic nephropathy [134].

Furthermore, apart from glucose, another important factor is insulin, since it has a directly modulating effect on a variety of cells. In patients with diabetes, this modulating ability is impaired, termed cellular insulin resistance. Cellular insulin

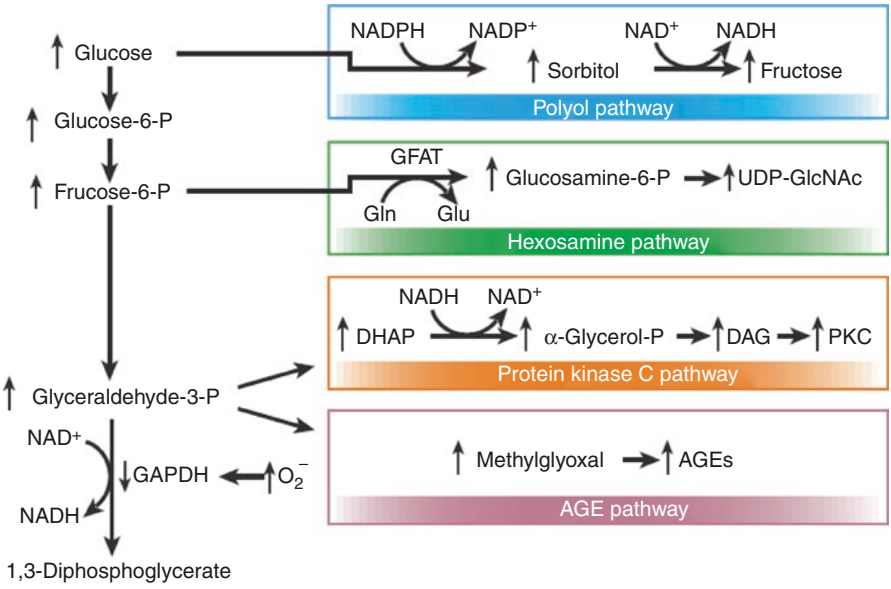

Fig. 16.6 The superoxide hypothesis. Excess superoxide as a linking mechanism for the polyol pathway, the hexosamine pathway, the protein kinase C pathway, and the AGE pathway. (Reprinted with permission from Springer Nature: Brownlee et al. [129])

resistance is associated with diabetic nephropathy [135] and microalbuminuria [136]. Another factor related to albuminuria, microvascular damage, and hyperglycemia is vascular endothelial growth factor A (VEGF-A). It is thought that in early diabetic nephropathy, VEGF-A is overexpressed [137]. Blockade of VEGF-A attenuates albuminuria [138]. With progression of diabetic nephropathy, VEGF-A expression decreases and rarefaction increases, possibly related to the angiogenic effect of VEGF-A [139]. At high glucose levels, the correlation between VEGF-A and angiogenesis seems disturbed [140]. Again, eNOS appears to play a central role, as this enzyme plays a role in angiogenesis but is influenced by glucose.

Concluding, there is a variety of hyperglycemia-associated molecular mechanisms that can cause microvascular damage in diabetic nephropathy. These mechanisms are comprehensive, complex, and subject to changing views. In Chapter 6, the different pathogenetic mechanisms are discussed in more detail.

Treatment Specific for Microvascular Damage in Diabetic Nephropathy

As discussed in this chapter, hyperglycemia as well as hypertension contribute to microvascular damage. Hence, it is not surprising that intensive glycemic control and intensive blood pressure control reduce albuminuria [141–143]. Evidence for a reduction of kidney failure is inconsistent, possibly indicating that more factors should be targeted [141–144]. In nondiabetics, intensive blood pressure control was shown to decrease risk for cardiovascular events and death [145], whereas in diabetics, intensive blood pressure control did not reduce cardiovascular morbidity and mortality [146]. Currently, standard of care in diabetic nephropathy involves RAS inhibitors. RAS inhibitors reduce the progression rate of renal disease independently of their antihypertensive effect, as was demonstrated by the Heart Outcomes Prevention Evaluation (HOPE) trial [34], the Reduction of Endpoints in Non-Insulin-Dependent Diabetes with the Angiotensin II Antagonist Losartan (RENAAL) trial [35], and the Irbesartan Diabetic Nephropathy Trial (IDNT) [36]. RAS inhibitors affect the microcirculation of the kidney by inhibiting the vasoconstrictive response of the RAS system on the efferent arteriole, thereby reducing intraglomerular pressure. In addition, both ARBs [147] and ACE inhibitors [147, 148] reduce capillary rarefaction. The mechanisms behind this are not yet fully elucidated and may include RAS-independent mechanisms like, for example, the bradykinin system [149]. Furthermore, overactivation of the aldosterone axis can occur in diabetes [31]. However, long-term RAS blockade with ACE inhibitors and ARBs may result in incomplete suppression of aldosterone, which is known as the aldosterone escape phenomenon [150]. Aldosterone has detrimental effects on the endothelium and is associated with endothelial dysfunction [151]. Yet, the effect of adding aldosterone antagonists, such as mineralocorticoid receptor antagonists, in chronic kidney disease has not yet been explored in large trials [152] but is currently under investigation.

The variety of mechanisms that are involved in diabetic nephropathy precipitates the availability of a wide range of drugs with different mechanisms of action, which are discussed in Chapter 19.1. A few examples of drugs that specifically target the microcirculation will shortly be discussed here. First, glycocalyx-restoring drugs are under investigation, which derives from the notion that in diabetic nephropathy the endothelial glycocalyx is damaged [153]. Monocyte chemotactic protein-1 inhibition restores the glycocalyx [93] and reduces albuminuria [107]. Also, endothelin receptor antagonists have an antiproteinuric effect which probably derives from their glycocalyx-restoring property [154]. Likewise, sulodexide, a chemical glycocalyx-mimetic consisting of 80% heparin-like substances and 20% dermatan sulfate, is able to restore the glycocalyx perturbation that is present in DM2 subjects [88]. The evidence for an albuminuria-reducing effect of sulodexide is conflicting [155, 156]. Sulodexide does have a blood pressure-lowering effect that is dependent on the state of the ESL as represented by the degree of albuminuria [157, 158]. The glycocalyx is closely linked to endothelial function, and as endothelial dysfunction is of major importance in the development of diabetic nephropathy, it is a very important target for treatment. Restoring eNOS activity is one of the main features of improving endothelial dysfunction. Examples include administration of L-arginine (the precursor of nitric oxide) or its essential cofactor BH4 or sepiapterin [159]. New classes of antidiabetic drugs like SGLT2-inhibitors, DPP-4 inhibitors, and GLP-1 receptor agonists have shown promising results with regard to glycemic control and/or renal outcomes but need to be further studied with regard to their effects on the renal microcirculation. Other future treatment may also target microRNAs, which are a class of small noncoding RNAs that are involved in nearly all pathophysiological processes. Overexpression of certain microRNAs has shown to be present in both diabetic nephropathy and endothelial dysfunction [160].

To end with, the mentioned drugs are only a small part of possible therapeutic options in diabetic nephropathy. This chapter particularly focused on drugs specifically targeting the microcirculation, since it is clear that the microcirculation is affected in diabetic nephropathy, and that drugs that target the microcirculation can induce substantial beneficial effects. However, due to the involvement of a wide variability of mechanisms, finding an optimal therapeutic agent remains complicated.

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Chapter 17
Coagulation and Hemostasis in Diabetic Nephropathy

Joris J. Roelofs

The Hemostatic System

The process of hemostasis is of vital importance since it enables us to close off damaged blood vessels while keeping the blood in a fluid state and to remove blood clots after vascular integrity has been restored. The hemostatic system is a highly conserved mechanism, in which blood clotting or coagulation plays a central role. Two thousand years ago, the Greek philosopher Plato already stated that the blood forms fibers once it leaves the body. He also invented the term "fibrin" to describe the fibrillary shape of clotted blood [1]. Most of the key components in the coagulation system were discovered during the twentieth century. Around the 1950s, virtually all coagulation factors had been characterized, such as von Willebrand factor (VWF), produced by endothelial cells, and factors (F) V, VII, VIII, IX, and XI, produced by hepatocytes. Deficiency in some of the factors were known to cause bleeding disorders, such as FVIII deficiency in hemophilia A and FIX deficiency in hemophilia B [1]. The exact mode of interaction between the several independently discovered coagulation factors remained elusive until 1964, when two independent groups designed a coagulation model which resembled a waterfall or cascade, resulting in the term "coagulation cascade" [2, 3]. In this cascade, each coagulation factor consists of a proenzyme which is converted to an active enzyme by the upstream activated coagulation factor (Fig. 17.1). Two different cascades converge in activation of FX. These are the intrinsic pathway, so-called because all its components are present in the blood, and the extrinsic pathway which requires a factor which is not present in the blood, namely, tissue factor (TF, derived from extravascular tissue).

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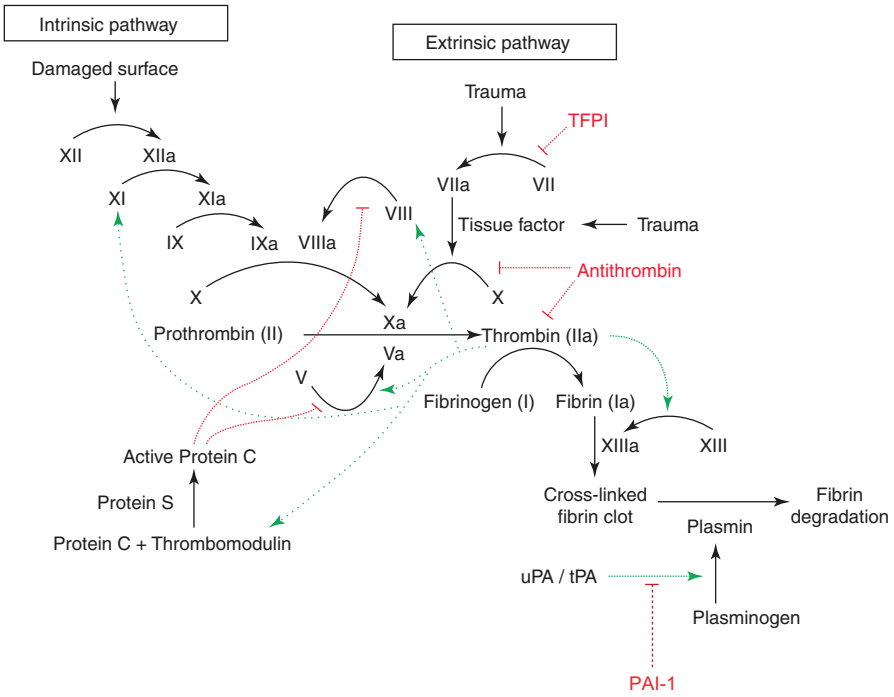

Fig. 17.1 Schematic representation of the hemostatic system, comprising the intrinsic and extrinsic pathway, as well as the fibrinolytic system. Green dotted arrows indicate activation; red dotted lines indicate inhibition

The end products of the coagulation cascade are fibrin fibers, which after cross-linking by thrombin-activated FXIII form the polymerized fibrin clot (Fig. 17.1).

Diabetes Is Associated with a Hypercoagulable State

Diabetes is associated with endothelial dysfunction (see Chaps. 10, 12 and 16 of this book), resulting in an increased risk of atherosclerosis (see Chap. 22) and a hypercoagulable state of the blood [4]. Coronary artery disease is a major cause of death in patients with diabetes, and thrombosis is the cause of death in up to 80% of diabetics [5, 6]. This hypercoagulability may be the result of an imbalance between circulating coagulation factors and the endothelial cell surface. The vascular endothelium is an essential player in the regulation of coagulation processes [7]. The major defense mechanisms that prevent inappropriate coagulation are found at the endothelial cell surface [8]. The endothelium itself is capable of producing both procoagulant factors such as vWF and anticoagulant factors such as thrombomodulin (TM). Various methods can be used to investigate endothelial

integrity or dysfunction. The plasma levels of biological markers, such as vWF, endothelin-1, and adhesion molecules, may reflect endothelial dysfunction [9]. Increased plasma levels of many different clotting factors including fibrinogen, FVII, FIX, FXII, and vWF in patients with type 2 diabetes are associated with vascular injury [4, 10].

Coagulation Factors in the Context of Diabetic Kidney Disease

There is increasing evidence that the above described hypercoagulability in diabetes patients can be causally linked to microvascular complications, among which diabetic nephropathy (DN).

Deviations of hemostatic parameters are more prominent in patients with DN than in diabetic patients without kidney disease [11–15]. Activated coagulation factors play important roles not only in coagulation but also in inflammatory processes, tissue remodeling, and fibrosis, mainly through interaction with protease-activated receptors (PARs) [16]. Below the most relevant coagulation factors, natural anticoagulants and PARs will be discussed in the context of DN.

Tissue Factor

Tissue factor (TF) is the primary cellular initiator of the coagulation cascade. Within the kidney, TF is expressed by tubular epithelial cells (TEC), parietal epithelial cells lining Bowman's capsule, endothelial cells, and in the interstitium [17]. Activation of coagulation through TF has been demonstrated in various – mainly inflammatory – kidney diseases such as lupus nephritis and crescentic glomerulonephritis [17–19]. Inhibition of TF with a blocking anti-TF antibody reduces glomerular fibrin deposition and crescent formation and slows down the development of kidney failure in a mouse model of experimental crescentic glomerulonephritis [19]. Studies in obese, ob/ob and db/db, mice with hyperinsulinemia and hyperglycemia – due to a genetic leptin deficiency, or leptin receptor deficiency, respectively – demonstrated higher intrarenal concentrations of TF mRNA in the mutant mice compared to controls [20]. Also, kidneys of streptozotocin (STZ)-treated diabetic mice showed a marked increase in TF-dependent clotting activity and presence of thrombin and fibrin immunostaining at 10 weeks after STZ injection [21]. Tubular epithelial cells, which were incubated with high concentrations of glucose, showed increased TF production [21].

Coagulation Factors Xa and Va

TF activates coagulation factor VIIa, which in turn activates FX into Factor Xa (Fig. 17.1). Increased levels of FXa have been found in diabetic subjects and in different animal models of DM. For example, in eNOS−/− mice, made diabetic by introducing the so-called Akita mutation in the insulin 2 gene, a model which mimics human DN [22, 23], FXa levels were increased [24]. The diabetic eNOS−/− mice displayed increased intrarenal FX mRNA, increased urinary FXa activity, and FX expression in glomerular macrophages. Treatment of these mice with edoxaban, an oral FXa inhibitor, strongly reduced the features of DN and reduced the expression of proinflammatory and profibrotic genes, such as plasminogen activator inhibitor type 1 (PAI-1) and tumor necrosis factor alpha (TNF-α) [24].

Comparable results were found by treatment of diabetic db/db mice with Factor Xa inhibitor fondaparinux [25]. At 20 weeks of age, the glomeruli of diabetic (db/db) mice showed significantly more of PAR-2-positive cells and larger amounts of fibronectin and collagen IV than glomeruli of control mice [25]. Diabetic (db/db) and normoglycemic mice were for the expression of PAR2, transforming growth factor (TGF)-β, fibrin, extracellular matrix (ECM) proteins, and CD31 at week 20. Fondaparinux treatment for 10 weeks significantly lowered proteinuria, glomerular hypertrophy, glomerular fibrin, and deposition of extracellular matrix proteins [25]. Taken together, these studies show that increased glomerular coagulation, FXa, and PAR-2 expression are implicated in the occurrence of DN and that Factor Xa inhibition may protect against development of DN.

FVa functions as a cofactor of FXa. Together, both factors form a prothrombinase complex on TF-expressing cells, which has the ability to convert prothrombin into thrombin (Fig. 17.1). Studies regarding the factor V Leiden (FVL) mutation have revealed an interesting and somewhat unexpected role for FVa in DN. The FVL mutation is a missense mutation in the FV gene (R506Q), which renders FVa relatively resistant to inactivation by activated protein C (APC) (Fig. 17.1). The prevalence of the FVL mutation in the Caucasian population is 4%–6% [26]. Wang et al. investigated the effect of heterozygous and homozygous FVL mutation in mice, which developed DN by injections with STZ [27]. In diabetic FVL mice, albuminuria and histological features of DN were significantly reduced compared with diabetic wild-type mice. This was associated with reduced podocyte apoptosis in the diabetic FVL mice. In vitro, it was shown that high-dose thrombin intensified, but low-dose thrombin prevented the glucose-induced apoptosis of podocytes [27]. The same group studied the effect of the FVL mutation in a large cohort of subjects with type 1 or type 2 diabetes. In these patients, the FVL mutation was associated with lower albuminuria levels, which was suggestive of a protective effect of low but sustained thrombin generation in the context of diabetes. Apart from the direct cytoprotective effects on podocytes, described above, the binding of thrombin to TM can also result in generation of APC, leading to cytoprotective effects on endothelial cells and podocytes by means of signaling via PAR-1 [28]. Thus, there seems to be a twofold, context-, and dose-dependent role of thrombin in chronic kidney diseases,

which may indicate an evolutionary benefit of the prothrombotic state associated with the FVL mutation [28].

Fibrin and the Fibrinolytic System

The end product of activation of the coagulation cascade is fibrin. Glomerular and interstitial fibrin deposition is a well described feature of DN [29, 30], and fibrinogen levels in the circulation of patients with type 2 diabetes are strongly correlated to the risk of developing DN [31]. Under physiological circumstances, fibrin deposits are dissolved by the fibrinolytic system. The key central in the fibrinolytic cascade is plasmin (Fig. 17.1), which degrades fibrin into fibrin degradation products. Plasmin is formed through conversion of its proenzyme, plasminogen. Two physiological plasminogen activators have been recognized: tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) [32], which are inhibited by their specific inhibitors PAI-1 and physiologically far more irrelevant PAI-2 [33]. Besides its role in regulating fibrinolysis, PAI-1 plays a role in a variety of processes dependent on plasmin activity. PAI-1 has the ability to inhibit plasmin-mediated proteolysis. Studies with transgenic mice have revealed a functional role for PAI-1 in processes that involve extracellular matrix turnover such as wound healing, atherosclerosis, and fibrosis [34–36].

Circulating PAI-1 levels are elevated in type 2 diabetes, and this elevation correlates with complications of diabetes, among which DN [37]. Festa et al., who followed 843 individuals for 5 years, reported the correlation between diabetes and changes of PAI-1 and fibrin turnover [38].

Normally, PAI-1 is only produced in trace amounts in the kidney, but significant upregulation of its expression has been reported in a wide variety of acute and chronic kidney diseases, including thrombotic microangiopathy [39], acute pyelonephritis [40], crescentic glomerulonephritis [41], membranous glomerulopathy [42], and DN [43]. In DN, classic Kimmelstiel-Wilson nodules contain PAI-1 protein [43]. A Turkish study in 92 T2DM patients, 16 of which had overt DN, showed that circulating levels of PAI-1 were significantly elevated in the DN group versus the diabetes patients without DN [6]. In STZ-induced DN, PAI-1−/− mice have reduced glomerular levels of fibronectin and lower amounts of albuminuria than WT mice [44]. In addition, spontaneously diabetic PAI-1−/− (db/db) mice, generated by crossbreeding PAI-1−/− mice with leptin receptor deficient mice, have lower albuminuria and less renal collagen accumulation than control mice [45].

Hagiwara et al. investigated the gene expression of uPA, tPA, and PAI-1 by real-time PCR in a rat model of type 1-associated DN (STZ-induced), as well as in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a type 2 diabetes model [46]. They found that in STZ-induced DN, the mRNA levels of uPA, tPA, and PAI-1 were increased by 60–80% and that insulin treatment reduced expression to baseline levels. In OLETF rats, the renal PAI-1 mRNA level was 250% of that in the control rats with comparable genetic background. Interestingly, tPA and uPA mRNA levels

were lower than those in controls. By in situ hybridization, PAI-1 mRNA was visualized in glomerular cells and TEC in both DN models. They concluded that the PAI-1 expression was upregulated in glomeruli and TEC in type 1- and type 2-associated DN, suggesting that modulation of fibrinolytic factors plays a key role in DN development [46]. Lassila et al. reported that PAI-1−/− mice are protected against STZ-induced DN [47]. Other studies using PAI-1−/− mice found that PAI-1 induces synthesis of ECM in DN by TGF-β activation and through reduced ECM degradation by inhibition of proteolytic activity of plasmin and other proteases [48]. A few PAI-1 inhibitors with possible clinical potential have been identified so far [49]. Huang et al. found that short-term administration of an inactive PAI-1 mutant (PAI-1R) slowed down the progression of glomerular sclerosis in db/db mice [50, 51]. They observed that PAI-1R increased glomerular availability of plasmin, reversed PAI-1-mediated inhibition of ECM degradation, and as such reduced symptoms of DN, compared to non-treated db/db mice [51]. Possibly, these effects were the result of PAI-1R-medited interference with binding of PAI-1 to vitronectin, which is known to extend the half-life of PAI-1.

Thrombomodulin

Thrombomodulin (TM, CD141) is a multi-domain type 1 transmembrane glycoprotein [52] which plays a critical role in both coagulation and inflammation [53, 54]. TM is constitutively expressed by endothelial cells, monocytes, and neutrophils [52, 54]. Interestingly, expression of TM by human urothelial cells has also been reported [55]. Structurally, TM consists of five domains: the N-terminal lectin-like domain (Led), six epidermal growth factor (EGF)-like repeats, a serine−/threonine-rich domain, a transmembrane, and a cytoplasmic domain [53]. The domain comprising the 6 EGF-like repeats is critical as a cofactor to thrombin for generation of activated thrombin activatable fibrinolysis inhibitor (TAFIa) and activated protein C (APC) [56]. APC has important anticoagulant as well as anti-inflammatory and cytoprotective properties [57, 58]. The lectin-like domain (also referred to as TMD1) displays a structural homology with the C-type lectin family and has direct anti-inflammatory effects [59].

Plasma TM was already long ago described as a marker for microvascular complications in diabetes mellitus, including DN [60]. TM plasma levels correlate with duration of diabetes in patients with type 1 and type 2 diabetes and are higher in patients with increasing numbers of complications (nephropathy, retinopathy, arterio-occlusive disease, neuropathy), while sTM levels have been described to normalize after simultaneous kidney-pancreas transplantation [61]. A prospective study of 200 T2DM revealed elevated levels of TM in patients with microalbuminuria, which was associated with cardiovascular disease and all-cause mortality [62].

TM orchestrates both coagulation and inflammation, largely through discrete domains. The Led domain inhibits complement activation, while the EGF-like domains independently enhance the formation of anticoagulant and cytoprotective

APC. The protective effect of APC in DN is established (see below). In addition, it has been shown that TM controls DN independent of APC, through the Led domain by regulating complement [63]. Wang et al. described that mice lacking TM's lectin-like domain (TMLeD/LeD) showed higher levels of albuminuria and other features of DN than control mice after STZ injections. Complement deposition (C3 and C5b-9) was strongly increased in glomeruli of diabetic TMLeD/LeD mice, while inhibition of complement with enoxaparin ameliorated DN and reduced podocyte injury in these mice. In vitro studies showed that the Led domain of TM limits glucose-induced complement activation on endothelial cells and podocytes [63].

Interestingly, therapeutic effects of gene therapy with adeno-associated virus (AAV)-delivered TM-Led domain were tested in db/db mice [64]. A single dose of AAV-TM-Led lowered albuminuria and reduced interstitial inflammation and glomerulosclerosis in db/db mice. These effects were associated with inhibition of the NF-κB-NLRP3-inflammasome-mediated inflammation and suppression of mitochondria-mediated apoptosis in the kidneys of treated mice [64].

VWF and ADAMTS13

Chronic hyperglycemia and oxidative stress-induced endothelial dysfunction in the context of DN are associated with accretion of ultralarge von Willebrand factor (ULVWF) multimers and thrombotic microangiopathy [65]. ULVWF multimers, which are stored in platelet α-granules and endothelial Weibel-Palade bodies, can be as large as 20,000 kDa and are very thrombogenic. Under normal circumstances, ULVWF multimers are immediately upon release cleaved by ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I repeats-13) into smaller VWF multimers, which are less active. ADAMTS13 is not only produced by liver cells and endothelium but also by podocytes [66, 67]. Several studies have described a correlation between plasma levels of ADAMTS13 and the severity of diabetic microangiopathic complications.

Taniguchi et al. measured the levels of VWF and ADAMTS13 in 86 type 2 diabetes patients and healthy control subjects [68]. They found that diabetics with DN had significantly lower levels of ADAMTS13 than controls. When the patients were divided into four groups according to eGFR and ADAMTS13 levels, the mean carotid intima-media thickness was significantly increased in patients with low ADAMTS13 levels in the same eGFR group, suggesting that reduced ADAMTS13 is associated with (micro)vascular disease [68].

Comparable findings were described by the international BErgamo NEphrologic DIabetes Complications Trial (BENEDICT) study group. It was found that in patients with diabetes, impaired ADAMTS13 activity, depending on different variants of the protease, accelerated renal disease [69]. In this study, 1163 normoalbuminuric type 2 diabetic patients were genotyped, and the Pro618Ala ADAMTS13 variants were determined. It was found that serum ADAMTS13

activity was significantly lower in Ala carriers than in Pro/Pro homozygotes. ADAMTS13 618Ala variant associated with less proteolytic activity and higher risk of renal and cardiovascular events and development of CKD [69]. From this, the authors concluded that screening for ADAMTS13 polymorphisms may help identify diabetes patients who may benefit from early reno- and cardioprotective therapy.

With respect to therapeutic potential of ADAMTS13 in the context of DN, Dhanesha et al. have described that ADAMTS13 slows down the progression of DN [70]. In this study, involving a model of STZ-induced diabetes, it was found that diabetic mice showed lower plasma ADAMTS13 activity and increased VWF levels, compared to nondiabetic controls. ADAMTS13−/− diabetic mice had increased albuminuria, creatinine and urea, mesangial ECM deposition, and more signs of intrarenal thrombosis than control mice. Deleting VWF in ADAMTS13−/− mice protected against development of DN, again suggesting a deleterious role for VWF in the pathogenesis of DN [70].

Anticoagulants in the Context of DN

Activated Protein C (APC)

APC is a natural anticoagulant, which provides feedback inhibition within the coagulation system (Fig. 17.1). The inactive zymogen protein C is efficiently activated by the thrombin-TM complex. The activation of PC by the TM-thrombin complex is approximately 20-fold augmented by the endothelial protein C receptor (EPCR) [71]. APC is not only a major anticoagulant but in addition has important anti-inflammatory and cytoprotective effects [72–74].

In mice with STZ-induce diabetes, it was found that the TM-dependent generation of APC in the renal microcirculation is decreased [75]**.** In a seminal paper Isermann et al. described that TM-dependent glomerular APC creation mediated cytoprotection in DN by inhibiting glucose-induced apoptosis in endothelial cells and podocytes [75]. They found that APC modulates glomerular cell apoptosis via PAR-1 and EPCR and that maintaining high circulating levels of APC protected mice against development of DN.

In a follow-up study, the same research group demonstrated how APC prevented glomerular oxidative stress and accumulation of the redox-regulating protein p66(Shc) in mice with DN [76]. These effects were most explicit in podocytes. In vitro, APC inhibited glucose-induced expression of p66(Shc) in podocytes via PAR-1- and PAR-3-mediated signaling, while this beneficial effect was not found in glomerular endothelial cells. Genetic deletion of p66(Shc) compensated for the observed loss of APC generation in mice with DN, normalizing renal damage markers and reducing oxidative stress. In addition, it was shown that APC controls the expression of p66(Shc) by epigenetic mechanisms, linking APC to mitochondrial function in DN [76].

Not only endogenous APC has a protective effect against the development of DN in mice but also administration of exogenous APC. Gil-Bernabe et al. have studied the effect of treatment with APC for 1 month in STZ-treated mice with DN [77]. It was found that APC-treated mice had a significantly improved renal function and lower levels of albuminuria and had significantly less renal fibrosis. Also, there were lower intrarenal levels of inflammatory and profibrotic cytokines and growth factors in APC-treated mice than in untreated animals. APC treatment resulted in less glomerular cell apoptosis and preserved expression of podocyte differentiation markers, such as podocin, WT-1, and nephrin [77].

The protective effect of APC against DN was found to depend on PAR-1- and EPCR-mediated signaling in mice. Lattenist et al. studied the expression levels of EPCR in 136 patients with diabetes and in kidney biopsies with features of DN [78]. EPCR activity can be regulated by proteolytic cleavage involving ADAM (a disintegrin and metalloprotease) types 10 and 17, yielding a soluble form of EPCR (sEPCR).

DN patients showed higher plasma and urinary levels of soluble (s)EPCR than diabetic controls without DN [78]. In kidney biopsies with DN, glomerular endothelial EPCR expression was markedly reduced in patients with DN, and this was associated with increased glomerular expression of proteases ADAM-17 and ADAM-10. In cell culture experiments involving human glomerular endothelial cells, it was shown that EPCR shedding was induced by high concentrations of glucose. This shedding was suppressed by ADAM-17 inhibition or silencing, which resulted in normalized expression of endothelial differentiation markers and reduced expression of markers of endothelial-mesenchymal transition (EndMT), such as TGF-β [78]. The authors concluded that ADAM-mediated shedding of EPCR contributes to development of DN, at least partly through induction of EndMT.

The net effect of PAR-1 in the context of DN was investigated by Waasdorp et al., who studied PAR-1-deficient mice in a model of STZ-induced DN [79]. They found that PAR-1−/− deficient mice were protected against development of DN, with lower albuminuria levels, less mesangial ECM deposition, and less tubular atrophy. Subsequent in vitro studies involving murine mesangial cells showed that hyperglycemic culture conditions resulted in an increased PAR-1 expression, accompanied by increased cell proliferation and expression of ECM proteins. In vivo, the PAR-1-deficient mice showed reduced mesangial cell proliferation and less glomerular fibronectin deposition in DN [79].

Protein S

Protein S (PS) is a natural anticoagulant factor that is best known for its cofactor activity of APC-mediated inactivation of FVa and FVIIIa (Fig. 17.1). Besides this role as anticoagulant, PS has been identified as a ligand for Tyro3, Axl, and Mer

(TAM) receptors [80, 81]. TAM receptors function as tyrosine kinase membrane receptors, activating cell proliferation and survival, cell adhesion. All three subtypes are expressed on macrophages, dendritic cells, and natural killer lymphocytes [82, 83]. PS induces multiple coagulation-independent effects via TAM receptor-dependent signaling. TAM receptors have been implicated in innate immunity, and elevated levels of circulating soluble TAM receptors (sTyro3, sAxl, sMer) are described in autoimmune disorders, such as lupus nephritis [84]. Ochodnický et al. investigated TAM and PS levels in the blood and urine of 126 patients with diabetes [85]. They found that diabetes patients with macroalbuminuria had higher circulating levels of sMer and more urinary sTyro3 and sMer than normoalbuminuric diabetics. In the same study, immunostainings for PS and TAM receptors were performed on kidney biopsy specimens from patients with DN and controls, which revealed that the increased clearance of sTyro3 and sMer was associated with loss of tubular Tyro3 and Mer expression in DN tissue. Also, biopsies with features of DN showed increased glomerular depositions of PS [85]. Next, TAM expression and shedding by TEC were investigated using human kidney cells in an in vitro diabetes model. It was found that under hyperglycemic conditions, human TEC had downregulation of Tyro3 and Mer mRNA and increased shedding of sTyro3 and sMer protein [85]. This study was the first to identify the kidney as a source of (s) TAM production and to implicate PS and possibly PS-TAM receptor interaction in DN. However, the authors could not establish an additional role for sTAM receptors as predictive biomarkers of renal functional decline.

Anticoagulant Drugs in DN

Most of the above described preclinical animal and laboratory studies seem to support the conclusion that coagulation factors and activation of coagulation promote or accelerate the development of chronic kidney disease in patients with diabetes, while administration of anticoagulants may protect against DN. On the other hand, evidence is presented that low but sustained thrombin generation has renoprotective effects in the context of diabetes. Unfortunately, patient studies which aim to dissect the net effects of coagulation and anticoagulants in DN are distinctly lacking.

Heparin has been promoted as a putative protective agent in DN, albeit not by virtue of its anticoagulant effects but due to its presumed ability to safeguard and restore the glycocalyx and heparan sulfate proteoglycans in the glomerular basement membrane (see also Chaps. 10 and 12 of this book) [86]. One Cochrane Review has addressed this issue, searching for relevant randomized controlled trials (RCTs) investigating the benefits and harms of heparin for preventing the onset of DN [87]. The authors of this review had to conclude that no studies met the inclusion criteria and that well-designed multicenter RCTs of heparin and related substances for preventing the onset of DN are still lacking [87]. Direct effects of other anticoagulants on renal diseases in general and DN in particular remain undefined

so far, but in view of the abundant use of all classes of anticoagulants worldwide, such effects may have significant clinical implications.

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Chapter 18
Renal Hemodynamics in Diabetic Kidney Disease: Relevance for Intervention

Marco van Londen, Niek Hessels, Annebelle Michielsen, Nicolien Kasper, and Gerjan Navis

Introduction

Diabetic kidney disease (DKD) is a major complication of diabetes and associated with a substantial burden of disease [1]. Its pathogenesis is multifactorial and driven by metabolic, inflammatory, and hemodynamic derangements. Hemodynamic factors have drawn interest already early on (1, reviewed 2) fueled by the typical biphasic course of renal function in diabetes with an early rise in glomerular filtration rate (GFR), typically accompanied by microalbuminuria, followed by progressive renal function decline later in the course of the disease, accompanied by macroalbuminuria, eventually leading to end-stage renal failure. The early rise in GFR is called hyperfiltration and is assumed to reflect glomerular hypertension [3]. The latter is assumed to be a main mechanism of progressive renal damage by intraglomerular hypertensive damage. In this concept, the glomerular damage leads to glomerular protein loss that elicits tubulointerstitial inflammatory damage, eventually leading to nephron loss and hence loss of renal function. The latter then can elicit a compensatory response in the remaining nephrons aimed at restoration of renal function, at the expense, however, of aggravation of intraglomerular hypertension, resulting in a vicious circle of progressive nephron loss.

This paradigm was developed in the last decades of the former century, mainly based on rat studies [4]. In rat strains with glomeruli located close to the kidney surface, the technique of renal micropuncture allows direct measurement of glomerular pressure and glomerular filtration rate at the single-nephron level. Studies in rat remnant kidney models firmly established the presence of glomerular hypertension and glomerular hyperfiltration in the remnant kidneys [5]. The pathogenetic role of the elevated glomerular pressure in these models was established by intervention studies with blockers of the renin-angiotensin-aldosterone system (RAAS)

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that decrease not only blood pressure but also glomerular pressure by specific effects on the glomerular microcirculation, i.e., efferent vasodilation. Thus, reduction of glomerular pressure was shown to exert marked renoprotective effects. These studies provided a rationale for the introduction of RAAS blockers as renoprotective agents [6]. In the decades that followed, the renoprotective properties of RAAS blockade in human chronic kidney disease were firmly established. Yet, direct evidence for a pathogenetic role of glomerular hyperfiltration in human kidney disease remained relatively sparse, as opposed to the overwhelming evidence that was obtained for proteinuria reduction as a renoprotective mechanism of RAAS blockade [7]. In fact, proteinuria and elevated glomerular pressure go often hand in hand, and the shift in emphasis in more recent studies is at least partly related to the fact that urinary protein loss can easily be measured in human, whereas measurement of glomerular hyperfiltration and in particular glomerular pressure is not.

Assessment of Glomerular Hemodynamics in Human

Lack of reliable and convenient assessment of the human glomerular microcirculation has been a substantial hurdle in substantiating (of refuting) the role for glomerular hypertension and hyperfiltration in human. In humans, glomerular hemodynamics has to be assessed by indirect methods. The feasible methods are based on clearance techniques that reflect GFR by endogenous (creatinine) and exogenous (inulin, iothalamate) [8] and, in dedicated settings, also effective renal plasma flow (ERPF) [9]. This poses substantial limitations on the assessment of glomerular hypertension and glomerular hyperfiltration, as the main (alleged) pathogenetic factor, glomerular pressure, is not measured at all. In dedicated settings, where ERPF is available, glomerular pressure is derived as the proportion of ERPF that is actually filtered as filtration fraction (FF) is GFR/ERPF. Of note, measurement of ERPF by clearance techniques assumes complete and stable tubular tracer extraction, and this assumption is not always met in diseased kidneys [10], rendering estimates of glomerular pressure unreliable, in particular in patients with CKD. Moreover, clearance data provide GFR and RPF for the sum of all functioning nephrons. Thus, once nephron loss has occurred, overall GFR may be normal due to hyperfiltration in the remaining nephrons. Accordingly, hyperfiltration can only be reliably established when the overall value of GFR is well above normal, very early in the course of the disease, and not in the phase of progressive nephron loss.

In clinical cohorts, GFR is usually assessed by creatinine-based methods, be it creatinine clearance from a 24-hour urine collection and a plasma sample or estimated GFR (eGFR) based on a plasma sample only. These estimates assume that renal excretion of creatinine is by filtration only and that tubular handling is negligible. Unfortunately, the accuracy of eGFR is poor in the higher range of GFR, making assessment of hyperfiltration in clinical cohorts difficult [11]. Finally, in subjects with high GFR, tubular secretion of creatinine is elevated. Accordingly,

when measuring a very high creatinine clearance, in the absence of assessment of measurement of a specific filtration marker, it goes unnoticed that a substantial part of the elevated creatinine clearance is due to elevated tubular secretion [12]. Taken together, these factors contribute to lack of reliable human data on glomerular hemodynamics, in particular during progressive renal function loss, and the elusive nature of hyperfiltration as a pathogenetic factor in progressive renal damage.

An exact definition of glomerular hyperfiltration has not been agreed upon, and accordingly, different cutoffs are used in the literature, i.e., ranging from GFR above 91 ml/min/1.73 m^2 to 175 ml/min/1.73 m^2, as reviewed recently [13]. Often hyperfiltration is defined as an increase of GFR over 130–140 ml/min/1.73m^2 or two standard deviations above normal in healthy individuals.

Clinical Determinants of Elevated Filtration

Renal hemodynamics are not static but display a diurnal pattern and respond to various triggers inherent to normal daily life in healthy individuals, such as posture, stress, and nutritional factors such as high protein intake and high sodium intake [3, 14]. In women, hormonal triggers exert pronounced effects on renal hemodynamics, as apparent from the changes over the menstrual cycle and from the effects of oral contraceptives. Prominent renal hemodynamic changes occur during pregnancy, with increases in ERPF as well as GFR denoted as hyperfiltration of pregnancy and considered as an adaptive response. After delivery, renal hemodynamics return to their baseline values [15].

Renal hemodynamics are consistently associated with body composition, with a higher FF in subjects with a higher body mass index (BMI), without a lower threshold for BMI [16]. At BMI values in the overweight or obese range, GFR is generally elevated: this is loosely denoted as obesity hyperfiltration and considered to contribute to the increased renal risk that is associated with overweight and particularly obesity [17]. Obesity hyperfiltration is reversible, albeit not fully, after weight loss by bariatric surgery and is partly responsive to RAAS blockade [18]. Body fat distribution is even more strongly associated with renal hemodynamics than with BMI, with a higher FF in subjects with a central body fat distribution [19]. Taken together, in healthy subjects, the highest GFR and FF are found in subjects with higher BMI and a central body fat distribution (Fig. 18.1). Of note, this pattern matches with epidemiological data on the correlates of albuminuria and long-term renal risk, respectively, with a more prominent risk related to body fat distribution than BMI per se [20, 21].

After nephron loss, as clearly illustrated by uninephrectomy, adaptive hyperfiltration occurs in the remaining kidney as a compensatory response to maintain overall GFR. In healthy kidney donors, this adaptive response has been well-documented during living kidney donor follow-up. On average in healthy, middle-aged donors, single-kidney GFR amounts to some 66% of prior two-kidney GFR [22], without excess risk for long-term renal damage [23]. The generally favorable long-term

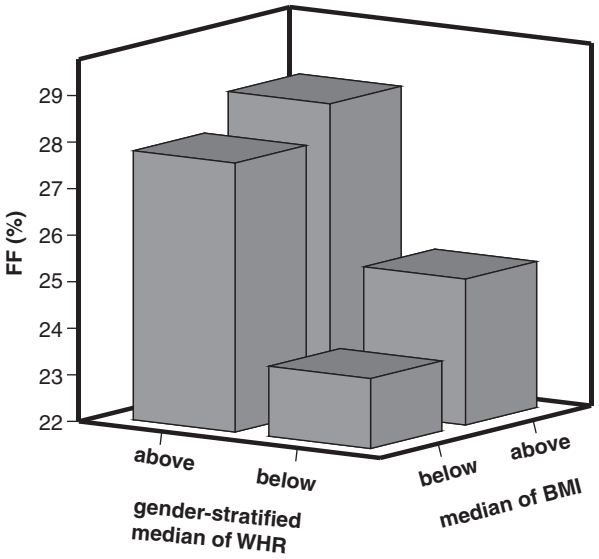

Fig. 18.1 Filtration fraction in healthy subjects by body fat distribution (waist-hip ratio, WHR) and body mass index (BMI). (From Kwakernaak et al. [19])

course of renal function in healthy kidney donors illustrates that even substantial hyperfiltration need not necessarily translate into an increased renal risk, provided it occurs in otherwise healthy individuals, hence the concept of adaptive hyperfiltration. Recent long-term follow-up data in kidney donors, showing a distinctly elevated albeit low absolute risk for long-term renal damage in kidney donors, may well reflect the liberalization of donor selection criteria and indicate that adaptive hyperfiltration is not merely innocent but can be a trigger to damage in (subclinically) compromised kidneys [24].

Elevated Filtration in Diabetes

Elevated GFR is reported to occur in early stages of type I diabetes (T1DM) as well as T2DM. Prevalence varies greatly, as reviewed recently [2], ranging from 10% to 67% in T1DM and 6% to 73% in T2DM. The wide variation may partly be due to the differences in assessment and definition, but more important is the notion that elevated filtration is not a fixed phenomenon but related to the severity of metabolic derangement, i.e., glycemic regulation, as well as concomitant derangements in volume status with corresponding hypertension. Elevated filtration is typically present in patients with poor glycemic regulation and expanded extracellular volume and responds to better glycemic regulation and correction of blood pressure and volume status [25, 26]. Whereas the underlying mechanism of elevated filtration is multifactorial, its reversibility illustrates that functional, hemodynamic alterations are important. Yet, functional mechanisms cannot be well dissected from structural alterations. Elevated filtration is associated with increased kidney and nephron size,

including glomerular as well as tubular hypertrophy, in diabetic as well as nondiabetic subjects. In healthy kidney donors, elevated single-nephron filtration rate was associated with larger nephrons as well as glomerulosclerosis, supporting an association with (propensity to) renal damage [27]. The changes in diabetes are attributed to the response of various growth factors to hyperglycemia, but other factors have been implicated as well. In particular, tubular hypertrophy has been linked to the increased proximal glucose and sodium reabsorption that characteristically occurs in conditions of elevated filtration [28]. Cause and effect relationships have not been well dissected, however, as elevated filtration and elevated tubular reabsorption can mutually influence each other. Increased proximal tubular reuptake of glucose and sodium is assumed to elicit hyperfiltration by a combination of intrarenal and systemic effects, namely, inhibition of tubuloglomerular feedback due to reduced distal tubular delivery of sodium, and consequent afferent vasodilation, combined with expansion of the extracellular volume and systemic hypertension, that can translate into glomerular capillary hypertension and hyperfiltration by the afferent vasodilation, in combination with elevated efferent vasomotor tone due to concomitant neurohumoral activation. Thus, multiple metabolic and vascular pathways are involved, as reviewed in detail elsewhere [2, 3, 26]. From a point of view of integrative physiology, it is relevant to note that elevated filtration apparently is due to concerted effects of dysregulated glucose metabolism/handling and deranged sodium and volume regulation.

Role of Elevated Filtration in the Pathogenesis of Progressive Renal Damage

As noted above, human evidence on a pathogenetic role of hyperfiltration in progressive renal damage is mainly indirect**.** Observational data support the notion that elevated glomerular filtration is followed by more rapid renal function decline [29, 30], but the effects of the elevated filtration per se are difficult to dissect from those of the concomitantly worse metabolic regulation [30]. As to the predictive effect of elevated FF for long-term renal function, only a single study is available that combines dedicated renal hemodynamic measurements in a large population with long-term outcome data [31]. In this study in renal transplant recipients, FF was higher in subjects with diabetes and in those with higher BMI; a higher FF was independently associated with worse renal outcome and graft loss, even after adjustment for confounders including GFR and proteinuria. At first sight, this could be a strong case for a pathogenic role of hyperfiltration in progressive renal damage. However, in this population, FF was similarly predictive for overall outcome and patient mortality, suggesting that FF is also a marker of an unfavorable overall risk profile, e.g., due to increased neurohumoral activation or metabolic factors, which might be involved in the worse renal outcome. The latter assumption is in line with epidemiological data showing an association of elevated filtration with elevated overall and cardiovascular risk [32, 33].

Another line of evidence derives from analyses of the early renal response to intervention as a predictor of long-term renoprotection. It is a consistent observation that an early decrease in glomerular filtration rate at onset of therapy – assumed to reflect alleviation of glomerular hyperfiltration – predicts a more favorable course of long-term renal function, in both diabetic [34, 35] and nondiabetic nephropathy [36], for different interventions, including protein restriction as well as pharmacological interventions with diverse mechanisms of action. The consistency of these data across different interventions supports a role of glomerular hyperfiltration in progressive renal function loss, in diabetic as well as nondiabetic renal disease. However, it must be noted that an early response of filtration rate to intervention can also be a reflection of renal responsiveness to therapy as such, rather than a reflection of a specific mechanism, so even after all these years, the jury is still out.

Role of Renal Hemodynamics in Renoprotective Intervention

Amelioration of hyperfiltration as a mechanism of renoprotection is currently going through a revival of interest. It fueled the introduction of RAAS blockers as renoprotective agents in the 1980s and 1990s of the former century and provided a rationale for dietary protein restriction as renoprotective intervention, as tested in the MDRD study in those days [37]. However, interest waned, due to practical barriers such as the impracticalities of reliably measuring human renal hemodynamics and to the difficulties in successfully and safely implementing long-term dietary protein restriction. Recent developments refueled interest in hyperfiltration as a target for intervention, be it pharmacological or dietary, as recently reviewed in detail elsewhere. In particular, the introduction of the SGLT2 inhibitors, acting by inhibition of tubular glucose and sodium reabsorption, provides a paradigm for reversal of the mechanisms underlying hyperfiltration [38]. Indeed, recent clinical data indicate an early reduction of glomerular filtration rate at onset of treatment, followed by a more favorable course of renal function and reduction of renal end points at 48 months [35]. The renal response to treatment is associated with weight loss, blood pressure reduction, and a decrease of volume markers such as atrial natriuretic peptide. The reduction in GFR is reversible after withdrawal, supporting its functional nature [39]. This renal function response pattern is strikingly similar to the response to RAAS blockade, as well as protein restriction (reviewed by 2). Other long-term studies are still under way. Whereas empirical long-term studies will have to prove whether this will provide real progress in terms of patient outcome, the dual action on glucose handling and volume status provides a theoretical and hopefully also clinical advantage over earlier modes of amelioration of hyperfiltration.

Interaction with Sodium Status: Time to Quit from the Sodium Paradox

As noted above, elevated filtration is associated with dysregulated glucose metabolism as well as deranged sodium and volume regulation. Whereas the role of metabolic dysregulation in hyperfiltration is firmly established, the role of sodium and volume status has remained somewhat contradictory. Whereas there is evidence for an association between volume overload and hyperfiltration in general, the findings of the so-called sodium paradox have substantially curbed enthusiasm for sodium restriction as an intervention for hyperfiltration. The sodium paradox refers to the phenomenon that, in rat models and in uncomplicated T1DM, stringent sodium restriction results in a rise in filtration fraction and/or GFR, allegedly due to a tubuloglomerular response to increased proximal reabsorption and reduced distal delivery of sodium [28]. Yet, it should be emphasized that these observations, albeit consistently and reliably reproduced in rat models and in uncomplicated T1DM [40], have never been reproduced in the major clinically relevant settings of today, namely, in T2DM, in the presence of renal damage, and against a background of RAAS blockade, the first-line therapy for these patients. In fact, we have refuted the presence of the sodium paradox in a sodium intervention study in T2DM with nephropathy, convincingly showing that a modest dietary sodium restriction in patients on RAAS blockade reduced blood pressure and albuminuria, along with a reversible reduction in creatinine clearance (Fig. 18.2, [41]). Corresponding effects were elicited by diuretic treatment, with a more pronounced effect by the combination of sodium restriction and diuretic. In none of these settings, the volume intervention was associated with a rise in creatinine clearance that would have occurred in case the sodium paradox paradigm would have been valid in this clinical setting. These findings

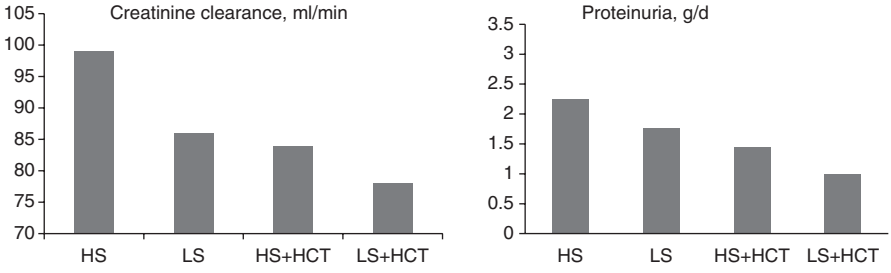

Fig. 18.2 Creatinine clearance (left panel) and proteinuria (right panel) during high sodium intake (HS) and sodium restriction (low sodium, LS) and their combination with hydrochlorothiazide (HCT) in patients with T2DM and CKD on RAAS blockade treatment. The data show that creatinine clearance is reduced in response to sodium restriction and/or diuretic, so the sodium paradox does not apply to this clinical setting. (Data from Kwakernaak, [41])

provide conclusive evidence against the relevance of the sodium paradox in the clinical setting that reflects today's burden of disease, namely, T2DM with CKD on renoprotective therapy. Accordingly, these data illustrate that sodium restriction is not to be avoided in T2DM with CKD because of fear of the sodium paradox, but, to the contrary, moderate sodium restriction should be part of the renoprotective intervention.

Interaction with Sodium Status: A Case for Early Intervention

Presentation of T2DM generally follows a long period of milder metabolic derangement, with overweight, obesity, and/or metabolic syndrome, and current preventive measures are aimed at targeting overweight and promoting a healthy lifestyle, as successful early intervention can reverse metabolic syndrome and hopefully prevent or postpone diabetes. Hyperfiltration can be considered to be part of the early phenotype of metabolic derangement [42]. Interestingly, lifestyle intervention data demonstrate favorable effects on renal hemodynamics as well, supporting the notion that early preventive measures aimed at prevention of diabetes and its cardiovascular complications have direct, favorable effects on the kidney as well, even before the development of overt renal damage. Correction of overweight/obesity by diet or bariatric surgery, as reviewed elsewhere, ameliorates hyperfiltration and reduces FF [18]. Interestingly, correction of overweight in hypertensive adolescents not only reduces blood pressure but also ameliorates sodium sensitivity [43]. The latter might well be related to weight loss-induced reduction of FF and the consequently altered peritubular Starling forces. Whereas such mechanistic data were not obtained, this classical and landmark study beautifully illustrates the deleterious interaction between overweight and volume overload, in line with epidemiological data showing added effects of overweight/obesity and high sodium intake on long-term outcome [44]. Of note, a renal hyperfiltration pattern can be elicited by liberal sodium intake even in young healthy subjects with very subtle overweight (Fig. 18.3, [45]). BMI appears to be a strong determinant of the responses of renal hemodynamics and extracellular volume (ECV) to a liberal sodium diet, to the extent that mildly overweight young men have a significantly higher ECV than their lean counterparts, associated with a renal hyperfiltration pattern (Fig. 18.3, [46]). Given the fact that BMI tends to track over the lifetime and that sodium intake in day-to-day life tends to be liberal, these data could implicate that by the time overt overweight or obesity has developed at middle age, the kidney has been exposed to mild hyperfiltration for more than two decades already. So far, we have no data on the clinical significance of these BMI-associated differences in renal hemodynamics and volume status, but it is remarkable that overweight phenotype of hyperfiltration and volume expansion can be fully corrected by dietary sodium restriction. This response pattern bears a striking similarity to the hyperfiltration response to high sodium in middle-aged hypertensives [47, 48], where it is associated with albuminuria, and it seems

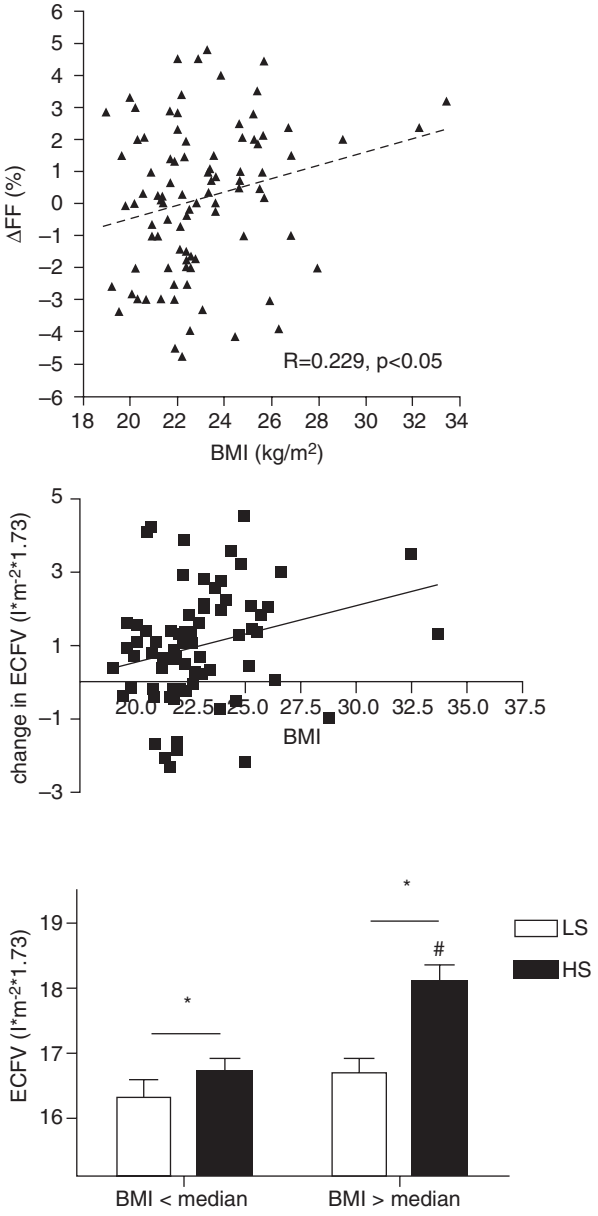

Fig. 18.3 Response of renal hemodynamics (filtration fraction (FF, upper panel, from Krikken et al., [45]) and extracellular volume (ECV, middle panel)) to a change in sodium intake from 50 to 200 mmol/day in healthy young men. The lower panel shows the resulting significant difference in ECV between lean (BMI < median) and mildly overweight (BMI > median) young men on high sodium (HS) only. (From Visser et al., [46])

logical to hypothesize that these phenotypes reflect early and later variants of the development of lifestyle-related renal damage over the life course. Whereas this assumption remains to be proven, these data strongly support to include focus on avoiding sodium excess in programs aimed at prevention of diabetes and its complications.

Conclusions

Glomerular hypertension and hyperfiltration are likely to contribute to progressive renal damage in diabetes. It is driven by intertwined effects of deranged glycemia and deranged sodium and volume status and provides a target for intervention by pharmacological and non-pharmacological measures, throughout the course of development of diabetes and its complications.

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Chapter 19
Microvascular Complications in the Eye: Diabetic Retinopathy

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Introduction

Diabetic retinopathy (DR) is a major cause of vision loss and blindness among persons with diabetes mellitus. It is estimated that approximately 35% of diabetes patients develop some form of DR [1]. DR is a progressive disease that is predominantly characterized by alterations in the retinal microvasculature. It may develop from an asymptomatic nonproliferative form associated with capillary nonperfusion, microaneurysms, and retinal hemorrhages, into a vision-threating disorder such as diabetic macular edema (DME) and proliferative DR (PDR).

Although DR and diabetic nephropathy (DN) are diseases that manifest themselves in different organs, the two diseases are strongly correlated as partly overlapping mechanisms are involved in the pathobiology of DR and DN, in particular

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microvascular alterations [2]. Compared to other tissues, the retina is highly vulnerable for the hyperglycemic milieu induced by diabetes, which is often attributed to the fact that retinal cells are not dependent on insulin for glucose uptake and to the unique anatomy and physiology of the eye. For instance, the density of blood vessels is low in the retina to prevent absorption of light. Yet, the retina has high metabolic demands, in particular in the dark-adapted state [3], which results in physiological retinal hypoxia [4]. As such, the retina has a limited capacity to adapt to metabolic stress, which may underlie its vulnerability to diabetes [3, 5]. It is often stated that DR precedes the development of DN in diabetic patients [6]. However, not all patients with advanced DR develop DN, underscoring that the pathobiology of both diseases are also different in a number of aspects [7].

A meta-analysis of patients with type 2 diabetes and renal disease indicated that DR can be used as a predictive biomarker to distinguish DN from nondiabetic renal disease [8]. In line with this observation, the identification of DR in diabetic patients with microalbuminuria can confirm the diagnosis of DN [9]. Traditionally, renal biopsies are required for the diagnoses of DN, but the potential to indirectly monitor DN by studying the retina via noninvasive ophthalmology techniques opens up the possibility for early detection of DN and may considerably improve the outcome for patients [2].

In this chapter, we discuss the clinical manifestation and disease progression of DR, focus on the main molecular and cellular mechanisms involved, and conclude by highlighting the current treatment options for DR.

Disease Progression of DR

DR develops gradually in patients with diabetes. The prevalence of DR increases with the duration of diabetes. After 20 years of diabetes, almost 80% of patients have some form of DR [1]. In the clinic, DR is broadly divided into two stages: nonproliferative DR (NPDR) and proliferative DR (PDR) (Fig. 19.1). However, DR is preceded by a long preclinical phase, which is associated with the development of several microvascular and other anomalies in the retina. It is likely that diabetes affects all cell types of the retina, but the major retinal vascular changes can be easily imaged, and therefore grading of the disease is based on the severity of the vascular anomalies [10]. Recent advances in imaging techniques also enable the detection of more subtle alterations such as neurodegeneration, altered distribution of cone photoreceptors and anomalies in the thickness of the neural retina [11–14].

The first anomalies that arise in the retina during the preclinical stage are thickening of the vascular lamina basalis, pericyte loss, increased vascular permeability, and formation of acellular capillaries. Progression of the disease into NPDR is recognized by the appearance of microaneurysms and hemorrhages, both associated with areas of capillary regression, vascular leakage, and hard exudates and spreading areas of capillary non-perfusion. When the disease pro-

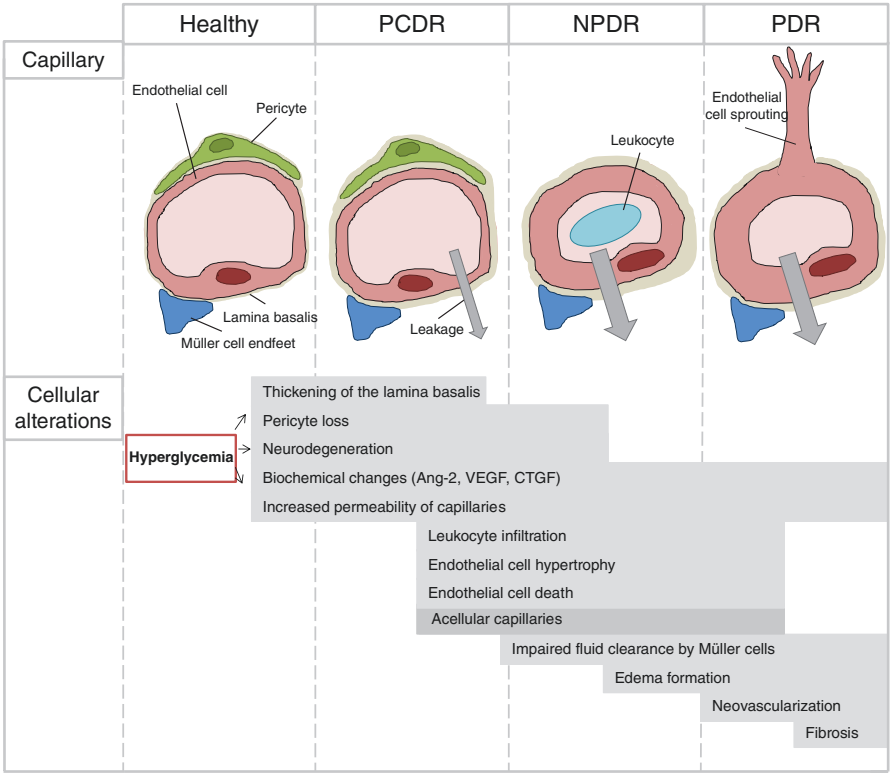

Fig. 19.1 Schematic overview of the microvascular changes occurring in retinal capillaries of diabetic patients. Hyperglycemia induces various molecular and cellular alterations ultimately leading to endothelial dysfunction, degeneration of retinal capillaries, and retinal ischemia. Before clinically relevant stages of diabetic retinopathy (DR) are manifested, thickening of the vascular lamina basalis, pericyte loss, neurodegeneration, upregulation of levels of growth factors, and increased vascular leakage occur. These processes lead to the induction of a pro-inflammatory microenvironment that is accompanied by increased leukocyte infiltration, endothelial cell hypertrophy causing narrowing of the capillary lumen and endothelial cell death, and subsequently formation of acellular, non-perfused capillaries. Nonproliferative DR is accompanied by further dysfunction of the retinal capillaries, increased leakage of capillaries and impaired fluid clearance from the retinal tissue to the circulation by Müller cells, ultimately leading to edema formation. In the more advanced stages, retinal ischemia may induce neovascularization, which can be accompanied by scarring and blindness. PCDR, preclinical DR; NPDR, nonproliferative DR; PDR, proliferative DR; Ang-2, angiopoietin-2; VEGF, vascular endothelial growth factor; CTGF, connective tissue growth factor

gresses to an even more advanced stadium, with widespread areas of retinal nonperfusion and ischemia, neovascularization can develop, which is the characteristic hallmark of PDR. PDR occurs more frequently in patients with type 1 diabetes [1]. PDR may lead to vitreous hemorrhage, fibrosis via the angio-fibrotic switch, fibrovascular membrane contraction, retinal detachment, and eventually blindness [15]. NPDR generally develops as a consequence of vascular damage caused

by hyperglycemia, whereas PDR develops as a direct result of retinal ischemia induced by capillary non-perfusion and is therefore not directly affected by metabolic control [16].

An important additional manifestation of DR is DME, which can occur in combination with NPDR and PDR. Blood vessels of the retina are highly selective in regulating the entry of molecules into the retinal tissue, comparable to vessels of the blood-brain barrier. DME is caused by the breakdown of these inner blood-retinal barrier (BRB) properties, which leads to leakage of fluid and plasma proteins from the vasculature into the neural retina and ultimately to edema formation [17]. DME in the central area of the retina, the macula, often leads to severe loss of visual acuity. DME is the most prevalent disease manifestation in type 2 diabetes and therefore represents the most common cause of vision loss in patients with diabetes [1].

Early Stages of DR

Hyperglycemia is a major factor that triggers the development of DR. It elicits the activation of molecular and cellular mechanisms suggested to be involved in the disease progression such as metabolic damage, inflammation, upregulation of levels of growth factors, and neurodegeneration. All these factors may induce damage to the retinal blood vessels and may eventually lead to vessel degeneration and formation of acellular, non-perfused capillaries. This process, which is also known as vasoregression, leads to the formation of an ischemic retina, and provides the basis for the formation of NPDR, DME, and PDR.

Metabolic Damage

High plasma levels of glucose lead to vascular damage due to the induction of oxidative stress, caused by mitochondrial overproduction of reactive oxygen species (ROS) [18]. Hyperglycemia also leads to endothelial dysfunction via activation of the polyol pathway, the formation of advanced glycation end products (AGEs), activation of protein kinase C (PKC) isoforms, and an increased flux through the hexosamine pathway [18]. These four pathways all lead to the production of ROS which are thought to be the cause of endothelial cell dysfunction and cell death. This subsequently leads to the vascular abnormalities observed during preclinical DR. For instance, increased levels of glucose activate the polyol pathway provides an alternative form of glucose metabolism in which glucose is converted to fructose [19]. However, this occurs at the expense of NADPH and NAD^+, which are important cofactors involved in redox reactions, making cells more sensitive for oxidative stress, as NADPH is the major substrate for the detoxification reactions of ROS [20]. Activation of the PKC isoforms leads to endothelial

cell apoptosis and the formation of acellular capillaries, whereas it also induces the expression of growth factors such as vascular endothelial growth factor (VEGF) [21, 22]. AGEs play a role in lamina basalis thickening by increasing the expression of proteins involved in synthesis of the extracellular matrix such as connective tissue growth factor (CTGF), but also induce the expression of other factors involved in the disease progression such as angiopoietin-2 (Ang-2) [23–25]. Activation of the hexosamine pathway, in which fructose 6-phosphate is converted into *N*-acetyl glucosamine, is associated with neuronal apoptosis [26]. Even when normal glucose levels are obtained in patients, progression of DR continues which suggests the existence of the phenomenon known as "metabolic memory" [27]. Oxidative stress plays an important role in the establishment of this metabolic memory, probably via modulating alterations in the epigenetic landscape [27, 28].

Inflammation

Several inflammatory mechanisms are considered to be involved in the formation of microvascular complications during the early stages of DR, which include, among others, activity of the pro-inflammatory transcription factor NF-κB, pro-inflammatory cytokines such as IL-1β and TNFα, and the intracellular adhesion molecule-1 (ICAM-1) [13, 29]. These inflammatory mediators may play a central role in the degeneration of capillaries, pericyte loss, vascular permeability, and neurodegeneration [13, 29]. Especially increased leukostasis, induced by cytokines and VEGF, has been suggested to cause the formation of early vascular lesions in the diabetic retina, via endothelial damage by FAS-FAS-L interactions, and progressive vascular occlusions and subsequent development of areas of non-perfusion [30, 31]. However, others have suggested that leukostasis is only an epiphenomenon of the retinal diabetic milieu and cannot explain by itself the pathogenesis of DR [32, 33].

Growth Factors

Several growth factors have been associated with the development of early vascular lesions in the diabetic retina, including Ang-2, VEGF, and CTGF.

Angiopoietin-2 (Ang-2) The angiopoietin (Ang)-Tie system plays an important role in maintaining vascular stability [25]. Ang-1 is secreted by perivascular cells and activates the tyrosine kinase receptor Tie2 on endothelial cells, thereby inducing increased cell survival, endothelial barrier function and vessel stabilization. In contrast, Ang-2 inactivates Tie2, leading to endothelial cell degeneration and vessel destabilization. It is thought that Ang-2 plays a key role in initiating vasoregression in preclinical DR [25]. Hyperglycemia induces Ang-2 expression, altering the balance between the two ligands and favoring the inhibitory effects of Ang-2 on the

Tie2 receptor [34]. High levels of Ang-2 lead to pericyte loss, which is one of the first morphological change observed in the diabetic eye [35]. In addition, high levels of Ang-2 lead to endothelial cell death and formation of acellular capillaries [25].

Vascular Endothelial Growth Factor (VEGF) VEGF is an important inducer of vascular permeability and the major pro-angiogenic factor [36, 37]. These functions are mainly mediated via the VEGF family member known as VEGF-A. In this chapter, we refer to this family member when discussing the functions of VEGF. The role of VEGF in the development of the advanced stages of DR such as PDR and DME is discussed below in detail. However, VEGF also plays an important role in the preclinical stage of DR. Hypoxia is a major inducer of VEGF expression, but hyperglycemia can upregulate VEGF expression as well [3]. VEGF induces vascular permeability, increases the adhesion of leukocytes to the vasculature, and translocates the insulin-independent glucose transporter-1 (Glut-1) from intracellular stores to the membrane which may further promote hyperglycemia-induced damage in the early diabetic retina [38, 39]. VEGF over-expression in the diabetic retina may also induce endothelial cell hypertrophy, leading to narrowing of the capillary lumen and thereby eventually be the cause of capillary non-perfusion [32]. This may even incite a vicious circle of disease progression, creating areas of local ischemia, followed by more endogenous VEGF production and further luminal narrowing. This vicious circle, which is an alternative or complementary mechanism to the paradigm of VEGF-induced leukostasis as the cause of capillary occlusion, could explain why vascular lesions spread from focal points in the diabetic retina, whereas other areas remain totally unaffected [31, 32].

Connective Tissue Growth Factor (CTGF) CTGF is a growth factor that regulates the expression of several other growth factors and extracellular matrix (ECM) proteins [40]. As a consequence, it is involved in a wide range of biological processes such as production of ECM components, angiogenesis, wound healing, and fibrosis [40]. Thickening of the lamina basalis of retinal capillaries is one of the first pathologically visible change in early diabetes, preceding the loss of pericytes [41]. CTGF appears to play a key role in this process [42, 43]. In preclinical DR, CTGF expression is upregulated by AGEs and VEGF [23, 43]. Besides the role of CTGF in lamina basalis thickening, recent findings suggest that CTGF also plays a role in inducing pericyte loss and the formation of acellular capillaries in the early stages of DR [44].

Neurodegeneration

Retinal vascular cells are closely associated with neurons and glial cells in the so-called neurovascular unit, which critically regulate their function. Before the onset of the typical microvascular lesions of NPDR, anomalies in the neuronal structure, and function of the retina can be detected [45]. For instance, in a longitudinal study

it was shown that thinning of the retinal neural layers precedes the development of microvascular changes [46]. Moreover, altered neuronal function is observed in the retina, and, as consequence, contrast sensitivity and dark adaptation is reduced in the early stages of DR [45]. The causal relationship between early neurodegeneration and vasoregression or the late vascular pathologies in DR remains unclear [46].

Ischemic Retinopathy

The ischemic milieu of the retina in NPDR induces the production of VEGF and other growth factors. High VEGF levels play a central role in the pathobiology of both DME and PDR.

Diabetic Macular Edema (DME)

Breakdown of the Blood-Retinal Barrier (BRB) Integrity of the BRB is essential for vision. Without a proper BRB, plasma proteins leak into the retinal tissue, leading to accumulation of proteins and fluid in the macula and other features of DME. In general, there are two main routes for molecules to cross the endothelial monolayer, that is, 1) via opening of the junctions between endothelial cells or 2) through the endothelial cell cytoplasm via vesicles or specific transporters. These pathways are known as the paracellular pathway and transcellular pathway, respectively. Small molecules and solutes can diffuse back and forth across the endothelium via the paracellular route, whereas molecules larger than 3 nm in radius cannot pass the BRB paracellularly and use the transcellular route. As described by the rules of Starling, the concentration of macromolecules is an important determinant of the interstitial osmotic pressure. Increased transport of macromolecules via vesicle-mediated transcytosis therefore plays an important role in the pathobiology of DME [17]. VEGF appears to be a major regulator of this process. VEGF has been shown to increase the number of caveolar vesicles in human retinal explants [47]. In addition, intraocular VEGF injections in monkey eyes shifted the distribution of vesicles from an abluminal localization to a luminal localization, without obviously altering the junctional integrity between cells [48]. This shift in distribution may reflect an altered direction of vesicular transcytosis, occurring from blood to tissue [48]. Thus, especially active transcytosis of plasma proteins via vesicles may alter the interstitial osmotic pressure, which in turn draws fluid from the leaky vessels into the retinal tissue causing DME. However, it should be noted that generally much more emphasis is put on the role of paracellular permeability in microvascular permeability and BRB breakdown in eye disease. Accordingly, disruption of the junctional interactions between retinal endothelial cells is most widely recognized as important causative factor in BRB breakdown and DME formation [17].

Inflammation The low-grade inflammatory milieu of the diabetic eye promotes breakdown of the BRB and further vascular leakage, in addition or as an alternative pathway to VEGF [49]. Pro-inflammatory mediators induce BRB breakdown primarily via the paracellular pathway. For instance, the pro-inflammatory cytokine TNFα alters the expression of several junctional proteins and increases the permeability for small molecules but not for large molecules [50]. However, the precise role of inflammation in the pathogenesis of DME remains still a manner of debate [33].

Impaired Fluid Homeostasis Besides increased protein and fluid extravasation in edema formation, impaired fluid reabsorption from the retinal tissue to the circulation also plays a role in edema formation. A correct balance between fluid extravasation and clearance is especially important in the retina, since the retina does not have a lymphatic system to remove excess fluid from the interstitium [51]. Müller cells, which are the glial cells of the retina, play an alternative role in this process. Müller cells regulate via facilitating transcellular water transport via water channels termed aquaporins (AQPs) [52]. The transport of water by these channels is tightly coupled to the potassium current in cells. In the diabetic retina, expression of the potassium channel Kir4.1 is downregulated, resulting in the accumulation of potassium in cells, increased water influx via AQP4, and swelling of Müller cells [52]. Moreover, altered expression of the AQPs subtypes in Müller cells of the diabetic retina may also impair fluid homeostasis, leading to cellular swelling [53, 54]. Thus, altered fluid clearance in the diabetic retina may contribute to both intracellular and extracellular edema and DME.

Proliferative Diabetic Retinopathy (PDR)

There is evidence that retinal ischemia in NPDR leads to a vicious circle of disease worsening by VEGF-induced leukostasis and/or endothelial hypertrophy [31, 32], and this may eventually lead to PDR, which only occurs when widespread areas of capillary non-perfusion have developed. In a response to counteract retinal ischemia, a pro-angiogenic response is initiated leading to retinal neovascularization and the development of PDR. However, these newly forming vessels are leaky, fragile, and prone to rupture, leading to hemorrhages in the vitreous. In addition, the newly formed vessels are the visible component of a coexisting wound-healing response, which develops into fibrous tissue formation and scarring and may cause retinal detachment and eventually total blindness.

Compared to the VEGF levels detected in the eyes of patients with NPDR, the mean concentration of VEGF in the vitreous of patients with active PDR are approximately 35 times higher [55]. However, it is important to note that high levels of VEGF are not sufficient to lead to neovascularization. Altered expression of its receptors in retinal vessels is also involved. In fact, one study showed that repeated high doses of intraocular VEGF injections did not induce retinal neovascularization in monkey eyes [32, 56]. In contrast, prominent neovascularization was detected in the iris [56]. This observation was explained by the notion that retinal capillaries express only

VEGFR1 in pericytes or on the abluminal side of endothelial cells, under normal physiological conditions, whereas the iris vessels constitutively express VEGFR2 [56, 57]. VEGFR1 has often been described to function only as a regulatory decoy receptor for the more important receptor VEGFR2 that mediates the angiogenic effects of VEGF. Thus, dysregulation of VEGFR signaling is necessary in the retina to facilitate neovascularization, but not in the iris. This may explain why retinal neovascularization only occurs in the advanced stages of DR, whereas increased VEGF levels are already observed in the preclinical phase of DR. In diabetic patients with established DR, altered expression of VEGFRs in the retina can be observed, showing prominent vascular expression of VEGFR1, VEGFR2, and VEGFR3 [57].

The induction of fibrosis in the fibrovascular membranes of PDR is dependent on an critical balance between VEGF and the pro-fibrotic growth factor CTGF [15, 40]. VEGF itself promotes CTGF expression. When the equilibrium between these factors reaches a certain threshold, fibrosis may overrule, initiating the angio-fibrotic switch [15].

Therapeutic Options

DR is a complex disease and many factors are involved. Hyperglycemia is the main underlying factor leading to vascular damage. Therefore, metabolic control is important for the management of DR. However, due to the phenomenon known as metabolic memory, and by the independent effects of established retinal capillary non-perfusion and ischemia, proper glycemic control alone is not effective to reduce the prevalence of DR or to treat DR when vision-threatening stages have developed [10, 58]. Other risk factors for DR include hypertension and hyperlipidemia, and management of these factors is relevant for DR, in particular for the clinical phases such as DME. Besides these general measures, there are only a few therapeutic options available for DR at the present.

Available treatments for DME are laser photocoagulation, anti-VEGF therapy, and corticosteroids. Focal and grid laser photocoagulation has been the standard care for DME for decades, but anti-VEGF agents have been shown to be superior [59]. Anti-VEGF therapy requires regular injections for 2 or more years and is associated with suboptimal responses in some patients. In addition, there are concerns that VEGF has important neuroprotective functions in the retina, suggesting the need for alternative treatment options [60]. Anti-VEGF treatment of DME has nevertheless led to a major improvement in treatment outcome in DME and has been shown not only to have a direct effect on macular edema but also a more long-term inhibitory effect on the progression of the underlying NPDR [61, 62]. The latter effect seems to underscore the presumed role of VEGF in the pathogenesis of the diabetes-induced damage leading to vasoregression and eventually NPDR.

Glucocorticoids have been shown to reduce DME, which generally is explained by their targeting of the inflammatory part of the pathogenesis and fluid clearance by Müller cells [52, 63]. However, glucocorticoids may have a direct restorative effect on the BRB [64, 65], providing an alternative explanation for their effectivity

in DME. Nevertheless, corticosteroids are associated with serious side effects such as cataract formation and glaucoma due to increased intraocular pressure [66, 67]. Therefore, treatment with these agents is only advisable in patients that do not respond to anti-VEGF therapy.

Patients with PDR are treated with panretinal laser photocoagulation and vitreoretinal surgery. During panretinal laser photocoagulation, focal thermal burns are used to destroy retina pigment epithelial cells and the overlying photoreceptors. Although this procedure sounds counterintuitive as a treatment option, it is significantly effective in reducing PDR, probably by downregulation of VEGF production induced by the reduction in oxygen consumption of the retina [68]. Vitreoretinal surgery may be necessary in later stages of PDR, when severe complications have been developed in the retina such as vitreous hemorrhage, tractional retinal detachment, and epimacular fibrovascular proliferations [10]. Recently, anti-VEGF therapy has also been shown to be a promising alternative treatment option for PDR targeting the pro-angiogenic phenotype of the retina [69]. However, targeting VEGF in PDR patients with advanced fibrovascular proliferations carries a risk of acceleration of the angio-fibrotic switch, inducing retinal fibrosis, contraction, and possibly retinal detachment [15, 70].

Screening

DR gradually progresses from an asymptomatic manifestation into a more advanced disease associated with vision loss. By the time clinical symptoms occur, several irrevocable cellular and molecular changes in the retina have already occurred. Although various treatment options exist for patients with DR, the currently available therapies are generally less effective in restoring vision loss beyond a certain stage of the disease. This indicates that early detection by screening of asymptomatic persons with diabetes is of uttermost importance. In addition, there is a great variability in the rate of disease progression and risk to develop clinically significant forms of DR among diabetic patients [71]. Some diabetes patients do not develop vascular anomalies and have good visual acuity after many years of diabetes, whereas others have a rapidly progressing form that does not respond to therapy [71]. An additional important aspect of screening is to identify the patients that are at risk at developing vision-threatening forms of DR. However, this remains a problem in the clinic.

Retinal Imaging

In the clinic, noninvasive imaging techniques are used to monitor the disease progression of DR. Commonly used techniques include ophthalmoscopy, fundus photography, fluorescein angiography (FA), and optical coherence tomography (OCT) [14]. Fundus photography allows detection of microvascular abnormalities within the retina, making grading of the disease possible (Fig. 19.2). To provide additional

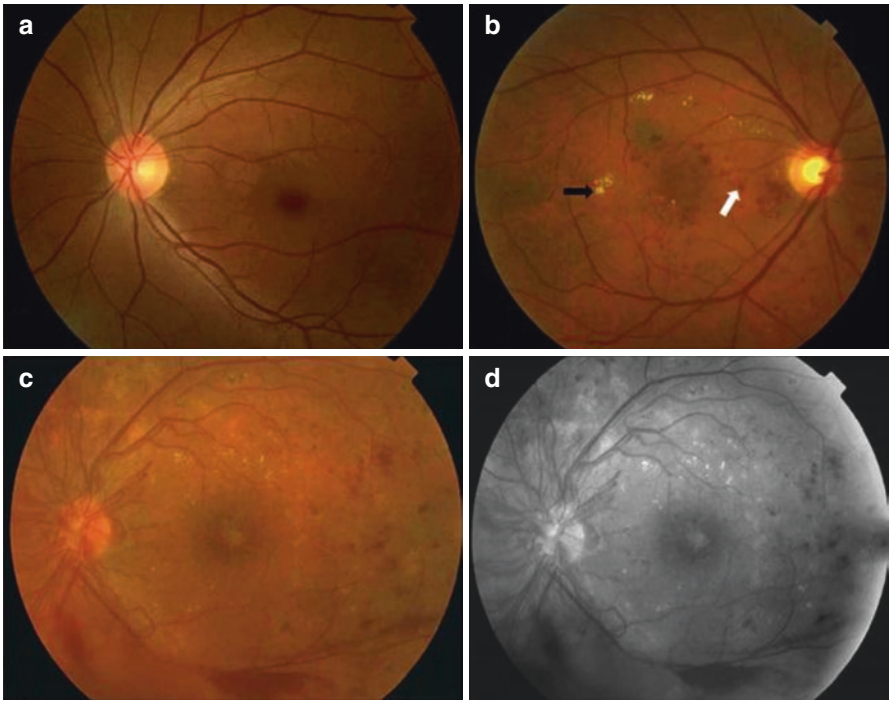

Fig. 19.2 Fundus images of patients with diabetic retinopathy (DR). Color or red-free fundus images of a normal retina (**a**), a retina with severe nonproliferative DR and maculopathy (**b**), and a retina with proliferative DR (**c**, **d**). Note intraretinal hemorrhages (**b**, white arrow) indicating retinal ischemia, yellow intraretinal hard exudates (**b**, black arrow) indicating vascular leakage, and neovascularization extending from the optic disk to the surface of the retina (**c**, **d**)

information, FA and angio-OCT are useful. During FA, the fluorescent dye fluorescein is administrated into the systemic circulation, and the vascular filling and extravasation of fluorescein from the retinal vasculature are assessed (Fig. 19.3). FA allows the detection of vascular leakage but also characterizes areas of capillary non-perfusion. OCT generates cross-sectional, three-dimensional images of the retina, whereas angio-OCT provides a detailed image of the perfused microvasculature. OCT makes it possible to determine the thickness for each individual layer of the retina, enabling detection of fluid accumulation and monitoring of treatment effects [72, 73].

Screening for DR is very cost-effective, and fundus photography and subsequent grading is the standard approach [74]. Addition of OCT imaging to screening programs considerably improves correct detection of DME and reduces the overall costs [75]. Implementation of OCT imaging in screening programs may therefore be useful for early detection of DR. Recently, automated image analysis employing artificial intelligence software, developed with deep learning algorithms, have been presented with high sensitivity and specificity outperforming human graders [76].

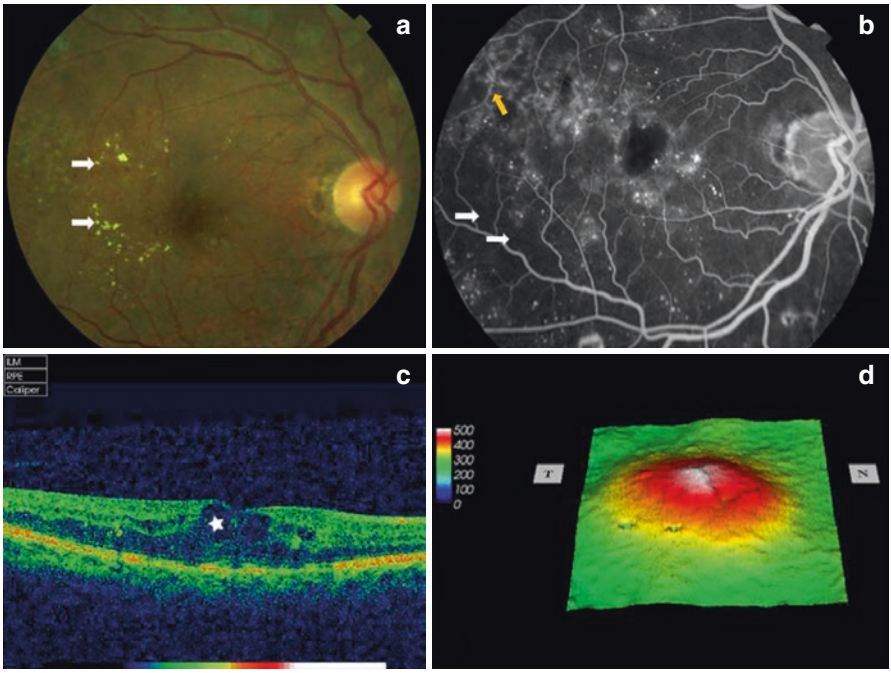

Fig. 19.3 Fundus image (**a**), fluorescein angiogram (**b**), optical coherence tomography (OCT) cross section (**c**), and OCT macular thickness map (**d**) of a patient with nonproliferative diabetic retinopathy and diabetic macular edema. Note hard exudates (**a**, white arrows), capillary nonperfusion (**b**, dark areas marked with white arrows) and leaky microaneurysms (**b**, orange arrow), retinal cysts (**c**, star), and thickened retina (**d**)

Although the specific sequence of events leading to the onset of DR remains a manner of debate, it is becoming clear that neurodegeneration can be observed in the retina long before the clinical recognized vascular lesions can be detected [45, 46]. OCT imaging can detect the associated thinning of the neural layers within the retina [46].

Biomarkers Due to the multifactorial nature of DR, it is likely that multiple biomarkers are needed in clinical practice for meaningful prognostic or predictive purposes rather than one individual biomarker [10]. Common biomarkers for DR are HbA1c, visual acuity, glucose levels, lipid levels, imaging characteristics, of which only HbA1c has a proven prognostic significance [77, 78]. Identification of novel biomarkers may have immense value for the clinic. For instance, the identification of increased VEGF levels in eyes of diabetic patients has significantly increased our understanding of the pathogenesis of DR, and the concomitant introduction of anti-VEGF agents and OCT imaging has revolutionized its treatment. The current search for novel biomarkers is mainly focused on the basic mechanisms underlying DR, which include AGEs, oxidative stress, endothelial dysfunction, inflammation, and pro-angiogenic factors [79].

Concluding Remarks

DR is the most common microvascular complication affecting diabetic patients. The threat of loss of vision from DR is one of the main concerns of persons with diabetes in relation to their disease [80]. It is becoming clear that DR should not be classified as a solely vascular complication of diabetes, as neurodegeneration and inflammation play an important role in the pathobiology of DR as well. Although significant progress has been made in the understanding of DR at a molecular and cellular level, there are still a limited number of therapies available at present. A better understanding of the basic mechanisms involved in the pathogenesis is essential for the identification of novel therapeutic targets and may explain why the currently available therapies are not effective in some patients.

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Part VI
Macrovascular Involvement

Chapter 20
Hypertension in Diabetic Kidney Disease

Gema Ruiz-Hurtado and Luis M. Ruilope

Introduction

High blood pressure (BP) is the main cause of death and disability globally [1, 2]. Arterial hypertension is very frequently observed in type 1 and 2 diabetic patients and greatly contributes to the enhanced cardiovascular and renal risk seen in these patients. Renal damage can have different origins in diabetic patients. In contrast to type 1 diabetes, when present in type 2 diabetes, practically all patients with renal involvement exhibit some degree of elevation in BP if prehypertension is considered [3].

An adequate control of BP is mandatory in order to diminish the risk of developing chronic kidney disease (CKD) and its evolution to end-stage renal disease (ESRD) in particular when diabetic nephropathy, and its faster evolution is present when compared with other etiologies of DKD. Simultaneously, a decrease in the risk of developing cardiovascular disease (CVD) is observed. In this chapter the inadequacy of office BP measurement in daily clinical practice as well as in clinical trials, the adequate target BP and the combination of different antihypertensive therapies, and the role of new antidiabetic drugs in BP control in CKD in type 2 diabetes mellitus (DM) generically known as diabetic kidney disease (DKD) will be reviewed [4].

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Blood Pressure as a Surrogate for CVD and CKD: Caveats of Office BP Measurement

BP constitutes a validated surrogate end point used in clinical trials as a substitute for a direct measure of how patient feels, functions, or survives. The validation of BP was based on data obtained by measuring this parameter in the office, and BP goals recommended by the most influential guidelines are mostly based on the outcome of trials where only office BP measurement was used. Some of these trials compared high versus low BP control and related it with the CV and renal outcome. In other trials changes in BP were not a part of the primary aim of the studies albeit the relationship with CV and renal outcomes was always analyzed.

For most patients with arterial hypertension treated with antihypertensive drugs, Guidelines of the European Society of Hypertension and the European Society of Cardiology (ESH/ESC) devoted to the treatment of arterial hypertension [5] consider that a systolic BP/diastolic BP (SBP/DBP) lower than 140/90 mmHg is the adequate goal. This number differs for diastolic BP in type 2 diabetics that is set at 85 mmHg. In patients with CKD and overt proteinuria, the goal is <130/90 mmHg. Guidelines developed specifically for subjects with CKD by the National Kidney Foundation in the USA, Kidney Disease Improving Global Outcomes (KDIGO) [6] and Kidney Disease Outcome Quality Initiative (KDOQI) [7], have maintained as the recommended goal, BP values lower than 130/80 mmHg for patients with diabetes. ESH/ESC guidelines considered this goal before the last guidelines were published in 2013; the absence of positive results in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial [8] forced the reconsideration of 140 mmHg as the adequate goal for SBP. In fact, a position statement on diabetes and hypertension, very recently published by the American Diabetes Association (ADA) [9], considers for most patients with diabetes, the adequate goal BP is <140/90 mmHg. Lower BP targets <130/80 mmHg may be appropriate for individuals at high risk of CV disease if they can be attained without treatment burden.

The measurement of BP in the office either in daily clinical practice or in clinical trials does not allow the recognition of two phenotypes of hypertension which are relevant from the point of view of CV and renal risk impeding an adequate control of elevated BP. These are the white-coat hypertension (WCH) and the masked hypertension (MH) that are frequently observed in untreated as well as in treated hypertensive (see Table 20.1 for definitions). Both phenotypes are particularly prevalent in diabetic patients [5]. Actual guidelines recognize the need in untreated patients to treat WCH, particularly in those with accompanying target organ damage, and to use antihypertensive therapy in all those presenting with MH that present with a risk similar to that of sustained hypertension [5]. Table 20.2 shows the prevalence of WCH and MH in treated hypertensives diagnosed as having CKD (characterized by an estimated glomerular filtration rate (eGFR) <60 ml/min/1.73 m^2 with or without albuminuria) in data obtained from the Spanish Ambulatory Blood Pressure REgistry (SABPRE) [12]. As it is shown in Table 20.2, MH is particularly prevalent with office BP level within the high-normal range, while WCH is particularly prevalent with office BP is between 130 and 160 mmHg. Actual guidelines do not mention

Table 20.1 Definitions of WCH and MH valid for untreated hypertensives according to the 2013 ESH position papers on ambulatory BP monitoring [10]

White-coat hypertension in treated and untreated patients with office BP ≥ 140/90 mmHg and
24-h ambulatory BP <130/80 mmHg
Daytime ambulatory BP <135/85 mmHg
Nighttime ambulatory BP <120/70 mmHg
Masked hypertension in treated and untreated patients with office BP < 140/90 mmHg and
24-h ambulatory BP ≥130/80 mmHg
Daytime ambulatory BP ≥135/85 mmHg
Nighttime ambulatory BP ≥120/70 mmHg

Table 20.2 Prevalence of WCH and MH in treated hypertensives with CKD

OBP	MH (%)	WCH (%)	*N*
<115	21.8	–	165
115–119	25.3	–	99
120–124	27.5	–	178
125–129	34.3	–	212
130–134	40.6	59.4	347
135–139	44.9	55.1	414
140–149	–	50.2	1141
150–159	–	38.0	1128
160–169	–	34.1	879
170–179	–	28.1	498
≥180	–	20.7	632

Data obtained from the SABPRE [11]
OBP office blood pressure, *MH* masked hypertension, *WCH* white-coat hypertension, *N* number of subjects

what to do with these phenotypes of hypertension in treated patients; the need for further treatment is necessary, although studies are needed.

How to Perform an Adequate BP Measurement

Recently published data from the SABPRE [13] indicate that in treated hypertensives WCH appears in 29% and MH in 32% of the patients. Thus, an inadequate evaluation of BP is observed in 61% of patients. The same percentages for diabetic patients are 33% and 24%, respectively [14], and for patients with CKD (including 25.6% of type 2 diabetic patients) 28.8% and 32.1% [11]. Both phenotypes can also be identified using home BP monitoring [15], but this method does not allow to detect another phenotype of great relevance particularly in diabetics, which is characterized by elevated night time BP recognized as the most risky phenotype of

arterial hypertension [5]. Recently we published that the presence of albuminuria, either high or very high, in hypertensive patients and particularly in diabetics is accompanied by a significant increase in nighttime BP [16]. An elevation in nighttime BP has been described as the trigger for the development of albuminuria in diabetes [17] and could constitute a very important promoter of the development of ESRD and of CV disease consequences in patients with DKD.

Under normal conditions and also in clinical trials, no consideration has been paid to the presence of the three previously quoted hypertension phenotypes in treated hypertensive patients. Therefore, inadequate therapy is commonly used in many hypertensive patients including those with DKD where the risk of progression of CV and of CKD is particularly elevated.

The Risk Accompanying High-Normal BP

The usual goal BP to be attained in hypertensive patients including diabetics (<140/90 mmHg) translates into the fact that most patients when considered as well-controlled stay within the high-normal BP stage (130–139/85–89 mmHg). Data published in 2008 in the general population [18] and recently confirmed [19] with the data of a pool analysis of three contemporary US cohorts the Reasons for Geographic and Racial Differences in Stroke (REGARDS) study, the Multiethnic Study of Atherosclerosis (MESA), and the Jackson Heart Study (JHS) have revealed that the burden of disease due to BP corresponds in 50% of the cases or more to prehypertension (120–139/80–89 mmHg), particularly in the high-normal range. The risk at 10 years in patients with high-normal BP accompanied by established CVD, diabetes, or both can be as high as 40% [20]. Additional CV risk reduction measures for adults with SBP/DBP <140/90 mmHg may be warranted. Among them, a more strict BP control has to be considered.

The recent data of the Systolic Blood Pressure Intervention Trial (SPRINT) [21], although diabetic patients were not included, is an argument in favor of reducing BP levels to values below 130 mmHg counteracting the risk accompanying high-normal BP [22]. Data from the ABPM sub-study of the SPRINT trial show a clearly better control of MH and nighttime BP in patients with a strict BP control [23].

Automated Office BP: An Adequate Method in Daily Clinical Practice Used in SPRINT Trial

Clinic BP was estimated in SPRINT trial through three automatic measurements using a validated oscillometric device with the patient isolated in the room [21]. It attained a mean value for SBP of 121 mmHg. This form of estimation, which in Europe is referred to as unobserved automated BP, was criticized because no previous trial had used it with the exception of the ACCORD, where SBP attained the mean

value of 119 mmHg [24]. This methodology is practically identical to the automated office BP (AOBP) proposed by Martin Myers [25, 26] using the BpTRUE device (BpTRUE Medical Devices, Ltd, Coquitlam, British Columbia, Canada) that estimates BP 5 times and minimizes the alarm reaction that promotes WCH. AOBP provides significantly lower values of BP compared to conventional measurement that in clinical practice consists simply in measuring BP twice. Methodologically it cannot be criticized and in fact gives a more real idea of BP in patients, and this is particularly relevant when the CV and renal risk are elevated, as is the case in DKD. Interestingly, the sub-study of ABPM in SPRINT trial [23] showed that when AOBP attains such a low value, daytime ABPM shows higher levels. Similar data have been shown in SABPRE [13] (see Fig. 20.1) where values of office BP were preferentially lower than those of ABPM below the line of coincidence of the mean value of SBP in both methodologies, represented by the red line in the figure. In the ARTS-DN trial [27], we investigated the capacity of finerenone, a nonsteroidal aldosterone antagonist, to decrease albuminuria in type diabetic patients with either high or very high albuminuria. Data obtained from the sub-study of ABPM [22] in the ARTS-DN study seen in Fig. 20.2 show that the presence of elevated nighttime BP and MH can be accompanied by an adequate office BP level obtained in observed automatic measurement, but the 24-h ABPM is clearly elevated promoting a relevant increase in CV and renal risk.

The caveat of AOBP is the time needed for each measurement which in case of the BpTRUE method takes 12 min for the six measurements performed of which only the last five are considered. Even so, in patients with elevated CV and renal risk, this time can be really cost-effective.

Future BP Goals in Diabetic Patients with CKD

In the near future, new guidelines for the treatment of arterial hypertension will appear in the USA first and then in Europe. Most probably, based on recent data from SPRINT trial [21] and from new meta-analysis [28–30], the adequate SBP goal will be lowered to values <130 mmHg for the hypertensive population including diabetes and CKD in agreement with previous data from KDOQI [7] and KDIGO [6]. This situation corresponds to observed automatic measure normally used in clinical practice and if AOBP is used should be around 5–10 mmHg [31]. Figure 20.3 shows the difference we found among the different ways to measure BP (office, BpTRUE, ABPM, and central BP) in a group of 500 type 2 diabetics (unpublished data from our Hypertension Unit). As can be seen, the difference between office BP and BpTRUE oscillates around 5 mmHg, while ABPM and BpTRUE show quite similar results.

Avoiding the presence of WCH and MH in clinical practice is important in DM. In this sense, the ADA recommends the utilization of HBPM in every untreated hypertensive diabetic in order to make an adequate diagnosis of WCH and MH [9]. This technique can also be used to identify these two phenotypes in treated hypertensive diabetics.

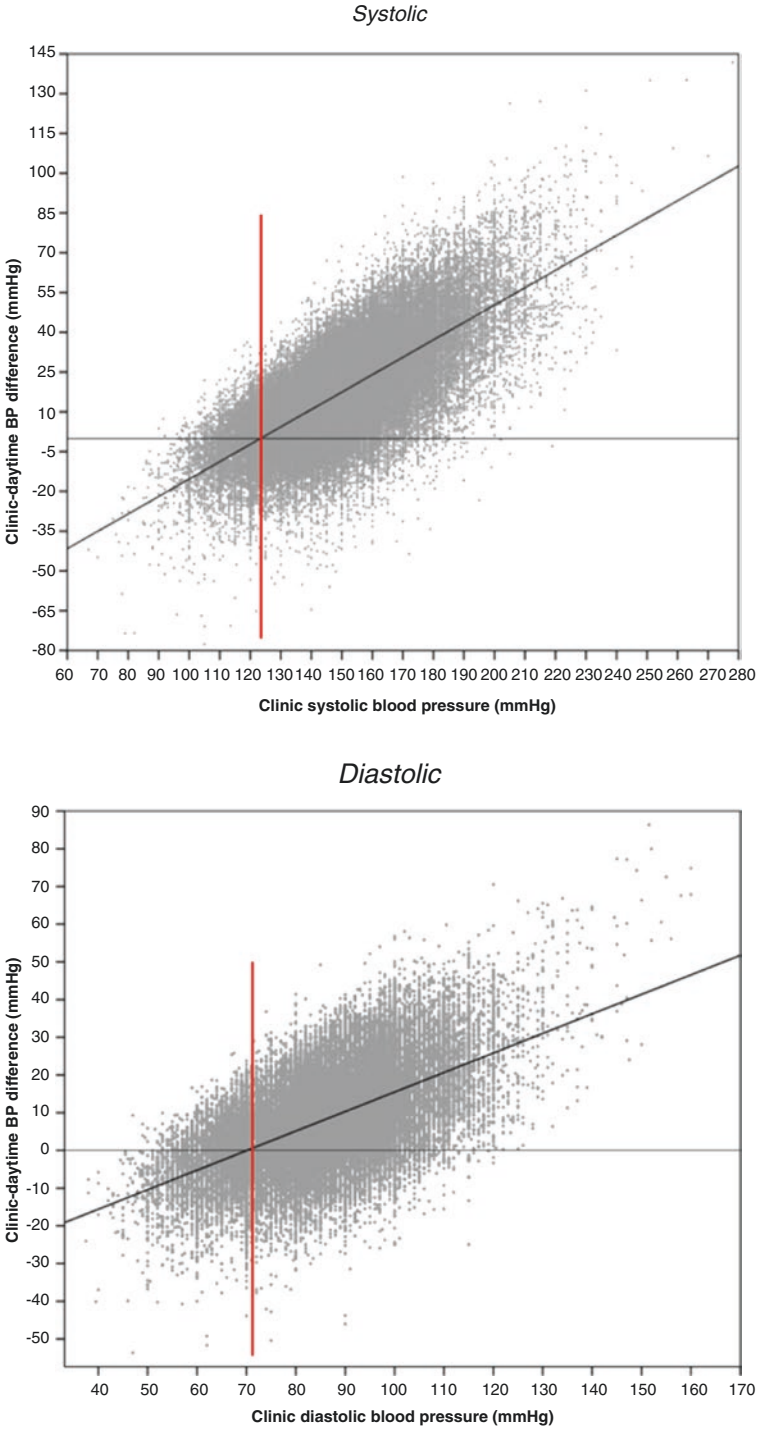
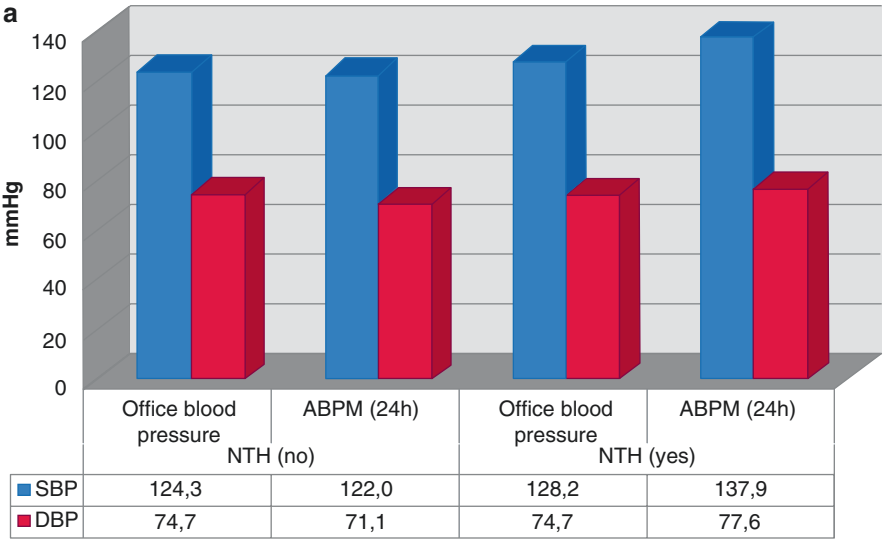


Fig. 20.1 Clinic minus daytime blood pressure differences according to clinic BP levels, in treated hypertensives. (Spanish ABPM Registry, 2016 [13])



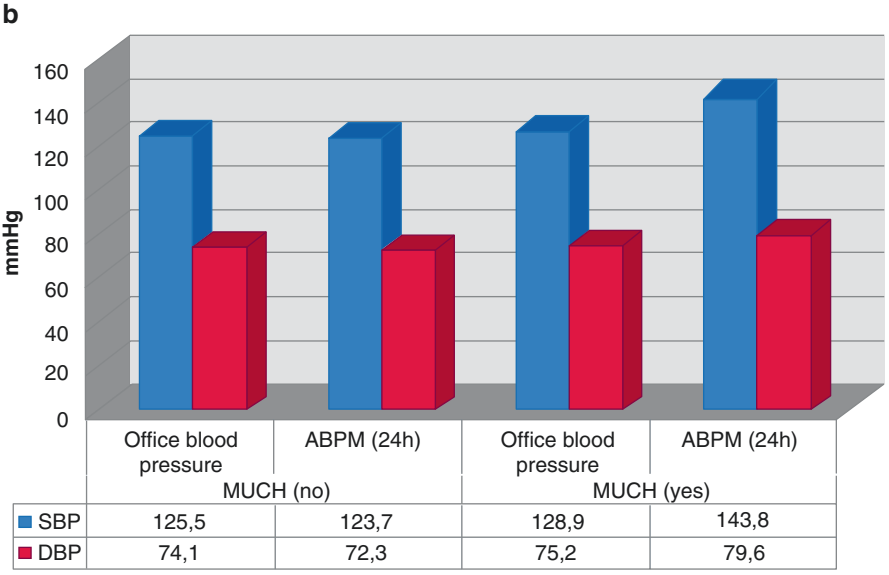


Fig. 20.2 Office BP and ABPM values in patients with and without elevated nighttime BP (panel **a**) and with and without MH (panel **b**). (Data obtained from ART-DM trial [22, 27])

Antihypertensive Treatment DKD: The Role of New Antidiabetic Drugs in the Control of BP

As can be seen in Fig. 20.4, the presence of albuminuria that determines the diagnosis of CKD requires as initial therapy an angiotensin-converting enzyme inhibitor (ACEi) or an angiotensin receptor blocker (ARB) to which a dihydropyridine

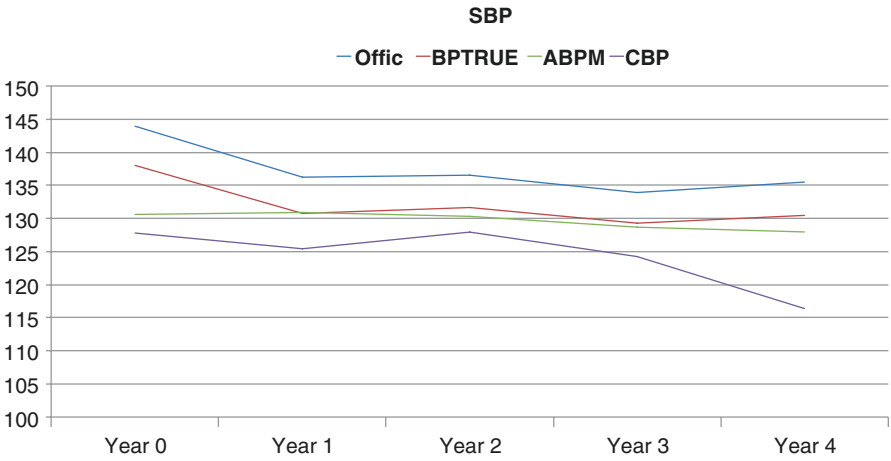


Fig. 20.3 Unpublished data of office BP, BpTRUE, ABPM, and central BP (CBP) in a group of 500 diabetic patients followed for 4 years in our hypertension unit

calcium channel blocker (CCB) and if needed a diuretic are added to attain the expected BP goal. Many patients with DKD will require the triple combination where the diuretic will be a loop diuretic if eGFR is <30 mL/min/1.73m^2. If adequate BP is not attained with the three drugs, the diagnosis of resistant hypertension is done, and as showed in Fig. 20.4, the use of a mineralocorticoid receptor antagonist (MRA) can be attempted provided eGFR is >30–45 mL/min/1.73m^2 [9].

Awaiting for new guidelines, the meta-analysis of Ettehad et al. that included the data from the SPRINT study [21] points to the possibility of treating diabetic patients to a goal lower than 130 mmHg either with or without DKD [28], albeit the benefit of such a low BP is lower when established CVD and CKD are present than when they are absent.

Ongoing studies reviewed by us [32] are investigating whether the value of new nonsteroidal MRAs with a significantly lower prevalence of hyperkalemia is positive for simultaneous CV and renal protection. These studies will be important because long-term therapy with ACEis or ARBs do not impede the development of new-onset albuminuria or the progressive increase in this parameter due to the escape of the effect counteracting angiotensin II allowing a breakthrough of aldosterone [32, 33].

Recently, the demonstration that new oral antidiabetic drugs significantly improve the CV and renal outcome of type 2 diabetic patients has introduced conceptually a radical change in the treatment of these patients. Studies like Empagliflozin Cardiovascular Outcomes, and Mortality in Type 2 Diabetes (EMPA-REG [34, 35]), the Canagliflozin Cardiovascular Assessment Study (CANVAS [36]), Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER [37, 38]), and the Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with

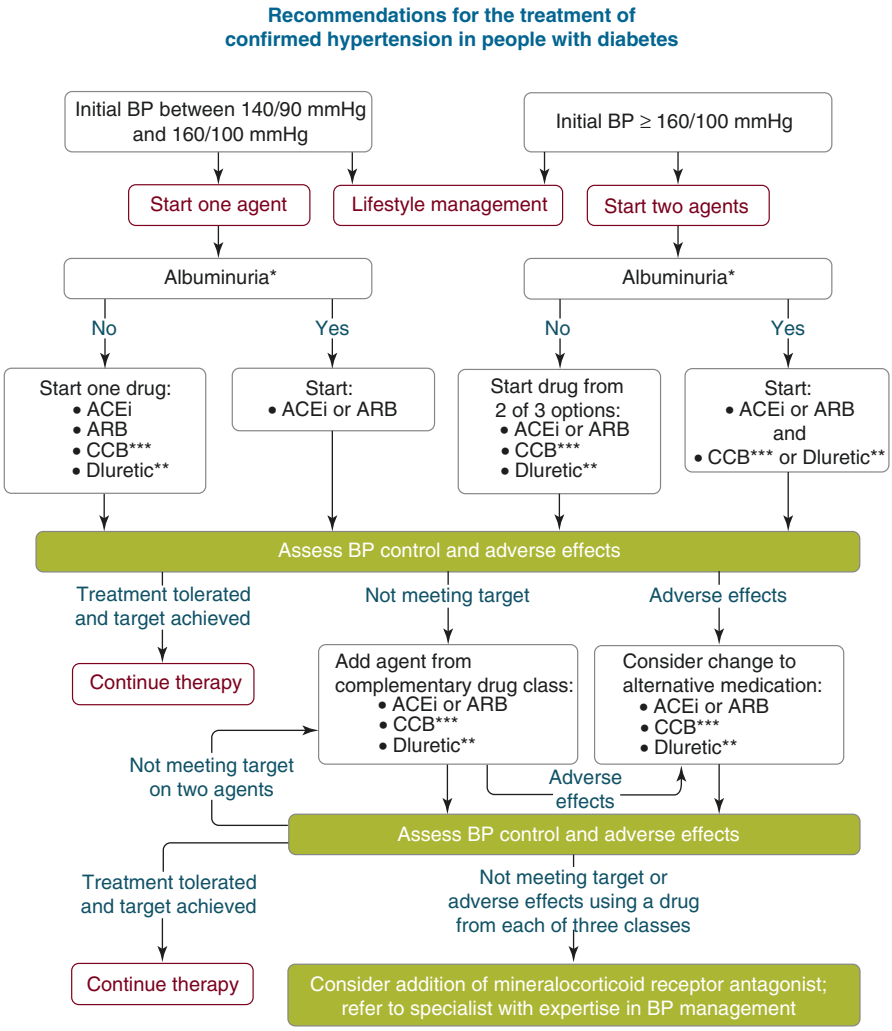


Fig. 20.4 Recommendations for the treatment of confirmed hypertension in people with diabetes. *An ACE inhibitor (ACEi) or ARB is suggested to treat hypertension for patients with UACR 30–299 mg/g creatinine and strongly recommended for patients with UACR ≥300 mg/g creatinine. **Thiazide-like diuretic; long-acting agents shown to reduce cardiovascular events, such as chlorthalidone and indapamide, are preferred. ***Dihydropyridine. (Obtained from de Boer et al. [9])

Type 2 Diabetes (SUSTAIN-6 [39]) trials have shown very positive results for major adverse cardiovascular events (MACE) and progression of renal disease which are accompanied among other positive mechanisms by a significant decrease in body weight and in BP. Both mechanisms participate in the improvement in MACE albeit the descent is not very significant (around 5 mmHg in SBP and less than 5% in body mass index) [40].

Concluding Remarks

In summary, arterial hypertension accompanies the great majority of cases of DKD considering prehypertension and in particular high-normal BP. An improvement in the way BP is measured through the use of AOBP, ABPM, or home BP is necessary to really optimize the antihypertensive treatment of these patients. In the majority of cases, double or triple combination will be required followed if possible by an MRA. New oral antidiabetic drugs SGLT-2 and GLP-1 agonists can be helpful at least in a part of the patients with DKD. More data are needed in patients with an eGFR <45 mL 7 min/1.73m^2.

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Chapter 21
Macrovascular Involvement in Diabetes: Renal Artery Stenosis

Bert-Jan van den Born and Fouad Amraoui

Introduction

Diabetes is characterized by an increased prevalence of micro- and macrovascular disease. Macrovascular disease occurs at least 15 years earlier in patients with type 2 diabetes, and they have a fourfold higher risk of developing cardiovascular complications compared to patients without diabetes after controlling for other cardiovascular risk factors [1, 2]. The increased risk for macrovascular disease also applies to the development of renal artery stenosis [3]. Patients with type 2 diabetes frequently have a combination of risk factors for macrovascular disease, including hypertension, dyslipidemia, and (central) adiposity. The duration of diabetes and the presence of other risk factors for cardiovascular disease contribute to the progression of macrovascular disease, including renal artery stenosis [4]. Whereas diabetes is one of the risk factors for macrovascular disease, including renal artery stenosis, the microvascular complications are more or less unique for patients with diabetes. Risk factors that accelerate macrovascular complications such as hypertension, glycemic control, and smoking also influence the rate of progression of microvascular complications [5–7]. In contrast to patients with type 1 diabetes, where microvascular complications usually develop somewhere within 20 years of the diagnosis [8], microvascular complications (retinopathy and nephropathy) in patients with type 2 diabetes can be present even before the time of diagnosis [9, 10]. Around 7% of patients already present with microalbuminuria at the time of diagnosis, while the incidence of new-onset microalbuminuria is 2% each year [10]. The development of microalbuminuria in patients with diabetes can be viewed as the first evidence of hemodynamic alterations in the kidney characterized by increased glomerular pressure and hyperfiltration that ultimately leads to glomerular changes and loss of

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functional nephrons. The hemodynamic consequences of diabetic nephropathy, from microalbuminuria to end-stage renal disease, result in increased vulnerability to increased glomerular pressure and decreased renal flow reserve. Impairment in renal flow reserve and regulation may further contribute to the renal damage observed in diabetic nephropathy [11, 12]. Because the interplay between microvascular and macrovascular complications affects renal blood flow in different ways, this may theoretically impact the benefit and risks associated with revascularization strategies for diabetic patients with renal artery stenosis.

Epidemiology and Pathophysiology

Prevalence of Renal Artery Stenosis in Diabetes

In a large autopsy study involving more than 5000 consecutive autopsy reports, atherosclerotic renal artery stenosis was present in 8.3% of all diabetic patients compared to less than 3% in persons without diabetes (odds ratio 3.5) [3]. In diabetic patients with a record of hypertension, the prevalence of renal artery stenosis increased to 10.1%. Vice versa, 53% of the patients with renal artery stenosis had diabetes, with 43% having bilateral disease compared to 30% in patients without diabetes. Reported prevalence rates for patients with diabetes and hypertension are even higher with 20–30% of patients having unilateral or bilateral renal artery stenosis [13–15]. The rate of progression of renal artery stenosis over time is two times higher in patients with diabetes compared to patients without diabetes [4]. In addition, diabetes is an independent risk factor for developing end-stage renal disease in patients with renal artery stenosis [16].

Unilateral Versus Bilateral Renal Artery Stenosis and Stenosis in a Single Functioning Kidney

In renal artery stenosis, the decrease in renal perfusion pressure leads to a reduction in renal blood flow and glomerular filtration rate. The decrease in stretch of renal afferents and diminished delivery of sodium chloride to the distal tubule stimulates renin production and the release of renin. This is further supported by the finding that in experimental models the rise in blood pressure (BP) is directly proportional to the increase in plasma renin activity [17]. In unilateral renal artery stenosis, the increase in BP results in increased pressure-mediated sodium excretion (“pressure natriuresis”) in the contralateral kidney. In the presence of intact renal autoregulation and as a result of a renin-mediated increase in angiotensin II, this natriuretic response is blunted, and a new equilibrium will follow at higher BP levels [18]. In bilateral renal artery stenosis or stenosis in a single functioning kidney, fluid

retention will be more pronounced, because pressure natriuresis cannot develop, while there is an increased risk of renal failure. Although the absolute risk may be small, and not much greater compared to patients with unilateral stenosis, acute kidney injury following treatment with renin-angiotensin blocking agents poses a real risk in patients with significant bilateral renal artery stenosis or a solitary functioning kidney [19].

Severity of Renal Artery Stenosis and Functional Consequences

The degree of renal artery stenosis, usually expressed as percentage luminal narrowing, that is required to cause a significant rise in BP or a decrease in renal function is subject of debate [20]. While the kidneys receive 20% of cardiac output, they require less than 10% of oxygen for metabolism [21]. The kidney is able to increase renal blood flow by almost 100% compared to baseline values [22]. This shows that a high degree of stenosis may be required to produce renal ischemia. In an experimental canine model, only luminal narrowing by >80% increased BP [23]. In humans, only severe renal artery stenosis is associated with reduced cortical blood flow on BOLD-MRI with accumulation of deoxyhemoglobin in the kidney cortex as a reflection of tissue hypoxia [24]. Reduction of renal blood flow therefore seems to be a late phenomenon that is associated with advanced stenosis.

Interplay Between Renal Artery Stenosis, Hypertension, and Renal Insufficiency in Diabetes

It is well established that renal artery stenosis is closely linked to hypertension and renal insufficiency [25]. This association is however not necessarily causal, especially in patients with diabetes. In patients with diabetes, the interplay between renal artery stenosis, hypertension, and renal insufficiency can be viewed from three different clinical and pathophysiological perspectives. First, diabetes is associated with a higher prevalence of both hypertension and renal dysfunction [26, 27], which is also more difficult to control [26]. The frequent coexistence obscures possible causal associations between renal artery stenosis, hypertension, and renal insufficiency. Second, diabetes is associated with impaired renal autoregulation in experimental models of diabetes [28–30]. In humans, renal autoregulation is difficult to assess. However, previous studies in patients with diabetes have shown that GFR decreases in diabetic patients with microalbuminuria and normal baseline renal function [11] and that the capacity to regulate glomerular filtration rate decreases with the duration of diabetes [31]. Impaired renal autoregulation may increase the risk of ischemic nephropathy in the stenotic kidney and could make the contralateral kidney particularly vulnerable to the detrimental effects of high BP. Finally,

diabetes is associated with renal microvascular disease resulting in diminished renal flow reserve in patients with diabetes compared to nondiabetic patients [12]. This may further limit possible beneficial effects of revascularization strategies in case of renal artery stenosis. Whether diabetic patients are more prone to the development of interstitial fibrosis and glomerular sclerosis as a result of reduced renal blood flow, however, remains to be determined.

Clinical Decision-Making

Given the increased prevalence of atherosclerotic renal artery stenosis in patients with diabetes, the relative contribution of other causes of renovascular disease, in particular fibromuscular dysplasia (FMD), will likely be lower compared to patients without diabetes. The predictive values of different clinical characteristics for atherosclerotic renal artery stenosis have not been specifically validated in diabetic patients. In a retrospective study that examined >1500 consecutive angiographies for the evaluation of renal artery stenosis, in most cases because of resistant hypertension, the presence of diabetes increased the odds of renal artery stenosis by ~80%. However, other clinical characteristics including age and evidence of atherosclerotic disease elsewhere (coronary artery disease, stroke) contributed more to the risk of renal artery stenosis with comparable odds ratios reported in other studies [32]. Therefore the same clinical clues that are used to identify patients with atherosclerotic renal artery stenosis can probably also be used in patients with diabetes (Table 21.1). Besides evidence of atherosclerotic disease clinical clues include the presence of an abdominal bruit, recent-onset (or worsening of preexisting) hypertension, and impaired kidney function. Next to these clinical characteristics, an increase in serum creatinine of more than 30% following renin-angiotensin system blocking agents, unexplained pulmonary edema ("flash edema"), and a difference of >1.5 cm in size between the two kidneys may be suggestive of the presence of a hemodynamically important renal artery stenosis. Resistant hypertension, defined as the presence of uncontrolled hypertension despite the use of three or more BP-lowering drugs that includes a diuretic, is generally regarded as an important clue for renal artery stenosis. However, to get BP controlled, the majority of diabetic patients need two or more drugs, and ~ 15% still have uncontrolled hypertension despite the use of three or more drugs [33]. Vice versa, in patients with resistant hypertension, diabetes is two times more common compared to patients who were controlled with three or less BP-lowering drugs [34]. Given the high prevalence of resistant hypertension in diabetic patients and the proven beneficial effects of treatment with mineralocorticoid receptor antagonists such as spironolactone [35, 36], it seems prudent to reserve diagnostic studies for renal artery stenosis for diabetic patients who remain to have uncontrolled hypertension despite lifestyle advice and four antihypertensive drugs, including a diuretic and a mineralocorticoid antagonist.

Table 21.1 Clinical clues for renal artery stenosis

Onset or development of hypertension before 30 years of age
Development of moderate to severe hypertension after age 55 years
Uncontrolled resistant hypertension or (recurrent episodes of) hypertensive crisis
Moderate to severe hypertension in a patient with atherosclerosis, a unilateral small kidney, or asymmetry in renal size of more than 1.5 cm that cannot be explained by another reason
Recurrent episodes of acute left-sided heart failure (flash pulmonary edema) associated with increased BP levels
Acute elevation in serum creatinine >30% after administration of renin-angiotensin blocking agents
Abdominal bruit in the upper abdominal or lumbar paravertebral region

Diagnostic Imaging

Conventional renal angiography is considered the gold standard for anatomical grading of renal artery stenosis (Fig. 21.1). Inter-observer agreement among experienced radiologists is good with a weighted kappa coefficient of 0.65–0.70 [37]. However, due to the invasive nature of this technique, serious vascular complications such as bleeding, dissection, and cholesterol embolization may occur. For initial testing, noninvasive imaging techniques can be used including computed tomography angiography (CTA, Fig. 21.2), magnetic resonance angiography (MRA), and duplex Doppler ultrasonography. Selection of the optimal imaging technique depends on patient characteristics, local experience, and availability. In general, sensitivity of these noninvasive techniques is lower than their specificity, meaning that failure to identify renal artery stenosis using either one of these noninvasive techniques does not rule out its presence. As for every diagnostic procedure, its performance highly depends on pretest probability following Bayes' theorem. Noninvasive testing should therefore only be performed in case of sufficient clinical suspicion.

The diagnostic accuracy of CTA for the detection of significant atherosclerotic renal artery stenosis (>50%) has been demonstrated to have varying sensitivity, ranging from 77% to 98% in different studies [38, 39], and a specificity of 94%. In patients with a high clinical suspicion for renal artery stenosis, the positive predictive value is estimated to be 68% and the negative predictive value 91%, while inter-observer agreement on the presence of renal artery stenosis is moderate. Given further improvements in the resolution and reconstruction of CTA, diagnostic accuracy is likely to be higher with newer imaging modalities. Disadvantages of CTA include radiation exposure and potential allergic reaction to iodinated contrast material, but the main concern is nephrotoxicity of intravenously administrated contrast, especially in patients with renal insufficiency and diabetes [40]. To avoid contrast-induced nephropathy, MRA may be preferred for diagnosing renal artery stenosis in diabetic patients with renal insufficiency. However, in patients with end-stage renal disease, gadolinium increases the risk of nephrogenic systemic fibrosis, a rare but life-threatening complication [41]. Two smaller studies, including a total

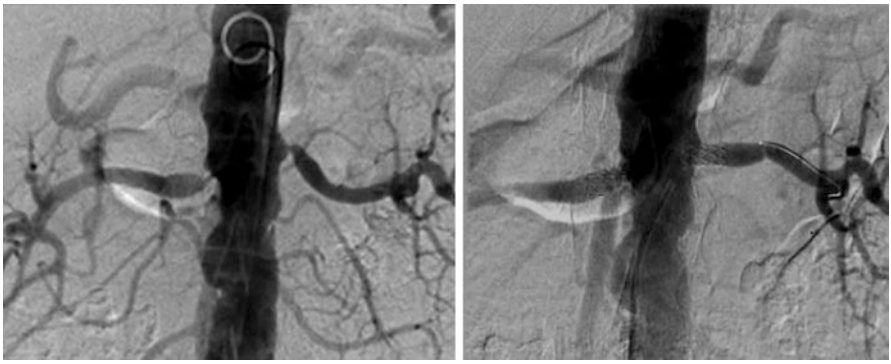

Fig. 21.1 Digital subtraction angiography showing severe bilateral ostial stenosis of the renal arteries with post-stenotic dilatation of the right renal artery. Situation before (left panel) and after revascularization (right panel)

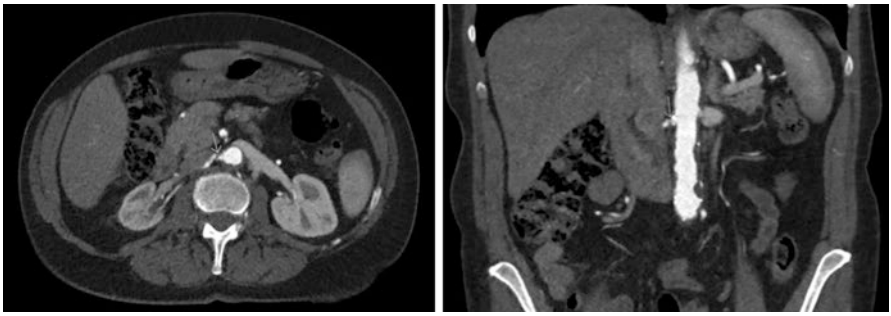

Fig. 21.2 CT angiography showing an ostial stenosis in the right renal artery in combination with a relatively small kidney and intravascular lumen. The abdominal aorta has an irregular aspect with multiple plaques and calcifications

of 68 patients, reported a 100% sensitivity of MRA for detection of renal artery stenosis (>50%) and a specificity of 71% and 96%, respectively [42, 43]. In a larger and blinded study, MRA was shown to be less accurate with a sensitivity of 78% and a specificity of 88%. Estimates of positive predictive value range from 49% to 89%, while the negative predictive value ranges from 90% to 100%. Flow-related artifacts may occasionally lead to overestimation of the stenosis and contribute to the lower positive predictive value range. Inter-observer agreement is less compared to CTA with a weighted kappa coefficient of 0.40–0.61 [38] and MRA fails to detect presence of accessory renal arteries in a substantial proportion of cases [42, 43], due to limited spatial resolution compared to CTA [44]. In addition, MRA cannot be performed in patients with pacemakers and other implanted electric devices and in patients with claustrophobia. Duplex Doppler ultrasonography is a widely available, inexpensive, and safe technique that may be used for diagnosing renal artery stenosis. Duplex Doppler ultrasonography allows the combination of direct

visualization of the renal artery with functional hemodynamic parameters. Using peak flow velocity measurements, sensitivity of duplex Doppler ultrasonography for detection of significant renal artery stenosis (>50%) is estimated to be 85% and specificity 92%. However, this technique is cumbersome, highly user dependent, and difficult to perform and may be less accurate in obese patients, limiting its use in clinical practice [45].

Functional Assessment

Renal artery stenosis is usually considered significant if the arterial lumen is narrowed by 50–70%. The clinical relevance of renal artery stenosis in terms of renovascular hypertension and renal function impairment is however difficult to determine by anatomical grading of the stenosis alone. By definition cure of renovascular hypertension after revascularization will establish the clinical relevance of a stenosis with certainty. Renovascular hypertension can however be superimposed on essential hypertension, which may impede even retrospective determination of the clinical relevance after revascularization. In these cases, hypertension will not be cured, but is easier to control with antihypertensive medication. Combining anatomic grading of renal artery stenosis with functional assessment may be helpful for clinical decision-making [46]. Several methods exist for estimation of the effect of renal artery stenosis on renal function and blood flow. Captopril renography measures the renal uptake and filtration of radionuclides (Fig. 21.3). In case of significant unilateral renal artery stenosis, the obtained renal scintigram will show decreased and delayed peak uptake of the radionuclide as a marker for reduced blood flow and delayed excretion as a measure of renal function in the affected kidney compared to the contralateral kidney. Angiotensin converting enzyme (ACE) inhibition with captopril, administered 1 hour prior to the procedure, is used to amplify the effect of renal artery stenosis on renal blood flow and function. The reported sensitivity of captopril renography as an initial diagnostic test, without prior imaging of the renal arteries ranges from 74% to 94% and specificity from 59% to 95% [47, 48]. A disadvantage inherent to the principle of this technique is that lateralization may only be evident in case of unilateral renal artery stenosis, whereas interpretation is hampered in bilateral renal artery stenosis or in the presence of asymmetric renal parenchymal disease. More importantly, abnormal captopril renography in patients with renal artery stenosis on conventional angiography was not found to predict favorable outcome of renal angioplasty with regard to BP or renal function [49]. This was however an exploratory study and not sufficiently powered to detect small differences. Because renal artery stenosis augments BP by activating the renin-angiotensin system, measurement of plasma renin activity seems an obvious strategy to assess presence of renovascular hypertension. However, peripheral vein measurement of plasma renin does not seem helpful, since a large proportion of patients who benefit from revascularization have low or normal

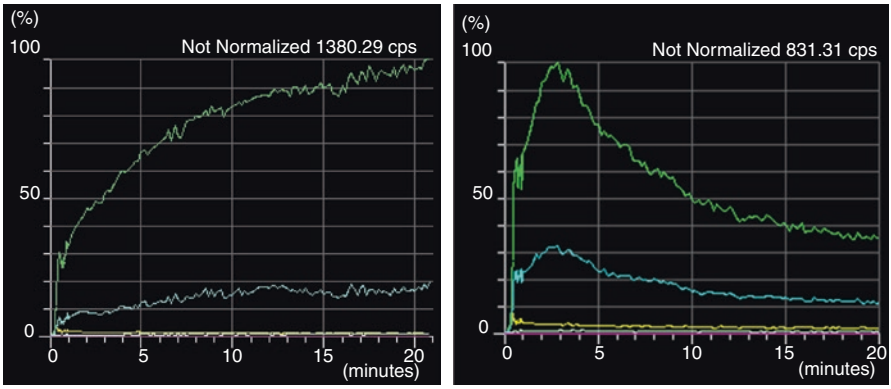

Fig. 21.3 Renogram with and without captopril in a patient with renal artery stenosis and a single kidney. The left panel shows a significantly delayed uptake of the tracer following administration of captopril, while the right panel shows normal renal uptake and excretion without captopril

peripheral plasma renin activity, whereas a large proportion of patients with essential hypertension have increased plasma renin activity [50, 51]. The renal vein renin ratio may provide more accurate information on the functional consequences of a renal artery stenosis. The principle of this method is that plasma renin activity measured in the vein of the stenotic kidney should be higher compared to the contralateral kidney [52, 53]. Using this method, renovascular hypertension was most reliably diagnosed in patients with totally occluded main renal arteries. Also, lateralization in patients with bilateral renal artery stenosis only occurred in the presence of total renal artery occlusion, indicating that this approach might be more suitable for detecting patients who would benefit from nephrectomy rather than from revascularization.

Instead of using markers for hemodynamic changes, direct invasive measurement of hemodynamic characteristics of a renal artery stenosis might provide more accurate information. Using sensor-equipped guidewires, the fall in pressure across a stenosis (FFR or fractional flow reserve) can be measured, while diffuse microcirculatory disease may be identified by measuring the renal flow reserve (RFR), defined as the ratio of intrarenal flow under hyperemic conditions and baseline intrarenal flow, prior to pharmacologically induced hyperemia (Fig. 21.4). This approach reflects daily practice in cardiology, where in case of uncertainty on the clinical relevance of a coronary lesion, pressure and flow measurements aid clinical decision-making. Identification of culprit lesions using this method allows direct revascularization therapy and has improved outcome of patients with coronary artery disease [54, 55]. Important physiological differences between the coronary and renal circulation may however preclude translation of this experience in cardiology to atherosclerotic renovascular disease. Firstly, the capacity of the coronary circulation to increase blood flow under

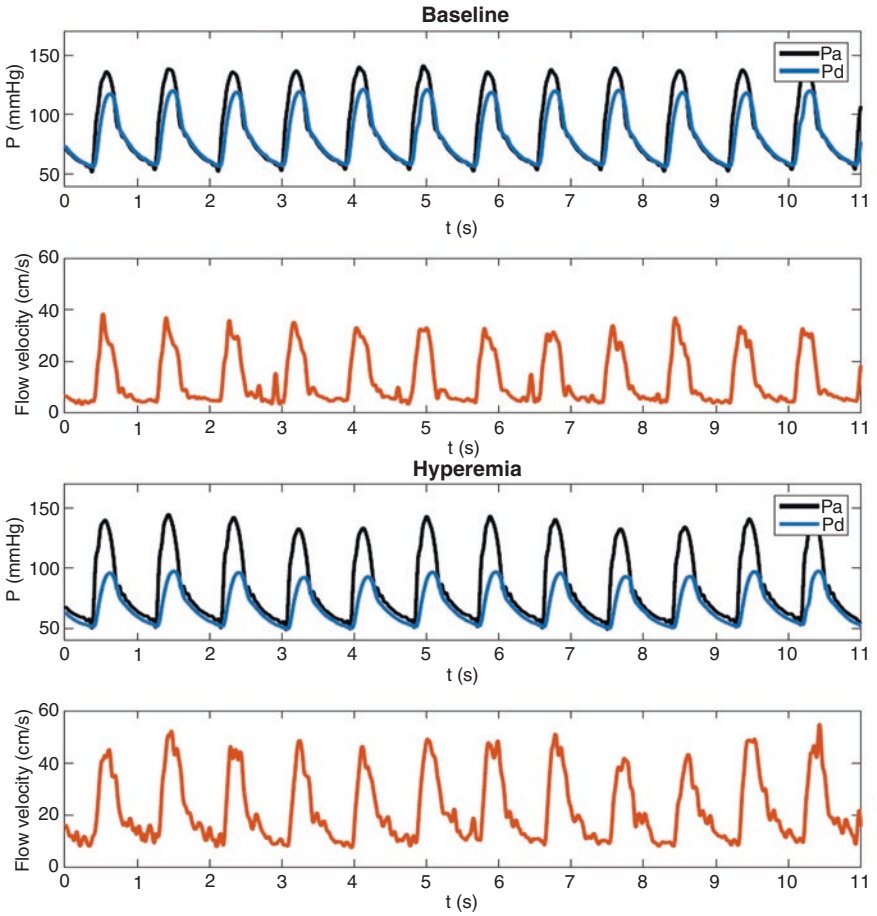

Fig. 21.4 Combined pressure/flow measurement in a patient with renal artery stenosis. At baseline proximal (aortic) pressure and distal (post-stenotic renal artery) pressure are similar. Under hyperemia following intrarenal dopamine, an increase in flow and a fall in distal pressure are observed

hyperemic conditions is larger than that of the renal circulation, allowing more room for detection of differences between patients with and without diffuse microvascular disease and ultimately identification of patients who might benefit from revascularization. Secondly, coronary autoregulation ensures blood flow across a wide range of perfusion pressures, while autoregulation of the renal circulation aims to maintain glomerular filtration pressure via angiotensin II-induced vasoconstriction of efferent arterioles, thereby allowing a decrease in renal blood flow. Measurement of the fractional flow reserve (FFR) alone, without hyperemia, does not seem to be helpful in identifying patients with renal artery stenosis

who will benefit from revascularization [56], while under hyperemic conditions the translesional pressure gradient has been shown to predict BP response after revascularization [57]. A combination of both FFR and RFR might better predict revascularization outcome [12], particularly in diabetic patients with renal artery stenosis, in whom diffuse microvascular renal disease is more common and may influence outcome of revascularization therapy.

Medical Treatment

While only selected patients with renal artery stenosis may benefit from revascularization therapy, medical treatment is indicated in all patients. There are however no randomized controlled trials that compared the effect of different treatment regimens on outcome of patients with renal artery stenosis. Activation of the renin-angiotensin system in patients with renal artery stenosis suggests that ACE inhibition or angiotensin receptor blockade (ACEI/ARB) is effective in lowering BP. Treatment of patients with atherosclerotic renovascular disease with ACEI/ARB has been shown to reduce the long-term risk of a composite outcome of death, myocardial infarction, and stroke [58]. This beneficial effect of ACEI/ARB in patients with atherosclerotic renal artery stenosis comes at the expense of a twofold increased risk of acute renal failure, especially in patients with diabetes, patients with chronic kidney disease, and patients concurrently treated with diuretics. Interestingly, the long-term risk of dialysis in the same study was reduced by ACEI or ARB. A possible explanation for this seemingly contradictory observation could be that renin-angiotensin system blocking agents induce potentially reversible acute renal failure in some patients, while the majority of patients with renal artery stenosis benefit from the well-established renoprotective effect of ACEI or ARBs [59]. In addition, renin-angiotensin blockade is of particular benefit in patients with diabetes for limiting the onset as well as progression of diabetic nephropathy [60, 61]. Thus, with careful monitoring of renal function, the available research supports the use of ACEI or ARB in diabetic and nondiabetic patients with renal artery stenosis. Even in patients with bilateral renal artery stenosis, ACEI/ARB is not contraindicated, if patients are carefully monitored. In a retrospective cohort study, patients with bilateral renal artery stenosis and a previous decrease in renal function following treatment with ACEI/ARB were successfully restarted after revascularization with a recurrence of acute kidney injury in only 10% of patients. In addition the risk of adverse effects related to ACEI/ARB was not higher in patients with unilateral compared to bilateral stenosis [19]. Most patients with renal artery stenosis will require combination therapy to achieve adequate BP control. In addition to ACEI or ARB, beta-adrenergic blockers, thiazide diuretics, and calcium-channel blockers are considered to be effective in patients with renal

artery stenosis [62]. Beta-blockers may be of particular value because of their ability to reduce plasma renin levels and sympathetic activity and has been associated with reduced mortality in patients with renal artery stenosis [63]. The beneficial effects of statins and aspirin in general populations with atherosclerosis support a role for these agents in the management of patients with renal artery stenosis [59, 64]. Statins have been shown to reduce the risk of progression of the atherosclerotic renal artery stenosis [65] and reduce the risk of restenosis after revascularization therapy [66]. In a retrospective analysis, statin use in patients with renal artery stenosis has been associated with a lower risk of mortality and progression of renal insufficiency compared to patients who were not treated with a statin, although major baseline differences limit interpretation of this study [67]. In an animal model of renal artery stenosis, statins were shown to preserve renal function and attenuate remodeling of the intrarenal microcirculation [68]. The effect of antiplatelet therapy in patients with atherosclerotic renal artery stenosis remains elusive, as no controlled trial has addressed this issue. Antiplatelet therapy does not seem beneficial in patients with chronic kidney disease [69], but has been associated with reduced mortality risk among patients with atherosclerotic renal artery stenosis in a single-center non-controlled study [63]. Given the presence of atherosclerosis, the use of aspirin in patients with renal artery stenosis seems prudent, provided the patient has no increased risk of bleeding.

Revascularization Strategies

Percutaneous transluminal renal angioplasty with stenting (PTRAS) is the preferred treatment for revascularization of renal artery stenosis and is superior to balloon angioplasty or surgical treatment [70, 71]. Most of these trials also included a significant number of diabetic patients. An overview of clinical studies that examined the effect of revascularization strategies in patients with renal artery stenosis is given in Table 21.2. Thus far, randomized controlled trials have failed to show a benefit of revascularization over medical management with regard to BP control and renal function improvement [72]. However, patients who are most likely to benefit from revascularization therapy (e.g., those with renal artery stenosis >70%) were mainly excluded from participation. Therefore revascularization therapy is still considered to be indicated in selected patients with renal artery stenosis (Table 21.3) [87]. Based on previously described pathophysiological characteristics, in particular the presence of microvascular disease, patients with diabetes may be considered to benefit less from PTRAS compared to patients without diabetes. However, this notion is not supported by previous trials comparing PTRAS and medical therapy [72]. In the CORAL trial, a trend toward less benefit from PTRAS was observed in patients with diabetes and in

Table 21.2 Clinical and anatomical findings favoring revascularization[a]

Severe bilateral renal artery stenosis or significant renal artery stenosis in a single kidney
Uncontrolled resistant hypertension or (recurrent) hypertensive crisis
Progressive renal insufficiency
Recurrent episodes of unstable angina, unexplained left-sided heart failure or sudden unexplained pulmonary edema ("flash edema")

[a]Adapted from the 2006 guideline recommendations of the American College of Cardiology/American Heart Association

those with "global ischemia," defined as stenosis of 60% or more of the diameter of all arteries supplying both kidneys (or stenosis of 60% or more of the diameter of all arteries supplying a single functioning kidney), although this was not significant [64]. Nonetheless, the presence or absence of renal microvascular disease in patients with renal artery stenosis may be a relevant predictor of PTRAS outcome. Indeed, in a post hoc analysis of the CORAL trial, urinary albumin-creatinine ratio was shown to predict outcome of PTRAS. The composite primary outcome including fatal and nonfatal cardiovascular and renal events (including >30% eGFR decline) occurred less often with PTRAS compared to medical treatment in patients with urine albumin-creatinine ratio < 22.5 mg/g. Follow-up systolic BP tended to be lower in these patients, irrespective of the presence of diabetes [88]. In addition, patients with an intrarenal resistive index (RI) >0.8 as marker of nephrosclerosis, were shown to have poor outcome after revascularization in one study, with no improvement in renal function or BP [89]. Together these observations suggest that PTRAS may be less effective in diabetic patients with overt renal microvascular disease but that diabetic patients without severe microvascular disease may still benefit from PTRAS. In a prospective study of 241 patients (41% diabetic) with more severe renal artery stenosis (>70%), the proportion of patients with improved renal function and BP control was shown to be similar in patients with and without diabetes. PTRAS reduced BP and plasma creatinine to a similar extent in diabetics and nondiabetics. Patients with a RI >0.8, indicative of nephrosclerosis, showed a lower and nonsignificant reduction of BP, while patients with a RI <0.8 had a significant reduction of BP after PTRAS [78]. Long-term renal function improved significantly in patients with moderate nephrosclerosis (RI 0.7–0.8). The decrease in plasma creatinine was less pronounced and not significant in patients without (RI <0.7) and with severe (RI >0.8) nephrosclerosis [90]. In a Japanese trial of 149 patients (61 diabetics), response rate with improvement in renal function and reduction of BP was shown to be similar in patients with and without diabetes [86]. Similar PTRAS outcome of diabetic and nondiabetic patients has also been demonstrated in smaller nonrandomized trials [79, 91]. Comparison of patients with bilateral renal artery stenosis or a unilateral stenosis of a solitary functioning kidney, with and without diabetes, showed that the proportion of patients with improvement of renal function was similar in both groups [92].

Table 21.3 Diabetes prevalence in clinical trials that examined the effect of PTRA with and without stent placement in patients with renal artery stenosis

Study/year	No. diabetics/total participants (%)	Effect on BP	Effect on renal function	Effect diabetes on outcome
Randomized controlled trials				
EMMA, 1998 [73]	10/49 (20%)	Less antihypertensive medication with PTA compared to medical treatment alone	No difference in creatinine clearance and adverse renal outcome	Not reported
DRASTIC, 2000 [74]	5/106 (5%)	No BP difference	No difference in eGFR	Not reported
STAR, 2009 [75]	33/140 (24%)	No BP difference	No difference in eGFR or end-stage renal disease	Not reported
ASTRAL, 2009 [76]	236/778 (30%)	No BP difference	No difference in improvement or deterioration of renal function	Not reported
Marcantoni C et al., 2012 [77]	32/84 (38%)	Similar reduction in BP with PTRAS or medical therapy	Similar stable eGFR after PTRAS or medical therapy	Not reported
CORAL, 2014 [64]	310/947 (33%)	No significant difference in BP	No difference in the prevalence of end-stage renal disease	No difference
Nonrandomized clinical studies				
Zeller T, et al., 2003 [78]	88/215 (41%)	Significant reduction in BP with PTRAS after 1 year	Significant drop of plasma creatinine with PTRAS after 1 year	No difference
Hanzel G et al., 2005 [79]	18/66 (27%)	Similar decrease in BP both after PTRAS and medical therapy	Higher proportion of patients with renal function improvement in PTRAS compared to medical	No difference
Arthurs Z et al., 2007 [80]	11/40 (28%)	Short-term decrease in BP after PTRAS, but no change after 12 months. Stable BP with medical therapy	Stabilization of plasma creatinine after PTRAS and slowly increasing creatinine with medical therapy	Not reported
Kane GC et al., 2010 [81]	39/100 (39%)	Decrease in BP and number of antihypertensives after PTRAS, but not with medical therapy	Higher proportion of patients with renal function improvement in PTRAS compared to medical therapy	Not reported
Kalra PA et al., 2010 [82]	286/897 (32%)	Decrease in BP both after PTRAS and medical therapy	Higher proportion of patients with renal function improvement in PTRAS compared to medical therapy	Not reported
Cianci R et al., 2011 [83]	28/93 (30%)	Nonsignificant decrease in BP after both PTRAS and medical therapy	Nonsignificant decrease in plasma creatinine after both PTRAS and medical therapy	Not reported
HERCULES, 2014 [84]	91/202 (45%)	Significant BP reduction with PTRAS	No change in average eGFR after PTRAS	Not reported
Ritchie J et al., 2014 [85]	151/467 (32%)	Significantly larger drop in PTRAS versus medical therapy in case of refractory hypertension	Similar eGFR after PTRAS and medical therapy.	Not reported
Fujihara, et al., 2015 [86]	61/149 (41%)	Significant BP reduction with PTRAS after 1 year	Stable eGFR with PTRAS after 1 year	No difference

Procedure Related Complications and Adverse Events

Procedural success rate of PTRAS, with residual renal artery stenosis less than 10%, is reported to be 95–100%. Re-stenosis occurs in 10–15% of patients within 1 year [71, 93]. Complications of PTRAS are rare but potentially severe. Procedure-related deaths did not occur in the latest and largest randomized controlled trial [64]. In smaller studies, mortality is reported to vary between 0% and 2% [76, 79, 94–99]. Two studies reported a higher mortality rate of 3.2% [75, 100]. Major complications including renal artery dissection, perforation, cholesterol embolization, and bleeding are reported to occur in 0–4% of patients in most studies [72]. The risk of procedural complications of PTRAS seems to be similar in diabetic and nondiabetic patients [92]. However, the risk of contrast-induced nephropathy is higher in patients with diabetes and renal insufficiency [101]. Hyperhydration with saline reduces the risk of contrast-induced nephropathy and is recommended in guidelines for percutaneous coronary interventions [102, 103]. Metformin does not increase the risk of contrast-induced nephropathy but may occasionally cause lactic acidosis in patients with renal insufficiency. Current guidelines therefore recommend periprocedural cessation of metformin [103].

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Chapter 22
Atherosclerosis and Diabetic Nephropathy

Raphael Duivenvoorden

Introduction

Atherosclerosis is commonly assumed to be a disease of modern age, related to our contemporary diet and sedentary lifestyle. However, CT imaging studies of ancient Egyptian and Peruvian mummies challenge this view by showing that about a third of them had developed atherosclerotic plaques [1]. Nowadays, the clinical manifestations of atherosclerosis, such as myocardial infarction and stroke, represent one of the greatest threats to human health worldwide [2]. The global cardiovascular disease (CVD) mortality rate is estimated to be 17.9 million people per year, representing a third of all global deaths [3].

Diabetes mellitus is a major risk factor for atherosclerotic cardiovascular events, and its global prevalence has increased at an alarming pace in the past decades [4]. The risk of coronary heart disease as well as stroke is twice as high in patients with diabetes mellitus as compared to those without [5]. Patients that developed diabetic nephropathy are at the highest risk of developing cardiovascular events [6]. In this chapter we focus on the cardiovascular risk diabetic nephropathy patients are facing and the mechanisms that confer this risk. Furthermore, we discuss therapeutic interventions that may ameliorate the soaring cardiovascular event rate in this population.

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Pathogenic Concepts of Atherosclerosis

Atherosclerosis is a protracted lifelong progressive disease of the artery wall, characterized by lipid accumulation, inflammation, and calcification [7]. The disease starts early in life. In fact, autopsy studies of 3832 US service members that perished in combat in Iraq, with a mean age of 25.9 years, showed that coronary atherosclerosis was present in 8.5% [8].

One of the earliest changes in the atherosclerotic disease process is endothelial dysfunction [7]. The endothelium is the monolayer of cells lining the artery wall and has important functions in regulating vascular tone, hemostasis, and inflammation [9]. Irritative stimuli such as dyslipidemia, hypertension, diabetes, and pro-inflammatory mediators can cause endothelial dysfunction and damage [9]. At sites of endothelial damage, lipids infiltrate the intima layer of the vessel wall, eliciting a disproportionate inflammatory response [7].

The activated endothelium expresses adhesion molecules and recruits leukocytes, predominantly monocytes, from the circulating blood [10]. Monocytes that infiltrate the plaque differentiate into macrophages, which produce proteolytic enzymes that digest the extracellular matrix and cause plaque rupture [10]. Recent experimental studies provided novel insight into the pathophysiology of atherosclerosis, showing that plaque macrophage content depends predominantly on local macrophage proliferation, rather than on previously assumed monocyte influx [11]. While atherosclerosis involves a complex immunologic response involving distinct roles for neutrophils, dendritic cells, mast cells, and T cells, macrophages are considered to be the dominant cell type driving this disease [10]. Macrophages ingest and digest the lipoprotein cholesterol that has infiltrated the artery wall and store it as cholesteryl ester in their cytoplasm as droplets. When many lipid-laden macrophages become apoptotic and release their cholesterol cargo in the extracellular space, the cellular debris and extracellular lipids can accumulate and form a lipid-rich necrotic core [7].

Another cell type that plays a pivotal role in atherogenesis is the proliferative/synthetic smooth muscle cell. These cells produce extracellular matrix molecules like collagen and elastin. Smooth muscle cells are responsible for the formation of a fibrous cap that covers the plaque and cause accumulation of extracellular matrix in the plaque [7]. Until recently, it was believed that proliferative/synthetic smooth muscle cells were dedifferentiated mature smooth muscle cells that migrated from the tunica media. However, a recent study disproved this theory and showed they are derived from the differentiation of multipotent vascular stem cells. In response to vascular injuries, these multipotent vascular stem cells become proliferative and differentiate into smooth muscle cells and chondrogenic cells, thus contributing to vascular remodeling and neointimal hyperplasia [12].

Plaques generally remain under the clinical horizon, either until a flow-limiting stenosis develops or when atheroemboli or thrombus formation causes downstream vessel occlusion. The latter may occur after physical disruption of the fibrous cap, exposing plaque content to the bloodstream, thereby triggering thrombosis [7].

Classically, atherosclerotic plaques with a large lipid-rich necrotic core and a thin fibrous cap were considered at high risk for rupture and atherothrombotic events. However, more recent studies challenge this "vulnerable plaque" concept [13]. In fact, coronary intravascular ultrasonographic imaging studies revealed that many thin-capped atheroma plaques do not cause clinical events [14]. Superficial plaque erosion is increasingly being identified as an alternative mechanism underlying atherothrombotic events and may be responsible for 30–40% of atherothrombotic events. In contrast to the classic vulnerable plaque, eroded plaques have a different phenotype, without a thin fibrous cap or large atheroma, a lack of significant inflammation, and an abundance of proteoglycan and hyaluronan matrix [15].

Enhanced Plaque Inflammation in Diabetes

Atherosclerotic phenotypes of plaques in diabetic patients do not differ markedly from nondiabetics; however the process does seem to be accelerated. In pathology studies in type 2 diabetics, atherosclerotic lesions in the coronary arteries had larger necrotic cores and more plaque load as compared to nondiabetics. Also more plaque macrophage content and apoptotic smooth muscle cells were observed in plaques of diabetics [16]. Experimental studies in mice corroborate these findings and showed that diabetes promotes atherosclerosis development and enhances plaque inflammation [17, 18].

Different effects of hyperglycemia contribute to increased plaque inflammation. Increased expression of adhesive proteins on the cell surface promotes monocyte recruitment to the plaque [19, 20]. There is also a direct effect of hyperglycemia on monocyte and macrophage function. Increased expression of cytokines, chemokines, and toll-like receptors, as well as increased nuclear factor-kB activity in response to hyperglycemia, has been demonstrated in myeloid cells in both experimental mouse models and humans [21–26]. Concomitant hyperglycemia and hyperlipidemia were shown to elevate the proliferation rate of plaque macrophages [27]. Furthermore, myelopoiesis in the bone marrow is induced by hyperglycemia and increases the number of circulating monocytes, while treatment of hyperglycemia reduces monocytosis and diminishes entry of monocytes into atherosclerotic plaques [28].

In addition to hyperglycemia, impaired insulin signaling also seems to play a role in atherogenesis. In a study in an atherosclerotic mouse model (apolipoprotein E knockout mice) in which the insulin receptor gene was deleted specifically in vascular endothelial cells, leukocyte adherence to the endothelium increased, and accelerated atherosclerosis was observed [29]. Impaired insulin signaling in macrophages was shown to predispose to foam cell formation due to increased binding and uptake of oxidized LDL as a result of increased CD36 expression and may have a role in enhanced atherogenesis [30–32].

Plaque inflammation can also be imaged in vivo in humans by 18F-fluorodeoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) [33]. In 216 patients that underwent cancer screening, insulin

resistance was an independent predictor of vessel wall inflammation [33]. Furthermore, plaque inflammation was shown to increase when components of the metabolic syndrome cluster [34, 35]. Patients with insulin resistance as well as type 2 diabetes have markedly increased vessel wall inflammation compared to nondiabetic controls [36]. In fact, in a study in 134 patients, with and without diabetes, the presence of type 2 diabetes was the strongest predictor for plaque inflammation. The magnitude of plaque inflammation increased with increments of fasting glucose levels [37].

Together these data from experimental and human studies indicate that hyperglycemia and impaired insulin signaling are causally involved in aggravating plaque inflammation. The inflamed plaque phenotype is considered prone to rupture and is associated with atherothrombotic events.

Endothelial Dysfunction in Diabetes

Loss of endothelial function precedes the development of diabetic vascular changes. In fact, ultrastructural changes of glomerular endothelium can be observed by transmission electron microscopy in diabetics, even before establishment of microalbuminuria [38, 39].

The formation of reactive oxygen species (ROS) plays a central role in the mechanism by which hyperglycemia induces endothelial damage [40]. Elevated glucose increases production of superoxide by the mitochondrial electron transport chain. The excessive mitochondrial ROS production has several effects. It induces protein kinase C isoforms, initiates the intracellular formation of glucose-derived advanced glycation end-products (AGEs) and increases glucose flux through the aldose reductase pathway causing sorbitol accumulation [40]. Furthermore, ROS triggers activation of poly (ADP ribose) polymerase, which is a nuclear enzyme implicated in the response to DNA injury. Its activation slows mitochondrial respiration causing cellular dysfunction [41]. ROS production induced by hyperglycemia also causes long-lasting epigenetic changes in the promotor of nuclear factor-κB which affects expression of adhesion molecules and chemoattractants [42]. These ROS activated pathways are critical mechanisms by which hyperglycemia induces endothelial dysfunction and play a pivotal role in developing diabetic vasculopathy [43–46].

The enhanced oxygen-derived free radical production observed in hyperglycemia inactivates nitric oxide (NO) bioavailability, causing impaired endothelial function and compromising endothelium-dependent relaxation [47]. Studies in endothelial NO synthase knockout mice showed that diabetic nephropathy development is accelerated [48]. This is corroborated by findings of a study in patients with an endothelial NO synthase polymorphism, which were shown to be at increased risk of developing end-stage renal disease due to diabetic nephropathy [49]. These data provide insight in the fact that both diabetic nephropathy and vascular disease share endothelial dysfunction and reduced NO bioavailability as a pathophysiologic process.

A way to assess endothelial function in vivo in humans is to quantify NO-dependent brachial artery flow-mediated vasodilation (FMD), which can be measured either invasively by forearm blood flow responses to methacholine chloride and nitroprus-

side or noninvasively by ultrasound [50]. In the Cardiovascular Health Study including 2792 adults aged 72–98 years that were followed for 5 years, FMD was an independent predictor of cardiovascular events [51]. In diabetic patients, forearm blood flow responses to methacholine chloride and nitroprusside were shown to be markedly attenuated when compared with nondiabetic subjects, indicating that NO-mediated vasodilation is impaired [52, 53]. Ultrasound studies measuring brachial artery FMD showed that hyperglycemia induced by an oral glucose load rapidly induces endothelial dysfunction in healthy subjects, as well as subjects with impaired glucose tolerance and diabetes mellitus type 2 [54–56]. The fact that children with type 1 diabetes already have a markedly decreased FMD illustrates that endothelial dysfunction is an early event in diabetic vascular disease [57].

Whether microalbuminuria is associated with endothelial dysfunction has also been a subject of investigation. In a study in type 1 diabetics, microalbuminuric patients were compared to normoalbuminuric diabetic patients under near-normoglycemic conditions using a euglycemic insulin clamp. They showed that microalbuminuric diabetic patients had markedly decreased FMD. Furthermore, the albumin excretion rate was inversely correlated with FMD [58]. The relation between microalbuminuria and FMD was confirmed in other studies in type 2 diabetics [59, 60]. Together these data indicate that endothelial function is impaired in diabetic patients but even more so if microalbuminuria is present.

Another endothelium-related topic that has gained interest in the last decade is the role of hyperglycemic damage to the endothelial glycocalyx. The glycocalyx is a glycosaminoglycan and hyaluronan-based endothelial surface layer that constitutes a gel-like permeability barrier preventing transvascular leakage of macromolecules. It has antithrombogenic properties and is involved in leukocyte trafficking [61]. Hyperglycemia induced in healthy subjects was shown to reduce glycocalyx volume and coincided with a decrease in FMD [62]. In patients with both type 1 and type 2 diabetes, the glycocalyx is markedly reduced when compared with normoglycemic subjects. Interestingly, the glycocalyx reduction was most profound when microalbuminuria was present [63, 64]. Other studies have established a direct link between glycocalyx disruption and the development of proteinuria [65, 66]. Collectively these data provide a pathogenic concept that places endothelial dysfunction and glycocalyx disruption at the center of the pathogenic process that drives both proteinuria as well as atherogenesis. This may provide an explanation why a markedly higher cardiovascular event rate is observed in diabetic patients with albuminuria as compared to diabetics with normoalbuminuria.

Cardiovascular Risk in Diabetes

Studies as early as the beginning of the twentieth century have indicated that patients with diabetes are at increased risk for developing cardiovascular events. Joslin reported in 1930 that 50% of diabetics died of atherosclerotic disease [67]. Later, the Framingham Heart Study reported on subjects that attended examinations in the 1950s and 1960s. The cardiovascular event rate was found to be doubled in diabetic

men and tripled in diabetic women, when compared to nondiabetics [68]. From 1973 to 1975 the Multiple Risk Factor Intervention Trial (MRFIT) examined and followed 361,662 men for an average of 12 years. Men with diabetes had a 3.2 times higher coronary heart disease mortality rate and 2.8 times higher stroke rate, compared to nondiabetics [69]. The INTERHEART study is a worldwide case-control study that included 15,152 patients with coronary artery disease and 14,820 controls between 1999 and 2003. Diabetes was again identified as an important risk factor of an initial acute myocardial infarction [70]. This was a consistent finding across all continents, with the lowest odds ratio in North America (1.75) and the highest odds ratio in China and Western Europe (5.07 and 4.29, respectively) [70]. Similar findings were observed in a meta-analysis, including 698,782 people from 102 prospective studies, showing that diabetes doubles the risk for coronary heart disease [5].

Collectively, these large observational studies provide a robust estimation of a two- to fourfold excess CVD risk in the diabetic population. This increased risk can in part be attributed to the harmful effects of hyperglycemia. In addition to hyperglycemia, various other risk factors contribute to the atherosclerotic cardiovascular event rate. Age, gender, smoking, and lack of exercise are important CVD risk factors in all populations and also apply to the diabetic population. Hypertension is important, which is extensively discussed in Chap. 20. Hypertension, dyslipidemia, and obesity are risk factors that frequently cluster in diabetic patients, which is commonly referred to as the "metabolic syndrome." All these risk factors apply to the general diabetic population. However, in the subgroup of patients with diabetic nephropathy, two other risk factors come into play: albuminuria and decline in renal function. These two risk factors are the major drivers of CVD risk in these patients.

Albuminuria, Decline in Renal Function, and Cardiovascular Risk

Microalbuminuria by itself confers a markedly increased CVD risk in diabetic patients. This is illustrated by the Heart Outcomes Prevention Evaluation (HOPE) trial, in which over 9000 patients with either a history of CVD or diabetes plus at least 1 CVD risk factor were included [71]. Patients with proteinuria, established diabetic nephropathy, or other significant renal disease were excluded. Microalbuminuria was defined as an albumin/creatinine ratio (ACR) of 2 mg/mmol or more. After a median follow-up of 4.5 years, the composite end point of myocardial infarction, stroke, and cardiovascular death occurred in 25% of diabetic patients with microalbuminuria, while this was 13.9% in diabetics without microalbuminuria. Interestingly, diabetics without microalbuminuria had an event rate that was similar to patients without diabetes or microalbuminuria (13.9% versus 13.8%), suggesting that diabetics only have excess CVD risk when microalbuminuria is present [71].

The fact that albuminuria and decline in renal function are major CVD risk factors independent of each other is well documented in various studies. One of them

is an elegant observational study using data from the UK Prospective Diabetes Study (UKPDS), in which 5097 patients were followed from diagnosis of diabetes through different stages of diabetic nephropathy. Ten years after the diagnosis of diabetes, 25% of patients had developed microalbuminuria, 5.3% macroalbuminuria, and 0.8% an elevated plasma creatinine level ≥ 175 umol/l or the need for renal replacement therapy. The annual cardiovascular mortality rate was 0.7% in patients without diabetic nephropathy, 2.0% with microalbuminuria, 3.5% with macroalbuminuria, and a staggering 12.1% for those with elevated plasma creatinine or need for renal replacement therapy [72].

These findings were corroborated by subsequent larger studies. In the Action in Diabetes and Vascular disease: preterAx and diamicroN-MR Controlled Evaluation (ADVANCE) study, Ninomiya et al. investigated the effects of urinary albumin-creatinine ratio and eGFR on CVD risk in 10,640 diabetic patients [73]. The average age was 66 years, the median duration of diabetes was 7 years, and the average follow-up was 4.3 years. The cardiovascular mortality rate was markedly affected by both the degree of eGFR as well as the degree of albuminuria. In diabetic patients with a normal kidney function (eGFR ≥90 ml/min/1.73m^2), microalbuminuria and macroalbuminuria were associated with a 1.96 and 2.87 higher cardiovascular mortality rate when compared to normoalbuminuria. In patients with an impaired renal function (eGFR <60 ml/min/1.73m^2), the cardiovascular mortality rate was 3.37 and 5.93 times increased if microalbuminuria or macroalbuminuria was present, when compared to diabetics with a normal kidney function and normoalbuminuria [73].

Convincing data on the association between impaired renal function, albuminuria and cardiovascular outcomes in diabetic patients are provided by the Chronic Kidney Disease Prognosis Consortium. They performed a meta-analysis in 1,024,977 participants from 30 general populations and high-risk cardiovascular cohorts and 13 chronic kidney disease cohorts. 128,505 of the participants were diabetics [74]. In diabetic patients with a normal kidney function, microalbuminuria (ACR 30–299 mg/g) and macroalbuminuria (ACR ≥ 300 mg/g) were associated with a 1.74 and 3.03 increased cardiovascular mortality rate, compared to diabetics without albuminuria. This is in line with the findings in the ADVANCE study described above [73]. The cardiovascular mortality was elevated even more if albuminuria was accompanied with an impaired renal function. With an eGFR of 45–59 ml/min/1.73m^2, the hazard ratios for microalbuminuria and macroalbuminuria were 2.27 and 3.24, respectively. These hazard ratios rise to 5.64 and 7.96 with an eGFR of 15–29 ml/min/1.73m^2. Patients with an eGFR <15 ml/min/1.73m^2 and macroalbuminuria had the highest cardiovascular risk. In fact, they had a 21.6 times higher risk of cardiovascular death. Of note, these hazard ratios apply to the comparison with diabetic patients with a normal kidney function without albuminuria. Interestingly, the cardiovascular risk seems to be mainly related to the decline in eGFR and presence albuminuria, and not so much by the presence or absence of diabetes [75]. In fact, cardiovascular mortality rates were very similar in diabetics and nondiabetics across the various ranges of renal function and albuminuria. This underscores the fact that is not so much the presence or absence of diabetes but instead the presence or absence of albuminuria and impaired renal function that dictates CVD risk.

Prevention of Atherosclerotic Disease

Cardiovascular risk factors are known to accumulate in diabetic patients, and therefore management of cardiovascular risk factors is paramount. When multiple risk factors are targeted simultaneously, with adequate glycemic control, renin-angiotensin system blockers, aspirin, and statins, marked benefit on cardiovascular mortality is observed [76]. The role and management of hypertension is discussed extensively in Chap. 20 and therefore not reviewed here.

Lifestyle

Lifestyle interventions are important to discuss with diabetic patients. Weight loss, increasing physical exercise, and dietary changes are of specific interest. These lifestyle adjustments can improve glycemic control as well as hypertension and are recommended by current guidelines. One would expect that the effect of lifestyle modification on glycemic control and hypertension would translate into benefit on cardiovascular outcomes; however the results of studies on this topic are disappointing. The Look AHEAD (Action for Health in Diabetes) study included 5145 patients with type 2 diabetes and a BMI > 25 kg/m2. Patients were randomized to standard intensive lifestyle modification or standard diabetes education. The intensive lifestyle modification consisted of caloric restriction, moderate-intensity physical activity, and weekly sessions with dietitians, behavioral psychologists, and exercise specialists. Weight loss medication and/or advanced behavioral strategies were used if weight loss goals were not achieved in the first 6 months. These efforts resulted in greater weight loss, better physical fitness, better glycemic control, and better blood pressure control. Nonetheless, after a median follow-up of 9.6 years, the occurrence of cardiovascular events was similar in both groups (HR 0.95, 95% CI 0.82–1.09) [77].

Probably the most effective lifestyle intervention for diabetic patients is smoking cessation. Smoking itself is associated an increased risk of developing diabetes and is known to aggravate insulin resistance, inflammation, and dyslipidemia [78]. Diabetic patients that smoke have a 50% higher total mortality and cardiovascular mortality rate as compared to diabetic patients that do not smoke [79]. Several studies have indicated that smoking is an independent risk factor for the development and progression of diabetic nephropathy [80–82].

Glycemic Control

Adequate glycemic control is of major importance for preventing CVD in both patients with type 1 and type 2 diabetes. How tight glycemic control should be has however been a topic of intense investigation.

For type 1 diabetes, important evidence is provided by the Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) follow-up period [83–85]. A total of 1441 type 1 diabetics were followed for an average of 27 years. During the first 6.5 years in the DCCT, patients were treated with either intensive insulin therapy or conventional therapy. At the end of the study, the mean HbA1c was 7.4% in the intensive-treatment group and 9.1% in the conventional-treatment group. The occurrence of a first cardiovascular event was reduced by 42% in the intensive therapy group. In the intensive group, less patients developed albuminuria, which in part explained the reduction in cardiovascular events [83]. Interestingly, there was a trend toward a sustained reduction in CVD due to the intensive insulin therapy during the first 6.5 years of the DCCT, despite the fact that there was no difference in glycemic control during the post-DCCT trial period [84, 85]. These findings are corroborated by a large observational study using the Swedish National Diabetes Register. A total of 33,915 type 1 diabetics were followed over an average of 8 years. Cardiovascular mortality was highest in patients with the highest HbA1c ($\geq$ 9.7%) and lowest in patients that achieved an HbA1c $\leq$ 6.9% [86].

Concerning glycemic control in type 2 diabetes, no clear benefit of intensive therapy on cardiovascular mortality has been established. The ACCORD and ADVANCE trials are large well-conducted randomized controlled trials that were unable to demonstrate a clear benefit of tight glycemic control on cardiovascular events, which has been nicely reviewed in a position statement by the American Diabetes Association, the American College of Cardiology Foundation, and the American Heart Association [87]. The ACCORD trial ($N = 10,251$) included patients with a history of CVD or high CVD risk. A median HbA1c of 6.4% was achieved in the intensive group versus 7.5% in the standard group. The study was ended prematurely because of an increased rate of mortality and cardiovascular death in the intensive therapy arm (HR 1.22; 95% CI, 1.01–1.46) [88]. The ADVANCE study ($N = 11,140$) also included patients with CVD or increased CVD risk. The median HbA1c level achieved in the intensive versus standard group were 6.3% and 7.0%, respectively. There was no difference in the overall or cardiovascular mortality between the groups, despite the fact that macroalbuminuria was markedly decreased in the intensive treatment arm [89]. In a meta-analysis including 34,912 patients from 28 trials, there was a reduction in the risk of nonfatal myocardial infarction (RR 0.87, 95% CI 0.77–0.98) with intensive glucose-lowering versus standard treatment [90]. However, intensive treatment did not significantly affect stroke, all-cause, or cardiovascular mortality [90].

European and American guidelines recommend an HbA1C goal of 7% (53 mmol/mol) for all nonpregnant adults. The evidence for this recommendation is strongest for type 1 diabetics and for patients with a short duration of type 2 diabetes. Less stringent HbA1C goals of <8% (64 mmol/mol) may be appropriate for patients with long-standing diabetes, especially if patients are prone for adverse events such as hypoglycemia [91, 92]. Recommendations for patients with diabetic nephropathy are the same as for diabetics that do not have diabetic nephropathy.

The effect of metformin on cardiovascular events in type 2 diabetic patients deserves specific mentioning. Cardiovascular mortality seems to be lower for metformin as compared to therapy with sulfonylureas [93]. Metformin is recommended as a first-line therapy in patients with type 2 diabetes [91, 92]. The use of metformin is however not recommended in patients with renal impairment [94].

Dyslipidemia

Dyslipidemia is frequently present in diabetic patients, especially in patients with type 2 diabetes. There is ample evidence that lipid lowering with statin therapy in diabetic patients is effective in reducing cardiovascular events. A meta-analysis of 14 randomized trials ($N = 18{,}686$) of statin therapy in diabetic patients, with a mean follow-up of 4.3 years, showed that for every 1 mmol/L reduction in LDL cholesterol, there was a 9% reduction in all-cause mortality and a 13% reduction in cardiovascular mortality [95]. In the subgroup of patients with diabetic nephropathy, effects of statin therapy on cardiovascular outcomes was similar as in diabetic patients without diabetic nephropathy [96]. Statin therapy is therefore recommended by European and American guidelines for all diabetic patients ≥40 years of age [91, 92]. There is paucity of evidence in patients younger than 40 years of age, but statin therapy is still recommended in these younger patients if they have an increased CVD risk profile or a history of atherosclerotic cardiovascular events [91, 92]. The initiation and intensification of statin therapy should be based on the patient's risk profile, rather than to aim for specific LDL cholesterol goals [91, 92]. Ezetimibe and PCSK9 inhibitors can be considered as adjunctive therapies in selected high-risk patients [91, 92]. Treatments with fibrates or niacin are not recommended [91, 92].

Antiplatelet Therapy

The use of antiplatelet therapy for primary prevention of CVD is controversial. The effect of aspirin is modest and comes at the cost of an increased risk of bleeding. A meta-analysis of 6 trials was performed and included 95,456 patients, of which 4% had diabetes [97]. In all patients (diabetic and nondiabetic), low-dose aspirin did not improve all-cause or cardiovascular mortality; however it did reduce the number of cardiovascular events (RR 0.87 (95% CI 0.79–0.96) [97]. In the subset of patients with diabetes ($N \approx 3800$), there was a nonsignificant risk reduction of 0.88 (95% CI 0.67–1.15) [97]. European and American guidelines suggest that low-dose aspirin (75–162 mg/day) can be considered for primary prevention of CVD in diabetic patients at increased cardiovascular risk (10-year risk >10%), if they are not at increased risk of bleeding [91, 92]. There is a well-documented benefit of aspirin therapy for secondary prevention.

Patients with diabetic nephropathy comprise a specific subpopulation, which confer an increased risk of CVD as well as an increased risk of bleeding. The evidence on the use of aspirin for primary prevention in this specific population is scarce. In a post hoc analysis of a randomized trial on primary prevention with aspirin in type 2 diabetics, Saito et al. did not observe a beneficial effect of aspirin in patients with an eGFR <60 ml/min/1.73m^2, but the number of patients in this analysis was too small to draw definite conclusions [98]. A recent meta-analysis of 3 trials including 4468 patients (diabetics and nondiabetics) with chronic kidney disease did not find a benefit of aspirin for primary prevention of cardiovascular disease [99].

Aspirin therapy for secondary prevention is widely recommended in diabetic patients [91, 92]. However, patients with diabetic nephropathy may have markedly impaired renal function in which the benefit versus harm of antiplatelet therapy may be different from the general population. In a Cochrane systematic review of 50 studies, including 27,139 patients with CKD, antiplatelet therapy (compared to placebo) reduced the risk of myocardial infarction (RR 0.87, 95% CI 0.76–0.99) but did not affect not-all-cause mortality or cardiovascular mortality. The risk of major bleeding was increased (RR 1.33, 95% CI 1.10–1.65) [100]. Furthermore, it is uncertain whether addition of glycoprotein IIb/IIIa inhibitors or clopidogrel to standard aspirin therapy is beneficial in patients with impaired renal function [101]. A meta-analysis of 9 trials included 9969 patients who had acute coronary syndromes or were undergoing percutaneous intervention. The addition of glycoprotein IIb/IIIa inhibitors or clopidogrel to standard aspirin therapy did not improve all-cause or cardiovascular mortality, but did increase serious bleeding [101].

This illustrates the fact that patients with diabetic nephropathy, especially those with more severe stages of CKD, comprise a specific population for which therapeutic benefit or harm may not necessarily be similar as in the general population. Good-quality data on the efficacy of primary prevention with aspirin in this population is lacking. Aspirin therapy for secondary prevention of CVD seems beneficial, but the benefit of adding glycoprotein IIb/IIIa inhibitors or clopidogrel to standard aspirin therapy is unclear. Benefit and harm should therefore be carefully evaluated for each individual patient.

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Part VII
Experimental Designs

Chapter 23
Animal Models of Diabetic Kidney Disease

Isabel Nguyen, Arianne van Koppen, and Jaap A. Joles

Introduction

This chapter focusses primarily on functional aspects of diabetic nephropathy (DN) as studied in animal models. As formal morphological proof by kidney biopsy of the development, presence or regression of DN is rarely indicated in the clinical setting, we decided to focus on functional rather than morphological aspects in animal models. Morphological characteristics of the most frequently used rodent models of DN are discussed in Chap. 8 of this book. In addition, a plethora of morphological studies in animal models of DN have recently and extensively been reviewed elsewhere [1–3]. Functional changes in DN are often termed diabetic kidney disease (DKD). Over time functional changes in DKD include characteristic early phases of normal glomerular filtration and hyperfiltration, followed by normofiltration and finally progressive loss of glomerular filtration rate (GFR; Fig. 23.1) [4]. However, this last phase is hard to achieve in most experimental models, because progression of the disease is hard to achieve in the short life span of rodents. Excess GFR in DKD is presumably at the expense of the renal functional reserve (RFR). The RFR can be assessed by infusing amino acids or dopamine [5, 6].

Nephron loss, which is continually masked by hyperfiltration, is believed to start in the early stages. Somewhere in the early phases, urinary albumin excretion (albuminuria, UAE) becomes evident and progressively increases until so many nephrons are lost that in the final stages UAE starts to fall. Theoretically the second

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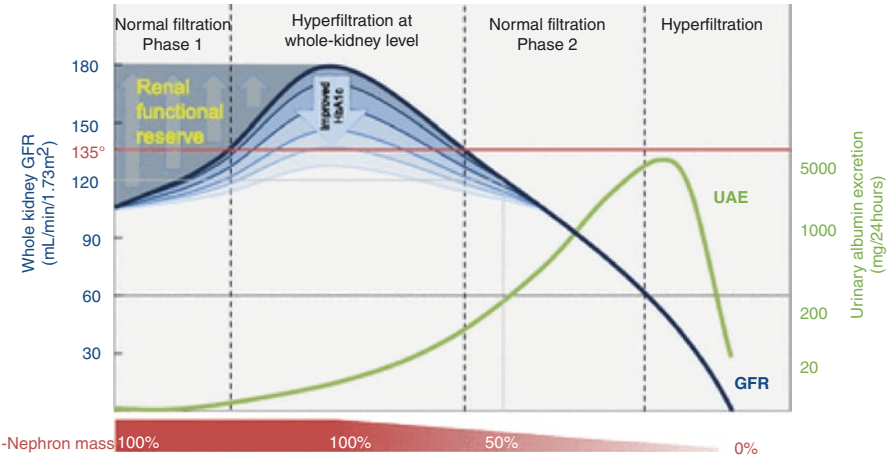

Fig. 23.1 Classic course of whole-kidney GFR and UAE according to the natural (proteinuric) pathway of DKD. (Reproduced with permission [4])

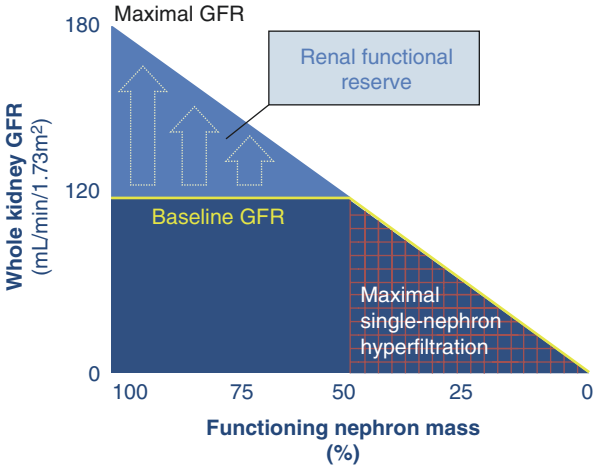

Fig. 23.2 Schematic representation of renal functional reserve in DKD. (Reproduced with permission [4])

phase of normofiltration can be realized at a 50% reduction of nephron number (Fig. 23.2) [4].

One of the recurring themes regarding animal models of DKD is that such models do not recapitulate every aspect of DKD (or DN) and often do not progress to renal failure [1, 2, 7, 8]. Probably, in the long run, many DKD models are progressive, but not fast enough to the investigator's liking. Be this as it may, we strongly feel that recapitulating every aspect of syndrome is not the purpose of an experimental model. On the contrary, an animal model should be tailored so that

it expresses a particular component or combination of components of the syndrome in a homogeneous fashion, allowing dissection and targeting of this aspect of the syndrome in isolation [9]. However, if one wishes to study biomarkers at every stage of DKD in diverse populations, then the optimal animal model by far is *Homo sapiens*. One should realize however that the first phases of the disease (stage 1–3) are clinically silent in terms of microalbuminuria, eGFR and other biomarkers, certainly when blood glucose and hypertension are controlled to some extent. Another misconception in our opinion is that a "good" animal model of DKD reliably predicts the effect of targeting a single component of DKD in diverse human populations. Such expectations will inevitably be disappointed. At best a tailored animal model of a certain aspect of DKD illustrates what one reasonably can expect in a specified human population of DKD where a specified aspect is also prominent.

One important consideration that should be taken into account is the way the disease is induced in experimental models. The best models should reflect the pathophysiological pathways involved in the development and progression of DKD in humans. Therefore, the main key processes leading to DKD should be considered when evaluating the most suitable tailored model. However, the mechanisms underlying the development of DKD and their sequential order are not entirely clear. In general, the main processes involved in progression of DKD besides hyperglycaemia include hyperlipidaemia/obesity and vascular changes induced by hypertension.

With these considerations as a leitmotif, we will scroll through the extensive literature on animal models of DKD. Naturally a selection of the thousands of published studies had to be made, and often we will limit ourselves to recent reviews. Per animal species we will discuss the most important models for type 1 and type 2 diabetes and where useful tabulate changes in the most important functional markers according to duration of diabetes following the sequence of events illustrated in Fig. 23.1.

Flies

Genetic ablation of insulin-producing cells located in the brain of *Drosophila melanogaster* can induce type 1 diabetes (T1D) [10]. Raising the larvae of *Drosophila* on a high-sugar diet induces obesity and insulin resistance in developing flies and many aspects of type 2 diabetes (T2D) [11]. Regarding DKD this model is particularly useful for the effects of DM2 on podocytes, illustrating typical loss of the Nephrin ortholog Sns [12]. Analogous changes in the pathway regulating Sns expression are documented in ob/ob mice (a model of DM2, see below) and in patients with DM2. Indeed, rescuing Sns even prolonged fly life

span. Diabetic cardiomyopathy has been quite extensively studied in diabetic flies [13]. However, besides the Sns (nephrin) pathway DKD remains to be explored in flies with DM. DM1 and DM2 models in *Drosophila* have recently been reviewed [14].

Worms

Refined carbohydrates (sugars) accelerate ageing and reduce life span in *Caenorhabditis elegans*, and much is known on dietary-induced changes in worm adipose tissue and more specifically on mitochondrial dynamics [15, 16]. However, although there is active research in *C. elegans* on renal disease caused by ciliopathies, no attention appears to have been focussed on DKD in these little worms [17].

Fish

The first excretory organs have evolved over 500 million years ago in fish. The main function of this organ was clearance of waste products via excretion and maintenance of an osmotic equilibrium in an aqueous environment. In a detailed review, Romagnani et al. described the evolutionary development of the kidneys in several species [18, 19]. The most remarkable difference between mammals and fish is the ability of continuous renal regeneration, e.g. to form new nephrons during adulthood. This makes the fish a very suitable model to study renal regeneration, but has as drawback that the fish a less likely model to study more chronic diseases. The zebrafish, *Danio rerio*, is the most extensive studied fish species. The complete genome is known, and their renal structure is simple, formed by a fused midline glomerulus with an ultrastructure that is indistinguishable from that in mammals, followed by one nephron consisting of a proximal and distal part adjacent to the each of the two cardinal veins while maintaining biological complexity inherent to the kidney of higher organisms.

Currently available models of renal failure in zebrafish have, due to spontaneous renal regeneration, a very short window of opportunity to test interventions. Other drawbacks of the models are that they are toxin-induced with the risk of overdosing, and toxin-induced damage is not cell type specific. In the field of diabetes, the zebrafish has been used to study glucose homeostasis and glucose (dis)regulation/hyperglycaemia. Diabetic complications include diabetic retinopathy and fatty liver disease. However, due to the regenerative nature of the kidney, these fish are not commonly used in the study of diabetic nephropathy. Mechanistic studies have been performed on podocyte damage on the role of CIN85 in podocyte damage in the zebrafish and the conserved nature of a DKD-inducing SNP which is abundant in both mouse and men [20, 21]. There is also a zebrafish model of diabetes mellitus and metabolic memory [22, 23]. These studies indicate that for mechanistic understanding of cellular and genetic events involved in DKD, the zebrafish can hold future study potential.

Rodents

Rodents are the most commonly used species in preclinical research and therefore the most commonly used animals in the study of diabetes. Although improvement in the management of hyperglycaemia and hypertension has reduced the number of patients reaching end-stage kidney disease (ESKD), DKD is still the most common cause of ESKD. As the underlying mechanisms are not fully understood, developing new therapies is a challenge. Rodent models can be used to gain insight in the sequential/temporal processes involved in onset, development and progression of the disease, which in turn can drive the development of targets and (pharmaceutical) interventions. However, this is hampered by the lack of reliable preclinical models. Using rodent models has advantages because their genetic blueprint is known and inducing genetic modifications such as knockout or overexpression is relatively easy. Furthermore, rodents breed rapidly and housing is relatively cheap. The utility of animal models in DKD has been constrained since most models fail to recapitulate important functional, structural and pathological features of advanced human DKD or only reach a mild disease stadium. We will discuss some of the most commonly used models of rodent T1D and T2D models and evaluate their utility. As mentioned above, our focus will be on functional readouts.

With regard to markers of renal function, rodent models of advanced DN should at least show a >50% decrease in renal function and at least a tenfold increase in albuminuria. However, for early DKD studies, this is too late [7] (Fig. 23.3).

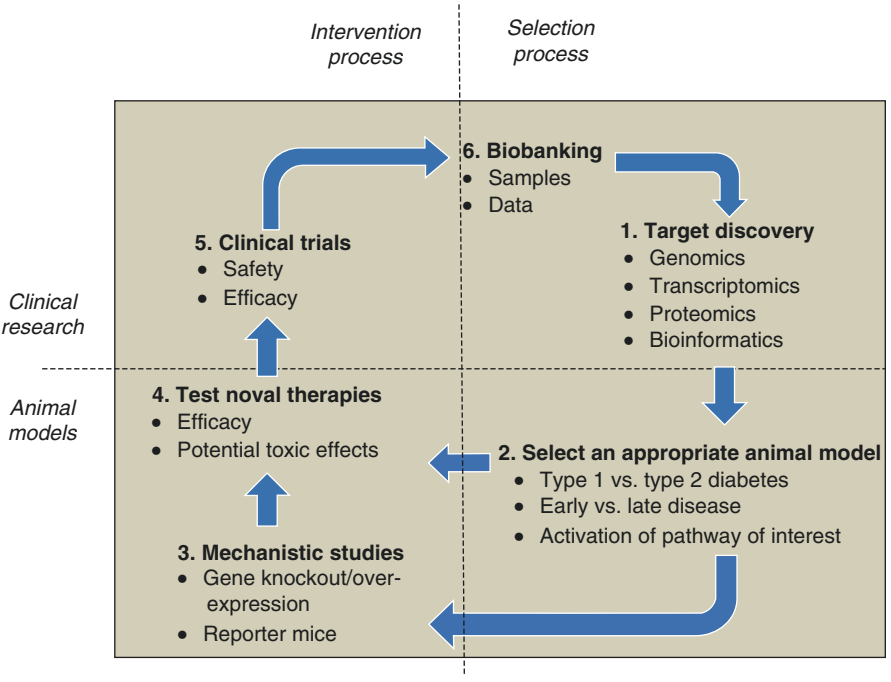

Fig. 23.3 Scheme for selection of appropriate model in studies on DKD. (Reprinted by permission from Springer in Betz and Conway [7])

Mice

One main drawback of using mice is that they are relatively resistant to the development of DKD. The C57/BL6 mouse is the most common strain used, and many genetic alterations have been performed on this background. However, the development of DKD and renal failure in this model is hard to achieve. Recent advances have focussed on accelerating renal injury either by performing knockout of key genes on C57/BL6 background or by identifying alternative strains that are more susceptible to develop DKD. Others try to mimic human disease by introducing additional factors involved in the development of DKD such as obesity or hypertension. Progression of disease development can induced by adding surgical, dietary or pharmaceutical interventions. Surgical interventions can include removal of renal mass, i.e. of one kidney (uninephrectomy) or even more (up to 15th/16th nephrectomy), hence increasing the burden on the remaining nephrons which might speed up disease progression. This requires surgical skills. Dietary interventions like high-fat diet or high-glucose diet are more easy to perform in large cohort studies but do not normally speed up disease development. Pharmaceutical interventions (ANGII, DOCA, etc.) also need surgery for implantation of pumps or pellets, and these agents must be carefully titrated. A large variation in efficacy has been observed in different strains of mice.

The minimal requirements of DKD model should be in accordance to the features observed in humans and therefore be characterized by the presence of hyperglycaemia and in the setting of T2D preferably in the context of metabolic overload/obesity/metabolic syndrome.

As in humans, DKD in mice is associated with increased risk for developing cardiovascular disease, and haemodynamic factors play an important role in de-progression of DKD. Diabetes induces vascular changes and dysfunction. Hypertension is not only a consequence of nephropathy but may also be involved in the development of DKD [4, 24]. Even a slight increase in blood pressure can already induce damage/leakage in the microvascular structure of the kidney, thereby causing albuminuria [25]. Inducing hypertension in mice is also quite complex. Several attempts have been made to achieve elevated blood pressure, but it is beyond the scope of this chapter to describe these in detail.

Type 1 Diabetes

Type 1 diabetes (T1D) is an auto-immune disease in which the immune system destroys the beta cells in the pancreas. In mice, this condition can be induced by using a toxin which also destroys the pancreatic beta cells. Many compounds have been tested to induce T1D in mice and rats; however, the most prominent and routinely used chemical is streptozotocin (STZ), which is a cytotoxic glucose analogue causing ablation of the pancreatic β-cells which then results in absolute insulin deficiency, hyperglycaemia and weight loss. STZ is taken up by

pancreatic β-cells via the glucose transporter GLUT2. Intracellular action of STZ results in changes of DNA causing its fragmentation. The main reason for the STZ-induced β-cell death is alkylation of DNA. STZ-induced DNA damage activates poly-ADP ribosylation. This process leads to depletion of NAD+, further reduction of the ATP content and subsequent inhibition of insulin synthesis and secretion, thereby causing insulin resistance. A high dose of STZ can be toxic and directly lead to organ injury, including the kidney. Repetitive injections with lower doses are therefore preferred. Besides insulin resistance, long-term STZ treatment induces weight loss and osmotic diuresis. STZ-treated mice do not develop hypertension.

An overview of studies performed in the most commonly used mouse of T1D-induced DKD is given in Table 23.1. The minimal requirements of the models are included. The models are ordered by strain.

C57Bl/6 Mice

C57Bl/6 mice are the most commonly used strain for target development and preclinical drug screening. The most common way of inducing DKD in this strain is by STZ injections. This results in mild to moderate albuminuria but does not reach a tenfold increase after 6 months (Table 23.1) [26]. After 5 weeks, hyperglycaemia was observed and reached a maximum of approximately 500 mg/dL. Albuminuria was only observed after 25 weeks, but GFR was increased after 5 weeks. Pathological changes include onset of mild glomerular damage, but there was no glomerulosclerosis by week 25. Slight tubular changes, but no tubular fibrosis, were observed [1, 26]. There are several genetic alterations on this C57BL/6 background.

Akita (Ins2+/C96Y) on C57Bl/6 Background

These mice have a mutation in the insulin gene which leads to a defect in folding of insulin and hence toxic accumulation [1]. These mice develop hyperglycaemia, mild hypertension, modest levels of albuminuria and pathology including mesangial matrix expansion and GBM thickening, without remarkable glomerular damage or tubulo-interstitial fibrosis, by 6 months of age [29]. This makes this model suitable to study early phases of DKD.

ApoE−/− on C57Bl6 Background

To induce hyperlipidaemia, ApoE−/− was induced on a C57Bl6 background. STZ-induced diabetes was combined with hyperlipidaemia due to Apo E deficiency. Besides hyperlipidaemia, these mice developed accelerated renal injury characterized by albuminuria and glomerular and tubulo-interstitial injury [28].

Table 23.1 The development of DKD over time is shown per mouse model

Model Strain and dose	Study length (weeks)	M/F	Blood glucose	BW	BP	GFR	UalbV/ UproV	GS	TI	Reference
C57Bl/6 + STZ 50 mg/kg i.p. single dose	5	M	↑	↔	N/A	↑ (FITC-inulin clearance)	↑	N/A	N/A	[26]
C57Bl/6 + STZ 75 mg/kg i.p. single dose	5	M	↑	↓	N/A	↑ (FITC-inulin clearance)	↓	N/A	N/A	[27]
Balb/c + STZ 67 mg/kg i.p. single dose	5	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↔	N/A	N/A	[27]
DBA/2J + STZ 40 mg/kg i.p. single dose	5	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	N/A	N/A	[26]
A/J + STZ 50 mg/kg i.p. single dose	5	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	N/A	N/A	[26]
FVB + STZ 50 mg/kg i.p. single dose	5	M	↑	↓	N/A	↑ (FITC-inulin clearance)	↓	N/A	N/A	[26]
MRL/MpJ + STZ 50 mg/kg i.p. single dose	5	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	N/A	N/A	[26]
129Sv + STZ 75 mg/kg i.p. single dose	5	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	N/A	N/A	[27]
KKH1J + STZ 50 mg/kg i.p. single dose	5	M	↑	↓	N/A	↑ (FITC-inulin clearance)	↑	N/A	N/A	[26]
C57Bl/6 + STZ 75 mg/kg i.p. single dose	10	M	↑	↓	N/A	↑ (FITC-inulin clearance)	↓	↔	N/A	[27]
Balb/c + STZ 67 mg/kg i.p. single dose	10	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	↑	N/A	[27]
129Sv + STZ 75 mg/kg i.p. single dose	10	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	↔	N/A	[27]
C57Bl/6 + STZ 50 mg/kg i.p. single dose	15	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↓	N/A	N/A	[26]

DBA/2 J + STZ 40 mg/kg i.p. single dose	15	M	↑	↓	N/A	↑ (FITC-inulin clearance)	↑	N/A	N/A	[26]
A/J + STZ 50 mg/kg i.p. single dose	15	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	N/A	N/A	[26]
FVB + STZ 50 mg/kg i.p. single dose	15	M	↑	↔	N/A	↑ (FITC-inulin clearance)	N/A	N/A	N/A	[26]
MRL/MpJ + STZ 50 mg/kg i.p. single dose	15	M	↑	↓	N/A	↑ (FITC-inulin clearance)	↑	N/A	N/A	[26]
KKH1J + STZ 50 mg/kg i.p. single dose	15	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	N/A	N/A	[26]
ApoE−/− on C57Bl/6 + STZ 55 mg/kg i.p. single dose	20	M	↑	↓	↔	N/A	↑	↑	↑	[28]
C57Bl/6 + STZ 50 mg/kg i.p. single dose	25	M	↑	↑	N/A	↔ (FITC-inulin clearance)	↑	↑	N/A	[26]
DBA/2J + STZ 40 mg/kg i.p. single dose	25	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	↑	N/A	[26]
A/J + STZ 50 mg/kg i.p. single dose	25	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↓	↔	N/A	[26]
FVB + STZ 50 mg/kg i.p. single dose	25	M	↑	↔	N/A	↑ (FITC-inulin clearance)	↑	N/A	N/A	[26]
MRL/MpJ + STZ 50 mg/kg i.p. single dose	25	M	↑	↓	N/A	↑ (FITC-inulin clearance)	↑	N/A	N/A	[26]
KKH1J + STZ 50 mg/kg i.p. single dose	25	M	↑	↓	N/A	↔ (FITC-inulin clearance)	↑	↑	N/A	[26]

Only the most common used models in which more than one time point was described in one study were included
Abbreviations: *M/F* male/female, *BW* body weight, *BP* blood pressure, *GFR* glomerular filtration rate, *UalbV*/*UproV* albuminuria/proteinuria, *GS* glomerulosclerosis, *TI tubulo-interstitial injury*, *N/A* not applicable

eNOS−/− on C57Bl/6 Background

STZ was used to induce diabetes in eNOS−/− mice. STZ dose and administration regime have been shown to be important factors involved in disease development [30]. Overall, eNOS deficiency leads to advanced nephropathic changes with features of progressive DKD, including pronounced albuminuria and glomerulosclerosis. This model can be used to study the more advanced stages of DKD development.

BALb/c

STZ in Balb/c mice induced glomerular damage but did not lead to increased GFR [1, 27].

DBA/2J

STZ induces albuminuria after 5 weeks and severe albuminuria at 25 weeks. DBA/2J mice develop more marked albuminuria than C57/BL6 [26]. However, long-term follow-up also shows development of features of IgA nephropathy. Pathological characteristics include nodular sclerosis and tubular damage but do not reach fibrosis. Mortality rate increased after 25 weeks. This model is a suitable model for mild to moderate disease.

Akita (Ins2+ C96Y0 on DBA/2J Background

This model develops hyperglycaemia to the same extent as the C57Bl/6 and 129Sv Akita mice but exhibits more albuminuria compared to C57Bl/6 and 129 SV mice without mesangial matrix expansion. Blood pressure in nondiabetic animals was elevated compared to other strains, but no further increase was seen under diabetic conditions [31]. Pathological changes were only mild.

OVE on FVB Background

Mutation in the calmodulin gene results in toxic accumulation of defective proteins in the beta cells. At 2 months of age, albuminuria is progressive, and GFR shows a decline from 5 to 9 months of age. SBP is increased from 3 months onwards. Histological evaluation revealed more advanced stages of DKD characterized by nodular glomerulosclerosis and tubulo-interstitial fibrosis [32]. Development of DKD can be further exacerbated by UNX [33, 34]. Although this model shows the advanced characteristics of DKD, it is currently not used for preclinical studies due to low viability.

TTRhRen on FVB Background

These transgenic mice express active human renin in the liver. STZ induced DKD with hypertension due to human renin overproduction. On an OVE26/FVB background, mice develop renin-dependent hypertension, and on OVE26 background they develop significant albuminuria, mesangial expansion, tubulo-interstitial fibrosis and decline in renal function at 20 weeks [35].

CD1 Mice

STZ in these mice leads to chronic renal failure and tubulo-interstitial fibrosis within 6 months. Albuminuria was more than tenfold increased [36]. Although nodular sclerosis was not observed, this model recapitulated the more advanced stage of DKD.

129/SV Mice

The 129/SV strain is known to be more prone to develop renal damage, possibly due to the appearance of two renin receptors [1, 37]. STZ induces mild to moderate albuminuria, but no advanced features such as nodular sclerosis or tubulo-interstitial fibrosis were observed. Despite their increased renal susceptibility, development of DKD is not progressive in wild-type 129/SV mice [36].

Akita (Ins2+/C96Y) on 129/SV Background

This model is also characterized by hyperglycaemia. Albuminuria was higher compared with the C57Bl/6 Akita strain but did not reach the albuminuria level found in the DBA/2J Akita strain. Mesangial matrix expansion was observed but no glomerulosclerosis. STZ increased systolic blood pressure compared to nondiabetic mice. Renal damage did not reach the advanced stages of sclerosis and fibrosis [1].

KKH1J Mice

STZ induced albuminuria and moderate glomerular damage [26]. Hyperfiltration was observed also indicating that this model reflects the early onset of DKD.

NOD Mice + STZ

Other models which are less common but might be of interest include the NONcNZO10/LtJ mice [38, 39]. This model shows mild features of DKD on a background of maturity-onset hyperglycaemia induced by obesity (see T2D).

Most mouse models of T1D represent the early phases of DKD development. Although there are models which are characterized by a more advanced DKD stage, these models are scarce or show comorbidity and/or low viability. Overall, it is clear that there is different susceptibility to DKD in inbred mice. The DBA/2J and KK/HIJ mice are in general more prone to develop DKD as characterized by pronounced albuminuria and morphological changes including glomerulosclerosis. Pronounced tubulo-interstitial damage was not observed which indicates that mouse models generally do not reflect the late-stage features of DKD. One might consider, however, that for studying these late stages, chronic kidney disease models in general might also be useful.

Type 2 Diabetes

Models of T2D typically use hereditary obesity which can be leptin deficient (ob/ob mice) or have inactivating mutations in the leptin receptor (db/db mice). These animals exhibit hyperphagia, obesity and insulin resistance and develop relative insulin resistance in the first 8 weeks of life. Dietary or pharmaceutical interventions on top of this genetic background can be used to further induce features of DKD. The degree of hyperglycaemia depends on the nature of the genetic modification and the strain and gender of the mice. Again, the C57BL6 mice are relatively resistant.

As there are many experimental models published in literature, an overview of the most commonly used T2D-induced DKD models which exhibit hyperglycaemia and obesity is given below. Dietary or pharmacological interventions to further progress disease development are not included in this overview.

Db/db on C57BL/Ks Background

The db/db mutation, on a C57BL/Ks background, was the first mutation described to influence the onset of diabetes in mice. Typical features resemble the early onset of human DKD including albuminuria from 8 weeks onwards, although this is not very progressive [40]. Cohen et al. described time course of the mesangial expansion [41]. Nodular lesions were observed from 22 weeks onwards. Further disease development can be induced by UNX [42]. This model represents a good model to study the early changes in human DN.

Db/db eNOS−/− on C57BL/Ks

DN is characterized by microvascular damage, targeting the endothelium, leading to more rapid progression. The db/db eNOS−/− mice develop albuminuria and pathological features resembling the human situation including nodular sclerosis. More importantly, decreased GFR is observed which parallels the later stages of DN in

human and is currently seen as the most promising model to study later phases of disease [43, 44].

Ob/ob Mutation

The ob/ob mutation exists on different backgrounds including the DBA2/J and C57Bl/6J, C57BL/Ks and FVB strains. Chua et al. summarized the differences in phenotype [45]. Overall, on the C57Bl/6j background, disease development is relatively mild, whereas on the FVB background, more pronounced morphological differences are observed [42]. This is a suitable model to investigate the onset of disease on a background of metabolic syndrome but lacks the progressive phase.

Ob/ob BTBR

Black and tan brachyuric mouse are naturally insulin resistant, and when the ob/ob mutation is placed in this strain, the mice exhibit sustained hyperglycaemia from 6 weeks onwards. This mouse model resembles classical features of human DKD including pathological changes as arteriolar hyalinosis, mesangial expansion, mesangiolysis, focal nodular glomerulosclerosis and reduction of podocyte number. The advantage of this model is that DKD is developed more rapidly compared to other strains. This presents an attractive model to study classical features of moderate DKD in humans. The mice are hypotensive though and difficult to breed and have high mortality rates around 24 weeks of age [46].

KK and KKay Mice

Combining the agouti gene which is related to obesity with the KK mice, known for their spontaneous hyperglycaemia, resulted in a model expressing two main pathologies related to human DKD. These mice develop albuminuria and increased blood pressure from 10 weeks onwards [47–49]. On pathology, they reach the early stages of DN which can be accelerated with UNX. A disadvantage is the spontaneous development of hydronephrosis at later age [50] (Table. 23.2).

Overall, there are models that do exhibit features of advanced human DKD, but all these models have their own disadvantages. The eNOS−/− db/db mice are a double knockout where only 1/4 of the offspring can be used, the ob/ob BTBR are difficult to breed and have high mortality rates at older age, and the KKAy develop obstructive uropathy at older age. This shows that there is no easy accessible and applicable model of advanced T2D which resembles characteristics of human DKD. In all these models, the increase in albuminuria is modest, and a progressive decline in renal function does not develop. Information on the presence of tubulointerstitial damage/fibrosis is often lacking, suggesting that this is not reached in

Table 23.2 The development of DKD over time is shown per T2D model

Model Strain and dose	Study length (weeks)	M/F	Blood glucose	BW	BP	GFR	UalbV/ UproV	GS	TI	Reference
Db/db on C57BL/ KS	4	M	↑	↑	N/A	N/A	↔	↔	↔	[51]
Ob/ob BTBR	4	M	↑	↑	N/A	N/A	↔	N/A	N/A	[46]
Ob/ob BTBR	4	F	↑	↑	N/A	N/A	↔	N/A	N/A	[46]
Db/db on C57BL/ KS	8	M	↑	↑	N/A	N/A	N/A	↔	↔	[40]
Ob/ob BTBR	8	M	↑	↑	N/A	N/A	↑	↑	N/A	[46]
Ob/ob BTBR	8	F	↑	↑	N/A	N/A	↑	↑	N/A	[46]
Ob/ob BTBR	8	F	↑	↑	↓	N/A	N/A	N/A	N/A	[52]
Ob/ob on C57BL6	10	F	↑	↑	N/A	N/A	N/A	N/A	N/A	[53]
Ob/ob on FVB	10	F	↑	↑	N/A	N/A	N/A	N/A	N/A	[53]
KKAy	10	F	↑	N/A	N/A	N/A	↑	N/A	N/A	[54]
Ob/ob BTBR	11	F	↑	↑	N/A	N/A	N/A	N/A	N/A	[52]
Db/db on C57BL/ KS	12	M	↑	↑	N/A	N/A	↑	↔	↔	[51]
Ob/ob BTBR	12	M	↑	↑	N/A	N/A	↑	↑	↑	[46]
Ob/ob BTBR	12	F	↑	↑	N/A	N/A	↑	↑	↑	[46]
Ob/ob BTBR	14	F	↑	↑	N/A	N/A	N/A	N/A	N/A	[52]
KKAy	14	F	↑	N/A	N/A	N/A	↑	N/A	N/A	[54]
Db/db on C57BL/ KS	16	M	↑	↑	N/A	N/A	↑	↔	↔	[40]
Ob/ob BTBR	16	M	↑	↑	N/A	N/A	↑	↑	↑	[46]
Ob/ob BTBR	16	F	↑	↑	N/A	N/A	↑	↑	↑	[46]
KKAy	18	F	↑	N/A	N/A	N/A	↑	N/A	N/A	[54]
Ob/ob BTBR	20	M	↑	↑	N/A	N/A	↑	↑	↑	[46]
Ob/ob BTBR	20	F	↑	↑	N/A	N/A	↑	↑	↑	[46]

Table 23.2 (continued)

Model Strain and dose	Study length (weeks)	M/F	Blood glucose	BW	BP	GFR	UalbV/ UproV	GS	TI	Reference
Ob/ob BTBR	20	F	↑	↑	↓	↑ (creatinine clearance)	N/A	↑	N/A	[52]
Db/db on C57BL/ KS	21–25	M	↑	↑	↑	N/A	↑	↔	↔	[40]
KKAy	22	F	↑	N/A	N/A	N/A	↑	N/A	N/A	[54]
Ob/ob BTBR	22–24	M	↑	↑	N/A	N/A	↑	↑	↑	[46]
Ob/ob BTBR	22–24	F	↑	↑	N/A	N/A	↑	↑	↑	[46]
Ob/ob BTBR	24	F	↑	↑	NA	↑↑	↑	↑	N/A	[55]
Db/db eNOS –/–	24–26	M	↑	↑	↑	↔	↓	↑	↔	[30, 43]
KKAy	26	F	↑	↑	N/A	N/A	↑	↑	N/A	[54]

Only the most commonly used models from which more than one time point was described in one study were included

Abbreviations: *M/F* male/female, *BW* body weight, *BP* blood pressure, *GFR* glomerular filtration rate, *UalbV/ UproV* albuminuria/proteinuria, *GS* glomerulosclerosis, *TI* tubulo-interstitial injury, *N/A* not applicable

most models. When it is found, the tubulo-interstitial fibrosis is only very mild. In contrast to T1D studies, for T2D studies, both male and female mice are used.

As there is a plethora of therapeutic interventions tested in both T1D and T2D in mice, an overview is not given in this chapter. Therapies include (but are not limited to) pharmaceutical compounds and biological compounds, e.g. plant/herb extracts and cellular therapies [56–59]. Suggested reviews covering this topic include [60–63].:

Rats

The large body of knowledge available on rat physiology makes it the species of choice for modelling aspects in DKD and for executing therapeutic strategies in vivo [2]. Body size (compared to mice) facilitates repeated blood sampling and monitoring of renal and cardiovascular function [7]. Furthermore, rats have shown to be more susceptible than mice to many cardiovascular diseases including hypertension, and for many traits, genetics and pathophysiology in rats have proven more relevant to human disease [7, 64].

In this part, we will provide an overview of the recent literature of widely available rat models used for DKD. As in the mice, a distinction will be made between T1D and T2D.

Type 1 Diabetes

An overview of studies performed on T1D-induced DKD is given in Table 23.1. Due to their predictable symptom onset and relatively low cost compared to breeding spontaneously diabetic rats, alloxan and streptozotocin (STZ) have been in use for many years.

Many compounds have been tested to induce T1D in mice and rats; however, the most prominent and routinely used chemicals are alloxan and streptozotocin (STZ), which are both cytotoxic glucose analogues. Although their cytotoxic effect is achieved via different downstream pathways, both ultimately cause ablation of the pancreatic β-cells which then results in absolute insulin deficiency, hyperglycaemia and weight loss.

The toxic effect of alloxan on pancreatic β-cells is the sum of several processes such as oxidation of essential thiol groups, inhibition of glucokinase, generation of free radical and disturbances in intracellular calcium homeostasis [65]. Alloxan exerts its diabetogenic actions when it is parenterally administered: intravenously, intraperitoneally or subcutaneously. The dose required for inducing diabetes depends on the animal species, route of administration and nutritional status. The range of diabetogenic dose of alloxan is quite narrow, and even slight overdosing may be very toxic.

Table 23.3 Overview of rat models of T1D with renal pathophysiology

Model Strain and dose	M/F	Age at start (weeks)	Study length (weeks)	Blood glucose	BP	GFR	UalbV/ UproV	GS	Reference
Wistar rat + STZ 55 mg/kg i.p single dose	M	N/A	2	↑	N/A	↑ (creatinine clearance)	↑	N/A	[66]
Wistar rat + STZ 50 mg/kg i.p. single dose	M	10–14	4	↑	N/A	↑ (sinistrin clearance)	↑	N/A	[67]
Wistar rat + STZ 60 mg/kg i.p. single dose	M	N/A	4	N/A	N/A	↓ (creatinine clearance)	↑	↑	[68]

Table 23.3 (continued)

Model Strain and dose	M/F	Age at start (weeks)	Study length (weeks)	Blood glucose	BP	GFR	UalbV/ UproV	GS	Reference
Wistar rat + STZ 65 mg/kg i.v. single dose	M	N/A	4	↑	↔	↓ (inulin clearance)	N/A	N/A	[69]
Wistar rat + STZ 60 mg/kg i.v. single dose	M	N/A	9	↑	N/A	↓ (creatinine clearance)	↑	↑	[70]
Sprague Dawley + STZ 60 mg/kg i.p. single dose	M	N/A	9	↑	↑	↑ (creatinine clearance)	↑	N/A	[71]
Wistar + STZ 60 mg/kg i.v. single dose	M	8–10	10	↑↑	N/A	↓↓ (creatinine clearance)	↑↑	↑↑	[72]
Wistar + STZ 60 mg/kg i.v. single dose	M	8–10	10	↑↑	N/A	↓↓ (creatinine clearance)	↑↑	↑↑	[73]
Wistar rat + STZ 65 mg/kg i.v. single dose	M	N/A	12	↑	N/A	↓ (creatinine clearance)	↔	N/A	[74]
Sprague Dawley + STZ 65 mg/kg i.p. single dose	M	N/A	13	↑	N/A	↑ (creatinine clearance)	N/A	↑	[75]
Sprague Dawley + alloxan 150 mg/kg i.p. single dose	M	N/A	6	↑	N/A	↓ (serum creatinine+urea)	N/A	N/A	[76]

Abbreviations: *M/F* male/female, *BP* blood pressure, *GFR* glomerular filtration rate, *UalbV/UproV* albuminuria/proteinuria, *GS* glomerulosclerosis, *N/A* not applicable

Table 23.3 illustrates that in Wistar rats, hyperfiltration is an early phenomenon and decreases in GFR are already observed 4 weeks after STZ. In contrast hyperfiltration persists up to 13 weeks in Sprague-Dawley rats. Albuminuria is increased at every stage which is in contrast with the situation in humans. There is a striking lack of studies in female rats, and very few studies of T1D measure BP.

These STZ-induced diabetic rat models have been used extensively to study therapeutic interventions to ameliorate DKD. Naturally, many of these studies focus on lowering the blood glucose levels in these animals. Although the glucose-lowering agents do not seem to have an effect on the glucose levels itself in Wistar rats, it seems to improve the hyperfiltration seen in this model (Table 23.4). Additionally, antihypertensive therapeutics lead to normalization of the hyperfiltration and a large improvement in GS.

Type 2 Diabetes

For rat models of T2D, a further distinction can be made between nonobese and obese rat strains. As in mice, obese models are comprised of strains that have leptin deficiency or inactivating mutations in the leptin receptor. An overview of T2D-induced DKD is given in Table 23.5.

Zucker Diabetic Fatty Rats

The obese Zucker fatty (ZF) harbours a homozygous missense mutation (fa) in the fa gene that encodes for the leptin receptor. This strain shows hyperphagia, obesity and hyperlipidaemia with only a mild elevation in blood glucose levels. By cross-breeding the ZF rat with Wistar Kyoto rats, a strain which is insulin-resistant and less tolerant to glucose, the Zucker diabetic fatty (ZDF) rat, was derived [86]. These rats exhibit obesity with diabetes and are widely used in the study of T2D and its complications [1]. Overt diabetes is found from an early stage in this model, despite compensatory hypersecretion of insulin, indicating insulin resistance [86]. Due to exhaustion of insulin secretion with impaired glucose tolerance, these rats become overtly diabetic between 8 and 10 weeks of age [113]. ZDF rats spontaneously develop DKD characterized by heavy proteinuria.

ZSF 1 Rats

The obese diabetic Zucker fatty/spontaneously hypertensive heart failure F1 hybrid (ZSF1) is derived by crossing rat strains with two different leptin receptor mutations (fa and facp): a lean female Zucker diabetic fatty rat (ZDF, fa) and a lean male spontaneously hypertensive heart failure rat (SHHF, facp). Both lean

Table 23.4 Overview of interventions in rat models of T1D with renal pathophysiology

Animal model	M/F	Age at start (weeks)	Drug (duration; weeks)	Intervention target	Blood glucose	BP	GFR	Chol	Trig	UalbV/ UproV	GS	TI	Reference
Wistar rat + STZ i.p. 65 mg/kg single dose	F	N/A	Aliskiren (50 mg/kg); 4	↓ BP	↓	↓	↓ (serum urea and creatinine)	N/A	N/A	↓	N/A	N/A	[77]
SD rat + STZ i.v. 65 mg/kg single dose	M	5	Exendin-4 (10 mg/kg); 8	↓ Blood glucose	↔	↔	↓ (creatinine clearance)	N/A	N/A	↓	↓↓	N/A	[78]
Wistar rat + STZ i.p. 65 mg/kg single dose	M	26	Enalapril (10 mg/kg); 8	↓ BP	↓↓↓	N/A	↓ (creatinine clearance)	N/A	N/A	↓↓↓	↓	↓↓↓	[79]
Wistar rat + STZ i.p. 65 mg/kg single dose	M	26	Valsartan (50 mg/kg); 8	↓ BP	↔	N/A	↓ (creatinine clearance)	N/A	N/A	↓↓↓	↓↓↓	↓↓↓	[79]
Wistar rat + STZ 45 mg/kg single dose	M	N/A	Liraglutide (0.03 mg/kg); 12	↓ Blood glucose	↔	↔	↓↓ (serum urea) ↓ (serum creatinine)	↓	↔	↔	↓↓	N/A	[80]
Wistar rat + STZ i.v. 30 mg/kg single dose	M	7	Metformin (70 mg/kg); 13	↓ Blood glucose	↓	N/A	↔ (creatinine clearance)	↓	↓	N/A	↓↓	N/A	[81]
Wistar rat + STZ 30 mg/kg single dose	M	7	Simvastatin (2 mg/kg); 13	↓ Cholesterol	↔	N/A	↑↑ (creatinine clearance)	↓	↓	N/A	↓↓↓	N/A	[82]
SD rat + STZ i.p. 60 mg/kg single dose	M	5–7	Vildagliptin (8 mg/kg); 24	↓ Blood glucose	↔	N/A	↑↑ (creatinine clearance)	N/A	N/A	↓↓	↓↓	N/A	[83]

Arrows indicate changes vs. diabetic rats without the intervention

Abbreviations: *M/F* male/female, *BP* blood pressure, *GFR* glomerular filtration rate, *Chol* cholesterol, *Trig* triglycerides, *UalbV/ UproV* albuminuria/proteinuria, *GS* glomerulosclerosis, *TI* tubulo-interstitial injury, *N/A* not applicable

Table 23.5 Overview of rat models of T2D with renal pathophysiology

Model strain	M/F	Age at start (weeks)	Study length (weeks)	Blood glucose	BP	GFR	UalbV/ UproV	Chol	Trig	GS	TI	Reference
ZSF1	M	8	<1	↑	↑	↔ (inulin clearance)	↑↑	↑	↑	N/A	N/A	[84]
ZDF	F	?	5	↑	↑	↑↑ (creatinine clearance)	↑↑	↑↑↑	↑↑	N/A	N/A	[85]
ZDF	M	7	6	↑	↔	↑ (inulin clearance)	↑	N/A	N/A	↔	↔	[86]
ZDF	M	6	8	↑↑	↓	↑ (creatinine clearance)	↑↑	↑↑	↑↑	↑	↑	[87]
WF	M	6	8	↑↑	↔	↔ (plasma creatinine)	↑	N/A	N/A	N/A	N/A	[88]
ZDF	M	13	8	↑	N/A	↓ (creatinine clearance)	↑	N/A	N/A	↑	↑	[89]
ZSF1	M	35	9	↑	↑	↓ (inulin clearance)	↑	N/A	N/A	N/A	N/A	[90]
SDT fatty	M	4	12	↑↑	N/A	↑ (creatinine clearance)	↑↑	↑↑	↑↑	↑	↑	[91]
OLETF	M	14	12	↑	N/A	↓ (creatinine clearance)	↑	N/A	N/A	↑	↑	[92]
OLETF	M	6	12	↑↑	↑/↔	↔ (creatinine clearance)	↔	↑	↔	↑↑	↑	[93]
ZSF1	M	8	12	↑↑	↑	↓ (creatinine clearance)	↑	↑↑	↑↑↑	↑	↑	[94]
GK	M	6	13	↑↑	N/A	↑ (creatinine clearance)	↑	N/A	N/A	N/A	N/A	[95]
ZDF	M	5–6	13	↑↑	↔	↑ (creatinine clearance)	↑	↔	↑↑	↑	↑	[96]
ZDF	M	6	13	↑↑	N/A	↓↓ (creatinine clearance)	↑↑	N/A	N/A	↑	↑	[97]
ZDF	M	7	14	↑↑	↔	↑ (inulin clearance)	↑	N/A	N/A	↑	↑	[86]
ZDF	M	8	16	↑	↔	↔ (creatinine clearance)	↑↑	↑↑	↑↑↑	N/A	N/A	[98]
ZSF1	M	9	16	N/A	↑	↓ (creatinine clearance)	↑↑	N/A	N/A	↑↑	↑↑	[99]
ZSF1	M	11	20	↑↑	↑	↓ (inulin clearance)	↑	↑	↑	↑	↑	[100]
GK	M	16	20	↑↑	N/A	↔ (plasma creatinine+urea)	↑↑	↑	↑↑	↑	↑	[101]
ZDF	M	9	22	↑↑	↑↑	↔ (inulin clearance)	↑↑	↑↑	↑↑	↑↑	↑↑	[102]
ZDF	M	7	23	↑↑	↔	↔ (inulin clearance)	↑	N/A	N/A	↑	↑	[86]
SDT fatty	F	6	24	↑↑	N/A	↑↑ (creatinine clearance)	↑↑	↑↑	↑↑	↑	↑	[103]

WF	M	6	24	↑↑	↔	↓ (creatinine clearance)	↑	↑↑	↑↑	↑	↑	[104]
ZSF1	M	8	24	↑	↔	↔ (inulin clearance)	↑	↑	↑	↑	↑	[84]
ZDF	M	12	26	↑↑	↑	↓↓ (creatinine clearance)	↑↑	↑↑	↑↑	↑↑	↑↑	[105]
ZF	F	6	26	↔	↑↑	↓ (plasma creatinine)	↑↑	↑↑↑	↑↑↑	N/A	N/A	[106]
ZDF	M	7	28	↑	N/A	↑ (creatinine clearance)	↑	N/A	N/A	↑	↑	[107]
SDT fatty	M	4	28	↑	N/A	↓ (creatinine clearance)	↑	↑↑	↑	↑	↑	[91]
GK	M + F	6	29	↑	↔	↔ (serum creatinine)	↔	↔	N/A	↑	↑	[108]
ZSF1	M	?	29	↑↑	↑	↓ (creatinine clearance)	↑	↑↑	↑↑	↑	↑	[109]
WF	M	27	39	↑↑	↔	↔ (creatinine clearance)	↑↑	↑↑	↑↑	↑↑	N/A	[110]
T2DN/ mcwi	M	12	40	↑	N/A	↓ (plasma creatinine)	↑	↑	↑	↑	N/A	[111]
T2DN/ mcwi	M	12	65	↑	↔	↓ (inulin clearance)	↑	N/A	N/A	↑	↑	[112]

Abbreviations: *M/F* male/female, *BP* blood pressure, *GFR* glomerular filtration rate, *Chol* cholesterol, *Trig* triglycerides, *UalbV/ UproV* albuminuria/proteinuria, *GS* glomerulosclerosis, *TI* tubulo-interstitial injury, *N/A* not applicable

and obese ZSF1 rats show elevated blood pressure as they inherit the hypertensive gene from the SHR strain. Only the homozygous obese ZSF1 rat with the two receptor mutations develops dyslipidaemia, hyperglycaemia and renal sclerosis and fibrosis [109]. It was recently demonstrated that the development of kidney disease in the ZSF1 rat model is largely independent of hypertension. Therefore the ZSF1 rat allows for separation of renal pathophysiology strictly due to obesity, hyperglycaemia and dyslipidaemia from changes due to hypertension [114]. Obese ZSF1 rats develop metabolic syndrome and diabetes as early as 8 weeks. Metabolic changes are associated with early signs of renal disease such as increased proteinuria and regression of glomerular and peritubular capillary density [99].

SDT Fatty Rats

The Spontaneously Diabetic Torii (SDT) fatty rat was derived by introducing the fa allele of the Zucker rat into the SDT normal rat genome. The SDT normal (non-fatty) rat is a useful model of nonobese T2D that spontaneously develops hyperglycaemia and glucose intolerance from about 20 weeks of age resulting from decreased insulin secretion accompanying β-cell degeneration [91]. The SDT fatty rat already develops diabetes from 5 weeks of age. SDT fatty rats of both sexes show significant hyperphagia and obesity. Serum glucose levels are elevated from 6 weeks of age and lipid parameters from 4 weeks of age [91].

Wistar Fatty Rats

The Wistar fatty rat (WF) is a congenic strain of the Wistar Kyoto (WKY) rat that has a fa/fa homozygous missense mutation in the leptin receptor gene. This model was derived by crossing the obese outbred Zucker rat with the WKY rats [115]. Only the male expresses T2D, characterized by hyperglycaemia, hyperlipidaemia, glucose intolerance, hyperinsulinemia and decreased whole-body insulin sensitivity similar to the ZF rats. The diabetes-induced changes appear to be caused by the combination of a predisposition for diabetes in the WKY rat and fa-induced obesity [115]. WF rats develop progressive insulin resistance, glucose intolerance and obesity between 3 and 8 weeks of age and become overtly diabetic between 8 and 10 weeks of age [1]. Wistar fatty rats at 14 weeks of age showed high levels of plasma glucose and insulin. Their diabetic state is however mild compared to ZDF rats. Renal disease is marked by the onset of albuminuria and decreased GFR at 20 weeks of age [113].

GOTO-Kakizaki Rats

The Goto Kakizaki (GK) rat is a nonobese and non-hypertensive model of spontaneous T2D [1]. The GK strain was developed from the Wistar rat through selective breeding of many generations of rats with elevated blood glucose levels. GK rats exhibit glucose intolerance as early as 2 weeks of age and develop mild hyperglycaemia and hyperinsulinemia between 3 and 4 weeks of age. By 12 weeks of age, GK rats develop T2D characterized by prolonged elevation of fasting glucose and insulin levels.

T2DN/mcwi Rats: Genetically Modified GK Substrain of Rats

The T2DN/mcwi rat is a genetic substrain of the GK rats that were developed from crossbreeding GK and fawn-hooded hypertensive (FHH) rats. The FHH rat is a model of hypertension-associated renal failure [116]. T2DN/mcwi rats develop T2D and progressive proteinuria by 6 months of age [1]. The urinary protein excretion increases progressively from 3 to 18 months of age [111].

Otsuka Long-Evans Tokushima Fatty Rats

The Otsuka Long-Evans Tokushima Fatty (OLETF) is an established model of T2D [1]. This model was derived from a spontaneous obesity development in an outbred colony of Long-Evans rats. OLETF and a control Long-Evans Tokushima Otsuka (LETO) lines were then developed by selective breeding. OLETF rats were initially studied as a model of late-onset T2D, as older OLETF rats were not only obese but also hyperglycaemic and insulin resistant [117]. In male OLETF rats, an impaired glucose tolerance was observed from 8 weeks of age, and plasma glucose levels became higher from 18 weeks of age [113]. Hyperglycaemia and hyperinsulinemia are exhibited in the early phases of the disease as a result of islet cell hyperplasia and peripheral insulin resistance [1]

Table 23.5 illustrates that in males hyperfiltration persists less long (up to 20 weeks, except for one study) than in females (up to 32 weeks). Studies in female rats, although present, are again underrepresented. As in T1D, albuminuria is increased at every stage which is in contrast with the situation in humans, where albuminuria first appears about 5 years after diagnosis [118]. In contrast, more attention is directed towards tubulo-interstitial injury than in T1D where this was aspect was ignored in practically all studies. This is important because in humans tubulo-interstitial injury is the best predictor of progressive loss of renal function in T2D [119]. Note that glomerulosclerosis and tubulo-interstitial injury invariably occur together in T2D in rats, which may not be the case in humans where the glomerular classification has no prognostic value regarding progression of DKD in T2D [119].

In type 2 diabetic rat models, blood glucose-lowering agents improve the hyperglycaemic state markedly (see Table 23.6). Additional effects can be found in

Table 23.6 Overview of interventions in rat models of T2D with renal pathophysiology

Animal model	M/F	Age at start (weeks)	Drug (duration; weeks)	Intervention target	Blood glucose	BP	GFR	Chol	Trig	UalbV/ UproV	GS	TI	Reference
ZDF	M	17	Ang (1–7; 100 ng/kg/min); 2	↓ BP	↓↓	↓↓	↑ (creatinine clearance)	N/A	↓↓	↓↓	N/A	↓↓	[120]
OLETF	M	22	Pioglitazone (10 mg/kg); 2	↓ blood glucose	↓↓	↔	↓ (creatinine clearance)	N/A	N/A	↓↓↓	N/A	N/A	[121]
ZDF	M	20	Sitagliptin (10 mg/kg); 6	↓ blood glucose	↓↓↓	N/A	↔ (serum creatinine) ↑ (serum urea)	N/A	↓↓↓	N/A	↓	↓↓↓	[122]
ZDF	M	12	ARB, Olmesartan (6 mg/kg); 10	↓ BP	↔	↓↓	↔ (inulin clearance)	↓↓	↓↓	↓↓↓↓	↓↓	↓↓	[102]
WF	M	5	Pioglitazone (3 mg/kg); 13	↓ blood glucose	↓	↓	N/A	N/A	N/A	↓	↓	N/A	[123]
ZSF1	M	12–13	Enalapril (3 mg/kg) 15	↓ BP	N/A	↓	N/A	N/A	N/A	↓	↔	N/A	[124]
ZSF1	M	8	Rosiglitazone (5 mg/kg); 24	↓ blood glucose	↓	↓↓↓	↔ (inulin clearance)	↓	↓	↓	↓↓↓	↓↓↓	[84]
ZSF1	M	8	Enalapril (60 mg/kg); 24	↓ BP	↑	↓↓↓	↔ (inulin clearance)	↓	↓	↓	↓↓↓	↓↓↓	[84]
OLETF	M	20	Enalapril (10 mg/kg/day); 26	↓ BP	↔	↓	↔ (creatinine clearance)	N/A	N/A	↓↓	↓↓↓	N/A	[125]
OLETF	M	20	Eplenerone (200 mg/kg/day); 26	↓ BP	↔	↔	↑↑ (creatinine clearance)	N/A	N/A	↓	↓↓	N/A	[125]
OLETF	M	20	Pioglitazone (10 mg/kg); 26	↓ blood glucose	↓↓	↔	↔ (plasma creatinine)	↔	↓	↓	↓	N/A	[126]

Arrows indicate changes vs. diabetic rats without the intervention

Abbreviations: *M/F* male/female, *BP* blood pressure, *GFR* glomerular filtration rate, *Chol* cholesterol, *Trig* triglycerides, *UalbV/ UproV* albuminuria/proteinuria, *GS* glomerulosclerosis, *TI* tubulo-interstitial injury, *N/A* not applicable

Table 23.7 Overview of rabbit models of T1D and T2D with renal pathophysiology

Model strain and dose	Study length (weeks)	M/F	Blood glucose	BP	GFR	UalbV/ UproV	GS	Reference
Type 1 diabetes								
Termond rabbit + alloxan 175 mg/kg IV single dose	3	M	↑	N/A	↓ (creatinine and urea)	↑	↑	[128]
New Zealand rabbit + alloxan 150 mg/kg IV single dose	12	M	↑	N/A	↓ (creatinine and urea)	↑	N/A	[129]
Japanese rabbit + alloxan 100 mg/kg IV single dose	12	M	↑	N/A	↓ (creatinine and urea)	↑	N/A	[130]
New Zealand rabbit + alloxan 65 mg/kg i.p. single dose	26	M	↑	N/A	↓ (creatinine and urea)	=	N/A	[131]
Type 2 diabetes								
New Zealand rabbit + high-glucose, high-cholesterol diet	12	M	↑	N/A	↓ (creatinine and urea)	↑	N/A	[129]
New Zealand rabbit + high-fat (15% extra fat) diet	8–12	F	↑	↑	N/A	N/A	N/A	[132]
Boscat white rabbit + high-fat (10% extra fat) diet	16	?	↑	↑	N/A	N/A	N/A	[133]

Abbreviations: *M/F* male/female, *BP* blood pressure, *GFR* glomerular filtration rate, *UalbV/ UproV* albuminuria/proteinuria, *GS* glomerulosclerosis, *N/A* not applicable

improvement of the lipid profile of these animals and the reduction in glomerulosclerosis and tubulo-interstitial injury. Antihypertensive interventions showed varying effects in improving the hyperfiltration but generally do lower lipid levels, proteinuria, glomerulosclerosis and tubulo-interstitial damage in these models.

Guinea Pigs and Rabbits

T1D in guinea pigs can be induced with streptozotocin [127]. Kidneys are enlarged, but changes in renal function have not been described. T1D in rabbits is mostly induced with alloxan (Table 23.7). Without exception only male rabbits have been studied. An early phase of hyperfiltration has not been reported. Blood pressure and

conventional renal injury are rarely measured. Renal inflammation (MCP-1, ICAM-1 and NG-kB expression) is increased [130].

T2D in rabbits is mostly induced by diet (Table 23.7). These rabbits tend to become obese, hyperglycaemic, hypertensive, hyperlipidaemic and hyperinsulinemic [132]. Information on the kidney is sparse. The kidneys become larger, develop lipid deposition [133, 134] and strike medullary accumulation of hyaluronan with thickened uroepithelium [135]. Renal perfusion is increased [136]. Interestingly, T2D has generally been studied in female rabbits. Whether switching the rabbits back to normal chow can reverse these striking morphological changes is unknown.

Cats and Dogs

Domestic cats occasionally present with DM in the veterinary clinic. Generally, they are not insulin-dependent. Urinary protein/creatinine ratios are increased, but GFR and blood pressure appear normal versus age-matched (~10-year-old) controls [137]. Domestic dogs with spontaneous DM are always insulin-dependent. They are sometimes present with hypertension and/or proteinuria, but these variables are generally stable. GFR appears normal versus age-matched (~7-year-old) controls [138].

Alloxan (40–100 mg/kg) has been used to induce insulin-dependent DM in dogs. After ~3 years, dogs with poorly controlled glycaemia (HbA1 9.3% vs. 5.6% in healthy controls) showed marked hyperfiltration (6.9 vs. 4.0 ml/min/kg), and those with moderate glycaemic control (HBA1 7.9%) were also hyperfiltering (6.2 ml/min/kg) [139]. Albuminuria is progressive, exceeding normal levels after ~5 years [140]. Alloxan (60 mg/kg) has been used in uninephrectomized beagle dogs by Brown et al. to study the effects of antihypertensive drugs (ACE inhibition, calcium antagonist or both) on progression of DKD over 1 year [141]. The dogs were insulin treated to maintain hyperglycaemia (400 mg/dl vs. 100 mg/dl in controls). Untreated diabetic uninephrectomized dogs were hypertensive, hyperfiltering and showed progressive proteinuria. After 12 months, all treatment regimens prevented hypertension and proteinuria even though hyperfiltration persisted. Notably, micropuncture studies performed by Brown after 12 months demonstrated marked glomerular capillary hypertension (63 vs. 54 mm Hg). Glomerular capillary hydrostatic pressure was only restored to normal in the group treated with ACE inhibition. Thus, in this model, calcium antagonists were renoprotective despite persistently abnormal glomerular haemodynamics (63 mm Hg vs. 63 mm Hg in untreated diabetics). All regimens prevented focal glomerular sclerosis, but only ACE inhibitors (without or with calcium antagonists) could prevent global glomerular sclerosis and reduce tubulointerstitial disease [142].

Dietary obesity is induced rapidly in laboratory dogs by feeding large amounts of cooked beef lard (~30 g/kg BW). It is accompanied within 1 month by hypertension, sodium retention renal hyperfiltration and hyperperfusion [143]. Although sur-

Table 23.8 Overview of interventions in dog models of diabetes with renal pathophysiology

Animal model	Drug (duration)	Intervention target	Blood glucose	BP	GFR	FF	UalbV/ UprotV	GS	TI	Reference
Alloxan, ♂&♀ beagles uninephrectomized	ACE inhibitor lisinopril (0.7 mg/kg) 12 months	↓ BP	= (with insulin)	↓	↔ (inulin clearance)	↓	↓	Focal ↓ Global =	↓	[141, 142]
Alloxan, ♂&♀ beagles uninephrectomized	Calcium antagonist TA-3090 (16 mg/kg) 12 months	↓ BP	= (with insulin)	↓	↔ (inulin clearance)	=	↓	Focal ↓ Global ↑↑	↑↑↑	[141, 142]
Alloxan, ♂&♀ beagles uninephrectomized	Lisinopril + TA-3090 (Same doses) 12 months	↓ BP	= (with insulin)	↓	↔ (creatinine clearance)	N/A.	↓	Focal ↓ Global =	↓	[141, 142]
Dietary obesity ♀mongrels	Bilateral renal DNX 5 weeks	↓ BP	N/A (insulinemia ↑)	↓	↔ (iothalamate clearance)	N/A	N/A	N/A	N/A	[143]
Dietary obesity mongrels	Aldosterone inhibitor eplenerone (20 mg/kg) 5 weeks	↓ BP	=. (insulinemia ↓then ↑)	↓	↓ (iothalamate clearance)	N/A	N/A	N/A	N/A	[146]
Dietary obesity ♂ mongrels	Baropreflex activation 7 days	↓ BP	=. (insulinemia ↑)	↓	↓ (iothalamate clearance)	N/A	N/A	N/A	N/A	[149]
Dietary obesity ♂ mongrels	Bilateral renal DNX 14 days	↓ BP	=. (insulinemia ↑)	↓	↑ (iothalamate clearance)	N/A	N/A	N/A	N/A	[149]
Dietary obesity ♂ mongrels	Bilateral renal DNX 8 weeks	↓ BP	=. (insulinemia ↑)	↓	↔ (iothalamate clearance	N/A	N/A	N/A	N/A	[150]
Dietary obesity ♂&♀ beagles	Bilateral renal DNX 6 months	↓ BP	=. (insulinemia ↑)	↓	↔ (creatinine clearance)	N/A	↓	↓ (not scored)	↓ (not scored)	[151]

Arrows indicate changes vs. diabetic dogs without drug treatment (other than insulin in T1D)
Abbreviations: *BP* blood pressure, *GFR* glomerular filtration rate, *FF* filtration fraction, *UalbV/ UproV* albuminuria/proteinuria, *GS* glomerulosclerosis, *TI* tubulo-interstitial injury, *DNX* Denervation, *N/A* not applicable

gical renal denervation in this model prevents hypertension, sodium retention, renal hyperfiltration and hyperperfusion all persist [143, 144]. Within 2 months, when the dogs are grossly obese (39 kg vs. 24 kg in lean controls), there is renal megaly, hyperinsulinemia, anaemia and enlarged Bowman's capsule and space characteristic of glomerular capillary hypertension and hyperfiltration [145]. All these functional and morphological changes could be reduced by eplerenone, an aldosterone antagonist [146]. In the inner renal medulla, there is accumulation of hyaluronic acid, as was also observed in rabbits [135, 147]. These medullary changes are accompanied by numerous changes in gene expression [148] (Table 23.8).

Recently, this model has been employed to study novel invasive antihypertensive strategies, namely, baroreflex activation by carotid sinus stimulation and catheter-based radiofrequency renal denervation [149, 150]. Interestingly, baroreflex activation reversed hyperfiltration, whereas surgical renal denervation did not, even though both interventions restored normal arterial pressure [149]. Once again, these observations in T2D suggest that hypertension can be therapeutically dissociated from hyperfiltration, as was also observed in T1D dogs [141]. Finally, 2 months after catheter-based radiofrequency renal denervation in dogs with high-fat dietary obesity, there were no effects on GFR [150], but after 6 months, albuminuria and urinary markers of tubular injury were ameliorated by the intervention vs. the sham-denervated obese dogs [151].

Pigs and Sheep

In pigs, T1D (alloxan, streptozotocin) and T2D (dietary carbohydrates and fats) are often combined. However, in analogy to permanent neonatal DM in Akita (ins2+/− C96Y) mice: mutation in insulin gene, resulting in a defect in protein folding leading to toxic accumulation of insulin (see section "Guinea Pigs and Rabbits"), a similar model has been developed in pigs [152], using the binary tet-on system [153]. At 4.5 months of age, kidney weight and glomerular volume were increased, but there were no ultrastructural changes in the glomerulus. Changes in kidney function were not reported. Alloxan (175 mg/kg) in combination with a high-fat diet was used in male Sinclair miniature swine, but after 12 weeks, there were no changes in kidney function [154]. Albuminuria was not measured.

The group headed by Dr. Lerman at the Mayo Clinic has pioneered studies on renal disease in pigs. Recently they documented in female domestic pigs that 16 weeks of a high-fat/high-fructose diet induced hypertension, hyperfiltration and increased renal blood flow, endothelial dysfunction and, notably, perirenal fat [155]. This is important because in the Framingham cohort, renal sinus fat area was associated with diabetes, hypertension and chronic kidney disease [156]. This model is also characterized by renal inflammation [157]. In a model in male Yorkshire pigs combining streptozotocin (50 mg/kg) and a high-fat diet, there was mild proteinuria after 19 weeks. Proteinuria and some aspects of renal injury were

prevented by injection of a monoclonal antibody against activation of integrin [158]. Feeding a high-sucrose/high-fat diet to male Chinese Bama minipigs induces T2D within 2 months and microalbuminuria after 5 months. However, creatinine clearance was unchanged. Renal lipid content was increased. A lipoprotein lipase activator prevented renal lipid accumulation and proteinuria [159]. In neutered male domestic pigs, adding a high-fat diet to streptozotocin (140 mg/kg) leads to an increase in GFR (decrease in creatinine) without albuminuria after 15 months [160]. Finally, feeding Bama minipigs a high-fat/high-sucrose diet for 23 months induced hyperfiltration and glomerular hypertrophy [161] . Albuminuria was not measured.

Pregnant sheep and sheep foetuses have been made diabetic with streptozotocin, but kidney function was not reported [162, 163].

Non-human Primates

Rhesus macaque monkeys have been rendered diabetic with streptozotocin and then used for pancreas xenotransplantation, but (reversal of) renal injury has not been studied [164]. Early DKD induced by streptozotocin (85 mg/kg IV) is dependent on glycaemia, and albuminuria appears after 36 months and is aggravated by high-salt (peanuts!) intake [165]. GFR only (start to) decrease after 42 months, but this is preceded by decreased renal blood flow as measured by angiography from 36 months and renal fibrosis from 24 months. Thus, in this model, structural changes precede measurable functional changes. Baboons with streptozotocin (65 mg/kg IV)-induced diabetes were followed for up to 10 years [166]. Renal biopsies were taken after 5 years. Albuminuria was only present in three out of nine monkeys after 10 years. However, these were also the three animals with increased glomerular basement membrane thickness and tubular connective tissue growth factor (CTGF) staining after 5 years, suggesting that CTGF may be a useful early marker in monkeys of DKD as has also been shown in humans [167].

In captivity, some ageing rhesus monkeys can become obese and develop metabolic syndrome or even overt T2D [168, 169]. At about 20 years of age, some of them have developed hyperinsulinemia, albuminuria and typical light and electron microscopic diabetic changes in glomeruli, but there was no hypertension or change in GFR (measured by creatinine, BUN or iohexol clearance).

Discussion

This overview illustrates that diabetes mellitus occurs throughout the animal kingdom. Few species appear to be exempt although in their natural habitat wild animals with T1D will probably not survive very long, and the balance between caloric intake and expenditure effectively prevents obesity and T2D. However, even horses

develop obesity and metabolic syndrome when food is in ample supply and physical activity limited [170]. The message is obvious.

Several general points can be made with respect to the use of animal models of DKD. First, although in humans tubulo-interstitial injury is the best predictor of progressive loss of renal function in T2D [119], very little attention is focussed on tubulo-interstitial injury in diabetic animals. Correlations (or their absence) between urinary markers of tubulo-interstitial injury and their histological substrate have been completely neglected, while we have freezers filled with the necessary material to explore this issue. This is particularly important because there is no clinical indication for renal biopsy in patients diagnosed with DKD, so we will probably never obtain such data in humans. Moreover, a considerable number of patients with diabetes do not develop albuminuria despite progressive loss of GFR. In this population, the search for an alternative non-invasive (i.e. urinary) and relatively inexpensive biomarker is even more urgent and an unmet need [171, 172]. This is a question that perhaps could be fruitfully addressed in dogs and pigs with dietary obesity that show all the features of DKD without albuminuria.

Second, although restoring insulin (by pancreas transplantation) can reverse glomerular [173] and tubulo-interstitial, but not arteriolar changes [174], in patients with T1D, very few (if any) animal studies address the important issue of reversibility of diabetic nephropathy in T2D. Classic experiments show restoration of salt sensitivity by weight loss in previously obese adolescents and dogs [175, 176]. However, whether weight loss is accompanied by decreases in perirenal fat [156], renal medullary matrix accumulation [135, 147] and a variety of cortical (glomerular and tubulo-interstitial) changes [177] is unknown. Such experiments are quite simple to perform. For instance, in a well-characterized model, one could perform uninephrectomy in both the obese and the lean animals when diabetic nephropathy is known to be present. Directly after uninephrectomy, one need only switch the obese animals back to the control diet. When the previously obese group has regained the weight of their lean counterparts, the experiment is terminated, and the contralateral kidneys are harvested.

Third, the bulk of the studies that we have reviewed utilize male animals. Admittedly there are studies that directly compare various aspects of DKD between sexes, but these are relatively scarce [178–181].

Fourth, multiple novel treatments have been tested in animal models of DKD. The literature is so extensive that it would certainly benefit from systematic meta-analysis. However, there are only a few focussed meta-analyses directed at a single novel therapeutic strategy in DKD [59, 182, 183].

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Chapter 24
(Clinical) Trial and Error in Diabetic Nephropathy

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Status of Trials in Nephrology

Relatively Low Number of Clinical Trials in the Field of Nephrology

The landscape of clinical trials in general faces considerable challenges. Attrition rates in late stages of drug development are increasing along with a continuous rise in drug development costs [1, 2]. A concerted effort is necessary to find out how to develop new effective and safe drugs in an efficient and cost-effective way. The area of nephrology does not only suffer from the general problems faced in clinical trials but also holds a number of other specific problems. These problems hold the smaller number of clinical trials in comparison to other specialties (Fig. 24.1), the sub-optimal quality of the trials and the small samples size to detect a realistic treatment effect [4].

Factors contributing to the low number of clinical trials in nephrology include the lack of visibility, lack of availability of new or more effective drugs, and the availability of patients willing to participate in clinical trials [3, 5–7]. However, these low numbers of trials do not adequately reflect the urgent need for new treatment strategies in nephrology.

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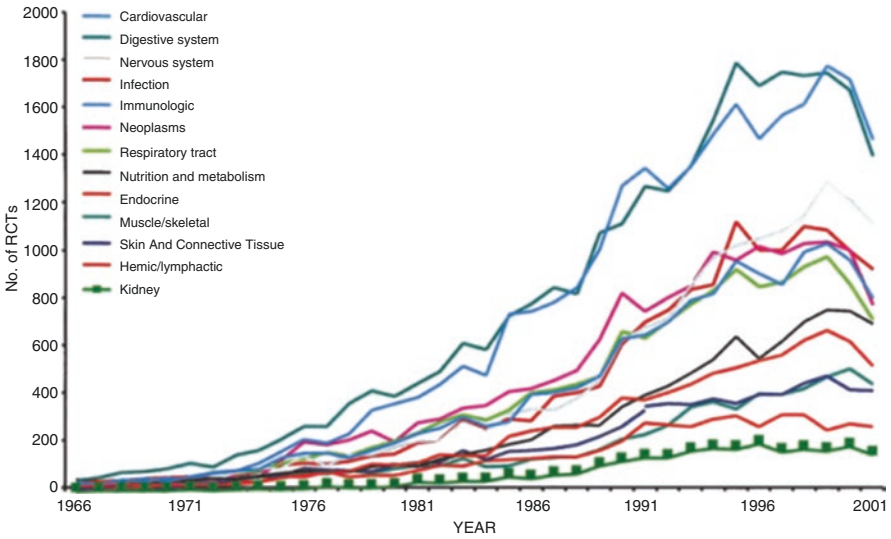

Fig. 24.1 Number of published randomized controlled trials (RCT) in nephrology, and 12 other specialties of internal medicine from 1966 to 2002 [3]

Chronic Kidney Disease Is a Large Public Health Problem

The number of people requiring dialysis for end-stage renal disease (ESRD) has been increasing rapidly across the world [8]. This increase closely parallels the ongoing growth in the prevalence of diabetes for which it is estimated that the number of diabetic patients will increase further from 415 million in 2015 to 642 million by 2040 [9]. Chronic kidney disease, in particular when advanced stages are reached, is associated with a high risk of premature mortality, has a huge impact on the quality of life of patients and their relatives, and places a heavy burden on national healthcare budgets [10, 11]. The high numbers of affected patients would suggest a high awareness to develop and test new interventions and thus a high number of clinical trials. Instead the opposite is seen. Nevertheless, new interventions are highly desired, particularly since the current-guideline recommended strategy of targeting the renin-angiotensin-aldosterone system (RAAS) is of proven benefit in preventing and treating diabetic nephropathy for some patients but by far not for all [12, 13].

Successful Trials

Trials in the past decades of nephrology research have provided insights in the targets to treat patients with type 2 diabetes mellitus and nephropathy. Some trials have shown that tight glycemic control has delayed the onset and progression of nephropathy in patients with types 1 and 2 diabetes mellitus. A fairly recent trial that has

investigated this statement was the ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation) trial. The ADVANCE trial showed that improved glucose control will improve major kidney outcomes in patients with type 2 diabetes by randomizing 11,140 patients to an intensive glucose-lowering strategy or standard glucose control [14]. A subsequent meta-analysis of four major clinical trials confirmed this finding [15].

There have also been successful trials indicating that blood pressure and albuminuria are targets to reduce renal risk in patients with diabetes. In particular blood pressure lowering with drugs intervening in the RAAS appeared to be successful. In the early 1990s, it was shown that the ACE inhibitor captopril delayed the progression of nephropathy in patients with type 1 diabetes [16]. Following this successful trial, the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) and Irbesartan Diabetic Nephropathy Trial (IDNT) showed a renal protective effect of the angiotensin receptor blockers (ARB) losartan and irbesartan in patients with type 2 diabetes and nephropathy beyond their ability to control blood pressure.

In the RENAAL study, 1513 patients with type 2 diabetes and nephropathy were enrolled to losartan or placebo on top of their antihypertensive treatment. After a follow-up period of 3.4 years, losartan significantly reduced the incidence of a doubling in serum creatinine and end-stage renal disease, by 25% and 28% respectively [17]. The IDNT (Irbesartan Diabetic Nephropathy Trial) included 1715 patients with type 2 diabetes and nephropathy. These patients were randomized to receive irbesartan (300 mg/day), amlodipine (10 mg/day), or placebo for the mean time of 2.6 years. Irbesartan treatment showed a significant relative risk reduction in doubling of serum creatinine and end-stage renal disease by 33 and 23% respectively, independent of its blood pressure-lowering effect [18]. Subsequent analyses from the IDNT and RENAAL trial showed that the reduction in albuminuria achieved in the first months of treatment with losartan or irbesartan is an important mediator of the long-term renoprotective effect [19–21]. This finding was subsequently confirmed in multiple clinical trials and has led to additional research to develop interventions targeting albuminuria, as discussed below and in Chapter 29.

In summary, the mentioned trials showed the importance of lowering glucose, blood pressure, and albuminuria as a mean to lower renal risk in patients with diabetes and nephropathy. In addition, the trials led to the marketing authorization of new interventions and have changed clinical practice guidelines. This has resulted in a stabilization of the incidence of ESRD in diabetes as reported in some countries [22, 23].

Residual Risk

Despite the promising and successful results from optimal RAAS inhibition in combination with tight glycemic and blood pressure control, many patients with diabetes and nephropathy still progress to ESRD [12]. The high residual risk is illustrated by the fact that the reduction of end-stage renal disease in the RENAAL was only 28% and not 100% [17]. In itself, this 28% is a considerable risk reduction compared to

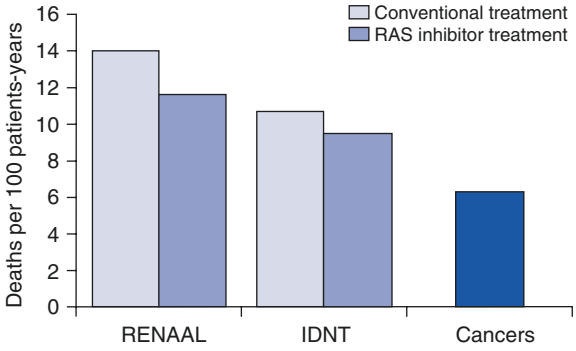

Fig. 24.2 There is a high unmet need in patients with diabetes and nephropathy. Renal risk reduction in two renal outcome trails RENAAL[1](losartan) and IDNT[2] (irbesartan) in comparison with treated cancer (1996–2003; US Cancer Institute Surveillance Epidemiology and End Results Database). The Y-axis indicates death with the assumption that in the absence of dialysis or renal transplantation, patients with end-stage renal disease would die. [15] RAAS = renin-angiotensin-aldosterone-system [24]

conventional therapy. Yet the starting absolute risk in this population was substantial and thus a high residual risk remains despite the large risk reduction.

Figure 24.2 summarizes the residual renal risk after interventions in the IDNT and RENAAL trial [24]. Post hoc analysis of the different trials showed that the high residual renal risk was associated with remaining increased renal risk markers [21]. In an analysis of the RENAAL study, Keane et al. showed that hemoglobin, serum albumin, serum creatinine, and albuminuria were independent risk factors for the progression of renal disease [25]. The high residual risk being associated with high residual risk markers highlights the urgent need for additional therapies that further lower the remaining risk factors [24]. The search for a solution was directed toward the discovery of new drugs and targets that lower the surrogates more efficiently than the current treatment options.

New Drug Targets and Intervention Strategies

The identified high residual risk requires new targets and therapies. Several options were tested: (i) further reduction of known risk markers by intensifying the inhibition of the RAAS with existing and new drugs, (ii) further reduction of the known risk markers by interventions beyond the RAAS, and (iii) identification and targeting of new risk markers.

Intensifying RAAS Inhibition

RAAS inhibition with monotherapy has been shown to be renoprotective in patients with diabetes and nephropathy. In an effort to inhibit the RAAS more stringently, existing therapies were combined. The ONTARGET (Ongoing Telmisartan Along

and in Combination with Ramipril Global Endpoint Trial) investigated the efficacy of combining the ACE inhibitor ramipril with the ARB telmisartan in a large population of 25,620 patients at high vascular risk. A total of 37.5% of patients were diagnosed with diabetes. The trial showed that if anything dual RAAS blockade did not reduce the risk of ESRD [26]. The outcome resulted in serious doubts and vigorous debates about the effectiveness of dual blockade. The ONTARGET was conducted in patients at high cardiovascular but not renal risk. It was thought that dual RAAS blockade could be effective in delaying progression of renal disease if it would be tested in a high renal risk population [27]. The VA-NEPHRON-D (Veterans Affairs Nephropathy in Diabetes) trial tested this hypothesis. The trial enrolled 1448 patients with type 2 diabetes and nephropathy to receive single RAAS blockade with losartan (ARB) or in combination with lisinopril. The trial was unfortunately terminated after 2.2 years due to an increased risk of hyperkalemia from 4.4% to 9.9% and an increase in acute kidney injury (AKI) from 11% to 18% [28]. At the time the trial was terminated, dual RAAS blockade offered no renal benefit, and the trial thus concluded that combination therapy is not recommended in the management of diabetes and nephropathy. Combining ACE and ARBs to increase their efficacy yielded no benefits. Other strategies to intensify RAAS blockade were then tested in an effort to further lower blood pressure and albuminuria.

The AVOID (Aliskiren in the Evaluation of Proteinuria in Diabetes) trial was conducted to test whether aliskiren (a selective direct renin inhibitor) could additionally lower albuminuria in patients with type 2 diabetes, who already received the maximal recommended dose of losartan. In the AVOID trial, 599 hypertensive patients with diabetes and macroalbuminuria were randomly assigned to receive aliskiren or placebo. Aliskiren compared to placebo reduced albumin-to-creatinine ratio by 20%. The study concluded that aliskiren may have renoprotective effects that are independent of the blood pressure-lowering effect [29]. These promising results led to the initiation of the Aliskiren Trial in Type 2 Diabetes Using Cardio-Renal Endpoints (ALTITUDE), a large clinical outcome trial to definitively test the efficacy and safety of aliskiren. Unfortunately, the excitement on the results from the AVOID study faded away when the ALTITUDE with 8561 patients randomized to aliskiren or placebo was terminated after 2.7 years because of increased rates of hyperkalemia, acute kidney injury, and fatality [30].

New Drugs Same Targets

New treatments to further lower albuminuria on top of single RAAS blockade were also tested in the last decade. Sulodexide is one of these new treatments. Several studies showed that this drug lowered albuminuria on top of ACE-ARB by an alleged effect on the glycocalyx (a thin gel-like layer covering the endothelium) [31–35]. These studies triggered the initiation of the SUN program which consisted of two large international randomized controlled trials to assess the efficacy and safety of sulodexide in patients with diabetes and nephropathy [36]. However, these trials failed to demonstrate a beneficial outcome with the use of this agent [37].

Research continued and another promising target emerged, namely, the endothelin system [38]. Specific endothelin receptor antagonists were developed soon after the discovery of the endothelin system. Avosentan and atrasentan are examples of drugs that antagonize endothelin receptors. These agents profoundly lowered albuminuria on top of RAAS inhibition in relatively small studies (RADAR for atrasentan, Endothelin Antagonist Evaluation in diabetic nephropathy study for avosentan) [39, 40]. A large outcome trial with avosentan was initiated as well. In this trial 1392 subjects were randomly assigned to receive 25 mg or 50 mg avosentan or placebo. The ASCEND trial was terminated early because of safety issues particularly congestive heart failure due to the sodium-retaining effects of this class of compounds [41]. However, important lessons were learned from the ASCEND trial. To effectively target the endothelin system in patients with diabetes and nephropathy, a very specific endothelin receptor antagonist should be used to minimize the sodium-retaining effects. Secondly, adequate diuretic therapy is needed to manage sodium/fluid retention, and perhaps most importantly one should only test these drugs in patients who beneficially respond to them. The SONAR trial incorporates these elements and tests the efficacy and safety of the selective endothelin receptor antagonist atrasentan [42]. The trial uses a trial design innovative to the field of diabetic nephropathy as discussed in more detail below.

New Targets

In addition to the trials above, not only known drugs and targets were tested, there were also completely new targets identified. Post hoc analysis of the successful RENAAL trial showed that hemoglobin levels were an important determinant of residual renal risk [25]. Increasing hemoglobin with erythropoietin analogues was a novel approach to reduce residual risk. This led to the CREATE and ACCORD studies in patients with nondiabetic chronic kidney disease. These trials reported no reduction in risk of renal outcomes associated with higher hemoglobin targets. After publishing the results from these studies, the TREAT study was designed. In this trial 4038 patients with type 2 diabetes and anemia were randomly assigned to darbepoetin-α or placebo to achieve a hemoglobin target of approximately 13 g/L. Unexpectedly, the trial showed that the use of darbepoetin-α in did not reduce the risk of renal and cardiovascular outcomes and was in fact associated with an increased risk of stroke.

Inflammation and oxidative stress emerged as important pathophysiological pathways that accelerate the development and progression of diabetic nephropathy. This knowledge has led to the development of specific anti-inflammatory antioxidative modulators such as bardoxolone methyl [43]. Bardoxolone methyl was initially developed as an oncolytic agent. In studies in cancer patients, marked increases in eGFR were noticed which supported the initiation of a renal development program. A phase II trial confirmed these beneficial effects and demonstrated in 227 type 2 diabetic patients with CKD stage 3b/4 that bardoxolone methyl at doses of 25, 75, or 150 mg increased eGFR compared to placebo [44].

These promising results led to the design of a large outcome trial, the so-called Bardoxolone Methyl Evaluation in Patients with Chronic Kidney Disease and Type 2 Diabetes Mellitus (BEACON). This trial was designed to confirm the efficacy of bardoxolone methyl in 2185 patients with type 2 DM and CKD stage 4. However, this study had to be terminated early again because of increased rates of congestive heart failure and mortality in the bardoxolone methyl arm. Although eGFR increased at a median of 9 months, no benefit in the composite outcome of ESRD or cardiovascular death was observed [45]. In light of these findings, further development of this drug class as a potential therapeutic modality in patients with diabetes and nephropathy has been halted.

Reason for These Failed Trials

The above summary is disappointing. None of the trials mentioned above have been able to identify a single new and effective treatment strategy for patients with diabetes and nephropathy despite the enormous human and financial resources that have been put into these large trials. Why did the trials fail to demonstrate a renoprotective effect? Several hypotheses have been put forward. One of them is that it is possible that several important trial design elements may have played a role [47, 48]. Post hoc analysis of unsuccessful trials showed that in almost all trials, lack of effect on the good risk markers (those leading to a good outcome) and/or too many effect on bad risk markers (those leading to poor outcomes) may have played a role in the failure of these trials as summarized in Table 24.1.

Specifically, a post hoc analysis of the ALTITUDE showed that although aliskiren decreased blood pressure and albuminuria, there were still many patients in the aliskiren treatment arm who did not have a reduction in albuminuria. Moreover, patients with a robust lowering of albuminuria (i.e., >30% reduction) during the first 6 months of treatment had a significantly lower risk compared to patients in whom albuminuria did not change [49]. This implies that if only these albuminuria responder patients were selected, the outcome of the trial would have been highly positive. In addition, aliskiren also increased the incidence of hyperkalemia which increases renal risk. Thus a good risk marker response may have been blunted by this poor risk marker response so that the ultimate trial results were negative because of the trial dilution of patients without a good risk marker response and inclusion of patients with a poor risk marker response.

A similar situation was seen in the BEACON trial which was terminated after 9 months because of high rates of congestive heart failure and mortality [45]. However, exclusion of patients at high risk of congestive heart failure by selecting a population with a low BNP level (<200 pg/mL) and without a history of congestive heart failure gave a completely different picture. In this selected population, bardoxolone methyl actually did not increase the risk of congestive heart failure and may even offer renoprotection [50].

Table 24.1 Overview of failed clinical endpoint trials in diabetic nephropathy

Trial	Type	Study arms	Primary outcome	Result	Failure
ONTARGET [27] (n = 25,620)	DM1 and 2	Telmisartan versus ramipril versus combination	Dialysis, DSCR, death	The number of events for the composite primary outcome was similar to telmisartan (n = 1147 [13.4%]) and ramipril (1150 [13.5%]; hazard ratio [HR], 1.00; 95% CI, 0.92–1.09) but was increased with combination therapy (1233 [14.5%]; HR 1.09, 1.01–1.18; p = 0.037)	Effect on surrogate but high side effect
SUN-MACRO [37] (n = 1248)	DM2	Sulodexide versus placebo Both on top of RAAS blockade	DSCR, ESRD or serum creatinine 6.0 mg/dl	The sulodexide group had a lower number of primary endpoints. But comparison was not statistically significant (hazard ratio: 0.85 [95% confidence interval: 0.50–1.44]; p = 0.54)	No effect of surrogate
TREAT [46] (n = 4038)	DM2	Darbepoetin-α versus placebo	ESRD, death or a cardiovascular event	Death or a cardiovascular event (hazard ratio for darbepoetin alfa vs. placebo, 1.05; 95% confidence interval [CI], 0.94–1.17; p = 0.41) Death or end-stage renal disease in darbepoetin alfa vs. placebo group (hazard ratio, 1.06; 95% CI, 0.95–1.19; p = 0.29)	Effect on surrogate but high side effect
ALTITUDE [30] (n = 8561)	DM2	Aliskiren versus placebo Both on top of RAAS blockade	ESRD, DSCR, death or time to cardiovascular death/first occurrence of cardiac arrest	After a median follow-up of 32.9 months, the primary endpoint had occurred in 783 patients (18.3%) assigned to aliskiren as compared with 732 (17.1%) assigned to placebo (hazard ratio, 1.08; 95% confidence interval [CI], 0.98–1.20; p = 0.12)	Low effect on surrogate but high side effect

Table 24.1 (continued)

Trial	Type	Study arms	Primary outcome	Result	Failure
VA NEPHRON D [28] (n = 1448)	DM2	Lisinopril versus placebo Both on top of losartan	ESRD, death, or the first occurrence of a change in the estimated GFR	Combination therapy offered no renal benefit but resulted in excessive risk of hyperkalemia (6.3 versus 2.6 events per 100 person-years; $P < 0.001$) and acute kidney injury (12.2 versus 6.7 events per 100 person-years; $p < 0.001$)	Low effect on surrogate and high side effect
BEACON [45] (n = 2185)	DM2	Bardoxolone methyl versus placebo	ESRD or death from cardiovascular causes	Primary composite outcome (hazard ratio in the bardoxolone methyl group vs. the placebo group, 0.98; 95% confidence interval [CI], 0.70–1.37; $p = 0.92$) Death from cardiovascular causes occurred in 27 patients randomly assigned to bardoxolone methyl and in 19 patients randomly assigned to placebo (hazard ratio, 1.44; 95% CI, 0.80–2.59; $p = 0.23$)	Effect on surrogate and high side effect
ASCEND [41] (n = 1392)	DM2	Avosentan 25 mg/avosent 50 mg versus placebo All groups on top of RAAS blockade	DSCR, ESRD or death	Avosentan reduced proteinuria compared with placebo but had excess adverse cardiovascular events, especially fluid overload (4.6%; $p = 0.225$), congestive heart failure (3.6%; $p = 0.194$), and death (2.6%)	Effect on surrogate but high side effect

In the Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT), the protocol included a forced dose titration to reach hemoglobin targets [51]. This led to a situation in which the patients who had a poor initial response, were also the patients who required a dose up-titration. These patients thus received high doses despite being unresponsive and having experienced a high risk of cardiovascular events [46]. If poor responders would have been removed from the study, and were not forced into a dose up-titration, the outcome of the trial may have been different.

Variability in Drug Response

Although the above examples are post hoc analyses and are prone to bias and confounding, they all point out that in the design of the clinical outcome trials in diabetic nephropathy, we failed to pay sufficient attention to the fact that all patients respond differently to a drug in terms of surrogates and clinical endpoints. Response variation may play a much bigger and more important role than originally anticipated in clinical trial design, and appropriate attention to it may be vital when it comes to the design of new trials (i.e., personalized medicine).

As far as variability in drug response is concerned, two types of response variation exist. The first is the so-called inter-individual response variation, which is the response variability *between* subjects in a single risk marker, for example, blood pressure. In other words, one patient experiences a robust reduction in blood pressure, and the other does not. This individual response variation in a single risk marker has been suggested by some to be a random measurement variation in the risk marker rather than a true pharmacological response variation. However, retrospective and prospectively designed studies showing strong correlations between first and second exposure to the same drug at the same dose strongly argue that the inter-individual response variation is a true phenomenon [52, 53].

The second type of response variation is called intra-individual response variation. This is based on the fact that many drugs, although being developed on the basis of targeting a single risk marker (e.g., blood pressure or HbA1c), often have effects on multiple risk markers. For example, an ARB is designed to lower blood pressure but can also lower albuminuria and at the same time increase potassium. Interestingly it turns out that individual patients respond differently in these multiple risk markers. This means that one individual can have a reduction in blood pressure without a reduction in albuminuria and an increase in potassium, whereas in another patient blood pressure does not change, but albuminuria shows a robust reduction without a concomitant increase in potassium [54]. Since each of the responses in risk markers is independently associated with renal outcome, it highlights the importance for clinical trials to always monitor the multiple risk marker effects of drugs in individual patients and to mitigate risk marker responses that increase renal risk such as an increase in potassium or increase in body weight (as a measure of sodium retention). Thus, post hoc analysis of large clinical trials have suggested, albeit post hoc, that by incorporating individual drug responses through appropriate patient selection, we would have been able to identify subgroups of patients in whom a drug may markedly slow progression of diabetic nephropathy. This adds to the belief that a negative trial might not be caused by poor or noneffective drug but might be the result of poor trial design.

Need for New Clinical Trial Designs

From Trial to Practice

In the development process of a new drug, clinical endpoint trials are required for the approval and registration of the compound. At the same time, trials should be representative for the patients to be treated. This is almost a contradictio in terminis, because in daily clinical practice, treatment of the individual patient does not resemble the way drugs are used in clinical trials. In real life, the dose of a drug is up-/down-titrated to reach a certain target, for example, a blood pressure target of 130/80 mmHg. If after a few weeks of treatment the physician does not see the blood pressure response to the drug, the dose will be increased, possibly multiple times. If no effect is observed or the patient is experiencing side effects, the drug will be stopped. Depending on the severity of the effect, the side effect will be managed, but if the side effect persists or is severe, the specific treatment is discontinued, and another antihypertensive drug will likely be tried in that patient. In trials, as opposed to what happens in real life, often the drug is not up-titrated to reach a target, but a fixed drug dose is used. In addition, if there is no target/risk marker response, the drug is continued since the patient is randomized in the trial and thus should continue to be followed according the intention-to-treat principle. If a patient experiences a side effect, it is recorded as part of the (safety) outcome of a trial.

The current situation shows a great gap between real-life drug use and the way drugs are developed and tested in clinical trials. If this gap is not closed in the near future, we will be left in uncertainty about the actual drug efficacy and safety in a real-life practice setting. In addition, the current clinical trial design of randomly assigning thousands of patients to a standard fixed drug and dose is no longer sustainable. This practice has led to unnecessary large and expensive trials and led to many trial failures and even to harmful effects in subgroups of patients. The trial approach needs to change by paying much more attention on how the individual patient responds to the drug. Thus, new designs are needed that integrate this personalized medicine concept, most likely resulting in much less trial failures and unnecessary exposure of patients to noneffective drugs.

Personalized Medicine in Diabetic Nephropathy Trials

Personalized medicine has been embraced in the oncology trials. Targeted therapies and careful patient selection based on biomarkers are common practice as illustrated by numerous examples [55–57].

The nephrology area should follow the example from the oncology area and start selecting (and deselecting) patients for trial enrollment. The SONAR trial, which is

mentioned previously in this chapter, is a first example of a diabetes nephrology trial in which patients are selected for trial participation based on their response to the drug. In this trial all patients receive the endothelin receptor antagonist atrasentan for 6 weeks. Patients in whom albuminuria decreases by more than 30% (responders) and in whom there are no signs of sodium retention (e.g., no increase in body weight) are randomized to treatment with atrasentan or placebo. The SONAR design is the first trial in nephrology that enriches the trial population for the individual drug response [42]. The specific patient selection and the new enrichment design of trials like SONAR trial will, if successful, set the stage for the next trials that will be developed for new compounds in this particular population. The results will support the current clinical practice and enhance the trends toward personalized/precision medicine in diabetes management. However, this type of enrichment design does not offer alternative treatments to patients who do not respond to the investigational drug. Other clinical trial designs are needed to test multiple interventions within the same clinical trial environment [58].

Future Trial Designs in Diabetic Nephropathy

The issue of non-response is actually not emerging from the execution of enrichment design trial, but the problem is embedded in our current clinical trial practice. A good example of this is the fact that we are currently running three important phase III renal outcome trials in diabetes and nephropathy: SONAR (endothelin antagonist), CREDENCE (SGLT-2 inhibitor), and FIDELIO (nonsteroidal mineralocorticoid). These trials are close to be running in parallel, and when they come with the results, we will hopefully have three drugs that reduce renal risk in this population. However, what we do not know is whether each of these drugs will add to the renal protection of the other when combined. Neither do we know whether the non-responders in the three trials (and there will very likely be a lot of non-responders) will respond better to any of the other two treatments. We thus need a solution in the direction of responder selection and testing new drugs on non-responders.

Platform Trials

A Platform Trial is characterized by a single protocol that holds the flexibility to add or remove treatment arms during the course of the study. These types of trials are already used in cancer research where there already is a strong focus on the integration of personalized medicine in trial design. Features from this platform approach can benefit trials in nephrology too, as it will allow more than one treatment arm in a study. If the trial would be designed in a way that a non-responder can switch treatment arm until a response, we are close to solving the problem we face regarding non-responders [59, 60]. The disadvantage of this design is the fact that one

needs to have all new drugs aligned in the same stage of development. The competitive order of which drug will be the first to test in the platform will be another challenge of this design.

Umbrella Trials

An Umbrella Trial is a trial that holds two phases. In the first phase, patients are randomized to a treatment arm, and in the second phase, efficacy is evaluated based on biomarker testing [61]. This is an element that is interesting for application to clinical trials in nephrology. By assigning the treatment based on the efficacy for a particular subgroup of patients, this may result in trials that are successful in reducing residual risk at least for a subgroup of patients as opposed to considering the drug as failed.

New Platform Sequential Design

A combination of platform and umbrella and then in "sequential" design may help. We would start with a platform of all patients with diabetes and nephropathy, a worldwide huge clinical trial database. In case a new drug comes to be ready for phase III clinical trial testing, patients are enrolled for response selection to the drug. Those that respond continue and enroll in the trial proper, the non-responders are available for when a next new drug comes to be ready for testing, and so on (Fig. 24.3).

	Drug A	*Drug B*	*Drug C*	*Drug D*	.....
Patiënt response type 1	**Response**				
Patiënt response type 2	No response →	**Response**			
Patiënt response type 3	No response	No response →	**Response**		
Patiënt response type 4	No response	No response	No response →	**Response**	
.....	No response	No response	No response	No response →	**Response**

Fig. 24.3 Illustrations of a new trial design adapting more to individual response variation and personalized/precision medicine. The response selection is followed by enrollment of patients into a randomized double blind clinical trial to determine ultimate drug efficacy preferably using established clinical outcomes

It is a challenging perspective, but it is time to bring the nephrology and diabetes community together and form a consortium in which we can tackle the residual risk by these new trial designs and offer those that come with new promising treatments a quick way of testing the efficacy of such drugs [62].

Conclusion

The important lesson learned from a decade of clinical trial failures in nephrology is that the one-size-fits-all approach does not fit everyone. Accordingly, much more emphasis should be placed on the individual and how the individual responses to the drug. The trial failures should thus mark a new era of clinical drug development with a trial design focused on individual patient selection. Despite the past clinical trial failures, there are still promising therapies at the horizon. Patients with diabetic nephropathy deserve that they are further developed and tested in the right way in individualized clinical trials.

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Part VIII
Therapy of Diabetic Nephropathy

Chapter 25
Treatment Goals in Diabetic Nephropathy

Gerald Vervoort

Introduction

Diabetic nephropathy is a devastating disease with increased morbidity and mortality due to progression of nephropathy and cardiovascular events [1–8]. As such, the optimal therapeutic approach involves intensive blood pressure management with emphasis on blockage of the renin-angiotensin system as well as management of hyperglycaemia, dyslipidaemia and albuminuria. Moreover, significant attention should be paid to diet modifications, exercise, weight control and smoking cessation [9–12] (see Table 25.1).

Finally we must devote sufficient notice to the impact of the disease on quality of life. Among patients with diabetes mellitus, those with comorbidities as nephropathy are predominantly prone to poor health-related quality of life [13].

Besides the treatment of diabetic kidney disease aiming at the deceleration of renal function loss and the prevention and treatment of its cardiovascular complications, prevention and treatment of secondary metabolic complications (anaemia, mineral and bone disorders and metabolic acidosis) must also be the subject of our treatment strategy (Table 25.2).

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Table 25.1 Treatment goals in diabetic renal disease

Blood pressure	<130/80 mmHg or even lower in younger patients, new-onset diabetes, no overt cardiovascular disease and no orthostatic symptoms
Albuminuria	Maximum reduction of albuminuria; in overt proteinuria <500–1000 mg/day but at least >60% reduction from baseline values; restrictions as mentioned in blood pressure management
Glycaemic control	HbA1c <7% in younger patients and new-onset diabetes with no overt cardiovascular disease and no hypoglycaemia; consider a higher HbA1c target for the elderly with cardiovascular and renal complications as well as those susceptible to hypoglycaemia
Dyslipidaemia	Treat dyslipidaemia (e.g. statin therapy); intensity depending on cardiovascular disease risk factors and age[a]; statin therapy in dialysis patients seems not justified
Diet	Dietary sodium restriction <100 mmol/day Protein restriction ±1 g/kg day
Weight	Advise all patients to lose weight if BMI >25 kg/m^2
Smoking	Advise all patients to quit smoking
Physical activity	Advise regular physical activity to promote a healthy weight, fitness and well-being; at least three times >30 min 1 h/week of brisk walking is recommended for all diabetes patients

[a]Statin therapy indications and intensity for patients with diabetes (see Table 25.2)

Table 25.2 Statin therapy indications and intensity for patients with diabetes

Risk factor	Age	Statin intensity
History of atherosclerotic CVD	All age groups	High dose
CVD risk factor[a]	<40 or > 75	Moderate or high
	40–75	High dose
No risk factor	<40	None
	40–75	High dose
	>75	Moderate of high

Abbreviations: *CVD* Cardiovascular disease
[a]CVD risk factors include LDL cholesterol ≥100 mg/dL (2.6 mmol/L), high blood pressure, smoking, chronic kidney disease, albuminuria and family history of premature atherosclerotic CVD

Blood Pressure Control

There is clear evidence now that antihypertensive treatment, especially with interference of the renin-angiotensin system, can reduce the progression of diabetic renal disease. As such there seems to be no difference to the effects seen in nondiabetic renal disease [9–11].

In type 1 diabetes, patients with albuminuria (and preserved renal function) as well as with overt nephropathy/advanced disease, a beneficial response was seen to the addition of angiotensin-converting enzyme (ACE) inhibitors, slowing the rate of progression in diabetic nephropathy [14–16].

Although in type 2 diabetes, the data on antihypertensive therapy in diabetic nephropathy is less convincing, strict blood pressure control seems also to be of great importance.

The United Kingdom Prospective Diabetes Study (UKPDS) was conceived to explore the role of intensive glycaemic and blood pressure control in 5102 newly diagnosed type 2 diabetes patients. This study showed that more intensive blood pressure management resulted in a reduction of renal complications. Each 10-mmHg reduction in mean systolic blood pressure was associated with a 12% decrease in the hazard ratio for the development of albuminuria and eGFR decline (<60 ml/min per 1.73m^2) [17].

The Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) study was performed in 11,140 patients with type 2 diabetes and an increased cardiovascular risk. The aim of this study was to investigate whether intensifying glucose control (HbA1c <6.5%) and blood pressure lowering would provide additional benefit in reducing the risk of micro- and macrovascular disease. In these patients with longer-lasting type 2 diabetes and/or albuminuria, the use of ACE inhibitors decreased the appearance or progression of albuminuria without significant effects on eGFR decline [18].

In type 2 diabetic patients with more advanced stages of diabetic renal disease, a benefit was demonstrated with respect to renal protection with the use of ARBs. Nevertheless, these patients will probably still evolve towards end-stage renal disease (ESRD), although the decline will be more slowly [19–21].

The KDIGO (Kidney Disease: Improving Global Outcomes) guidelines recommend the use of ACE inhibitors or ARBs and a blood pressure goal <130/80 mmHg in all patients with renal diabetic disease [9, 10]. The combination of an ACE inhibitor and an ARB offers no clear benefit and increases the risk of severe side effects (hyperkalaemia and acute renal failure) [22].

Target blood pressure goals have recently been challenged by large clinical trials (SPRINT, ACCORD) in which intensive blood pressure control (<120 mmHg) was pursued [23–25]. In the Systolic Blood Pressure Intervention Trial (SPRINT), 9361 adults aged >50 years with systolic blood pressure of 130 mm Hg or higher and at least one additional cardiovascular disease risk factor (but no diabetes) were investigated to determine whether treating blood pressure to a target of <120 mmHg is superior to treating to <140 mmHg. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial involved 10,251 middle-aged and older type 2 diabetes patients who were at risk for cardiovascular events because of existing cardiovascular disease or additional conventional risk factors. In ACCORD three medical treatment strategies were tested (stringent glycaemic control HbA1c <6.0% (10,251 patients); systolic blood pressure <120 mmHg (4733 patients); and lipid trial with additional fenofibrate (5518 patients)), to reduce cardiovascular morbidity and mortality.

Although a composite endpoint of cardiovascular events and death was significantly reduced in the SPRINT trial, the effects on renal endpoints were less convincing and even showed an increase in the decline of GFR. In the ACCORD trial, no significant effects on cardiovascular endpoints were seen, and the same drawbacks were appreciated with respect to renal function [26].

It seems reasonable to assume that the blood pressure goals should be individualised. Orthostatic hypotension and a very low diastolic blood pressure (<60 mmHg) should be avoided in older patients with diabetes (and known cardiovascular disease) due to the increased risk of falling and a decrease in effective cardiac blood flow, respectively. On the other hand, blood pressure goals can probably be adjusted downwards without untoward side effects in younger people without evident cardiovascular disease although this remains to be demonstrated [9–11, 27].

Overall, the use of renin-angiotensin system (RAS) blockade should be advocated in combination with salt restriction and/or loop or thiazide diuretics in manifest diabetic renal disease with a blood pressure goal of <130/80 mmHg unless serious side effects. If blood pressure (or albuminuria) is not at goal, titrate ACE inhibitor or angiotensin receptor blockers (ARB) to maximum dose tolerated. If goals are still not met, nondihydropyridine calcium-channel blockers (verapamil or diltiazem) could be used if patients are not on beta-blockers (in that case the suggestion is adding a long-acting dihydropyridine calcium-channel blocker) [9–11, 27, 28]; mineralocorticoid receptor antagonists might reduce proteinuria as well as blood pressure and are certainly an important possibility if targets are not achieved [28].

Glycaemic Control

In patients with recent onset diabetes (type 1 as well as type 2), intensive blood glucose control reveals long-lasting favourable effects on the risk of diabetic kidney disease development [29–35]. This suggests that this so-called legacy effect or metabolic memory can prevent irreversible renal damage due to hyperglycemia-induced alterations like epigenetic modifications, dysregulation of (mitochondrial) metabolism and a persistent increase in inflammatory pathways [36, 37].

In patients with type 1 diabetes, poor glycaemic control is an independent predictor of progressive diabetic kidney disease. Strict metabolic control targeting an HbA1c ≤ 7% reduced the 9-year risk of albuminuria significantly, an effect that persisted during the following >10 years, also reducing the risk of eGFR loss [31, 32].

Similarly, in patients with new-onset type 2 diabetes, >10 years of intensive glycaemic control targeting an HbA1c of 7% reduced the development of diabetic kidney disease characterised by a decrease of albuminuria and reduction of eGFR (doubling of serum creatinine) [34].

The importance of intensive glycaemic control after the onset of manifest renal complications (or even in long-standing diabetes mellitus) has not been shown to have a serious impact on the risk of diabetic renal disease or improve overall clinical outcomes [12].

In more advanced stages of diabetic nephropathy in type 1 diabetes, strict glycaemic control reduces the progression of diabetic kidney disease, although one can appreciate that the effect is less pronounced than in less advanced stages of diabetic renal disease. Mainly, less progression in albuminuria was shown, but the effects on decrease in GFR were small and not of impressive clinical importance [30, 31].

In patients with type 2 diabetes and (high risk of) cardiovascular disease or manifest, even early-stage, renal complications, targeting low HbA1c did not benefit all cause and cardiovascular mortality. The effects on renal endpoints showed reductions in albuminuria, but again the effects on GFR decline (doubling of serum creatinine), or the development of ESRD were small and rather insignificant [38–40]. Participants without significant renal disease at entry of the study showed the greatest benefit of intensive blood glucose control to prevent progressive and ESRD, stretching the importance of good glycaemic control early in the course of the disease.

Tight glycaemic control results in an increased risk for hypoglycaemia, which is fairly undesirable in patients with high cardiovascular risk.

So intensive glycaemic control appears to have both risks and (small) benefits. As such, a more tailor-made personalised approach is designated which should focus on age, comorbidities, risk for hypoglycaemia and life expectancy of individual patients.

Particularly in this aforementioned group of patients, the unmet need, with respect to reduction in renal complications, is high.

The fact that pancreatic transplantation improved and even reversed glomerular and tubular structural lesions emphasises the importance of strict metabolic control and the imperfection of the current glucose-lowering therapies [41, 42].

From that point of view, interestingly, there is growing evidence that certain glucose-lowering medication classes show renal protection independent of diabetes control. Sodium-glucose cotransporters 2 (SGLT2) inhibitors may be able to correct the disengagement of the tubuloglomerular feedback in diabetes, favouring a renal protective effect by normalising filtration pressure and attenuation of the loss of podocytes and nephrons. Besides the direct renal effects, SGLT2 inhibitors also have effects on systolic and diastolic blood pressure, body weight and uric acid as well as inhibitory effects on the inflammatory and fibrotic responses of proximal tubular cells to hyperglycemia [43–45].

The Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients (EMPA-REG OUTCOME) was conducted in 7020 patients with type 2 diabetes at high cardiovascular risk. The addition of empagliflozin reduced major cardiac events and had significant effects on renal endpoints. The EMPA-REG OUTCOME trial reported a 39% reduction in incident or worsening kidney disease including doubling of serum creatinine and ESRD [46]. The recently published Canagliflozin and Cardiovascular and Renal Events in Type 2 Diabetes (CANVAS-(Renal)) trial, involving 10,142 participants with type 2 diabetes and high cardiovascular risk and receiving canagliflozin or placebo, lowered the rate of eGFR decline by 40% in patients with type 2 diabetes and a high risk of cardiovascular disease [47].

However, although the results are very promising with respect to renal protective effects, the efficacy of SGLT2 inhibitors is reduced in patients with an eGFR <45 ml/min per 1.73m^2. They also have been associated with urinary and genital (yeast) infections, euglycaemic ketoacidosis and remarkably an increased risk of lower-limb amputations [43, 46, 47].

Also glucagon-like peptide-1 receptor agonists (GLP1-RAS) have shown favourable renal effects. The underlying mechanistic pathways by which GLP1-RAS exert

their effects are speculative and may be multifactorial [45, 48]. Renal GLP-1 receptors have been identified, but their exact physiological role is incompletely understood. Its antihypertensive action may be related to a GLP-1-induced natriuresis through inhibition of the sodium-hydrogen ion exchanger isoform 3 in the proximal tubule. Glomerular blood flow and filtration rate are also regulated by GLP-1, but the mechanisms are complex and may depend on, e.g. glycaemic conditions.

Inhibition of renal inflammation and oxidative stress probably mediates this protection.

To assess the long-term effects of liraglutide on cardiovascular outcomes and other clinically important events, the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial was initiated in 2010. In the Liraglutide and Renal Outcomes in Type 2 Diabetes trial, 9340 type 2 diabetes patients and high cardiovascular risk, renal outcome was reported in a trail in which patients were assigned to receive liraglutide or placebo on top of usual care. It was shown (in a prespecified secondary analysis) that the addition of liraglutide to standard care resulted in lower rates of the development and progression of diabetic kidney disease although mainly driven by new-onset macroalbuminuria [49]. Mean decline of eGFR was less with liraglutide among patients whose baseline eGFR was between 30 and 60 ml/min per 1.73 m^2.

In the Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with Type 2 Diabetes (SUSTAIN-6), 3297 with type 2 diabetes and established cardiovascular or chronic kidney disease were treated (on top of usual care) with either semaglutide or placebo. In the semaglutide group, new or worsening nephropathy was reduced by 36% (3.8% in the semaglutide group and 6.1%in the placebo group). The effects on albuminuria onset and progression were more pronounced than the effects on eGFR [50].

Dyslipidaemia

Dyslipidaemia is common in patients with diabetes mellitus [51]. Although more pronounced in type 2 diabetes, it is increasingly recognised that dyslipidaemia is also prevalent in type 1 diabetes [52]. The tendency of hyperlipidaemia is increased by the development of renal failure [53, 54]. The degree of lipid abnormalities should therefore be considered important in the management of patients with diabetes and renal disease to prevent cardiovascular disease due to systemic atherosclerosis. In addition it has also been suggested that dyslipidaemia may contribute to accelerate the development of diabetic renal disease [55].

Most clinical guidelines recommend tight control of dyslipidaemia, especially in high-risk patients since diabetes is considered to be a coronary heart disease equivalent [9–11]. The new American Diabetes Association (ADA) statement has not specified any LDL goals for statin therapy. Statin is dosed based on patients' atherosclerotic cardiovascular disease (CVD) risk factors rather than single LDL levels.

Treatment recommendations beyond lifestyle alteration and optimization of glycaemic control are for the use of high-intensity statin therapy in patients of all ages with overt coronary heart disease, and those aged 40–75 years with additional risk factors, and moderate intensity statin therapy patients aged over 40 years without additional risk factors. Clinical judgement should guide the use of moderate- or high-intensity statin therapy in patients younger than 40 years or older than 75 years with additional risk factors [56]. It is justified to accept that in patients with diabetes and more advanced stages of diabetic renal kidney disease, the risk for cardiovascular disease is high and consequently should be treated. Whether or not the same applies for patients in this age group with (micro)albuminuria and preserved renal function has yet to be sorted out although evidence is pointing out into that direction [57–60].

The evidence that treatment of dyslipidaemia has a significant impact on progression of diabetic kidney disease is still to be proven. A meta-analysis did not show improvement of kidney outcomes with statin therapy [61], but fibrates did reduce the risk of albuminuria progression [62].

Albuminuria

Treatment or interventions that produce a durable decrease in albuminuria may slow the progression of diabetic kidney disease even in the absence of increased blood pressure [15, 21, 63–68]. Nevertheless, most patients with diabetes and albuminuria do have hypertension.

In patients with normotension and normoalbuminuria, there seems to be no conclusive evidence that treatment with RAS blockade is useful. It does not prevent the development of microalbuminuria or the progression of early diabetic renal lesions [69].

Although there is now increasing consensus that a significant decrease in albuminuria has prognostic importance for improving renal outcomes, the natural behaviour of the course of albuminuria is not well predictable. Spontaneous remission of microalbuminuria is well recognised at least in type 1 diabetes, while a significant proportion of the remaining patients do not progress to more advanced stages of diabetic kidney disease [70, 71]. It should also be noted that combination therapy with an ACE inhibitor and an ARB or a direct renin inhibitor (aliskiren) plus losartan was associated with a greater reduction in albuminuria than monotherapy, in the absence of renovascular advantages [22, 72].

As such the use of (reductions in) albuminuria as a beneficial surrogate marker for diabetic kidney disease is more and more challenged [73].

In clinical practice, changes in albuminuria should be used together with changes in eGFR, blood pressure, lipids and the development of other vascular complications, to monitor kidney status and to distinguish those patients more likely to develop or progress to more serious diabetic renal disease. In patients with overt nephropathy/proteinuria, the general aim is to reduce protein excretion less than 500–1000 mg/day with a minimum reduction of 60% of baseline values [9–11]. In

the case of persistent (and progressive) microalbuminuria despite blood pressure < 130/80 mmHg, additional antihypertensive therapy may be useful, although the precautionary measures described in the chapter on blood pressure control are also applicable here.

Of interest is the proposed additional effect of mineralocorticoid (MC) receptor antagonists on albuminuria as an effect "beyond blood pressure reductions" [74–77]. Besides effects exerted via the MC receptor (decrease of renal sodium reabsorption), there is increasing evidence that profibrotic effects of aldosterone due to activation by, among others, TGF-beta play an important role in the development and progression of diabetic renal disease [78]. This could be abolished by MR blockade. Indeed, meta-analysis has shown that the use of MC receptor antagonists has an antiproteinuric effect, but there are no long-term data regarding beneficial effects on GFR decline or the prevention of ESRD in diabetic nephropathy [79].

Lifestyle Interventions

Diet modifications that are particularly related to renal outcomes are salt and protein restriction and a decrease in caloric intake in view of weight reduction.

Salt restriction enhances the effect of RAS blockade on proteinuria, independent of blood pressure [80, 81]. Recent data indeed demonstrated that moderately lower sodium intake in diabetic chronic kidney disease (CKD) patients is associated with substantially better long-term outcome of RAS blockade [82]. Restriction of sodium intake ≤ 100 meq/day is nowadays advocated in most guidelines [9–11]. It should however be recognised that this is not always and under all circumstances feasible. Addition of diuretics can correct for the intake of high salt [83].

The role of protein restriction is still uncertain in diabetes patients especially in view of their additive role beyond the use of RAS blockade and strict control of blood pressure and glycaemia.

It is suggested that the effects of strict dietary protein restriction (0.5–0.85 g/kg/day) in diabetes patients are beneficial with respect to maintenance of GFR and reductions in proteinuria [84]. However, a long-term prospective trial is certainly needed to establish the efficacy and compliance with strict protein restriction in diabetic kidney disease. Especially safety issues have to be taken into account in more advanced stages of diabetic kidney disease. Undernutrition may be an important explanation for increased mortality in these patients due to protein-energy wasting, inflammation and cachexia [85]. Diabetes patients with serious kidney disease are often catabolic. This risk is particularly high during periods of illness. As such it seems reasonable to avoid a high-protein diet by limiting daily protein intake to about 1 g/kg .

Obesity per se is known to confer an increased risk of the development of advanced kidney disease [86]. Weight reduction in overweight, mainly type 2 diabetes patients, can cause marked reductions in albuminuria [87]. The possibility of achieving a significant weight reduction seems to be much greater with surgical intervention than with pharmacological or dietary measures [88]. Nevertheless, a

rather low number of prospective studies have addressed the role of intentional weight loss as a means of stopping or reversing diabetic kidney disease. In that light, it is of interest to emphasise the effects newer blood glucose-lowering agents like SGLT2-inhibitors and GLP1-RAs appear to have on weight [46, 47, 49, 50, 89].

Smoking is without any doubt an important risk factor for the development and progression of diabetic kidney disease. This risk increases with increasing dose of smoking [90, 91]. Whether smoking cessation can indeed reduce the risk of renal kidney damage remains however to be determined.

Physical activity and exercise is promoted in diabetes patients with the intention to improve metabolism, support weight loss and preserve cardiopulmonary functionality. Overall, there is a lack of evidence that physical activity can improve cardiovascular outcomes or have a serious impact on renal impairment except for slight decreases in albuminuria. On the other hand no evidence of harm was shown when promoting increased physical activity leading to a reduction in fat mass and an improvement in quality of life [9, 92].

Combination Therapy

An aggressive combined therapeutic approach focused on the above-mentioned factors underlying the disease, including hypertension, hyperglycaemia, dyslipidemia and lifestyle, can provide optimal protection against (progression of) diabetic renal disease.

Such a combined approach was highly successful in type 2 diabetes patients with a significant reduction of GFR decline and the development of ESRD after a follow-up >20 years [58–60]. Although the evidence is less vigorous in type 1 diabetes, the results of smaller studies point in the same direction [93].

Quality of Life

End-stage complications like diabetic kidney disease have the greatest perceived burden on quality of life [13]. On the other hand, the adherence to multiple medications and interventions on a routine basis also represents a significant burden for many diabetes patients [94]. Recognising individual patient treatment preferences may be one of the keys to translating findings from clinical trial populations to general patient populations.

Treatment-related quality of life will be likely to improve by simplifying current treatment modalities through treatment innovations. Without such novelties, we may still be able to lighten patient concerns by schooling patients early in their disease and telling them about the true nature of optimal diabetes care, by incorporating their preferences into treatment decisions and by acknowledging patient preferences and quality-of-life concerns in public health efforts to improve the quality of diabetes care.

Secondary Metabolic Derangements in Diabetic Kidney Disease

Anaemia

Anaemia is common in diabetes patients with chronic kidney disease and accounts for an increased (cardiovascular) morbidity and mortality. Early identification and treatment of anaemia are an important therapeutic approach to improve outcomes in these patients. This approach should be timely and needs to be individualised according to patient's clinical status. Apart from vitamin B_{12} and folate deficiency, lower erythropoietin (EPO) and iron levels are considered principal factors responsible for anaemia in diabetic kidney disease. Follow-up and maintenance of adequate levels of EPO and iron are of importance.

In general it is recommended to maintain haemoglobin (Hb) levels between 10 and 12 g/dL in all diabetes patients with chronic renal failure. The Hb target should be individualised for each patient considering variables such as age, physical activity and comorbidity. Increase of Hb to more than 13 g/dl should be avoided due to its association with increased mortality [95, 96].

Iron deficiency is a common cause of anaemia as well as an incomplete response to ESAs in diabetic kidney disease. It is recommended to treat iron deficiency before starting ESAs. Iron can be replenished by oral or intravenous route, requiring monitoring of the ferritin levels (>100 mcg/l) and transferrin saturation (>20%) [96].

Mineral and Bone Disorders

Preventive and therapeutic approaches to chronic kidney disease-mineral bone disorder (CKD-MBD) do not differ between diabetic and nondiabetic patients [97]. Markers of altered bone metabolism (hyperphosphataemia, elevated FGF-23 and alkaline phosphatase and diminished Klotho concentrations) as well as reduced vitamin D levels have been linked to an increased risk of cardiovascular events and progression of renal failure [98]. The treatment of CKD-MBD is targeted at lowering high serum phosphate towards the normal range and avoiding hypercalcaemia. Furthermore treatment of abnormal PTH levels is recommended although the optimal PTH level is not known. However, it is suggested that patients with levels of intact PTH progressively rising or persistently above the upper normal limit for the assay be evaluated for modifiable factors, including hyperphosphataemia, hypocalcaemia, high phosphate intake and vitamin D deficiency. Routinely use of vitamin D is not recommended and reserved for patients with serious kidney failure and severe and progressive hyperparathyroidism [97].

Metabolic Acidosis

Chronic metabolic acidosis in diabetes patients with chronic kidney disease may produce multiple pathophysiologic changes like bone resorption, aggravation of secondary hyperparathyroidism and systemic inflammation [99]. Observational studies have described a significant association of metabolic acidosis with increased mortality and progressive loss of renal function [100, 101].

In diabetes patients with CKD and metabolic acidosis, alkali therapy (usually with sodium bicarbonate 0.5–1.0 meq/kg/day) should be used to maintain the serum bicarbonate concentration in the normal range (23–29 meq/L). The justification behind this approach is based upon randomised studies showing beneficial effects of alkali therapy on the progression of chronic kidney disease, bone health as well as nutritional status [102].

Renal Replacement Therapy

As for any other patient with ESRD, diabetes patients with ESRD can be offered renal replacement therapy. Temporary and careful explanation of the therapeutic options and modalities of renal replacement therapy to patients and their immediate environment is recommended in an early stage of renal failure. There is no reason for starting much earlier with renal replacement therapy in diabetic patients compared to nondiabetic patients [9].

Diabetes patients requiring renal replacement therapy have the following options:

1. Haemodialysis (e.g, facility haemodialysis, home haemodialysis).
2. Peritoneal dialysis (e.g, machine-assisted intermittent peritoneal dialysis, continuous ambulatory peritoneal dialysis, continuous cyclic peritoneal dialysis).
3. Renal transplantation (e.g, cadaver donor kidney, living related donor kidney, living unrelated donor kidney [emotionally related donor], living unrelated donor kidney [unrelated by family or emotionally; the so-called altruistic donor]), pancreas plus kidney transplantation or pancreas-after-kidney transplantation (both in type 1 and at least insulinopenic patients).
4. Islet transplantation (after kidney transplantation; in type 1 diabetes or at least in insulinopenic patients).

A detailed description of these topics is beyond the scope of this chapter.

However, timely preparation for renal replacement therapy and education on the different options of transplantation and their expected outcomes are indicated and require necessary expertise and attention for individual needs of patients as well as their immediate environment.

Conclusions

To prevent progression of diabetic renal disease and concomitant cardiovascular morbidity and mortality, it is important to adapt treatment goals to the individual needs and characteristics of patients, depending on age, comorbidity, vascular complications and life expectancy.

A multifactorial, aggressive approach (blood pressure management, glycaemic control, lipid management, lifestyle changes) is needed to improve the prognosis of patients with diabetic nephropathy, taking into account the disadvantages in frailty patients.

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Chapter 26
Kidney Transplantation and Diabetic Nephropathy

Jesper Kers and Frederike J. Bemelman

Introduction

Diabetes after renal transplantation or posttransplant diabetes mellitus (PTDM) is a common problem and is associated with significant morbidity and mortality. PTDM can be either a manifestation of pretransplant existing diabetes mellitus or a truly new-onset diabetes mellitus after transplantation (NODAT). It is not so clear yet to what extent PTDM increases the risk of cardiovascular complications in transplanted individuals. In a single-center study, only the presence of pretransplant diabetes mellitus increased the risk for posttransplant major cardiovascular events, and NODAT did not [1]. Whether PTDM is an entity on its own or just a variation of type 2 diabetes mellitus (T2DM) is a matter of debate. An important mechanism of PTDM is, like in T2DM, insulin resistance, but pancreatic ß-cell dysfunction leading to insulinopenia and the effects of immunosuppressive drugs also contribute to hyperglycemia. PTDM has some unique distinguishing features as compared to T2DM. It varies over time after transplantation, and it is strongly associated with the use of immunosuppressive drugs in a dose-dependent way. Treatment of PTDM with antidiabetic medication can result in drug interactions with cytochrome P450 inhibitors, and hypoglycemic agents increase the risk for sudden death through paroxysmal rhythm disorders.

Definitions of diabetes are based on the 2003 consensus guidelines of the American Diabetes Association [2].

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The Diagnosis of Posttransplant Diabetes Mellitus

The oral glucose tolerance test (OGTT) is the gold standard to establish PTDM. Since this test is laborious and cumbersome, a fasting and a postprandial glucose measurement often replaces it. However, a substantial number of patients with PTDM demonstrate normal glycemic control prior to transplantation. When relying solely on fasting plasma glucose levels, some of these patients are underdiagnosed. In a study in 72 dialysis patients, 56 (78%) showed blood values in the diabetic range upon OGTT with normal fasting plasma glucose levels [3]. In the early period after transplantation, elevated glucose levels are ubiquitous, and hyperglycemia is often transient. Fasting plasma glucose levels in this period are also insensitive, and even OGTT might miss hyperglycemia erroneously when performed early in the morning after an overnight fasting. The maximal diabetogenic effect of steroids is 7–8 h after ingestion. Capillary blood glucose testing in the afternoon and evening in a cohort of renal transplant recipients 6 weeks after transplantation demonstrated hyperglycemia in 46% of cases, whereas all recipients had normal fasting glucoses and only 12% a disturbed OGTT [4]. HbA1c levels have appeared to be unreliable in the first 2 months following transplantation as well. These HbA1c levels can be confounded by blood transfusions. However, after 3 months, HbA1c performs better in the detection of PTDM. An HbA1c of >6.5% was in concordance in almost 90% of cases with the OGTTs in the diabetic range [5].

A consensus statement proposed restriction of the diagnosis of PTDM to patients with stable transplant function, on maintenance immunosuppression, and in the absence of infection or rejection [6]. This definition might enable comparisons in the prevalence and incidence of PTDM between centers, and it will facilitate future clinical trials.

Epidemiology of Posttransplant Diabetes Mellitus

The reported incidence of PTDM varies between 9% and 39% of renal transplant recipients using the abovementioned criteria. The wide variability can be partly explained by variation in frequency and in time after transplantation of testing blood glucose levels. Stress factors related to surgical procedures, infections, episodes of rejection, and immunosuppression can all lead to hyperglycemia. The effects of immunosuppressive drugs are dose-dependent; many immunosuppressive regimens are calcineurin inhibitor (CNI)-sparing, and target trough levels of CNIs have become lower throughout the years as compared to the earlier studies. This can explain why in some reports, the incidence of PTDM is declining in the last 10 years [7]. Lower rejection rates might also contribute to this trend.

Mechanisms of Posttransplant Diabetes

Both endocrine pancreatic beta-cell dysfunction and insulin resistance have a causal role in PTDM, but the relative contribution of each of these causes remains unclear. It is unknown how transplantation affects the gluconeogenesis in the kidney. In normal conditions, 100% of the plasma glucose is filtered through the glomeruli of which more than 90% is reabsorbed in the proximal tubuli by the sodium-glucose linked transporter (SGLT)-1 pathway. Furthermore, pathophysiological mechanisms are probably variable between patients, vary even within an individual patient, and depend on external factors such as rejection and immunosuppressive drug regimens [8]. Figure 26.1 provides a schematic overview of the proposed pathophysiological mechanisms that take place in PTDM (adopted from [9]).

Risk Factors for Posttransplant Diabetes Mellitus

Non-modifiable risk factors are age over 45 years [10], ethnicity, heredity, and family history. Modifiable risk factors are obesity, dyslipidemia, hypertension, hypomagnesemia, hepatitis C infection, cytomegalovirus infection, and immunosuppressive agents.

A retrospective study in the US Renal Data System showed a 35% higher risk in Hispanics and a 68% higher risk in African-Americans. Patients with a family history of diabetes had a sevenfold risk [11]. Most nucleotide variants leading to enhanced susceptibility for T2DM affect the cell cycle of the pancreatic beta cells, and only a minority is associated with insulin resistance [12]. Obesity, defined as a body mass index (BMI) > 30 kg/m^2, increases the risk of PTDM with 75% and is often already present prior to transplantation, commonly followed by further weight gain after transplantation. Hepatitis C virus and cytomegalovirus are both associated with PTDM, possibly via inflammatory cytokines and increased oxidative stress enhancing insulin resistance [13]. High doses of statins also enhance the risk for developing PTDM with 9% after 1 year [9, 14]. Magnesium has a facilitating role in the sodium-potassium

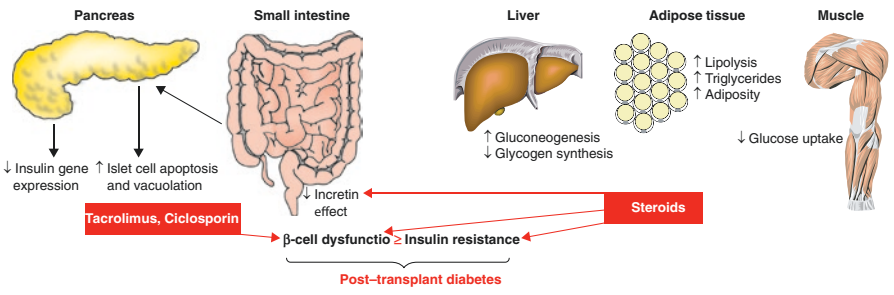

Fig. 26.1 Proposed pathophysiology of PTDM. (The figure was adopted from Sharif and Cohney [9])

gradient enabling glucose transport. Low magnesium levels are both in the normal population and in the transplanted population, which can be a risk factor for developing diabetes mellitus [9, 15]. The use of both proton pump and calcineurin inhibitors can lead to hypomagnesemia [16]. However, well-designed intervention trials exploring putative beneficial effects of magnesium suppletion have still to be performed.

Histological Changes in Posttransplant Diabetes Mellitus

After transplantation, diabetic nephropathy develops at an average of 5.9 years after transplantation, but histological changes can be seen as early as 1 year after transplantation [17–21]. Pre-existing diabetes mellitus from donor origin is associated with a worse short- and long-term graft outcome, at least in recipients from a deceased donor [22–24], and diabetic changes can be observed in biopsies acquired during the surgical transplantation procedure. Histological changes that are observed in renal transplant biopsies overlap with patterns of damage that are seen in diabetic nephropathy of the native kidneys (as described in Chap. 8); however after transplantation they can co-occur with features of T cell- and antibody-mediated rejection, viral infections, and patterns of injury related to drug toxicity. Especially the differential diagnosis with CNI toxicity, which is characterized by hyaline vascular changes as well, can be difficult. Early diabetic nephropathy is characterized by thickening of the glomerular and tubular basement membrane, mesangial matrix expansion due to extracellular matrix deposition, and mesangial hyperplasia [25]. Nyumura and colleagues showed with morphometric analysis of renal transplant biopsies that in patients with diabetic nephropathy who underwent renal transplantation, the level of blood HbA1c after transplantation associated with the progressive development of glomerular basement membrane thickening and mesangial area increases [21]. Also, glomerular capillary number, capillary area, and the size of the glomeruli were higher in patients with an HbA1c > 7.0%. In patients with a lower HbA1c on the contrary, these features were not different from control protocol biopsies from patients matched for clinical variables, indicating that glycemic control is mandatory in recipients with pre-existing diabetes mellitus to prevent the development of PTDM [21]. Patients with NODAT were excluded from these analyses. During the course of diabetic nephropathy, mesangial matrix expansion progresses, and eventually characteristic nodular fibrotic and hyalinized mesangial areas that compress the glomerular capillary tuft, so-called Kimmelstiel-Wilson nodules, will form. At an early stage, most probably due to mesangial matrix remodelling and mesangiolysis, glomerular capillaries can form micro-aneurysmatic dilatation. In diabetic nephropathy, both afferent and efferent glomerular arteriolar hyalinosis can be seen, which might differentiate PTDM from calcineurin inhibitor toxicity, which is associated with hyalinosis of the afferent glomerular arteriole exclusively. Comparative studies of CNI toxicity versus diabetic nephropathy have not been conducted, and the fact that the use of tacrolimus and to a lesser extent cyclosporine A is associated with PTDM makes a comparison difficult. One should bear in mind that the reproducibility of scoring arteriolar hyalinosis is low, which means that subtle features of both diseases

can be missed [26]. In diabetic nephropathy, proliferation of extra efferent arterioles, termed glomerular polar vasculosis, has been described as well [21, 25]. In the late stage of diabetic nephropathy, non-specific segmental and global glomerulosclerosis can develop, and at that stage, overt proteinuria has developed in most cases. In patients with overt proteinuria, hyaline changes of the Bowman's capsule can be seen, so-called capsular drops. Some studies describe such changes as being specific for diabetic nephropathy [27–29], although capsular drops have been described in the context of other glomerular diseases associated with proteinuria, including membranous nephropathy [30], which can also occur in transplanted kidneys (either as recurrent disease, de novo, or in the context of antibody-mediated rejection). Histological examples of diabetic nephropathy after transplantation are depicted in Fig. 26.2.

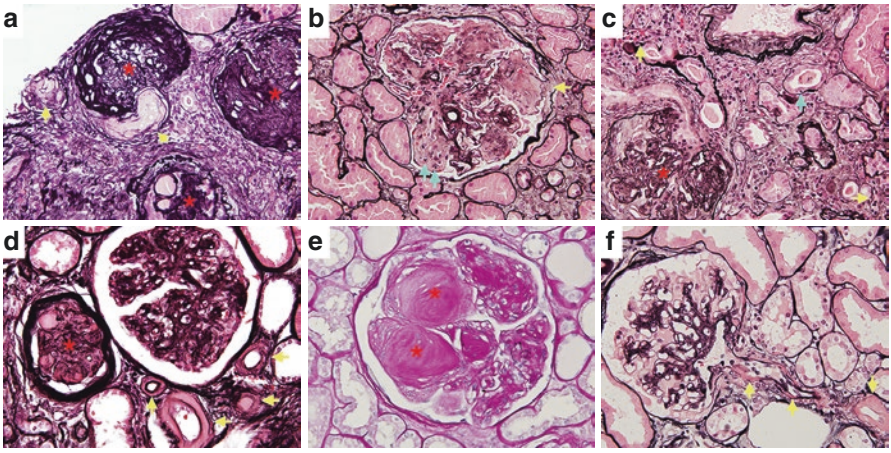

Fig. 26.2 Light microscopic, immunohistochemical, and electron microscopic microphotographs of PTDM. (**a–c**) This 59-year-old male developed PTDM 3 years after deceased kidney transplantation. His urinary protein concentration was 5.28 g/l, eGFR was 29 ml/min, and the HbA1c was 64 mmol/mol (8.0%). At the biopsy taken 16 years after transplantation, there is extensive arteriolar hyalinosis (**a**, yellow arrows) with globally sclerosed glomeruli (**a + c** red asterisks) and nodular mesangial sclerosis with a FSGS tip lesion (**b**, yellow arrow) but also some glomerulitis (**b**, blue arrows), peritubular capillaritis (**c**, yellow arrows), and some tubulitis (**c**, blue arrow). There was no C4d deposition on peritubular capillaries as evidence of complement activation. The patient was diagnosed with chronic active mixed rejection on a background of diabetic nephropathy. Because of the extensive chronic damage, the patient was treated conservatively with ACE inhibition and switched from dual immunosuppressive therapy to triple therapy. (**d**, **e**) This 66-year-old female was known for years with T2DM, which had led to end-stage renal disease. She acquired a renal transplant, and after transplantation she developed a stenosis of the proximal ureter with recurring transplant pyelonephritides. She was treated with insulin, prednisolone, and a calcineurin inhibitor. Her HbA1c was relatively stable at ~70 mmol/mol (8.6%). At 12 years after transplantation, transplantectomy was performed, which showed very extensive thickening of the Bowman's capsule with global glomerulosclerosis (**d**, red asterisk), arteriolar hyalinosis (**d**, yellow arrows), and Kimmelstiel-Wilson nodules (**e**, red asterisks). (**f**) This 30-year-old female, without a history of diabetes mellitus, acquired a non-heart-beating deceased kidney transplant from a donor with T1DM. Under dual therapy with prednisolone and a calcineurin inhibitor, the recipient remained with a HbA1c of 36 mmol/mol (5.1%), 1 g per 24 h urinary protein, and an eGFR of 79 ml/min. The reperfusion biopsy shows extensive arteriolar hyalinosis of donor origin (**f**, yellow arrows)

Immunofluorescence has no added value for the diagnosis of PTDM, since it is not considered an immune-mediated process. When immunofluorescence is performed in case of suspicion of de novo or recurrent primary immune-mediated renal disease, nonimmunological entrapment of mostly IgG and albumin and to a lesser extent IgA, IgM, and C3c in a linear pattern along the thickened glomerular and tubular basement membrane can be observed [31, 32]. Linear IgG staining is not as intense as in heavy chain deposition disease, or anti-glomerular basement membrane nephritis, which can develop as an "alloimmune" response posttransplantation in patients with Alport disease [33, 34]. Although linear immunoglobulin staining is regarded as a-specific entrapment in sclerosed glomerular basement membranes in diabetic nephropathy, Mise and colleagues suggested that a higher intensity of linear IgG staining along the glomerular basement membrane is associated with a higher hazard for the development of end-stage diabetic nephropathy of native kidneys [35]. A higher linear IgG score is associated with more proteinuria, a lower estimated glomerular filtration rate, more insulin use, and numerically thicker glomerular basement membranes on electron microscopy, indicating a profile of more advanced diabetic nephropathy in patients with a higher linear IgG score. Whether similar findings can be extrapolated to PTDM is not known. In advanced-stage PTDM, like in diabetic nephropathy of the native kidneys, there can also be non-specific entrapment of IgM and/or C3c in areas of nodular sclerosis [31, 32]. On electron microscopic evaluation, no electron-dense deposits are present [31].

Glomerular changes can develop at different rates, and the differential diagnosis with other posttransplant vascular diseases like antibody-mediated rejection, also characterized by glomerular basement membrane thickening and/or multilayering, and hypertensive vascular changes, either of donor or recipient origin, can be difficult when patterns are subtle. It is to be advised to correlate biopsy finding with clinical features of underlying disease entities, including (HbA1c) levels for PTDM, drug pharmacodynamics (i.e., blood trough levels) for CNI toxicity, and the existence of donor-specific antibodies for antibody-mediated rejection. In case of chronic active antibody-mediated rejection, there should be evidence of (micro) vascular inflammation (i.e., glomerulitis, peritubular capillaritis, arteritis) and/or evidence of complement activation (C4d deposition on peritubular capillaritis) [36–38]. Over the past years, a lot of effort has been put into the study of molecular changes that occur during rejection and how transcriptomic platforms can be of help in diagnostics [36]. It is however not clear whether some of these molecular changes can also be observed during diabetic nephropathy [39], since diabetic nephropathy, like antibody-mediated rejection, is characterized by endothelial damage with overlap of transcripts with the endothelial cell-associated transcripts (ENDAT) that are enriched during humoral rejection [40–43].

Besides glomerular and vascular changes, also tubulointerstitial damage can develop in the course of diabetic nephropathy after transplantation. These features are also not different between diabetic nephropathy of the transplanted kidneys and diabetic nephropathy of the native kidneys. Interstitial fibrosis and tubular atrophy can be seen as the consequence of a final common pathway of damage and are

observed in all progressive renal diseases, including diabetic nephropathy [44]. A feature that can be seen in practically all patients with interstitial fibrosis and tubular atrophy is interstitial inflammation. Tubulitis is not a feature that is typically seen in fibrotic or non-fibrotic areas during diabetic nephropathy, and according to a recent report by Lefaucheur and colleagues, inflammation in scarred areas represents T cell-mediated rejection rather than a non-specific and indolent resorption infiltrate [45]. A report by Borda and colleagues found a higher percentage of subclinical T cell-mediated rejection in patients with PTDM (22%) as compared to patients with an increased fasting glucose/impaired glucose tolerance (8%) and controls (7%) [19]. The authors did not investigate whether the percentage of antibody-mediated rejection also differed between these metabolic groups, although they did not observe a difference in glomerulitis. The cause of this association is not clear, and larger longitudinal studies should define the temporal interaction between glycemic control and renal transplant rejection in more depth. Although anti-glycemic drugs are not the typical causal agents of tubulointerstitial nephritis, a hypersensitivity reaction to recently introduced drugs should always be considered as a potential cause.

Genetic Variants and Molecular Pathways Associated with Posttransplant Diabetes Mellitus

Various studies have identified single nucleotide polymorphisms (SNPs) that are associated with the prevalence of PTDM. Benson and colleagues performed a meta-analysis and concluded that three variants were significantly enriched in patients who had developed PTDM compared to matching controls: *CDKAL1* rs10946398, *KCNQ1* rs2237892, and *TCF7L2* rs7903146 [46]. All of these variants have previously been linked to the presence of T2DM in the general population from different ethnical backgrounds, although with conflicting results [47]. All three variants have high minor allele frequencies in the control groups (ranging from 18.41% to 43.39%) with a higher enrichment in patients with PTDM: odds ratios (95% confidence intervals) of 1.43 (1.11–1.85), 1.43 (1.10–1.86), and 1.41 (1.07–1.85), respectively. This meta-analysis shows that the relative contribution of these genetic variants for the development of PTDM is limited. How could these genes contribute to the development of PTDM?

TCF7L2 encodes for transcription factor 7-like-2 (TCF7L2) and is the most studied gene involved in PTDM. It appears that the *TCF7L2* rs7903146 variant associates with PTDM independent from other known risk factors, including the use of tacrolimus, body mass index, age, and corticosteroid pulse treatment for acute rejection [48]. In the same study, Ghisdal and colleagues describe that the *TCF7L2* rs7903146 variant is enriched in patients with PTDM versus euglycemic controls but not in the group with an impaired fasting glucose versus euglycemic controls, which suggests that the function of TCF7L2 is more important in the progression from prediabetes to PTDM than from euglycemia to prediabetes. TCF7L2 is a

transcription factor that is expressed in multiple organs, including the brain, the liver, the intestine, and adipocytes and beta cells of the pancreas [49]. It has been suggested that genetic defects in *TCF7L2* can render a person more susceptible to T2DM by decreasing the production of glucagon-like peptide-1 (GLP1), which is an important hormone responsible for glucose-dependent secretion of insulin from islands of Langerhans [50, 51].

CDKAL1 is a gene that encodes for cyclin-dependent kinase 5 regulatory subunit-associated protein 1-like 1 (CDKAL1). CDKAL1 (Cdkal1 in mice) is a member of the methylthiotransferase family and was described to be of importance in pro-insulin translation in pancreatic beta cells [52, 53] and mitochondrial function in adipose tissue in mice [54, 55], and Cdkal1 inhibits adipocyte differentiation via activation of Wnt signaling [56]. Cdkal1 in zebrafish has also been shown to promote pancreatic beta-cell differentiation via inhibition of Cdk5 [57].

KCNQ1 encodes for potassium voltage-gated channel subfamily Q member 1 (KCNQ1, also known as $K_V7.1$ or KvLQT1). KCNQ1 is best known for its importance in cardiac myocyte repolarization [58, 59], but it has a broader tissue distribution including neurons [60], where it is important for excitability, and renal and gastrointestinal epithelial cells where it is important for sodium absorption, gastric acid secretion, and jejunal chloride secretion [61]. Missense variants can lead to familial atrial fibrillation [62] and long QT syndrome [58, 59]. Patients with KCNQ1 loss-of-function-associated long QT syndrome also have hyperinsulinemia and symptomatic hypoglycemia [63]. An inverse phenotype is observed in pancreatic beta cells from patients with T2DM, attributed to a reduction in depolarization-evoked insulin exocytosis [64, 65].

When data from all the genes that have been described to be associated with PTDM in at least three studies (as presented in the meta-analysis by Benson and colleagues) is plotted in a protein-protein interaction network [46], there is evidence of interactions among proteins, either by text mining, by co-expression in previous studies or from curated databases (Fig. 26.3). This network shows

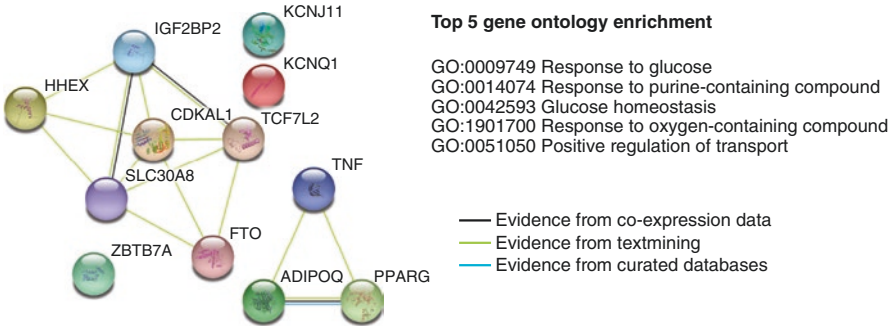

Fig. 26.3 Protein-protein interaction network based on single nucleotide variants associated with PTDM. The network is enriched in genes known to regulate the response to glucose and glucose homeostasis and the response to purine- and oxygen-containing compounds and regulation of molecular transport. (The figure was generated with the use of STRING version 10.5 [67])

functional enrichment of response to glucose and glucose homeostasis, response to purine- and oxygen-containing compounds, and positive regulation of cellular transport as the top five enriched gene ontology pathways [66]. Beyond genetic association of single nucleotide variant enrichment in patients with PTDM, there are no other molecular studies available that investigated PTDM on the level of epigenetic regulation, gene transcription, posttranscriptional regulation, protein translation or posttranslational modifications, and protein-protein interaction. Future holistic systems biology approaches will further increase our knowledge on the molecular pathways that lead to PTDM and how these pathways relate to immunosuppressive drug protocols.

Treatment Options for Posttransplant Diabetes Mellitus

Lifestyle Modifications

Lifestyle modification might be of help in the prevention of PTDM [68], but few studies have been done to provide solid evidence. On average, transplant recipients gain 4 kg of weight after transplantation. This increase of weight is often attributed to the use of steroids. Some studies do not show differences between the steroid-free and the steroid-containing arms. Increased appetite and less dietary restriction probably also have a role [69]. It is assumed that target levels of HbA1c should be similar to those in the general population, but no studies support this assumption. Smoking increases the risk for ischemic heart disease after transplantation.

Bariatric Surgery

In non-transplanted patients with a BMI > 40 or > 35 with serious obesity-related comorbidity, bariatric surgery is the standard of care and has proven to be very successful in terms of weight loss and reduction of metabolic complications. However bariatric surgery can result in increased absorption of oxalic acid from the gut leading to nephrolithiasis, acute kidney injury, and rarely oxalate nephropathy. Especially patients with already impaired renal function are vulnerable to these complications [70, 71]. Successful weight loss after bariatric surgery has also been reported in renal transplant recipients. Decreased absorption of immunosuppression appears not to be of clinical significance. Colomb et al. reported the outcomes of laparoscopic sleeve gastrectomy in 10 renal transplant recipients with a mean age of 57 years, a median preoperative BMI of 42 kg/m^2, and a median follow-up of 14 months. There was no mortality, graft rejection, or dysfunction. After 6 and 12 months, the BMI had decreased to 31 and 29 kg/m^2, respectively [72]. In an analysis of the USRDS database from 1991 to 2004, 188 cases were found, of which 101 underwent bariatric surgery cases prior and 87 after transplantation. Median weight loss was 31–61%,

similar to the general population, but 30-day mortality post-bariatric surgery was 3.5% [73]. Bariatric surgery can also be a bridge to renal transplantation since many centers do not wait-list renal transplant candidates with a BMI > 35 [74].

Anti-glycemic Medication

Although often antidiabetic therapy follows the guidelines for T2DM [75], studies seeking evidence for the optimal treatment are scarce. Metformin, which inhibits glucose production by the liver and decreases insulin resistance, is often the prime therapy and seems safe in patients with an eGFR >30 ml/min [76]. The safety of administration of this drug in patients with an eGFR <30 ml/min remains a matter of debate [77]. SGLT2 inhibitors were shown to reduce weight gain, reduce cardiovascular mortality, and lower blood pressure in the non-transplant population [78]. These drugs hold promise to be beneficial also in the transplanted population but need to be further explored. One concern is the profile of side effects, especially an increased incidence of urinary tract and genital infections as well as volume depletion. There is one randomized trial with the DPP-4 inhibitor vildagliptin compared to placebo in stable renal transplant recipients, in which postprandial glucoses were better at 3 months in the treatment arm [79]. There are no randomized trials on the benefits of GLP1 agonists in renal transplantation.

Immunosuppressive Medication

There are no official recommendations for immunosuppressive strategies in patients at risk for contracting PTDM. Calcineurin inhibitors have dose-dependent diabetogenic properties. They increase insulin resistance, are directly toxic to the pancreas, and inhibit glucose uptake by peripheral tissues [80] by lowering the amount of GLUT4 at the surface of adipocytes. Tacrolimus in higher doses with target trough levels between 10 and 15 microgram/L has a five times stronger diabetogenic effect than cyclosporine A [81] due to a dose-dependent direct toxic effect on pancreatic beta cells. Low levels of tacrolimus did not seem to increase the risk of PTDM [82, 83]. Steroids inhibit insulin signaling, inhibit the synthesis of GLUT4, and are toxic for the pancreatic beta-cell load. In a retrospective study in the United States, the combination of tacrolimus and the mTOR inhibitor sirolimus was the most diabetogenic and increased the mortality risk by 20% [84].

Data on the effects of early steroid withdrawal are contradictory. In a randomized trial including 177 patients, the risks and benefits of early steroid withdrawal as compared to long-term maintenance therapy were studied. A similar risk in developing PTDM was observed in both treatment arms, and therefore no benefit was observed for early steroid withdrawal [85]. Another prospective randomized double-blind placebo-controlled trial comparing corticosteroid withdrawal at day 7 after transplanta-

tion to maintenance therapy with low-dose steroids showed no benefits of corticosteroid withdrawal on allograft survival and cardiovascular mortality [86]. However, a meta-analysis did show a decrease in cardiovascular risk of withdrawing steroids [87]. The lack of well-designed clinical studies in renal transplant recipients had led to recommendations based on expert opinion. To reduce cardiovascular morbidity, HbA1c levels should be targeted <6.5%, blood pressure < 130/89 mmHg, and LDL cholesterol <2.6 mmol/L. Metformin is the prime therapy followed by a second oral drug, which can be either a sulfonylurea derivative, a glinide, DPP-4, or a GLP1 agonist. If hyperglycemia persists, insulin therapy may replace the second oral drug. Hypertension should be treated preferably by angiotensin-converting enzyme (ACE) inhibitors and dyslipidemia with a low-dose statin treatment and when necessary with ezetimibe.

Conclusions

PTDM and transplant diabetic nephropathy, either via pre-existing diabetes mellitus or via the development of de novo diabetes mellitus after transplantation, commonly complicate the follow-up of renal transplantation. Patients are at risk for cardiovascular events, but also graft survival is negatively influenced by PTDM. Many clinical risk factors have been identified, including the adverse effects of immunosuppressive agents. There seem to be overlapping genetic variants between T2DM and PTDM, which suggests similar genetic predisposition, but there are likely also molecular differences since transplant recipients can develop true NODAT. Diabetic nephropathy after transplantation develops with similar histopathological features as diabetic nephropathy of the native kidneys, although it seems more rapidly progressive. It is not yet known how other transplant diseases that cause endothelial damage, including antibody-mediated rejection and CNI toxicity, can influence the progression of glucose-mediated renal damage. An interesting direction for future research would be to investigate the molecular interaction of immunosuppressive drugs, the gastrointestinal tract (including the liver and pancreas), and peripheral sites like adipose tissues and muscle. Better understanding of these molecular interactions can help to lower cardiovascular risk, and it will allow the development of treatment options that block the diabetogenic but not the immunosuppressive capacity of current drug regimens.

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Part IX
Future Developments

Chapter 27
Health Programmes in Low- and Middle-Income Countries

Maria Pallayova, Gopesh K. Modi, and Indranil Dasgupta

Introduction

Despite recent scientific breakthroughs and the availability of novel treatments, diabetes mellitus remains a significant global health challenge associated with development and progression of serious micro- and macrovascular complications affecting multiple organ systems [1]. Diabetic kidney disease (DKD) has a particularly devastating impact on affected individuals and is very costly to health services and society while being one of the main causes of end-stage renal disease worldwide [2, 3].

DKD affects about 30% of type 1 and type 2 diabetic patients with increasing estimated prevalence of up to 50% in those with long-standing diabetes. Approximately 20% of all diabetic patients will develop end-stage renal disease (ESRD) without intervention [4–6]. Although the rates of diabetic complications in developed nations have stabilized as a result of improved cardio-metabolic management, the absolute actual numbers of individuals affected by diabetes and its complications are rapidly increasing particularly in developing nations [7].

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Steadily, increasing global population and improvements in survival leading to aging of the population over the past three decades have contributed to the increasing prevalence of chronic kidney disease (CKD) throughout the world [8]. The main driver of this escalating global CKD burden is the rapidly rising prevalence of diabetes and hypertension which is running parallel to increasing longevity, urbanization and lifestyle changes including change in dietary habits, lack of physical exercise, increasing body mass index and smoking [9]. However, premature mortality is still high in low-income countries despite the global shift from premature death to disability due to disease [10]. Additionally, patients from low- and middle-income countries (LMIC) are often the least able to deal with the burden of diabetes and CKD, and the healthcare facilities of these countries are least able to cope with the demand for equitable access to complex diabetes and renal therapies [11]. A comparative risk assessment of global mortality from cardiovascular disease, CKD and diabetes in 1980–2010 has demonstrated that the mortality burden of cardiometabolic risk factors has shifted from high-income to low- and middle-income countries [12]. The epidemiological shift to non-communicable diseases (NCD) as the major cause of morbidity and mortality is increasingly evident even in the poorest nations [13]. The current age-specific rates of NCD mortality rates in LMIC are twice as high as high-income countries. At present, four out of the five total deaths globally occur in LMIC. It has been projected that by 2030, three out of the four leading causes of death will be attributable to NCDs [14, 15]. Death and disabilities from NCD already exceed that due to communicable diseases, maternal and child health issues and nutritional causes combined in South Asia [13]. Combination of all these factors has positioned DKD as an important global health challenge, especially in LMIC.

In this chapter, we will discuss how the population and epidemiological changes have influenced DKD and may impact its future across different populations and clinical settings in LMIC. We will also review the current health programmes in LMIC and discuss multifactorial approaches based on collaborative efforts to optimize future management of DKD and associated cardio-metabolic risk factors in order to reduce their sequelae.

Definition of Low- and Middle-Income Countries

For the 2018 fiscal year, low-income economies were defined as those with a gross national income (GNI) per capita calculated using the World Bank Atlas method [16] of $1005 or less in 2016. The middle-income economies are those with a GNI per capita of more than $1005 but less than $12,236. Lower middle-income and upper middle-income economies are separated at a GNI per capita of $3956. The low-income countries recognized by the World Bank Group's classification include 31 countries, the lower middle-income group comprises 53 countries, and the upper middle-income group consists 56 countries [17]. Therefore, almost two-thirds of all the nations on the globe are LMIC countries and home to more than three-fourths of

the global population [19]. The size of LMIC population and the increasing burden of DM and CKD make it inevitable that the overall impact of DKD in the future will be related to LMIC populations.

Clinical and Economic Burden of Diabetic Kidney Disease

At present, diabetes mellitus is estimated to affect more than 415 million people (8.8% of adults) worldwide of whom about 75% live in LMIC [20]. The prevalence of diabetes continues to increase globally with the largest increases being in the regions where economies are moving from low-income to middle-income levels [20]. It has been estimated that by 2030, the prevalence of hypertension and diabetes will rise by over 80–100% in LMIC and by 20–50% in high-income countries [20]. Moreover, it is estimated that as many as 193 million people remain undiagnosed worldwide, of whom about 81.1% live in LMIC where resources are often lacking and governments may not prioritize screening for diabetes, e.g. in sub-Saharan Africa [20]. There is lack of reliable recent data on prevalence of prediabetes, diabetes, hyperglycaemia in pregnancy and diabetic complications in LMIC where diabetes appears to be increasing rapidly. The parallel increases in the prevalence of hypertension and obesity globally are likely to multiply the risk of development of DKD.

With respect to kidney disease, the gaps in the current literature include a lack of standardized reporting of DKD, which is the leading cause of end-stage renal disease. In a recent global survey, only 7.7% of the countries reported having registries for non-dialysis CKD [21]. Even, dialysis and kidney transplantation registries are non-existent in most LMIC. Despite the sparse evidence and fewer available data on DKD prevalence in LMIC, a systematic analysis of the worldwide global burden of CKD in 2010 [22] has demonstrated that CKD affects as many as 11% of the population worldwide. This consisted of 8.6% in men and 9.6% in women in high-income countries and 10.6% in men and 12.5% in women in LMIC [22]. Therefore, the burden of CKD in general is higher in LMIC placing even more strain on their limited resources. Furthermore, economic development is inversely related to CKD: poverty increases the risk of developing CKD and risk of adverse outcomes related to CKD [21].

While much of the increased prevalence of renal impairment can be attributed to an increasing prevalence and/or insufficient control of diabetes and associated known risk factors, an overlap with other forms of progressive kidney disease, including nondiabetic glomerular disease, hypertensive and ischemic nephropathy related to vascular damage, nephrotoxin exposure (including some traditional and alternative remedies) and CKD of unknown cause is increasingly being observed in populations of LMIC [23–25].

Diabetes is now the major cause of ESRD in most countries of the world. The USRDS figures reveal that 44% of all incident ESRD patients are diabetic [26]. In Australia and New Zealand, 25% of incident ESRD is due to diabetes [27] and

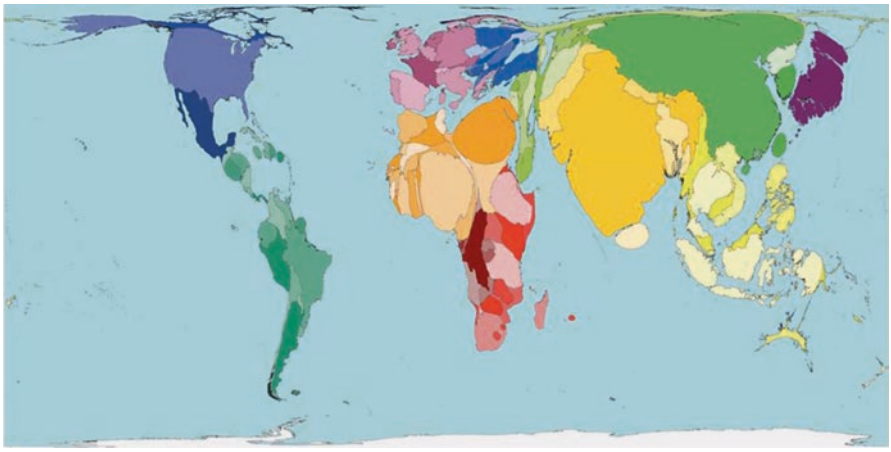

Fig. 27.1 Geographic burden of kidney disease in the world. Size of countries depicted in proportion to annual mortality due to kidney disease [30]

between 15% and 33% in the European Renal Registry [28]. Lately, LMIC are reporting ESRD attributable to diabetes in a substantial proportion of patients. In a survey across Asia, diabetic nephropathy was the most common cause of ESRD in nine out of ten countries surveyed [29]. India, home to the largest number of people with diabetes and hypertension in the world, is likely to face a catastrophic CKD burden (Fig. 27.1) [30].

Both diabetes and kidney disease impose a very high economic burden on individuals and society. About 2–3% of the healthcare expenditure of developed nations is spent on the treatment of ESRD patients even though they comprise less than 0.2% of the total population [31]. The costs of treating earlier stages of CKD are even higher. The Australian Institute of Health and Welfare reported health expenditure on CKD in 2004–2005 to be $898.7 million, which is 1.7% of total expenditure. This was reportedly an increase of 33% since 2000–2001 [32]. The UK National Health Service spending on CKD in 2009–2010 was £1.45 billion, 1.3% of all health spending [33].

The implications for LMIC are obvious if they are to provide care parallel to these economies. Experience in LMIC that offer enhanced access to ESRD care illustrates this. Thailand started universal coverage for dialysis in 2008. The total spending on dialysis spiralled up from 160 million bahts (US $4.8 million, 0.2% of the total budget) in 2008 to 3.9 billion bahts (US $118 million, 3.4% of total budget) in 2012 [34]. Uruguay reported spending 3% of its total health budget on dialysis [35]. Similarly, the Brazilian Ministry of Health spent US $500 million on renal replacement therapy (RRT) in 2004, and 28% (US $100 million) of the Egyptian healthcare budget was spent on government-sponsored RRT in 2008 [36, 37].

The public sector healthcare spending is extremely low in several LMIC. As a result, patients are forced to make out of pocket expenditure, and this drives many families below poverty lines. In one Indian study, the cost of dialysis resulted in

catastrophic healthcare expenditures for 70% patients [38]. Therefore, only a minority of ESRD patients in developing countries is able to receive long-term RRT.

ESRD represents the terminal point in the natural history of DKD. Limited and often insufficient access to publicly available medical services, health insurance, specialist care, diagnostic tests including blood glucose test strips, insulin and other antidiabetic agents, cardiovascular disease medicines and medications that attenuate the course of CKD and its consequences is a significant challenge in healthcare in LMIC [39]. The Prospective Urban Rural Epidemiology (PURE) study [39] has demonstrated a very low use of four key cardiovascular disease medicines (aspirin, beta-blockers, angiotensin-converting enzyme inhibitors and statins) that are unavailable or unaffordable for 0.14% of households in high-income countries, 25% of upper middle-income countries, 33% of lower middle-income countries, 60% of low-income countries (excluding India) and 59% households in India. Further, only 19% of global health expenditure on diabetes is spent in LMIC, where 75.4% of people with diabetes live; the State of Africa Region has the lowest total health expenditure amongst all regions [20]. A gap between health expenditures and the cost of diabetes care may promote a high frequency of complications, disabilities and premature mortality [40]. The shortage of nephrological personnel is a critical issue [41]. The findings from recent survey revealed <5 nephrologists per million of the population (pmp) in most of LMIC (with many sub-Saharan Africa countries having <1 nephrologist pmp) in comparison with >15 pmp in the high-income countries [21].

These realities translate into wide gaps in the treatment of diabetes and kidney disease in LMIC. A recent systematic review has shown that in 2010 while 2618 million people received life-saving RRT worldwide, at least 2284 million people might have died prematurely because of lack of access to RRT [42]. The largest treatment gaps were noted in low-income countries, particularly in Asia (1.907 million people needing but not receiving RRT) and Africa (432.000 people) [42].

Health Programmes in LMIC

Comprehensive healthcare programmes centred around CKD and, more specifically DKD, are almost non-existent in most of the LMIC. The main body of information available currently is on ESRD and RRT utilization. A recent estimate showed that globally 2.618 million people received RRT in 2010, but 44% of these patients were in just five countries (USA, Japan, Germany, Brazil and Italy) that are home to 12% of world population. A meagre 7.2% of RRT recipients were from LMIC [18].

The absence of state-supported renal replacement programmes and lack of health insurance make care for ESRD practically unaffordable in large parts of the world [43]. In Nigeria, the cost of one haemodialysis session is US$100. This is twice the minimum monthly wage paid to government workers in the country [44]. It costs US$14,300 per patient per year for dialysis treatment in China. Lately, commitments are being made to support the cost of ESRD care in many countries. The

Chinese government has instituted a variety of insurance schemes for rural and urban populations. However, patients have to make co-payment of as much as 35–45% of the cost, which is prohibitive for most people [45]. In India, the cost of a single dialysis session varies from US$20 to $60. Government-supported insurance schemes have been started in some states for the poor, but coverage remains limited and sometimes capped at $500 per year [46]. The government of India recently launched the 'National Dialysis Services Programme' to provide dialysis services for the economically weaker sections of the society in all district hospitals of the country under a public-private partnership model [47].

To tackle the problem at an earlier stage, several countries have started CKD prevention programmes. Taiwan started a kidney health promotion project aimed at spreading awareness and augmenting research in 2003 with a budget of US$15.0 million/year. In 2007, a programme of integrated care for patients before they developed ESRD was instituted (budget US$1.5 million/year) [48]. A decline in the number of incident ESRD cases has been noted since 2009 leading to a savings of US$36 million per year [48]. The government of Cuba has been running a programme that supports epidemiological research, continuing education for medical professionals and reorientation of primary healthcare towards service delivery, surveillance and intervention in CKD [49]. The Ministry of Health of Mexico set up a network of health services for managing CKD with an outlay of $US50 million with a view to reducing the number of patients with ESRD by 50% by 2025 [50]. Uruguay and Chile have adopted similar programmes [51, 52]. In Uruguay, the incidence and prevalence of end-stage kidney disease declined from 1.6% and 5.4%, respectively, in 1994–2003, to 0.13% and 1.6% in the following decade [53]. Similar decrements were reported from Chile. The annual incidence and prevalence of end-stage kidney disease reduced from 13.3% and 14.5%, respectively, in 2005–08 to 1.9% and 4.6% in 2009 [52]. However, these official country statistics need to be independently validated and the overall effects of these programmes judged appropriately. More importantly, these programmes will need to sustain to be able to make a difference in the long-term.

A recent survey of 130 countries elucidated the level of global preparedness for handling CKD. It reported gross unavailability of kidney care services in most countries in all aspects: detection, diagnosis, treatment programmes, data management and access to RRT. For CKD monitoring in primary care, serum creatinine with estimated glomerular filtration rate and proteinuria measurements were available in only 21 (18%) and 9 (8%) countries, respectively. For instance, no country from the low-income and lower middle-income categories reported complete public funding for medications for non-dialysis CKD care (including angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, other antihypertensive agents, statins and glucose-lowering agents). Even in the high- and upper middle-income nations, 32% reported complete public funding for medications in non-dialysis CKD care [21].

The awareness of CKD remains very low—both amongst patients and the physicians. A cross-sectional survey of community-based adults in Northern Tanzania [54] demonstrated a very limited knowledge of CKD despite the operation of

national kidney disease prevention programme. In a sample of Chinese adults, the prevalence of CKD was 13.6% but only 8.3% of them were aware of the condition [55]. Even in developed countries, the awareness of CKD is lacking. In a study from the USA involving around 90,000 adults at high risk of CKD, the prevalence and awareness rates were around 20–30% and 6–11%, respectively [56]. Awareness is low amongst non-nephrologist physicians. Italian general physicians were able to correctly identify only one patient out of eight patients with CKD; a nephrology consultation was requested in only 5% of patients with overt CKD (GFR 60–30 ml/min). Referral rate to a nephrologist was <50% even in the more advanced stages of CKD (GFR 30–15 ml/min) [57]. One can assume similarly low CKD awareness amongst physicians in LMIC.

CKD care and follow-up require trained manpower with varied skills such as nephrologists, renal nurses, dialysis technicians, nutritionists and renal social workers. The availability of most of these personnel is very limited in the LMIC. Reports suggest that in Latin America, the number of nephrologists varies from 1.7 per million of the population in Honduras to 53.9 per million of the population in Uruguay [58]. The wide variance is evident in Asia as well. In Southeast Asia, there is one nephrologist pmp, and the numbers vary from 25 pmp in Brunei to 0.2 pmp in Indonesia and Myanmar [58]. India, with a population of over 1.25 billion, has only 850 nephrologists as reported in 2009 [58]. Many countries in Africa have fewer than ten nephrologists [41]. This makes it imperative to involve general practitioners in dealing with management of DKD.

Most patients with CKD are managed by primary care providers in the LMIC with only a few making it to specialist nephrology care [25]. Innovative methods such as telemedicine could be useful to facilitate access to medical advice, guide management of CKD and thus also improve the quality of follow-up care [25]. The situation is more grim because there is a shortfall of even trained general physicians in these countries. The best answer would be judicious task-shifting, aided by the use of technology, whereby electronic decision support systems help nonphysician healthcare workers to deliver up-to-date guideline-based care in the community with appropriate referrals to physicians. A systematic review suggests that compared with standard care, the involvement of community health workers in health programmes has the potential to be effective in LMIC, particularly for tobacco cessation, blood pressure and diabetes control [59]. However, such approaches will need proper training and retraining of staff, adequate referral channels and support structures to ensure success. Training can be facilitated by accessing nephrologists from various countries for hands-on training, setting up community screening programmes or staff training for task-shifting through the International Society of Nephrology Educational Ambassador programme (www.theisn.org/programs).

Taking this forward, it has also been shown that algorithm-driven, primary care disease management programmes may reduce the rate of renal function loss in patients with CKD [60]. To improve the cost-effectiveness, self-management interventions for patients with CKD may be implemented. This is being tested in an ongoing randomized controlled trial (RCT)—BRinging Information and Guided Help Together (BRIGHT) [61]. Technology using e-health platforms,

telemedicine and mobile phone networks can increase the reach and scope of such interventions for training and care delivery. Technology will play a big role in LMIC because of its speed, reach, reliability, reduced chance of corruption and availability. Examples of such innovations are ASHA (accredited social health activists) programme for tuberculosis in India and M-DOK mobile health system that allows rural community health workers in the Philippines to send patient information over text messages to specialists in urban areas, who then advise on accurate diagnosis and appropriate treatment [62]. Some of other such examples are TeleDoctor in Pakistan, which provides access to physicians through a telephone hotline; E Health Point in India, which facilitates patient-doctor interactions in rural areas through video-conferencing; Nacer which uses telephone and Internet technology to allow health workers in Peru to collect data on various populations and share it remotely with medical experts for data analysis; and the use of live stream video to monitor dialysis centres and patients in district hospitals of India. These technological advances are of particular benefit for data capturing, monitoring and analysis [63].

While global guidelines assume the availability of sophisticated laboratory assays and treatments, it is particularly challenging to identify people undiagnosed with diabetes and those with DKD in many of the LMIC in a timely manner. In many of these countries, the data required to develop diabetes risk prediction scores for their populations is limited or non-existent [20], and laboratory testing is infrequent or unaffordable. As a result, blood glucose levels and kidney function are often unknown, which hampers the diagnosis and delivery of targeted therapies. Thus, there is a pressing need for the increased use of existing diagnostic tests. Affordable point-of-care testing of blood glucose, albuminuria and estimated glomerular filtration rate with reliable accuracy could improve the ability to detect patients with diabetes and DKD early and monitor those with advanced DKD at minimal cost, especially in rural areas of LMIC. The ability to identify these patients will allow opportunities for intervention, follow-up and specialist referral if required. Assessing the practicality and sustainability of the long-term use of point-of-care devices in these settings should be a high priority for future research and could provide an incentive for public-private partnerships [25].

The cornerstone for all these strategies is early detection of DKD. While screening in diabetics has been borne out by most analyses and is part of standard guidelines, general population screening for CKD has not been found to be cost-effective. A case can be made for general population screening in LMIC, where the population incidence of diabetes and hypertension is high, and as many as 50% or more patients are with undiagnosed diabetes. Another reason could be the presence of as yet unknown risk factors and lack of resources for managing CKD or ESRD. As part of the WHO's Package of Essential Noncommunicable (PEN) Disease Interventions for primary healthcare, model-based economic evaluation was performed for screening for diabetes and hypertension in Bhutan. While it upheld the current guidelines for screening in high-risk groups, in resource-limited settings, universal coverage (i.e. screening 100% of population) was found to be more

cost-effective [64]. It is tempting to speculate that such a result is expected for universal CKD screening as well which could be carried out in primary healthcare as part of comprehensive NCD strategies in LMIC for precisely similar assumptions.

It is worthwhile pointing out that the cost-effectiveness threshold varies according to the level of economic development. On the basis of the cost of the intervention per disease-adjusted life-year saved, interventions are classified as highly cost-effective (cost less than the per capita GDP), cost-effective (1–3 times the per capita GDP) or not cost effective (>3 times the per capita GDP) [65]. This will warrant local cost-effectiveness analysis based on more specific and relevant variables.

In parallel, the discussions around the cost of CKD or ESRD care often take place around the out-of-pocket costs incurred by the patients. According to health economists, the economic consequences should be evaluated under three subheads: social welfare costs, the value that people place on better health; macroeconomic costs, the GDP losses countries incur due to ill health in the population; and microeconomic costs, related to household financing of care, changes in consumption patterns and forgone earnings of individuals and households due to the ill health amongst members. These domains are obviously different for all countries and societies and determine the cost-benefit figures for interventions [66].

In 2016, the International Society of Nephrology (ISN) identified and prioritized key activities for the next 5–10 years in the domains of clinical care, research and advocacy and created an integrated comprehensive action plan and performance framework to close gaps in global kidney care, research and policy and thus benefit people who are at risk for or affected by CKD worldwide [25]. The plan was based on ten themes focused on the following four key areas: (i) improve the identification of CKD and reduce risk factors for CKD, (ii) improve the understanding of causes and consequences of CKD, (iii) improve outcomes with current knowledge, and (iv) develop and test new therapeutic strategies [25]. Since 2014, several international initiatives have emerged under the ISN leadership to foster collaboration in observational and interventional research, including Kidney Disease: Improving Global Outcomes (KDIGO), ISN Advancing Clinical Trials (ISN-ACT) and International Network of CKD cohort studies (ISN-iNET CKD), the CKD Prognosis Consortium (CKD-PC) and the Kidney Health Initiative (KHI) [67]. ISN also is active in advancing nephrology in developing worlds, training nephrologists and fostering sister centres across the world for development of centres to deliver kidney care. Since its inception in 2006, the World Kidney Day (WKD), a joint initiative of the International Society of Nephrology (ISN) and the International Federation of Kidney Foundations, has become the most successful effort mounted to raise awareness amongst decision-makers and the general public about the importance of kidney disease. The ISN Clinical Research Program has shown that early detection and prevention programmes can be carried out cost-effectively in very resource-poor settings using the CKD, Hypertension, Diabetes and Cardiovascular Disease (KHDC) template [68].

The implementation of renal health programmes is urgently needed. The lack of human and financial resources has hampered nephrology programmes in the

detection, prevention and treatment of CKD in many LMIC [41]. The first step to making progress in improving implementation of health programmes in LMIC is to make sure the programmes are tailored appropriately for different settings. There is also a need to ensure that guidelines and treatment strategies are tailored to LMIC and that decision-makers and funders understand the clinical and socioeconomic benefits of improving access to care [25]. Strategies to reduce burden and costs related to CKD need to be included in national programmes for non-communicable diseases [9]. The essential elements to develop and execute a successful programme for NCD and CKD are given in recent reviews [69, 70].

Similarly, national and regional collaborations are important elements for mounting a response to the emerging epidemic of DN. For example, the Latin American Society of Nephrology and Hypertension is fostering a cardiovascular, cerebral, renal and endocrine-metabolic health programme in which 12 countries in the Latin American region implement different strategies, including allocation of national funds and strengthening of transplant programmes with the focus on promotion, prevention, rehabilitation, research and teaching [71].

For any successful healthcare intervention at the community and societal level, a comprehensive approach is needed. And, the most important component for this is the recognition of CKD as a public health problem by the governments and then subsequent support at policy, administrative and financial levels. The WHO proposed Innovative Care for Chronic Conditions as a new model of health systems to help manage the global increasing epidemic of chronic diseases, including both communicable and non-communicable diseases (Fig. 27.2) [72].

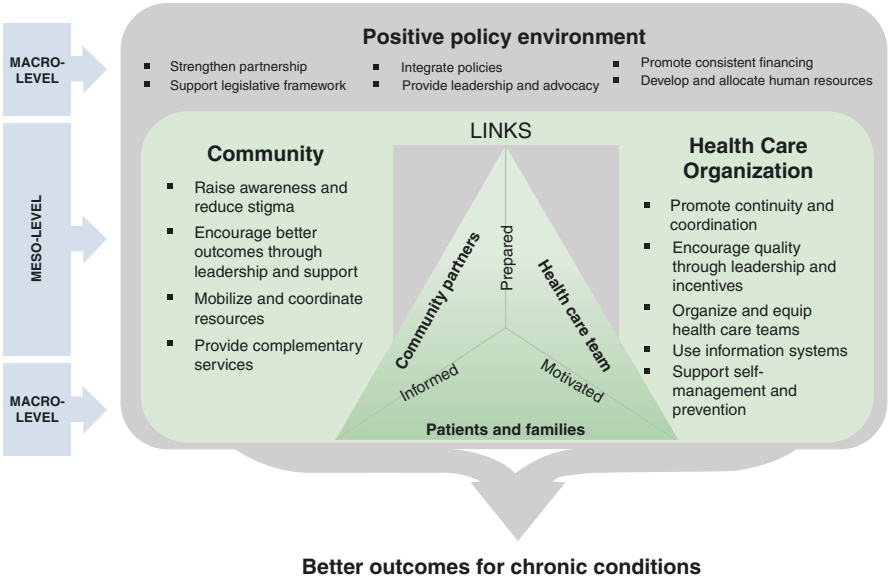

Fig. 27.2 The World Health Organization Innovative Care for Chronic Conditions framework [72]

Future Directions

It is evident that LMIC are looking at a catastrophic situation if the burden of DKD grows unabated, as the numbers would predict. The existent gap between the need, access and resources is too wide to be addressed by mere allocation of funds because even the latter approach cannot be sustained. Healthcare costs continue to increase with 12% of global health expenditure dedicated to diabetes treatment and related complications that account for the majority of the total expenditure [11]. The expected population growth and the growth in prevalence of type 2 diabetes in low- and middle-income countries mean that without effective strategies to support better management of diabetes, it is likely that there will be large increases in the rates of diabetic complications leading to further increases in future health expenditure [11].

Given the skewed distribution of burden of DKD in LMIC and a general lack of preparedness to handle the problem, the future of DKD will be defined by its impact and its handling in the LMIC. Based on the discussion in Sect. 27.4, a list of domains that would require action is shown in Table 27.1.

Successful prevention and treatment of DKD is one of the Sustainable Development Goals (Goal 3) [73] aimed to reduce premature mortality from non-communicable diseases (by one-third) by 2030. Screening, intervention and implementation of management strategies can prevent advanced DKD and reduce the incidence of end-stage kidney disease. In many LMIC, the optimal and sustainable diabetes control has, however, not yet been achieved; and health service delivery, access and effective coverage and access to affordable care are limited. Improvements to the availability and affordability of key medicines as part of many national programmes are also likely to enhance their use and help

Table 27.1 Major domains for action to deal with DKD burden in LMIC

Recognition of diabetic kidney disease (and CKD in general) as a public health problem
Increasing awareness about DKD (and CKD in general) in population
Dissemination of knowledge about DKD (and CKD in general) to a wide range of healthcare workers and allied professionals
Training of kidney experts, including increasing number of nephrologists
Task-shifting across the range of healthcare workers to enhance reaches for preventive programmes
Tailor-made clinical practice guidelines for LMIC
Wider availability of affordable diagnostic tests
Screening and follow-up strategies
Innovative use of technology for all domains
Reducing cost of care of diabetes mellitus and DKD and sustained funding for programmes instituted
Collaboration between professional societies and nations for global effort
Development of newer modalities for prevention and treatment of DKD
Objective monitoring and evaluation of programmes

towards achieving the WHO's targets of the 50% use of key medicines by 2025 [39]. The growing burden of diabetes prompted the United Nations General Assembly to unanimously pass Resolution 61/225 to label diabetes as a global public health issue.

Besides the need for diabetes and renal care, the ongoing complex management of complications of advanced DKD is critical to reduce the increased all-cause and cardiovascular mortality, cardiovascular morbidities, kidney disease progression and kidney failure, cognitive decline, anaemia, mineral bone disease, fractures and impaired quality of life. Guidelines advising on the management of CKD-related abnormalities such as hypertension, anaemia, and metabolic bone disease suffer from limited evidence from LMIC [74–76], where underlying causes of these abnormalities can vary by country [25]. Therefore, guideline development must be tailored appropriately for different settings and complemented by effective knowledge translation efforts aimed at care providers, patients and their families [25]. Nephrology-specific implementation activities should also target building nephrology-specific capacity such as formal curricula and the creation of training positions within nephrology residency programmes [25]. Implementation science also has a potential to maximize the efficiency of health service investment as well as outcomes for patients in LMIC [25].

Healthcare costs continue to increase with 12% of global health expenditure dedicated to diabetes treatment and related complications that account for the majority of the total expenditure [20]. The expected population growth and the growth in prevalence of type 2 diabetes in LMIC means that without effective strategies to support better management of diabetes, it is likely that there will be large increases in the rates of diabetic complications, including DKD, leading to further increases in future health expenditure [20]. Surveillance for DKD especially amongst those with long-standing diabetes is, therefore, an important goal and should be of a high priority in LMIC.

Ensuring access to comprehensive, integrated primary and secondary specialist care and health services through better health financing methods may help to ensure that persons with diabetes fully understand their health conditions and are able to act early to minimize risks associated with poor metabolic control.

Conclusion

The prevalence and associated burden of DKD are rising worldwide with the fastest growth occurring in LMIC [25]. Advanced DKD is a significant risk factor for ESRD, other severe health outcomes and considerably reduced quality of life and life expectancy of populations of LMIC. On the basis of the current findings, greater attention should be placed on the development and implementation of diabetes and nephrology health programmes tailored appropriately for different settings. With the improved identification and management of diabetes in LMIC, a substantial proportion of DKD-related adverse outcomes could be avoided.

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Chapter 28
Omics in Diabetic Kidney Disease

Massimo Papale, Francesca Conserva, Paola Pontrelli, and Loreto Gesualdo

Introduction

We know that cells are highly complex systems regulated at different hierarchical levels. Omics study the pool of molecules within the same hierarchical level; thus, for instance, genomics refers to the study of all the DNA sequences present in our genome, while metabolomic studies the pool of metabolites present in a specific sample type (cell, tissue, biofluid, etc.). Within each omics branch, molecules are similar in structure; therefore the techniques used for their characterization are the same and have evolved through time, becoming high-throughput and affordable.

In this chapter we will describe the main omics fields: genomics, epigenomics, transcriptomics, proteomics, and metabolomics. A brief introduction will cover the main discoveries that contributed to the evolution of each field, and then the reader will be guided through the most popular techniques and methods used to characterize and study the molecules within each omics (Fig. 28.1). Finally, a few examples of omics studies in diabetic kidney disease and diabetic nephropathy (DN) will be presented and discussed. For a complete review on the main omics findings in DN, we refer to [1].

The reader should be warned that, although lots of literature has been published and lots of money is currently invested in omics, we are only scratching the surface of this vast, exciting, and revolutionary new field of research. Along with the development of omics sciences, a new area of research has quickly emerged: Systems Biology. It is expected that the huge amount of omics data will be processed even better by computer models that can simulate the functioning of systems through

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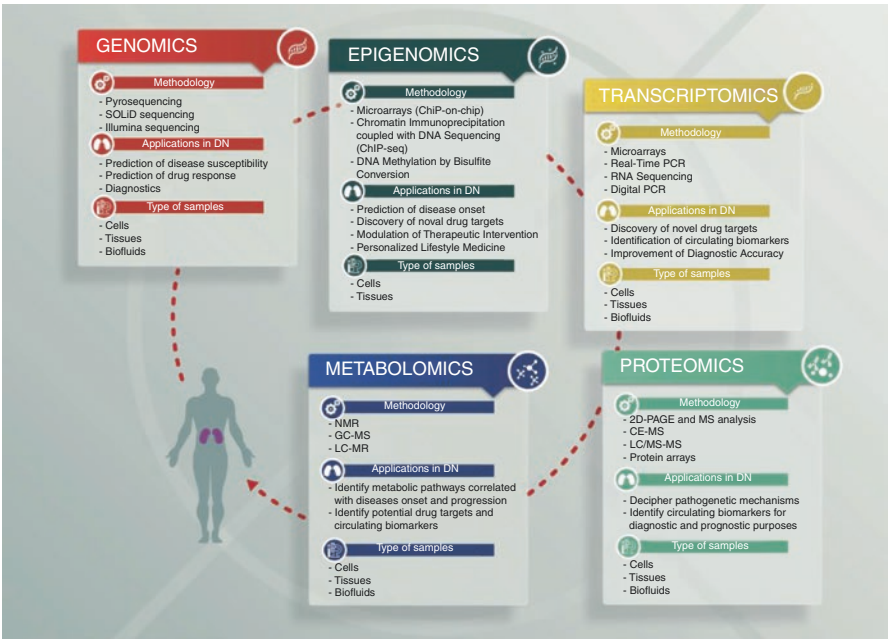

Fig. 28.1 Overview on the main omics fields The figure summarizes the principal techniques employed in omics studies. Specific applications in DKD along with applicable biological samples are also reported for each omics field. The ideal workflow begins with the screening of disease susceptibility through genomic analysis and follows the evaluation of disease initiation through epigenomics. Finally, transcriptomics, proteomics, and metabolomics can be used to identify dynamic biomarkers

new algorithms able to manage lots of variables. This would finally allow the identification of new qualified biomarkers and the development of more accurate point-of-care testing for personalized medicine.

Overview on Genomics

Genomics is the science that studies the genetic material of an organism (genome) and its interactions with the environment. The study of a genome contemplates essentially two tasks: DNA sequencing and sequence analysis.

The first DNA sequencing methods were developed by Allan Maxam and Walter Gilbert, and in parallel by Frederick Sanger and colleagues, who were awarded the Nobel Prize in Chemistry in 1980. Although revolutionary, DNA sequencing using these methods was expensive, required hazardous reagents and only produced a handful of information.

Given these limitations, in 1990, a joint effort was undertaken, and different research labs across the world joined the Human Genome Project with the goal to uncover the

genetic blueprint of our species [2]. By 2003, the first human genome was sequenced; it costed around 3 billion dollars, and only about 98% of the genome was sequenced. The remaining 2% (mostly structural variations containing repetitive elements) was impossible to resolve with the sequencing technology available at that time.

Over the following years, researchers also worked to refine the reference human genome sequence, which was initially coming from one single individual. It became clear that a single reference genome was not representative of the human population diversity, as evidenced by the observation that some ethnic groups are more susceptible than others to certain diseases. With the hope to take a leap forward in the understanding of genetic variation in the population, the 1000 Genomes Project was undertaken in 2008 [3]. The data collected during this project allowed to understand that some genetic variations are common and probably irrelevant to disease susceptibility, while others are more rare, occasionally restricted to precise geographical areas, and sometimes linked to human disease.

In the past, family members were sequenced for a number of specific single nucleotide polymorphisms (SNPs) in order to identify those genetic traits that were inherited along with the disease. This approach, known as genetic linkage, allowed to identify mutations responsible for single gene disorders. In the case of more complex diseases such as diabetes, we know today that it is rather the combination of multiple SNPs within the same individual to confer disease susceptibility. For this reason, studying of the whole genome and looking for patterns of genetic variations instead of single candidate genes could be more informative [4]. Sequencing technologies have continued to develop since the completion of the Human Genome Project, and today, sequencing an entire human genome is much faster and less costly [5]. Of note, the systematic understanding of every single nucleotide in the genome and what a mutation leads to also required an advancement in data analysis and computational algorithms.

The recent advances in the field of genomics have a tremendous potential on human health. For instance, pinpointing a specific mutation responsible for the onset and/or progression of a disease allows a more precise pharmacological intervention. The advancement of sequencing technologies allows us today to focus on new areas of genomics such as the genome of the microbial population in our bodies to understand how this changes during health and disease.

In the next paragraph, we will briefly describe some of the methods that can be used for DNA sequencing.

Methods for Studying Genomics

Several methods have been developed to perform DNA sequencing and could be grouped into:

1. First-generation DNA sequencing.
2. Advanced DNA sequencing.
3. Next-generation DNA sequencing.

First-generation DNA sequencing methods include the Sanger method and the Maxam-Gilbert method. These methods are only suitable for short sequences (100–1000 bp).

In the *Sanger sequencing* method, also known as the chain termination method, DNA is preamplified using PCR. Amplified DNA is exposed to heat to produce a single-strand DNA template, and, along with associated primer, it is aliquoted into four reaction tubes for a second amplification. The four tubes contain a DNA polymerase and dNTPs for second amplification; each tube also contains a modified ddNTP that, when added to the nascent strand, terminates the sequence. The resulting DNA fragments with different lengths are then loaded onto a polyacrylamide gel for electrophoresis, and DNA sequence can be retrieved through the analysis of the band pattern within the gel.

In the *Maxam-Gilbert sequencing* method, also known as chemical cleavage method, single-strand DNA is tagged at its 5′ end with radiolabeled phosphate (32P). Radiolabeled DNA is then aliquoted into four reaction tubes, each containing different chemical agents. These chemical agents cleave DNA selectively at a specific base, thus producing different fragments that are loaded into a gel for electrophoresis. Finally fragments are analyzed using autoradiography, and the original DNA sequence is retrieved.

The *shotgun sequencing* method was developed to analyze long DNA sequences, and it was employed to sequence the human genome during the Human Genome Project. The principle is to randomly fragment long DNA sequences into smaller fragments, sequence the fragments individually (e.g., using Sanger method), and then find overlapping terminal regions to reconstruct the original DNA sequence. Overlapping regions, also known as contigs, are assembled by a computer software. One big disadvantage is that this method is unsuitable for the sequencing of particular genomic regions such as repetitive elements.

The currently used sequencing technologies have been developed over the last decade and are known as "next-generation." They have the advantage to be cost-effective and high-throughput and include pyrosequencing, SOLiD sequencing, and Illumina sequencing. Next-generation sequencing (NGS) systems have some features in common:

- Sample preparation – all NGS platforms require a library. This is obtained either by amplification or through ligation with adapter sequences.
- Sequencing instrument – each fragment of the library is amplified on a solid surface coated with DNA linkers complementary to the library adapters. When amplification starts, clusters of DNA are obtained, each originating from a single library fragment.
- Data output – at the end of the sequencing run, each instrument provides the raw data, a collection of DNA sequences originated from each cluster.

The differences between the NGS platforms are mainly related to the technical aspects of the sequencing reaction.

In *pyrosequencing*, nucleotides are added one at a time. When a nucleotide is complementary to the target DNA, this is incorporated, and pyrophosphate is

released. The release of pyrophosphate ultimately determines light emission. Light emission is finally detected by a camera which records the sequence.

The *SOLiD sequencing*, also known as "sequencing by ligation," is based on the dual measurement of each base through the hybridization and ligation of different sequencing primers and fluorescently labeled oligonucleotide probes. A big disadvantage of this method is that it leads to very short sequencing reads.

In the *Illumina sequencing*, also known as the "sequencing-by-synthesis" method, purified DNA is fragmented, and adapter molecules are added to the ends of the DNA fragment to be sequenced. DNA is then loaded onto a slide (flow cell) coated with two different oligos that are complementary to the DNA adapters. DNA fragments bind the oligos through the adapter sequence forming a bridge-like structure, and clonal amplification occurs. Once clonal amplification is completed, several steps allow to retain only the forward strand that can be hybridized by a sequencing primer complementary to the adapter region. With each cycle, fluorescently labeled nucleotides compete for addition to the growing chain, and a specific fluorescent signal is emitted; thus sequencing occurs during synthesis. This method is high-throughput, and today, an entire human genome can be sequenced within hours. The main disadvantage of this method is related to the requirement of highly trained personnel for the experimental setup and data analysis.

Genomics in Diabetic Kidney Disease

We know that certain ethnic groups are more susceptible to DKD than others [6] and that diabetic siblings of patients with DKD have a higher risk of developing this condition [7]. Several studies have tried to elucidate the genetic determinants of DKD susceptibility, and the recent development of NGS platforms allowed researchers to shift from candidate-based studies to genome-wide scans. To date, several genome-wide association studies (GWAS) have been published on DKD patients. Different research groups identified a strong association between the engulfment and cell motility 1 gene (ELMO1) polymorphism and DKD susceptibility. ELMO1 is suggested to regulate the expression of ECM protein genes and to promote phagocytosis of apoptotic cells. The association between polymorphisms of the ELMO1 gene and higher DKD susceptibility was found in several ethnic groups, including Caucasians, Japanese, Pima Indians, African Americans, and Chinese patients [8–11].

In a recent study, Germain et al. employed a multistage-based GWAS to search for novel susceptibility genes associated with DKD in patients with type 1 diabetes. Authors claim that SORBS1 might be a gene involved in DKD; interestingly SORBS1 encodes for sorbin, a protein found to be highly expressed in the renal tubule [12] but whose function still remains largely unknown.

As for most complex diseases, genetic association studies in DKD produced inconsistent results, and today it is still impossible to precisely categorize diabetic patients according to their risk for developing DKD. Of note, a big limitation of

genome-wide scans lies in the great number of patients and controls that are needed to achieve genome-wide significant results. In addition, researchers and nephrologists should keep in mind that not all diabetic patients with compromised renal function can be correctly classified as diabetic nephropathy unless renal histology is precisely characterized [13, 14].

Overview on Epigenomics

The epigenome represents the interface between the genome and the environment. Epigenetic marks are structural changes within the DNA that modulate gene expression. Epigenetics confers structural and functional diversity to cells in our body, and today we know that perturbations in the epigenetic landscape can drive disease progression. Unlike DNA mutations, epigenetic modifications are dynamic and reversible; thus they open a new path for therapeutic intervention.

As elegantly described by Berger et al. [15], epigenetic changes are usually initiated by signals coming from outside the cell (e.g., environmental factor) and defined as "epigenators." These epigenators cause deregulation of specific intracellular pathways and lead to the activation of effector molecules (such as DNA-binding proteins and noncoding RNAs) defined as "epigenetic initiators." Finally "epigenetic maintainers" are capable of preserving the maintenance of the newly generated epigenetic marks even in the absence of the epigenators.

Epigenetic modifications include DNA methylation and histone modifications. In eukaryotes DNA methylation promotes transcriptional silencing and is extremely important for the regulation of tissue-specific genes. DNA methylation consists in the covalent attachment of a methyl group to the C5 position of specific cytosine residues within the DNA. This process is catalyzed by the enzyme DNA methyltransferase (DNMT) and occurs mainly at specific sites named CpG islands (1000–2000 bp) where cytosine and guanine residues are repeated.

Histone modifications include methylation, acetylation, phosphorylation, and ubiquitination. These marks serve as signals for the opening and compaction of the chromatin, as well as for recruiting factors that promote and antagonize transcription.

During histone methylation, the enzymes histone methyltransferases (HMTs) promote the transfer of one, two, or three methyl groups to a lysine or arginine. Lysine methylation of H3 and H4 is implicated in both transcriptional activation and repression depending on the methylation site, while arginine methylation promotes transcriptional activation [16].

During acetylation, the acetyl group of acetyl coenzyme A is added to specific histone lysine residues by the enzymes histone acetyltransferases (HATs); another set of enzymes known as histone deacetylases (HDACs) regulate the removal of specific acetyl groups. Acetylation is generally associated with gene activation [17].

During histone phosphorylation, protein kinases phosphorylate specific serine, threonine, or tyrosine residues on histones. Histone phosphorylation is a critical

step in chromosome condensation and often occurs during mitosis. Phosphorylation of histones can also be a sign of DNA damage; phosphorylation of histone H2AX at S139 occurs in the presence of DNA double-strand breaks and serves as a recruiting point for DNA damage repair proteins [18].

Finally, histones can undergo mono- and polyubiquitination. Histone ubiquitination can promote gene activation, transcriptional repression, and recruitment of DNA repair proteins at the DNA damage site; the effects of histone ubiquitination are different according to the substrate and type of ubiquitination [19].

In conclusion, epigenetic marks modulate the accessibility of the chromatin, thus regulating gene expression. Epigenomics consists in the genome-wide mapping of DNA methylation and histone modifications and the integration of this information with gene expression.

Methods for Studying the Epigenome

Several methods exist for the analysis of the epigenome. One of the most used techniques for the analysis of histone modifications, also employed to study DNA-protein interactions, is known as *chromatin immunoprecipitation* (ChIP). ChIP is based on the use of an antibody that is highly specific to the histone modification of interest. The technological advances of the last decades have made it possible to associate ChIP to high-throughput technologies such as microarrays (ChIP-on-chip) and next-generation sequencing (ChIP-seq) allowing to perform epigenome-wide association studies (EWAS). In ChIP-seq experiments, the DNA is cross-linked to its associated proteins using formaldehyde. Sample then undergoes shearing to obtain small fragments (200–600 bp), and the DNA-protein complex is immunoprecipitated using an antibody specific to the epigenetic modification of interest. Once the complexes of interest have been isolated, cross-linking is reversed, and the resulting DNA is used to prepare a library for next-generation sequencing. Enrichment of specific DNA sequences indicates the epigenetic modification of interest is likely to be present.

A broadly employed technique for the identification and quantification of DNA methylation at single nucleotide resolution is *bisulfite conversion*. The principle of this method is that, following treatment with bisulfite, cytosine residues, unlike 5-methylcytosine residues, are converted to uracil. The DNA resulting from bisulfite conversion can then be hybridized onto arrays containing predesigned probes to distinguish between methylated and unmethylated cytosine residues. NGS can also be used to perform whole genome bisulfite sequencing (WGBS), unmethylated cytosine residues will be read as thymine (T) upon sequencing, while methylated cytosine residues (protected from conversion) will still be read as cytosine. It is important to keep in mind that in the presence of closely related samples, the degree of the methylation differences could be subtle, and the robustness of the results will depend on the sequencing coverage [20].

Epigenomics in Diabetic Kidney Disease

Epigenetics is essential in the regulation of tissue-specific genes, and recently, several experimental studies showed that certain disease states are characterized by specific perturbations of the epigenome. Metabolic memory in particular appears to be the results of long-lasting epigenetic modifications that are important in driving DKD progression [21].

Starting from DNA isolated from peripheral blood cells, Bell et al. used an array-based approach to analyze the genome-wide methylation of T1D patients. Authors identified a CpG island proximal to the UNC13B gene correlating with DKD progression [22]. Importantly, this gene has been suggested to induce apoptosis contributing to renal cell injury during hyperglycemia [23].

The genome-wide DNA methylation pattern of diabetic patients with end-stage renal disease and diabetic patients without nephropathy was also investigated by Sapienza et al. Preliminary results revealed that several genes, mostly involved in oxidative stress and fibrosis, displayed different methylation profiles within the two study groups [24].

Finally, it was recently shown that common drugs are capable of modifying the epigenetic landscape of a cell. The angiotensin II receptor antagonist, losartan, in particular was able to reverse histone modifications characteristics of *db/db* mice, regulating the expression of key inflammatory and profibrotic genes [25].

Overview on Transcriptomics

Transcriptomics represents the study of the transcriptome, defined as the complete set of RNA molecules that are transcribed by the genome in a given cell population or under certain specific physiological or pathological conditions; these RNA molecules include messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), and noncoding RNA (ncRNAs). Gene expression is dynamic and can be modulated by different factors, over time and under different conditions; thus studying the transcriptome is important as it can reveal the pathological mechanisms underlying disease progression. The characterization of the transcriptome, for instance, allows to quantify changes in the expression level of genes during development or under different conditions such as in different classes of patients; it can be applied to identify the molecular pathways affected by specific drug treatments or to find molecular markers able to discriminate between similar conditions; it can also be useful to clarify the biological functions of transcribed genes or to discover all species of transcripts (gene fusions, splice variants). In the human genome, not all genes are expressed in the same way: some, known as housekeeping genes, are essential for very basic cellular functions and are expressed in every cell type all the time. Other genes are expressed in particular cell types or during particular stages of development and can be activated or inhibited by signals, such as hormones, that circulate through the body. The regulation of gene expression is very complex, and

transcriptional regulation can be operated at different levels (mRNA transport into the cytoplasm, translation control, mRNA degradation control) and by different proteins such as transcription factors and proteins involved in RNA processing (5′ capping, RNA splicing, 3′ polyadenylation).

Aside from those RNA species commonly known to be involved in protein synthesis, such as mRNA, tRNA, and rRNA, the transcriptome also includes other RNA species that were discovered over the last decades and known as noncoding RNA (ncRNAs). ncRNAs transcripts are encoded within a large number of genomic sequences that are not meant to be translated; these are mainly implicated in cell homeostasis and in epigenetic control. ncRNAs form a heterogeneous group of RNA molecules that can be classified according to their length and function into three categories: very small RNA, ranging in length from 18 to 25 nucleotides, which includes short interfering RNAs (siRNAs) and microRNAs (miRNAs); small RNAs, from 20 to 200 nucleotides, such as small nucleolar RNA (snoRNA), PIWI-interacting RNA (piRNA), and others generally acting as transcriptional and translational regulators; and medium and large RNAs, up to (and even more) 10,000 nucleotides, which have a structural role and act in different ways to both repress and activate target gene expression [26].

The role of ncRNAs in the modulation of physiological and pathogenic processes in several organs, included the kidney, is emerging exponentially in the last years. Among them, the role of miRNAs and their involvement into the pathological mechanisms underlying diseases have been extensively studied [27]. miRNAs are short ncRNAs, highly conserved between vertebrates and invertebrates, which act as gene regulators, modulating the expression of about 1/3 of mammalian gene products. Each miRNA can target hundreds of mRNAs that can be degraded or blocked in their translation. In almost 25 years, from their first identification in 1993 in *Caenorhabditis elegans*, research on miRNAs brought from their isolation to target determination, to the analysis of their regulation leading to their clinical applications that include miRNAs as diagnostic and prognostic markers for several diseases and the development of anti-miRNAs as therapeutic agents to treat pathological conditions. Moreover miRNAs are present in biofluids, thus representing ideal biomarkers, indicators of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Changes in miRNA can be correlated with gene expression changes in development differentiation, signal transduction, infection, aging, and diseases such as renal pathophysiology [27].

Methods for Studying the Transcriptome

As mentioned previously, the transcriptome is dynamic and can change over time and under different circumstances. The recent discovery that RNA transcripts can act as messenger molecules circulating through the body opened a window on the possibilities of using these circulating RNA species as biomarker of disease, therapy, and prognosis.

There are several methods used for studying RNA: microarrays, quantitative reverse transcription PCR (qRT-PCR), digital PCR, and next-generation sequencing (NGS) technologies. There are some technical issues that should however be addressed regardless of the method of choice when studying RNA: the integrity of the ribonucleic acid, the absence of genomic DNA, the absence of reaction inhibitors, and the methods employed for data normalization. RNA is degraded easily because RNAse enzymes are ubiquitous; therefore caution should be used to prevent contamination of samples with non-RNAse-free equipment and reagents. It is also strongly recommended to compare samples that were processed similarly to exclude the presence of confounding variables.

Microarray technology is based on the concept of hybridization between complementary DNA strands. Spotted samples known as probes are immobilized on a solid support (a microscope glass slides or silicon chips or nylon membrane) and are representative of thousands of genes simultaneously. For expression analysis experiments, the immobilized spots can be single-strand cDNA or oligonucleotides that are representative of the pool of RNA species under investigation. Different types of chips can be purchased according to the sample type that needs to be studied; these chips contain DNA probes that are complementary to the known RNA species present in the studied sample. It is important to know that microarray studies allow the measurement of relative RNA expression levels; thus this approach can be used for comparative studies, such as healthy subjects vs disease or drug treatment vs untreated and so on. Moreover this approach has a short time of execution, an exceptional quantitative accuracy and fair simplicity of data generation and data analysis. Of note, the number of transcripts recognized depends on genome annotations; there could be issues linked to cross-hybridization and saturation, and also sensitivity could be limited.

Real-time PCR is probably the most used method to investigate the transcriptome for several reasons. It allows to perform large-scale but also small-scale experiments (e.g., validations); it has evolved through time to ensure a good specificity and sensibility, does not require expensive equipment, and is relatively fast. In real-time PCR, RNA is converted to cDNA, and specific primers allow to amplify the regions of interest using PCR. Amplification is detected through the emission of fluorescence, and fluorescence emission is measured in real time during amplification. These real-time measurements allow to identify the exponential phase of amplification and associate it to a specific PCR cycle (also known as threshold cycle or CT). The sooner this cycle is reached, the more abundant the RNA target was in the initial sample. This method is used to compare samples obtaining a relative quantification, but it can also be employed for absolute quantifications when creating a standard curve. The main limitation of real-time PCR is that it can only be used to measure known RNA molecules because the PCR reaction occurs through primer amplification.

RNA sequencing using NGS technology is the method of choice when the goal is to obtain very detailed information on the RNA sample under investigation. The method is similar to that described for DNA so we will not discuss it in detail; one difference consists in the additional step required for the isolation of the RNA species of interest before sequencing as we know that different RNA molecules are

present in the cell (mRNAs, miRNAs, tRNAs, etc.). It is important to know that the great advantage of using RNA-seq in transcriptomics relies on its ability to detect novel RNA transcripts and structural variations. In addition, this approach is not limited by saturation or background noise and is applicable for quantitation of alternative splicing or discovery of novel splicing isoforms. Sample preparation and data analysis are however more complex.

Digital PCR is a very recent PCR technology that can be used for quantification of RNA (but also DNA) target sequences. The method involves the massive partitioning of the sample into thousands of equally sized droplets, so that each contains no more than one target molecule. Endpoint PCR reaction is then performed on each drop to determine the presence or absence of target template. This generates a series of data that allows to precisely determine the number of targets present in the initial sample.

Among the major advantages of digital PCR, there is the possibility to obtain an absolute quantification without the need for standard curves or reference assays; this can be extremely useful when there is no information on the most suitable reference candidates for data normalization. Also this method is not affected by the presence of sample inhibitors and is very sensitive to extremely rare targets.

Transcriptomics in Diabetic Kidney Disease

The first transcriptomic profile of glomeruli from DN patients was published in 2004 by Baelde et al. By using an oligonucleotide microarray approach on control and diabetic glomeruli, authors found several differentially expressed genes, whose altered expression levels were linked to vascular damage, mesangial matrix expansion, proliferation, and proteinuria [28]. In 2011 Woroniecka et al. performed the transcriptome analysis of human diabetic kidney disease biopsies, analyzing separately the gene expression profiles of control and diseased glomeruli and tubuli. In this work, researchers identified several pathways specifically modulated in diseased glomeruli and tubuli such as Ras homolog gene family member A, Cdc42, integrin, integrin-linked kinase, and canonical complement signaling pathway and vascular endothelial growth factor signaling in diseased glomeruli and inflammation-related pathways and the canonical complement signaling pathway in the tubulointerstitial compartment, opening the scenario on novel genes and pathways that could be involved in the pathogenesis of diabetic kidney disease or could serve as biomarkers [29]. Several other research groups analyzed the gene expression profiles of both the glomerular and tubular compartment of kidney biopsies from diabetic patients with DN, highlighting the importance of several other pathways involved in the pathogenesis of DN (for a complete description, see References [1, 30]). Also animal models have been largely used to characterize transcriptomic profiles of diverse experimental models of DN, highlighting other pathways that could play an important role in the pathogenesis of DN but also offering the possibility to investigate particular mechanisms of interest and design therapeutic intervention studies to reduce the progression of kidney damage under diabetic conditions [30].

Other than in kidney tissues, gene expression profiles have been also characterized in urinary sediment of patients with DN. Specific changes in the urinary mRNA levels of Glu-Leu-Arg (ELR)-negative CXC chemokine ligand (CXCL), in particular CXCL9, have been suggested as markers for risk stratification of DN [31]. Quantification of gene expression in urinary sediment was also indicated as a non-invasive strategy to search novel biomarkers associated to DN [32]. By this approach, mRNA markers of epithelial-mesenchymal transition were correlated with the progression of DN [33] such as urinary mRNA profiles of podocyte-associated markers that were found to increase with the progression of DN [34].

Together with the analysis of gene expression profiles, in the last decades, several research groups investigated the involvement of ncRNAs in kidney diseases included DN [35] and in particular the role of miRNAs in the pathogenesis of renal complications in diabetic patients.

ncRNAs, including miRNAs, modulate several physiological and pathogenic processes in the kidney, and their role in the pathogenesis and progression of DN has been largely described [1, 35–37]. Characterization of miRNA expression profiles has been described in vitro in different types of kidney cells and in vivo in animal models of DN and human biopsies, thus identifying several miRNAs targeting important processes identified as involved in the pathogenesis of the disease [37, 38]. One example is represented by miRNA-21 that is now widely recognized as a promoter of tissue fibrosis in many conditions [39–42]; circulating miRNA-21 levels were shown to correlate with the degree of interstitial fibrosis and tubular atrophy on allograft biopsies from transplant recipients [43], and upregulations of miRNA-21 were recently found in kidney biopsies of patients with DN compared to patients with minimal change disease [44]. The pharmacological modulation of some miRNAs could represent a novel approach to inhibit specific processes involved in the progression of renal damage in diabetic patient such as glomerular hypertrophy or extracellular matrix depositions in the glomerular or tubule-interstitial compartment [45]. One example is represented by miRNA-29c whose inhibition in vivo significantly reduced albuminuria and kidney mesangial matrix accumulation in a *db/db* mice model [46].

In addition, the possibility to quantify miRNAs expression in biofluids, where they can also be included into exosomes, supported their use as biomarkers to predict the progression of DN. Several clinical trials are actually ongoing using miRNA as prognostic and diagnostic biomarkers in diabetic patients (www.clinicaltrials.gov). Moreover, in the last period, specific oligonucleotides targeted against miRNA, known as anti-miR, have been developed, able to bind their targeted miRNA with high affinity and specificity [27]. Inhibition of microRNA function by anti-miR oligonucleotides represents a novel strategy for treating human disease and in the future, thanks to the increasing knowledge in molecular mechanisms involved in the pathogenesis and progression of DN, will open novel scenarios in therapeutic intervention strategies. Our future understanding of the disease and the advance of personalized medicine will benefit from increased knowledge of human transcriptional regulatory mechanisms especially for the opportunity to create novel therapeutic approaches.

Overview on Proteomics

The completion of the Human Genome Project has allowed us to define that there are approximately 22,300 [47] protein-coding genes in human beings, the same range as in other mammals. This result seems to indicate that gene analysis is not sufficient to explain the complexity of human beings compared to other species. However, there is no doubt that genomic sequencing has contributed decisively to develop new technological solutions aimed at better analyzing not only human genes but also their downstream products including transcripts, proteins, and metabolites. The development of *ionization sources* (i.e., matrix-assisted laser desorption/ionization [MALDI] [48] and electrospray ionization [ESI]) [49] and *bioinformatic tools* has rapidly provided new technological platforms for the analysis of complex protein datasets and the interpretation of the cross-linked relationship among the differently expressed proteins allowing to decipher their functional role in physiological and pathological conditions. Although proteomics offers the benefit of studying biological systems by analyzing the main regulators of biological functions, namely, proteins, the feasibility of a proteomic study suffers from the impossibility of amplifying proteins with the same ease of nucleic acids. Proteomic studies in humans have been focused mainly on *biological fluids*, and nephrology is not an exception. One of the few ways to obtain kidney tissue is to run kidney biopsy, but it is mainly used for diagnostic purposes rather than for research. In contrast, urine is, among the biological fluids, the richest in renal-derived proteins, and, consequently, it is also the most commonly used sample in proteomic-based studies on renal disease [50].

Methods for Studying the Proteome

The proteomic analysis of biological samples may be pursued by distinct and complementary strategies that allow separating the protein mixtures and identifying the key disease-related molecules by mass spectrometry analysis. Both protein separation and MS analysis can be carried out by distinct and sometimes complementary strategies. As we will see, there is no perfect method, but choice depends on the objective of the study. *Mass spectrometers* consist of three main components, an ion source, a mass analyzer, and an ion detection system so that the analysis of proteins requires protein ionization and generation of gas-phase ions, their separation onto the mass analyzer according to their mass-to-charge ratio, and the detection of ions [51]. *MALDI-ToF mass spectrometry* is generally the method of choice when the separation of the protein mixture is performed through *two-dimensional gel electrophoresis* (*2-DE*) that represents the most popular gel-based approach for analyzing the proteome. 2-DE allows double protein separation according to the isoelectric point (pI) and the molecular mass (MW) [52] and provides, for each sample, a characteristic proteomic map showing the separated proteins as protein spots or spot

trains due to the presence of protein *posttranslational modifications* (*PTMs*). After the acquisition of the proteomic maps of an appropriate number of cases and controls, their comparative analysis by dedicated software may allow identifying differently expressed protein spots that are further excised from the gel, digested into small peptides mixtures, and analyzed by mass spectrometry (MALDI-TOF MS, nanoHPLCESI-MS/MS) to obtain the protein ID. Two-DE allows immediate visualization of protein isoforms that describe the presence of PTMs (e.g., phosphorylation) playing an important role in the pathogenic mechanisms of many diseases. However, this approach may be laborious and expensive without providing satisfactory results; in fact it underestimates protein complexity of the sample since less expressed proteins, proteins having a molecular weight lower than 10 kDa and higher than 250 kDa, and transmembrane (hydrophobic) proteins are difficult to visualize. For the above reasons, 2-DE is the methods of choice for the analysis when low sample to sample variation is expected (e.g., in ex vivo studies on cells) or when the study is carried out on a restricted and well-characterized cohort of patients in order to identify putative disease-associated biomarkers. Proteomic analysis of biological samples has been theoretically simplified by the development and diffusion of a new generation of high-resolution mass spectrometers [53] that can be coupled to various separating techniques, namely, *liquid chromatography* (LC) [54] and *capillary electrophoresis* (CE) [55]. The most powerful approach to carry out gel-free proteomic analysis is based on LC/tandem MS instruments that are capable of separating and fragmenting a high number of precursor ions, an approach that permits to assign many precursor ions to a unique protein ID, thus improving the accuracy of the analysis. A combination of different mass analyzers in tandem such as *hybrid ion trap-orbitrap*, *quadrupole-TOF*, and *quadrupole-ion trap* can combine the individual strengths of different types of mass analyzers and greatly improve their capabilities for proteomic-based analysis. Although these strategies can identify, in a shortened time, many putative biomarkers ready to be validated, the complexity of the datasets asks, as for other omics approaches, a proper management by means of statistical and bioinformatic tools to finally allow the recognition of reliable disease-specific biomarkers before proceeding with their validation. Over the last years, another emerging technology for studying the proteome is represented by the so-called protein arrays that provide a versatile, high-throughput, and sensitive platform for biomarker discovery [56]. Mainly a protein array/microarray consists of a microscopic slide-based surface on which individually purified proteins are chemically immobilized for various uses. Protein arrays can be classified into three major categories: analytical, functional, and reverse phase protein arrays (RPPAs) [57]. Briefly, *analytical protein arrays* contain specific affinity molecules such as antibodies, lectins, and aptamers that allow the detection and quantification of many proteins within a biological sample. They can be theoretically customized to investigate the activation of a high number of protein patterns, but, in practice, their accuracy depends on the availability and specificity of the affinity reagents. *Functional protein arrays* contain individually purified recombinant proteins specifically encoded within a given organism in order to study proteins to proteins or proteins to nucleic acids or lipids interactions as well as to dynamically

evaluate proteins' PTMs, such as phosphorylation, ubiquitination, and acetylation. Among the described applications, the field of serological biomarker identification is the most rapidly expanding one. In fact, protein arrays are currently used as screening platforms to investigate the presence of autoantibodies in serum or plasma samples and conversely to characterize new autoantigens in human diseases. Finally, *RPPAs* use an inverse approach since the samples to be investigated are spotted onto a glass slide at high density and subsequently incubated with known set of antibodies that are generally chosen to permit the analysis of specific intracellular signaling pathways. All the discussed gel-free approaches have permitted medium- to high-throughput analysis of thousands of biological samples and appear to be the most appropriate for clinical proteomic studies that need the multicenter collection of numerous samples and their rapid analysis in order to identify a new set of confident biomarkers. In the next paragraphs, we will discuss the main results related to the proteomic-based analysis of kidney tissue and biofluids of diabetic patients and how they are contributing to understand the pathogenesis and to identify diagnostics and prognostics biomarkers of diabetic kidney disease.

Proteomics in Diabetic Kidney Disease

Most of the proteomic studies on kidney tissues have been conducted in animal models of diabetes and diabetic nephropathy. However, none of the models used so far have been able to reproduce faithfully the complexity of molecular and structural events observed in human DN that is, in fact, recognized as part of a more heterogeneous disease overall referred as diabetic kidney disease [58]. For this reason, we will only report the results of human studies to avoid confusion resulting from potential biomarkers described in animal models that have not yet been fully validated in humans. Although renal biopsy is rarely carried out on diabetic patients, the recent development of new strategies for the extraction of intact and unmodified proteins from *formalin-fixed paraffin-embedded (FFPE)* samples has made available the use of the archives of kidney tissues for proteomic analysis [59]. Some recent studies [60, 61] on glomeruli isolated from FFPE samples have shown increased expression of nephronectin, a protein implicated in the assembly of extracellular matrix and nephrogenesis, and accumulation of complement C3 and the membrane attack complex C5b9 together with a significant reduction of podocyte-associated proteins and antioxidant proteins, in DN. Interestingly, a recent proteomic analysis on postmortem glomeruli isolated from type 1 diabetic patients with long duration of diabetes (over 50 years) identified some enzymes involved in the processing of free intracellular glucose, glycolysis, and in the TCA cycle as key protective molecules for the development of DN [62]. The elevated levels of such enzymes involved in glucose metabolism would preserve glomerular function by reducing free intracellular glucose and its metabolites and, correspondingly, lowering plasma metabolites from these pathways. The authors concluded that, in the protected group, the increased activation of aldose reductase and sorbitol

dehydrogenase may enhance the metabolism of excess intracellular free glucose, thus protecting podocytes and glomeruli from hyperglycemic toxicity. The importance of the regulation of the metabolic flux to neutralize the toxic effects of hyperglycemia was correlated to PKM2 activation that would normalize high glucose-induced elevation of toxic glucose metabolites in podocytes and even partially preserve their mitochondrial function. However, DN in type 1 diabetic patients has a different presentation than in type 2 diabetes; thus the above findings need to be confirmed in larger cohorts of subjects with multiple stages of DN and shorter durations of both T1DM and T2DM to determine whether they are also observed in individuals with classical presentation of DN. It is worth noting that although kidney biopsy can give information on microscopic structural changes, the analysis of urine proteome may give information on global protein changes in the kidney, which could be more closely associated with the molecular changes in disease. *Urine* has been described in many studies as the most appropriate biological fluid for biomarker discovery in kidney diseases including DN [63] since it may be collected in noninvasive way at different time points and it is rich in kidney-derived (about 70%) and plasma-derived (about 30%) proteins. The most consistent data on DN urinary biomarkers derive from studies conducted by Mishack and colleagues in both type 1 diabetes and type 2 diabetes. They identified by CE-MS a panel of 273 urine biomarkers called classifier 273, mainly composed of collagen fragments, that showed both diagnostic and prognostic value since it was able to predict the transition from normo- to microalbuminuria or from micro- to macroalbuminuria in diabetic patients [64] and has been validated in multicenter independent cohorts of CKD patients [65]. The molecular characterization of DN has just begun, since CE-MS analysis may describe only partially the urinary proteome, limiting itself to small polypeptides. In fact a recent comparative bioinformatic analysis of 31 urinary proteomic papers has clarified that these differently excreted peptides deriving from extracellular matrix elements together with the acute phase reactant proteins (a1-acid glycoprotein 1, haptoglobin, clusterin, a2-HS-glycoprotein, and mannan-binding lectin serine protease 2) and the Apo proteins implicated in the transport and metabolism of lipids and cholesterol may represent confident biomarkers of incipient diabetic nephropathy [66]. Furthermore, the assessment of collagen fragments, inflammation, cell adhesion molecules, and the enzyme inositol pentakisphosphate 2-kinase may exert prognostic value for the progressive renal function decline in incipient diabetic nephropathy [67]. Interestingly, other mechanisms like early changes of tubulointerstitium that correlates with increased urinary excretion of uromodulin and osteopontin, or the activation of coagulation and fibrosis, start already in uncomplicated diabetes and potentially represent the activators of the molecular mechanisms described in incipient nephropathy. It is thought that the application of *LC/MS/MS* to proteomic analysis can be decisive to obtain full knowledge of the biomarkers associated with each stage of kidney damage in diabetes. In this context, the recent use of *isobaric tags for relative and absolute quantification (iTRAQ)* and LC/MS/MS has allowed to quantify and identify a set of urinary proteins differentially excreted between normoalbuminuric and microalbuminuric T2DM patients that, when included in a multiplex assay, have shown over 92%

accuracy for the classification of normoalbuminuric and microalbuminuric T2DM patients [68]. As already reported, DN is the most frequent, although not the only one, histological manifestation of the DKD. The limit of current proteomic studies is thus the lack of a proper histological classification of patients with DKD prior to analysis. In a pilot study conducted on a small cohort of diabetic patients, histologically classified as DN or other nondiabetic chronic kidney disease, it has been obtained a finer proteomic-based urine characterization of kidney damage in diabetes that highlighted a potential role of protein ubiquitination in the onset of DN [69]. This finding has recently been associated to the pathogenesis of the DN [70], thus suggesting the assessment of urinary ubiquitinated proteins as new way to discriminate DN from other chronic kidney diseases in type 2 diabetes. The application of proteomics to the study of kidney disease in diabetes is therefore extremely promising, but only the combination of two critical factors, namely, the correct selection of patients and the screening of samples by high-throughput technologies and the focusing on the role of posttranslational modification, will allow to fully identify molecular changes that appear already in the early stages of kidney disease in diabetes.

Overview on Metabolomics

The link between metabolism and renal function is historically well known since the *clearance of serum creatinine*, a by-product of muscle metabolism, is currently used, together with urinary albumin excretion to assess renal function. However, several studies have shown that, even with normal urinary albumin excretion, the eGFR may be deeply reduced. Of note, serum creatinine has poor sensitivity in the early stages of renal impairment, and GFR may deteriorate up to 50% prior to a significant rise of this metabolite. Consequently, serum creatinine concentration may not be considered a good biomarker for detecting mild-to-moderate kidney failure.

The development of metabolomics, a systems approach useful for profiling in vivo metabolic status through the analysis of small molecules (i.e., metabolites), may allow identifying fundamental biochemical insights into disease pathways, drug toxicity, and gene function, thus overcoming the limits of the current biomarkers. Metabolomic analysis of biological samples can be pursued by targeted and untargeted approaches. The *targeted profiling* indicates the analysis of sets of few metabolites generally included in specific metabolic pathways. It is, generally, a quantitative approach that allows quantification of each metabolite of an interested metabolic pathway through the use of isotope-labeled standards [71]. *Untargeted analysis* provides, instead, a comprehensive evaluation of the metabolome without any *a priori* hypothesis on the metabolic pathways, and it is more suitable for biomarker discovery studies since the whole metabolic profile of cases and controls may allow the identification of disease-correlated biomarkers. As obvious, the complexity of the datasets generated by untargeted analysis needs

extensive data analysis through bioinformatic and statistical methods in order to construct disease-specific metabolomic classifier further sequenced by mass spectrometry. Most of the metabolomic studies analyze, in well-defined groups of cases and controls, the metabolic profile at a single snapshot, while a more thorough understanding of the individual pathways would require a time-course analysis to assess the metabolic flux of a defined pathway to quantify the key enzymes. In an ideal workflow, this approach would be particularly useful, after untargeted analysis, to achieve quantitative assessment of sets of correlated metabolites. Of note, in the last years, the optimization of the separation techniques has allowed the selective purification of specific classes of metabolites such as phospholipids and fatty acids, leading to the development of new more focused untargeted analysis such as "phospholipidomics."

Methods for Studying the Metabolome

Metabolomic data are currently generated by using *nuclear magnetic resonance (NMR)* and *mass spectrometry (MS)* coupled with *gas (GC-MS)* or *liquid chromatography (LC/MS)* [72]. *NMR* is a quantitative, fast, and highly reproducible spectroscopic technique that is based on the energy absorption and reemission of the atom nuclei due to variations in an external magnetic field [73]. Hydrogen is the most commonly targeted nucleus in NMR analysis (*1H-NMR*) since it is naturally abundant in biological samples and provides information on the amount of each metabolite and on its chemical structure. The complexity of the spectral data generated by NMR analysis may be managed by one (*1D-NMR*) and two (*2D-NMR*) frequency axes. *1D-NMR* is the most commonly used method in high-throughput metabolomic studies. Conversely, *2D-NMR* spectra are mostly used to characterize those compounds that cannot be identified by *1D-NMR* spectra. Furthermore, *2D-NMR* allows to separate otherwise overlapping spectral peaks and, therefore, gives additional and important information on the chemical properties of the metabolite [74]. Unfortunately, the *NMR* application to biomarkers' discovery studies is limited by its low sensitivity (about micromolar concentration) that prevents the confident identification of low-abundant metabolites [75].

Unlike *NMR*, *GC-MS and LC-MS* are very sensitive techniques which, however, have other drawbacks that limit their wide application. For example, *GC-MS* is suitable for the analysis of *volatile and thermally stable metabolites* because the separation of the ionized sample occurs through a carrier gas that works at high temperatures. Otherwise, readily volatile samples need chemical derivatization to make them suitable for further GC-MS analysis. This process is time-consuming and thus represents one of the major limits of GC-MS since it may affect the reproducibility of the results due to the multistep procedure in sample preparation, the incomplete derivatization, and the formation of adducts. Furthermore, *GC-MS* is able to analyze only medium polar compounds with relative small mass (molecular ion mass-to-charge ratio, m/z, <800), while for metabolites with higher mass or

polarity, *LC-MS* is now considered the gold standard. Although this approach requires minimum sample preparation (i.e., sample deproteination by solvent precipitation or solid matrix extraction when protein-rich samples as serum/plasma are analyzed), it suffers from matrix effects that may interfere with spectra resolution and cause ion suppression, thus preventing the ionization of potentially interesting compounds [76].

Despite the techniques used, metabolomic profiling generates, as other -omics approaches, complex datasets that require proper management by statistical univariate (one variable analyzed at a time) or multivariate (two or more variables analyzed) methods. Univariate analysis includes the t-test, the analysis of variance, the analysis of covariance, and the univariate linear regression, while the most commonly used multivariate methods are multivariate linear regression, multivariate analysis of variance, cluster analysis, and principal component analysis (PCA). Of note, multivariate analysis may generate *false-positive results* that must be minimized by applying specific correction methods such as the *Bonferroni*, which corrects for family-wise error rate (FWER), and the *Benjamini-Hochberg*, which corrects for false discovery rate (FDR). A number of specific work [77, 78] can be consulted for an in-depth study on the statistical methodologies applied to the study of complex datasets. The potentialities of metabolomic analysis for the identification of confident biomarkers of DN and DKD will be discussed in the following paragraphs focusing on specific applications in urine and serum/plasma samples that have shown Krebs cycle, lipid metabolism, amino acid metabolism, urea cycle, and nucleotide metabolism as strongly associated with DKD.

Metabolomics in Diabetic Kidney Disease

Most of the metabolomic studies applied to diabetic nephropathy have been conducted on biological fluids such as urine and serum/plasma and will be discussed below.

Urine Metabolomics Metabolomic analysis of urinary samples in DKD patients may have different purposes, from the early detection and differential diagnosis to the identification of the molecular mechanisms responsible for disease development up to the identification of new potential therapeutic targets. This is made possible by the peculiarities of the kidney, a metabolic organ capable to concentrate and excrete in the urine a variety of metabolites coming from biochemical pathways linked to kidney dysfunction. The reduction of renal function (lower eGFR) in T2DM normoalbuminuric patients has recently been associated to a specific metabolic pattern that also included indoxyl sulfate, a well-known uremic toxin. Furthermore, metabolic analysis of urine samples of T2DM patients identified a set of 13 metabolites implicated in organic anion transport, TCA cycle, and amino acid metabolism that were differently excreted between T2DM patients and HS and a subset of 5 metabolites that were useful to discriminate T2DM patients with and without CKD [79].

The majority of the less excreted metabolites in DN group were water-soluble organic anions and functional analysis correlated them to impaired mitochondrial function in DN. The metabolomic approach has also been used, on both type 1 and type 2 diabetes cohorts, to characterize specific patterns potentially associated to the progression of renal damage. However, current knowledge seems to suggest a limited capacity of the metabolomic profile in predicting the transition from normalbuminuria to microalbuminuria. In type 2 diabetes, no significant differences were found in normoalbuminuric patients who developed microalbuminuria over time, while in type 1 diabetic patients, an increased excretion of metabolites linked to fatty acid metabolism, detoxification system, and gut microbiome have been described, which, however, allow to predict the evolution of renal damage with an accuracy below 75% [80]. A pilot study carried out by GC-MS on type 2 diabetic patients including 21 micro- to macroalbuminuria case/controls pairs described the possible usefulness of 3 urinary metabolites (hexose, glutamine, and tyrosine) assessed together with 2 plasma metabolites (butenoylcarnitine and histidine) to highlight the progression from micro- to macroalbuminuria on top of the traditional renal risk markers, namely, baseline urinary albumin excretion and baseline estimated glomerular filtration rate [68]. The model reached a 99% accuracy and suggested the key role of the impaired mitochondrial oxidation of fatty acids, inflammation, and oxidative stress in DN progression.

Serum and Plasma Metabolomics Plasma and serum metabolomic studies on DN showed, at least in part, overlapping results to those on urine by stressing key role of amino acids, phospholipids, and fatty acid metabolism in the progression of DN.

Targeted metabolomic analysis on amino acid metabolites demonstrated a significant increased concentration of uric acid, xanthine, and adenosine in serum of DN vs matched healthy controls [81]. The analysis of the phospholipidic subset allowed to characterize a significant decrease of phosphatidylinositol and a linear increase of sphingomyelin in T2DM patients with DN [82]. The role of fatty acids in DN pathogenesis seems to be even more important in light of their abnormal accumulation in parenchymal cells of multiple tissues, a phenomenon called lipotoxicity, which is now considered one of the triggers of T2DM and its chronic complications [83]. Defective fatty acid oxidation has recently been described as one of the mechanisms responsible for renal fibrosis, because renal tubular cells use fatty acid oxidation of as a fuel for their functions. The reduced expression, in renal fibrotic tissue, of two isomers of carnitine palmitoyl transferase (CPT 1 and 2) prevents, in fact, the fatty acid uptake that in turn leads to their accumulation [84]. Specific metabolomic screening of FAs, called lipidomics, may allow to understand the role of fatty acid impairment in various settings. Lipidomics analysis of T2DM patients with and without DN showed high discrimination power on different stage of DN and correlated the disease progression to plasma levels of arachidonic acids; this suggests a key role of the inflammation in the progression of DN [85]. Of note the changes of the gut microbiome have been associated to the progression of DN to ESRD through the production of uremic toxins like p-cresol and indoxyl sulfate that are normally excreted by the kidney; this correlates with the decline of renal

function toward ESRD. A recent study showed that increased baseline levels of uremic toxins may predict, in DN patients, the development of ESRD up to 10 years before any clinical evidence [86]. Although it is not yet clear whether the accumulation of uremic toxins precedes or is a consequence of the renal damage, it suggests a critical role of the gut microbiome in the progression of diabetic kidney disease and emphasizes this "super organ" as a possible new therapeutic target [87].

Point-of-Care Testing: The Future of Personalized Medicine

Although omics analyses have notably increased our knowledge of this diabetic kidney disease, their impact has currently reached an intermediate stage since the ultimate goal of understanding the temporal sequence of predisposing and triggering events the renal damage will still require further improvements. For example, one of the greatest efforts of the scientific community in the coming years will have to be the development of joint and multicenter initiatives that may enable the development of well-designed clinical studies that take into account at least the following aspects:

- The definition of *consensus protocols for collection, processing, and analysis* of samples to achieve comparable and reproducible results among different studies.
- The setup of *large prospective studies* on an appropriate number of patients to obtain reliable data and biomarkers capable of predicting the onset of renal damage.
- The *improved selection of the histological phenotypes* within the general group of diabetic kidney disease.

Moreover, although bioinformatics allows to pinpoint a set of molecular pathways to focus attention on, *current algorithms are still unable to contemplate the presence of protein PTMs into data analysis*; thus they provide an incomplete picture of what is really happening into a living system.

In this context the *systems medicine* approach may finally allow the analysis of intricate networks, integrating specific components from different layers (e.g., genomics, transcriptomics, proteomics, and metabolomics) that are dynamically coordinated and actively interacting. In fact, their integration uniquely characterizes living systems as a whole, and the study of perturbation of their relationship is specifically associated with a disease state and pathophysiological processes [88, 89]. Integration of data coming from omics studies will allow to define, at a system level, the biological activity of the distinct key molecular targets involved in DN onset and progression by leveraging the information of data coming from different sources (Fig. 28.2).

We must be aware that the identification of really *qualified biomarkers* that would be able to accurately describe the complexity of the clinical and histopathological phenotype asks for the above reported conceptual and practical improvements.

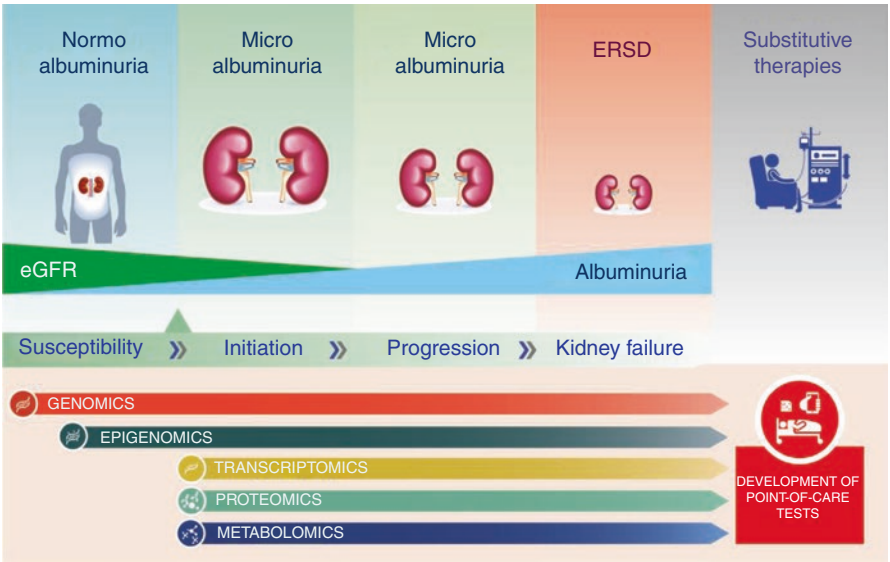

Fig. 28.2 The figure depicts the natural history of chronic renal failure in patients with T2D (upper box) and the corresponding omics approaches that could be used to identify the different stages of disease progression (lower box). Genomics and epigenetics identify genetic susceptibility, while transcriptomics, proteomics, and metabolomics provide powerful tools for early detection of renal damage initiation and monitoring of disease progression toward kidney failure. Omics are promising as they allow the identification of novel biomarkers of diagnosis, prognosis, and response to therapy. The identification of a biomarker signature as a tool for precision medicine will promote the development of point-of-care testing, allowing to monitor patient's status in real time

Of note, the incredible amount of new biomarkers might critically contribute to develop *new and even more accurate test for precision medicine*. In fact, when biomarkers will be validated as qualified biomarkers, it will be possible to integrate them in small, easy-to-use, and robust diagnostic devices to be used in *point-of-care (PoC) testing*.

Many biomedical companies are working in this direction by exploiting the principles of microfluidics to develop new reliable and accurate biosensors. If it is already possible for diabetic patients to monitor glucose directly at home, the identification of novel qualified biomarkers will allow, in the near future, to create a new generation of PoC to monitor, through blood or urine analysis, the risk of kidney disease progression. In the end, the future of medicine seems to be linked to the development of *information and communication technology (ICT)*, which today allows to integrate many biological parameters and analyze their meaning through sophisticated neural networks to obtain more accurate information on state of health and illness of each individual. We are in the midst of a technological and cultural revolution that seems to be a step away from taking off. One of the most exiting challenges of the coming years will certainly be the ability to correctly and critically interpret the ever-growing amount of information that will

be generated by these approaches. This will require always a careful check by physicians to validate the predicted reliability in the pathophysiological context of each disease.

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Chapter 29
Future and Novel Compounds in the Treatment of Diabetic Nephropathy

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Introduction

The last trials leading to licensed new drugs to slow the progression of kidney function decline showed that the ACE-inhibitor captopril in type 1 diabetes and the angiotensin receptor blockers losartan and irbesartan in type 2 diabetes delayed the onset of a doubling of serum creatinine, dialysis, or renal transplantation in patients with diabetic nephropathy (DN) beyond their effects on blood pressure [1–3]. This is 16 years ago. Today, angiotensin receptor blockers (ARBs) as well as angiotensin-converting enzyme inhibitors (ACEi) are still considered the standard of care for DN, in addition to lifestyle interventions and control of metabolic status and blood pressure [4, 5]. This is due to the fact that all subsequent efforts to lower renal risk in the population with diabetes were unsuccessful. Many large clinical trials have been conducted in the last decade to test the efficacy and safety of novel treatment strategies to slow the progression of DN. Many of them demonstrated a lack of benefit or even unfavorable effects in the active treatment group, as reviewed in Chap. 24.

The failure of finding new therapies beyond ACEi or ARB left us with a very high residual risk for most patients with DN. For example, the dialysis and mortality rates in the RENAAL and IDNT trials, two landmark clinical trials that demonstrated the renal protective effects of losartan and irbesartan in patients with DN, exceed 10 patients per 100 patients per year during ARB treatment and are at least as high as the average mortality rates of all cancers [6]. These numbers illustrate the large unmet need in this area.

The search for new compounds to improve the prognosis of patients with DN continues, and a couple of promising drugs have reached late-stage clinical

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development. This chapter will review several drug classes that are currently tested in phase 3 clinical trials and, secondly, highlight novel therapies in early-stage clinical development. Finally, future directions and challenges for development and clinical application of DN therapies will be briefly reviewed.

Novel Compounds for Diabetic Nephropathy in Late-Stage Development

Recently, several promising therapies emerged from phase 2 clinical trials and are currently being tested in large phase 3 clinical programs (Table 29.1). Agents that have shown (potential) renoprotective effects in patients with DN include SGLT-2 inhibitors, endothelin type A receptor antagonists (ERAs), and mineralocorticoid receptor antagonists (MRAs). These approaches are targeting new pathways, while they are still aimed at lowering the main residual risk factors for the ongoing renal progression, including blood pressure and albuminuria [6, 9].

SGLT-2 Inhibitors

Sodium-glucose cotransporter type 2 (SGLT-2) inhibitors are novel glucose-lowering agents that inhibit the SGLT-2 transporter in the proximal tubule. SGLT-2 transporters are responsible for reabsorption of nearly all (90%) of the glucose filtered by the kidneys [10]. Treatment with SGLT-2 inhibitors reduces fasting blood glucose and HbA1c by increasing urinary glucose excretion proportional to the level of hyperglycemia and the glomerular filtration rate [11, 12]. Recent trials in diabetic patients reported beneficial metabolic effects of SGLT-2 inhibition [13–15], which were more pronounced than those of alternate hypoglycemic agents in terms of HbA1c lowering, weight reduction [16–18], and lowering insulin dose requirement [19–21].

SGLT-2 inhibitors are developed and registered as antihyperglycemic drugs. However, it has been shown that SGLT-2 inhibition confers additional effects which can contribute to long-term CV and kidney protection. Since the reabsorption of sodium by SGLT-2 transporters is linked to that of glucose, SGLT-2 inhibition reduces the reabsorption of sodium as well, thereby inducing modest natriuresis and diuresis (Fig. 29.1). This may explain the reduction in blood pressure and body weight along with an increase in hematocrit observed with SGLT-2 inhibition. Furthermore, the reduced reabsorption of glucose and sodium leads to an increased distal sodium chloride (NaCl) delivery [22], which restores tubuloglomerular feedback and thereby mitigates renal hyperfiltration [23]. This mechanism may contribute to the potent albuminuria-lowering properties of SGLT-2 inhibitors that were observed in recent clinical trials [7, 8, 14]. Additionally, many other systemic

Table 29.1 Novel compounds for diabetic nephropathy in late-stage development

Drug class	Compound	Current status	Study information
SGLT-2 inhibitors	Empagliflozin (BI, Eli Lilly)	Phase 3 completed (November 2015)	EMPA-REG OUTCOME study: Tested effects of empagliflozin vs. placebo in 7020 patients with T2DM and a history of CV disease Empagliflozin was associated with significant reductions the composite endpoint of CV mortality over a median follow-up of 3.1 years [7] Adverse events: Increased rate of genital infection in empagliflozin group
	Canagliflozin (Janssen Research and Development)	Phase 3 completed (August 2017)	CANVAS and CANVAS-R study: Tested effects of canagliflozin vs. placebo in 10,142 patients with T2DM and elevated CV risk Canagliflozin was associated with a lower risk of CV events [8] Adverse events: Higher risk of amputation
		Phase 3 ongoing	CREDENCE study (NCT02065791): Is evaluating the effects of canagliflozin vs. placebo on cardiorenal outcome in 4464 patients with T2DM, DN and macroalbuminuria
	Dapagliflozin (AstraZeneca)	Phase 3 ongoing	Dapa-CKD study (NCT03036150): Is evaluating the effects of dapagliflozin vs. placebo on renal outcomes and CV mortality in 4000 patients (estimated) with DN and with or without T2DM
ERAs	Atrasentan (AbbVie)	Phase 3 ongoing	SONAR study (NCT01858532): Is testing the long-term renal effects of atrasentan vs. placebo in patients with T2DM and DN After an enrichment period, patients with a response in albuminuria and without signs of excess fluid retention will be randomized
MRAs	Finerenone (Bayer)	Phase 3 ongoing	FIDELIO-DN study (NCT02540993): is evaluating the effects of finerenone vs. placebo on long-term renal outcome in an estimated population of 4800 patients with T2DM and DN at high renal risk FIGARO-DN study (NCT02545049): is evaluating the effects of finerenone vs. placebo on long-term CV outcome in an estimated population of 6400 patients with T2DM and DN at high CV risk

As of September 2017. *SGLT-2,* sodium-glucose cotransporter 2; *ERA,* endothelin receptor antagonist; *MRA,* mineralocorticoid receptor antagonists; *T2DM,* type 2 diabetes mellitus; *CV,* cardiovascular; *BI,* Boehringer Ingelheim; *DN,* diabetic nephropathy

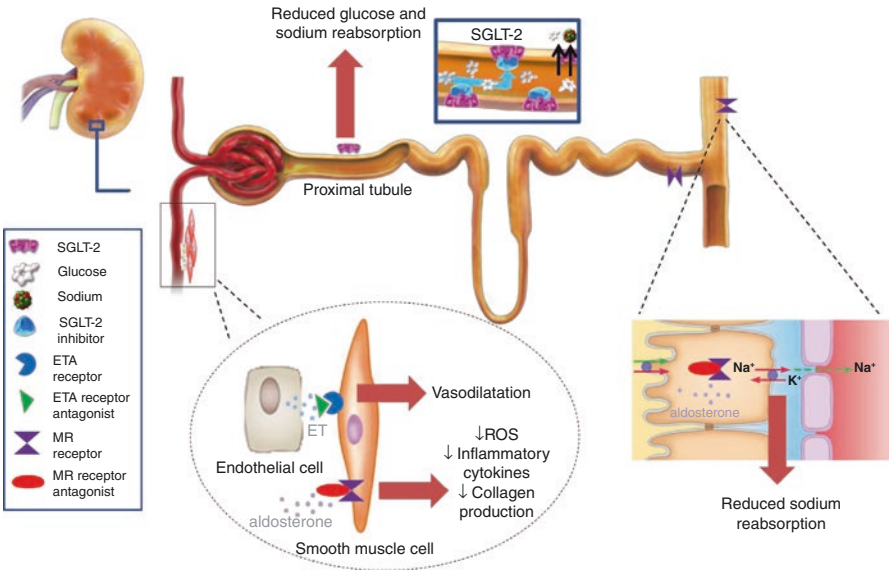

Fig. 29.1 Renal effects of SGLT2-inhibitors, ETA receptor antagonists, and MR receptor antagonists. (SGLT-2 sodium-glucose cotransporter 2, ETA endotheline type A, MR mineralocorticoid receptor, ROS reactive oxygen species)

and renal physiological effects of SGLT-2 inhibition, such as uric acid lowering [24], improving arterial stiffness, and reducing intrarenal inflammation [25, 26], are thought to contribute to the beneficial renal and CV effects of SGLT-2 inhibitors.

Two large, phase 3 trials with SGLT-2 inhibitors are now completed and have shown that the beneficial effects on renal and CV risk markers translate into long-term CV protective effects and potential beneficial effects on kidney outcomes. The empagliflozin, cardiovascular outcomes, and mortality in type 2 diabetes (EMPA-REG OUTCOME) study investigated the effects of the SGLT-2 inhibitor empagliflozin in 7020 patients with type 2 diabetes and a history of CV disease. The addition of empagliflozin to standard care (glucose-lowering therapy) was associated with significant reductions in the primary endpoint (a composite of CV mortality, nonfatal myocardial infarction, and nonfatal stroke), CV and all-cause mortality, and hospitalizations for heart failure during a median of 3.1 years [7]. In a further pre-specified analysis assessing the long-term renal effects of empagliflozin versus placebo, empagliflozin was associated with slower progression of nephropathy and lower rates of clinically relevant renal events [27]. Among patients receiving empagliflozin, there was an increased rate of genital infection but no increase in other adverse events.

The Canagliflozin Cardiovascular Assessment Study (CANVAS) program recently reported on two trials (CANVAS and CANVAS-R) in which 10,142 patients with type

2 diabetes and an elevated risk of CV disease were randomly allocated to the SGLT2 inhibitor canagliflozin or placebo. Patients treated with canagliflozin had a lower risk of CV events than those who received placebo but a greater risk of amputation, primarily at the level of the toe or metatarsal. The trial also suggested a possible benefit on the kidney. Canagliflozin reduced the risk of progression of albuminuria and, importantly, reduced the incidence of the composite outcome of a sustained 40% reduction in the estimated glomerular filtration rate, the need for renal replacement therapy, or death from renal causes [8]. However, just like the EMPA-REG trial, the CANVAS trials were not primarily designed to test effects on the kidney. Prospective trials to confirm these promising findings are therefore required. However, unlike empagliflozin, canagliflozin was associated with a higher rate of lower limb amputation.

Several large clinical outcome trials are ongoing to characterize the long-term renal and CV protective efficacy and safety of SGLT-2 inhibitors. To this end, the Evaluation of the Effects of Canagliflozin on Renal and Cardiovascular Outcomes in Participants with Diabetic Nephropathy study (CREDENCE; NCT02065791) is evaluating the effects of canagliflozin in patients with type 2 diabetes mellitus, stage 2 or 3 chronic kidney disease, and macroalbuminuria, in addition to standard of care plus an ACEi or ARB. The Study to Evaluate the Effect of Dapagliflozin on Renal Outcomes and Cardiovascular Mortality in Patients With Chronic Kidney Disease (Dapa-CKD; NCT03036150) evaluates the effects of dapagliflozin versus placebo on renal outcomes and CV mortality in patients with or without type 2 diabetes and nephropathy who are already receiving an ACEi or ARB.

Endothelin Antagonists

Renal endothelin-1 production is almost universally increased in patients with nephropathy and is associated with albuminuria and renal function loss [28, 29]. Binding of endothelin to the endothelin type A (ETA) receptor causes pronounced vasoconstriction, while activation of the ETB receptor induces vasodilatation via nitric oxide and prostaglandin release. ETB receptors also reduce arterial pressure by inhibiting tubular sodium and water reabsorption [30]. The pathologic effects of endothelin-1, including vasoconstriction, sodium retention, inflammation, and renal fibrosis, are mediated by the ETA receptor [31, 32]. Accordingly, initial studies involving acute intravenous endothelin receptor blockade suggested that ETA, but not ETB, blockade exerts protective renal and vascular effects in patients with nephropathy [33, 34].

The initial studies raised interest in this drug class, and renal effects of ERAs were investigated in larger clinical studies. Avosentan, an endothelin ETA receptor antagonist (ERA), significantly reduced proteinuria in patients with type 2 diabetes and nephropathy. However, a large phase 3 trial, testing the effect of avosentan on hard renal outcomes (ASCEND), was terminated prematurely because of an excess of congestive heart failure (CHF) and mortality in the avosentan treatment arm [35].

These results emphasize the importance of preventing the retention of fluid and sodium during treatment with ERAs. A post hoc analysis of the ASCEND trial showed that the increase in body weight during the first weeks of avosentan treatment, as marker of fluid retention, was associated with CHF development, indicating that careful body weight monitoring could provide an early signal of CHF development. A further analysis of the ASCEND trial indicated that appropriate diuretic therapy can mitigate the risk of fluid retention and worsening of CHF [36]. Above all, the failure of the ASCEND trial underscores the importance of careful patient selection and monitoring, appropriate ERA dosing, and the use of concomitant diuretic therapy. In addition, the use of ERAs with a higher selectivity for the ETA receptor, such as sitaxsentan and atrasentan, is preferable since these agents are less likely to induce side effects related to volume overload and edema.

The ERA atrasentan, which has a higher endothelin receptor A selectivity than avosentan, is currently tested for the treatment of DN. Atrasentan was originally developed for prostate cancer but has been repositioned for the treatment of DN. Promising effects have been observed with this agent in the Reducing Residual Albuminuria in Subjects with Diabetes and Nephropathy with Atrasentan (RADAR) trial. The RADAR trial was a randomized placebo-controlled trial involving 211 patients with DN and overt proteinuria, which studied the effects of atrasentan (0.75 mg/day and 1.25 mg/day) on albuminuria during a 12-week period [37]. Low- and high-dose atrasentan induced a comparable, significant decrease in albuminuria (35% and 38%, respectively), whereas atrasentan 1.25 mg/day elicited more fluid retention, reflected by a significant increase in body weight and a decrease in hemoglobin. However, it did not result in a higher rate of fluid retention-related adverse events compared to placebo. Based on the favorable efficacy-safety profile of these atrasentan compounds, which was supported by smaller randomized trials [38, 39], the currently ongoing Study Of Diabetic Nephropathy With Atrasentan (SONAR) study (NCT01858532) was initiated to characterize the long-term renal effects of atrasentan 0.75 mg/day in patients with type 2 diabetes and nephropathy on top of standard care with an ACEi or ARB plus diuretic therapy. Eligible patients will proceed to a 6-week enrichment period, after which patients with a response in albuminuria (>30% reduction) and without unacceptable rise in body weight (<3 kg) or B-type natriuretic peptide (<300 pg/ml) will be randomly assigned to long-term treatment with atrasentan or placebo. The enrichment design of the SONAR trial is unique for clinical trials in DN and potentially enables selection of a patient population with maximal benefit and minimal adverse effects. As discussed in Chap. 24, these types of study designs with careful patient selection may be the future for clinical trial design and conduct in DN.

Since many patients with nephropathy are treated with ARBs, combination therapies with ARBs and ETAs are also developed. Sparsentan is such a combination therapy consisting of irbesartan with an ETA. This drug combination has been shown to lower albuminuria in preclinical studies [40, 41]. An ongoing phase 2 study is testing whether combination therapy with sparsentan has superior albuminuria-lowering properties in comparison to single therapy with irbesartan in patients with primary focal segmental glomerulosclerosis (FSGS) [42]. An interim

analysis of the secondary endpoint showed that a significantly greater proportion of patients receiving sparsentan ($n = 64$) achieved a more than 40% reduction in proteinuria from baseline, compared to irbesartan-treated patients ($n = 32$) [43].

Mineralocorticoid Receptor Antagonists

The steroidal mineralocorticoid receptor (MR) plays an important role in the renin-angiotensin-aldosterone system (RAAS). The MR binds several ligands, including aldosterone and cortisol. Although most attention has focused on the association of angiotensin II (ATII) and end-organ damage, it has become increasingly clear that aldosterone is an important mediator of both CV and kidney injury, beyond the influence of renin and ATII [44, 45]. In patients with nephropathy, the activity of the MR receptors is upregulated, eventually driven by increased levels of circulating aldosterone, altered cortisol activity, and/or elevated local expression of the MR. [46] Additionally, treatment with ACEi and/or ARBs results in an incomplete suppression of aldosterone levels in some patients, in particular during prolonged treatment. This phenomenon, also known as "aldosterone breakthrough,' [47] blunts the efficacy of ACEi and ARB therapy [48]. In patients with DN who developed aldosterone breakthrough and expressed residual albuminuria while treated with an ACEi or ARB, administration of the MRA spironolactone resulted in a considerable reduction in albuminuria [49, 50].

Current clinically approved steroid-based MR antagonists (MRAs), including spironolactone and eplerenone, mimic the molecular structure of the natural MR ligands. Over the past years, trials have demonstrated that MRAs further reduce albuminuria and blood pressure in patients with diabetic and nondiabetic nephropathy when added to a RAAS inhibitor [51–54]. Addition of MRAs subsequently has been purported to avoid aldosterone breakthrough and to provide additional protection against adverse renal and CV events in patients with nephropathy. However, addition of spironolactone or eplerenone to RAAS inhibition increased the risk of hyperkalemia in patients with early and advanced nephropathy as much as twofold and three- to eightfold, respectively [51, 55]. In addition to other deleterious conditions such as cardiac dysfunction, increased serum potassium levels are associated with an elevated risk for progression of nephropathy in patients with DN [56].

Next-generation, nonsteroidal MR antagonists were introduced in an effort to more selectively target the MR receptor while retaining a similar receptor affinity as compared to spironolactone. The Mineralocorticoid Receptor Antagonist Tolerability Study-Diabetic Nephropathy (ARTS-DN) study was carried out to compare the effects of finerenone, a novel nonsteroidal MRA, with placebo in patients with type 2 diabetes and nephropathy [57]. A total of 823 patients were randomized to receive once-daily doses of finerenone (7.5, 10, 15, or 20 mg) or placebo, in combination with a RAAS inhibitor. Finerenone decreased albuminuria in a dose-dependent manner: a placebo-adjusted reduction of 21–38% was observed from baseline to 90 days. The occurrence of hyperkalemia was 1.8% versus 0% in the placebo group.

Of note, this trial included a population with limited risk for hyperkalemia: only patients with potassium levels <4.8 mmol/L were eligible, and the prevalence of advanced DN was relatively low. Further research in particular in broad populations in early and advanced DN is needed to assess whether addition of finerenone confers long-term renal and CV benefits and whether it results in a more favorable risk benefit profile in comparison to other registered MRAs. The efficacy and safety of finerenone in patients with DN is currently being tested in the ongoing finerenone in reducing kidney failure and disease progression in diabetic kidney disease (FIDELIO-DKD; NCT02540993) and the finerenone in reducing cardiovascular mortality and morbidity in diabetic kidney disease (FIGARO-DKD; NCT02545049) studies.

In addition to MR blockade, the reduction of plasma aldosterone concentrations by aldosterone synthase (CYP11B2) inhibition has emerged as a new strategy for the treatment of CV and nephropathy. Current evidence from phase 2 clinical trials indicates that LCI699, the first orally active aldosterone inhibitor, initiates a dose-dependent decrease in plasma aldosterone levels and significantly reduces blood pressure, although these reductions were smaller than those observed with MRAs [58–60]. The effects of LCI699 on the glucocorticoid axis limit the use of higher doses because of the loss of selectivity for CYP11B2. Novel agents with a higher selectivity for CYP11B2 that facilitate to test this approach at higher doses are now in early phase clinical development.

Novel Compounds for Diabetic Nephropathy in Early-Stage Clinical Development

Novel agents targeting pathways that may be involved in the development and progression of DN, such as glomerular hyperfiltration, inflammation, and fibrosis, have been a major focus for the development of new therapies (Table 29.2).

Anti-inflammatory Compounds

CCR2 Antagonists

Studies in human and experimental DN have shown that inflammatory processes largely contribute to the development and progression of kidney injury [67–69]. The production of C-C motif-ligand 2 (CCR2; also called monocyte-chemotactic protein 1) by diabetic kidneys plays a major role in renal macrophage accumulation, thereby initiating an inflammatory process. The secretion of CCR2 is stimulated by

Table 29.2 Novel compounds for diabetic nephropathy in early-stage development

Mechanism of action	Compound	Current status	Study information
Anti-inflammatory			
CCR2 antagonists	Emapticap pegol NOX-E36 (Noxxon)	Phase 2 completed (February 2014)	RCT in 75 patients with T2DM and nephropathy Subcutaneous administration of 0.5 mg/kg emapticap twice weekly for 12 weeks conferred a 26% reduction in UACR [61]
	CCX-140 (ChemoCentryx)	Phase 2 completed (December 2014)	RCT in 332 patients with T2DM and nephropathy Oral 5 mg and 10 mg CCX140-B during 52 weeks decreased UACR with 16% and 10%, respectively [62]
VAP1-inhibitors	ASP-8232 (Astellas)	Phase 2 completed (March 2017)	12-week RCT in 125 patients with T2DM and nephropathy evaluating the anti-albuminuric effects of ASP8232 (NCT02358096)
Anti-fibrotic			
JAK 1/2 inhibitors	Baricitinib (Eli Lilly)	Phase 2 completed (June 2017)	Multidose RCT in130 patients with T2DM and nephropathy Oral baricitinib led to a decrease in UACR after 24 weeks (baricitinib 4 mg/day conferred a reduction of 40%) and also induced a decline in hemoglobin levels (NCT01683409)
TGFα/EGF ligand inhibitors	LY-3016859	Phase 1/2 completed (August 2015)	Placebo-controlled RCT in 61 patients with type 2 diabetes and nephropathy Tested effect of different doses of LY-3016859 i.v. On proteinuria change from baseline to 25 weeks
TGFβ/PDGF inhibitors	TGF-β1 mAb	Phase 2 completed (March 2017)	Placebo-controlled RCT in 416 patients with type 1 or 2 diabetes and nephropathy testing the effects of TGF-β1 mAb s.c. On serum creatinine levels Treatment was well-tolerated, but after 8 months study was terminated due to futility [63]
Integrin α5/β3 antagonists	VPI-2690B	Phase 2 completed (March 2017)	RCT in 165 patients with type 1 or 2 diabetes and nephropathy Tested the effects of different doses of VPI-2690B i.v. On proteinuria change from baseline to 50 weeks (NCT02251067)

(continued)

Table 29.2 (continued)

Mechanism of action	Compound	Current status	Study information
Vasoactive			
Dual AT_1 and ETA receptor antagonists		Clinical	The dual AT_1 and ETA receptor antagonist sitaxsentan led to additional Reno- and cardioprotection in diabetic rats [40, 41]
PDE5 inhibitors	PF-00489791	Phase 2 completed (August 2013)	Placebo-controlled RCT in 256 patients with type 2 diabetes and nephropathy PF-00489791 20 mg/day during 12 weeks reduced UACR with 15.7% [64]
sGC activators	Cinaciguat	Preclinical/ early clinical	sGC activation elevated renal cGMP levels in a dose-dependent manner and was highly efficacious in preventing the progression of DN in a rat model [65]
NEP inhibitors	LCZ696 (sacubitril/ valsartan)	Phase 3 ongoing	HARP-III trial: Multicenter, double-blind RCT in 414 patients with chronic kidney disease, testing the effects of sacubitril/valsartan vs. irbesartan on renal function change after 12 months [66]

As of September 2017. *CCR2,* C-C chemokine receptor type 2; *FSGS,* focal segmental glomerulosclerosis; *RCT,* randomized controlled trial; *T2DM,* type 2 diabetes mellitus; *JAK,* Janus kinase; *TGF,* transforming growth factor; *EGF,* epidermal growth factor; *PDGF,* platelet-derived growth factor; *AT_1*, angiotensin 1; *PDE5,* phosphodiesterase type 5; *UACR,* urine albumin/urine creatinine ratio; *sGC,* soluble guanylate cyclase; *DN,* diabetic nephropathy; *NEP,* neutral endopeptidase; *VAP1,* vascular adhesion protein 1

a variety of pro-inflammatory stimuli, including proteinuria [70], and has been suggested to be a risk marker of kidney disease progression [71].

Until now, several agents that specifically inhibit CCR2 have been investigated in clinical trial setting. Emapticap pegol (NOX-E36) was investigated in an exploratory phase 2 double-blind, randomized clinical trial involving 75 patients with type 2 diabetes and albuminuria. Twice-weekly subcutaneous treatment with emapticap versus placebo over 3 months seemed to have beneficial effects on top of standard of care, based on a reduction in urinary albumin/creatinine ratio (UACR) and a reduction in HbA1c. The reduction in UACR was most pronounced at 8 weeks after discontinuation of treatment (26%, $p = 0.06$) [61]. Another double-blind, placebo-controlled trial in 332 patients with type 2 diabetes and proteinuria assessed whether CCX140-B could further reduce albuminuria in addition to standard care [62]. Treatment with oral CCX140-B 5 mg and 10 mg lowered UACR with 16% and 9%, respectively. The albuminuria-lowering effect persisted for several weeks after the drug was stopped. This suggests that the effects of CCR2 may not be solely hemodynamic. In both trials, CCR2 inhibitors were generally safe and well-tolerated.

VAP-1 Inhibitors

Vascular adhesion protein 1 (VAP1, also known as AOC3) is a membrane-bound glycoprotein that functions as an adhesion molecule for lymphocytes, regulating leukocyte migration into inflamed tissue [72]. In a phase 1 randomized clinical trial (NCT02358096), the VAP-1 inhibitor ASP8232 proved to be safe in patients with type 2 diabetes and nephropathy. A randomized, placebo-controlled phase 2 study (ALBUM: A Study to Evaluate ASP8232 as Add-On Therapy to Angiotensin Converting Enzyme Inhibitor or Angiotensin Receptor Blocker in Reducing Albuminuria in Patients With Type 2 Diabetes and Chronic Kidney Disease; NCT02358096) evaluated the efficacy of once-daily oral administration ASP8232 as add-on therapy to RAAS inhibition in reducing UACR after 12 weeks in patients with type 2 diabetes and nephropathy. The results of this trial are not reported.

Anti-fibrotic Compounds

JAK 1/2 Inhibitors

A prominent pathway associated with progression of DN is the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway [73]. Baricitinib is a selective JAK1 and JAK2 inhibitor, originally developed and now registered for the treatment of rheumatoid arthritis. The albuminuria-lowering effect of the drug was investigated in a phase 2 randomized, double-blind trial (NCT01683409) in 129 participants with type 2 diabetes and nephropathy. Patients were allocated to baricitinib at low-to-high daily doses (0.75 mg, 1.5 mg in single or divided dose, 4 mg) or placebo for 24 weeks, on top of standard care with an ACEi or an ARB. Baricitinib treatment resulted in a reduction in UACR at 6 months (in the highest-dose group this reduction accounted 40% versus placebo). After 4 weeks of study drug washout, the UACR reduction was sustained in the medium- and high-dose baricitinib groups. Moreover, the investigators observed a significant decrease in hemoglobin at 6 months in the high-dose treatment group, which may have been attributable to the dependence of erythropoietin signaling on JAK2 activation [74]. Although no unexpected side effects were detected, the long-term use of baricitinib might worsen pre-existing anemia in patients with DN. Further studies will need to examine the effects of baricitinib on hard renal endpoints and safety outcomes.

TGF-β PDGF Inhibitors

Transforming growth factor beta (TGF-β) has long been considered another molecular mediator involved in progression of DN. TGF-β protein synthesis is induced by a variety of messengers, including platelet-derived growth factor (PDGF).

Increased exposure of human proximal tubule cells to high glucose concentrations stimulates TGF-β protein synthesis in the presence of PDGF [75]. Activation of TGF-β subsequently stimulates downstream pathways, which results in glomerular basement thickening, glomerulosclerosis, and ultimately progression of DN [76]. A number of agents were developed to antagonize TGF-β and PDGF. A randomized, double-blind, phase 2 study assessed whether modulating TGF-β1 activity with a TGF-β1-specific monoclonal antibody (TGF-β1 mAb) was effective in slowing renal function loss in patients with DN on a stable dose of RAASi. Treatment with different doses of subcutaneous TGF-β1 mAb was well-tolerated, but the study was terminated after 8 months as the change in serum creatinine levels did not differ between placebo and TGF-β1 mAb [63].

Integrin α5/β3 Antagonists

Integrin A5β3 stimulates insulin-like growth factor 1 (IGF1) signaling and also plays a role in podocyte interaction with matrix proteins [77]. Experimental studies found that expression of α5β3 and vitronectin is increased in hyperglycemic rats [78]. VPI-2690B is a monoclonal antibody that binds to α5/β3 integrin and thereby supposedly blocks the action of α5/β3 integrin on IGF1 signaling. This antibody has been shown to reduce albuminuria in diabetic rats and atherosclerosis in diabetic pigs [79, 80]. VPI-2690B is currently in phase 2 clinical testing for the treatment of DN. This placebo-controlled trial is evaluating the anti-albuminuric effects of subcutaneous administration of VPI-2690B every other week during a total period of 48 weeks (NCT02251067).

Vasoactive Compounds

Guanylate Cyclase Activators

Endothelial dysfunction has been associated with disease progression in patients with DN [81]. Recent studies have highlighted a role for impaired nitric oxide (NO) production/signaling in the progression of DN [82–85]. Soluble guanylate cyclase (sGC) is an enzyme which is activated by NO binding to catalyze GTP into cGMP (Fig. 29.2) [86]. The increased formation of reactive oxygen species found in diabetic patients causes oxidation and subsequent loss of the prosthetic heme group of sGC, rendering it nonresponsive to NO [87]. This finding has led to the development of pharmacological compounds aimed at reactivating oxidized or heme-free sGC [88, 89]. As such, a class of synthetic compounds that activates defective sGC (also known as sGC activators) has been demonstrated to promote NO signaling and restore cGMP generation [90].

sGC activators have been proposed as a potential drug class to retard the progression of nephropathy. This is supported by the finding that NO plays an

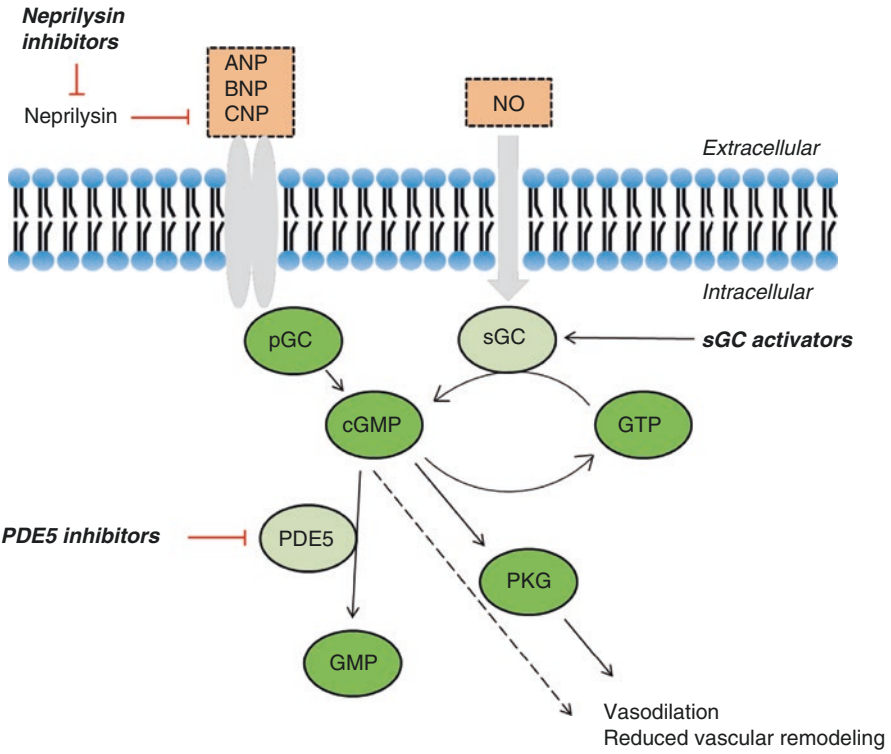

Fig. 29.2 Involvement of novel vasoactive compounds in the nitric oxide signaling pathway. The nitric oxide signaling pathway plays a major role in the renal vascular system. The activation of sGC and/or pGC and the subsequent rise in cGMP concentration induce an NO signal to the downstream elements of the signaling cascade, including PKG and cGMP-regulated phosphodiesterase. This eventually results in smooth muscle relaxation and vasodilation. PDE-5 inhibitors, neprilysin inhibitors, and sGC activators each enhance NO signaling by targeting different processes in this pathway. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; NO, nitric oxide; pGC, particulate guanylate cyclase; sGC, soluble guanylate cyclase; GMP, guanosine monophosphate; cGMP, cyclic guanosine 3′5’-monophosphate; GTP, guanosine 5′-triphosphate; PDE5, phosphodiesterase type 5; PKG, protein kinase G

important role in the autoregulation of renal blood flow, which is moderated by several mechanisms including tubuloglomerular feedback (TGF) and myogenic response [91–93]. A preclinical study found that the effects of NO on TGF and microvascular autoregulation are predominantly mediated by the sGC-/cGMP-dependent signaling pathway [94]. According to recent phase 2 trials, intravenous administration of cinaciguat, a sGC activator, in patients with decompensated heart failure led to substantial improvements in hemodynamics and symptoms with preservation of renal function [95–97]. However, two studies were prematurely terminated due to an increased occurrence of nonfatal hypotension [95, 96]. Few studies have explored the utility of sGC activators for the treatment of nephropathy. In preclinical NO-deficient models with different etiologies of nephropathy,

several sGC activators have consistently shown renoprotective effects [98, 99]. Another study in a rat model of type 2 diabetes-induced nephropathy that expressed markers of oxidative stress showed that sGC activation elevated renal cGMP levels in a dose-dependent manner and was highly efficacious in preventing the progression of DN [65]. Clinical studies translating these preclinical findings to humans are currently ongoing.

PDE5 Inhibitors

Another mediator of cGMP metabolism is the cGMP-hydrolyzing enzyme phosphodiesterase type 5 (PDE5). Preclinical studies suggest that elevating the cGMP intracellular pool through inhibition of PDE5 might exert renoprotective effects in DN [100–102]. The first clinical study to suggest translation of the PDE5-related preclinical findings demonstrated that administration of a PDE5 inhibitor once daily for 30 days significantly reduced albuminuria in microalbuminuric patients with type 2 diabetes [103]. A subsequent randomized, double-blind, placebo-controlled trial involving 256 patients with type 2 diabetes and nephropathy assessed the antialbuminuric effects of the selective PDE5 inhibitor PF-00489791 on top of standard care with ACE inhibitors or ARBs. Administration of 20 mg PF-00489791 once daily for 12 weeks significantly reduced UACR with 15.7%. The investigators concluded that the safety and efficacy profile supports further investigation of PF-00489791 as a novel therapy to improve renal outcomes in DN [64].

NEP Inhibitors

Natriuretic peptides, especially B-type natriuretic peptide (BNP), have primarily been regarded as biomarkers in heart failure (HF). The natriuretic peptide (NP) system has potent counter-regulatory effects on the RAAS system, which lower blood pressure but also mediate renal beneficial effects [104]. Neutral endopeptidase or neprilysin (NEP) is responsible for degradation of natriuretic peptides and a range of other vasoactive peptides including bradykinin, substance P, ATII, and endothelin [104, 105]. Stand-alone NEP inhibitors increase levels of NPs and bradykinin, resulting into vasodilation, natriuresis, diuresis, and reduction in blood pressure [106, 107]. However, they also increase levels of ATII and endothelin, which in turn undermine the beneficial effects of the upregulated NP production [108, 109]. Dual ACEi and NEP inhibitors (such as omapatrilat) were reported to be protective against hypertension, heart failure, and nephropathy in preclinical and early clinical studies [104, 110, 111]. Its further clinical development was hampered due to the increased the risk of angioedema, most likely elicited by an increase in bradykinin [112–114]. These observations gave rise to the development of angiotensin receptor neprilysin inhibitors (ARNi). The substitution of the ACEi for an ARB reduces bradykinin release and lowers the risk of angioedema. The first compound in this drug class, LCZ696 (sacubitril/valsartan), was tested in a large clinical

trial involving patients with heart failure, the Prospective comparison of ARNI with ACEI to Determine Impact on Global Mortality and morbidity in Heart Failure (PARADIGM-HF) trial. LCZ696 showed a doubled rate in angioedema compared with enalapril; however, fewer patients in the LCZ696 group stopped their study medication because of adverse effects. LCZ696 conferred a better cardioprotection than enalapril, leading to its approval by US Food and Drug Administration for the reduction of hospitalization due to HF [115].

Although no large-scale human trials have to date been conducted with NEPi/ARB in DN, experimental animal studies showed cardiac and renal protective effects of these agents in diabetes and nephropathy [116–120]. The ongoing UK Heart and Renal Protection III (HARP-III) trial is a multicenter, double-blind, randomized controlled trial in 414 patients with chronic kidney disease. After 12 months of treatment, the effects of sacubitril/valsartan on renal function change in comparison to those of irbesartan will be assessed [66].

The Future: The Case for Personalized Medicine

The ultimate question remains how to further develop and apply these novel compounds in order to confer optimal benefit for individual patients. DN is a highly heterogeneous disease with multiple pathophysiological processes involved in disease progression. The novel drugs described in this chapter target these pathophysiological processes. From all the established as well as novel drugs for the treatment of DN, it is already known that individual patients show a marked variation in the way they respond to them (Fig. 29.3). The goal will thus be to tailor drugs to individual patients in whom the pathophysiological process targeted by the drug is deregulated, thus personalizing treatment.

Biomarkers can help to personalize treatment in several ways. First, biomarkers can be used to identify subgroup of patients who are more likely to benefit from a drug or develop side effects before drug exposure. This type of biomarker is labeled as a predictive biomarker. Second, the change of the biomarker during short-term treatment – couple of weeks – can also be used to predict the effect of the drug on clinical outcomes. This type of biomarker is defined as a dynamic biomarker. Above all, dynamic biomarkers can be used to find the right drug dose for the right patient. Several research programs in Europe and the United States are currently ongoing aiming to discover and validate predictive and dynamic biomarkers for the treatment of DN.

The importance to personalize treatment for the future development of new drugs and treatment of DN is highlighted by the failure of late-stage confirmatory clinical trials aiming to develop new drugs for DN as discussed in Chap. 24. Notably, although these trials failed to demonstrate a protective effect at a population level, post hoc analyses revealed various important aspects. First, detailed (post hoc) analyses of the unsuccessful trials have suggested that a distinct set of patients do benefit from the experimental drug, but many others do not. This variation in response

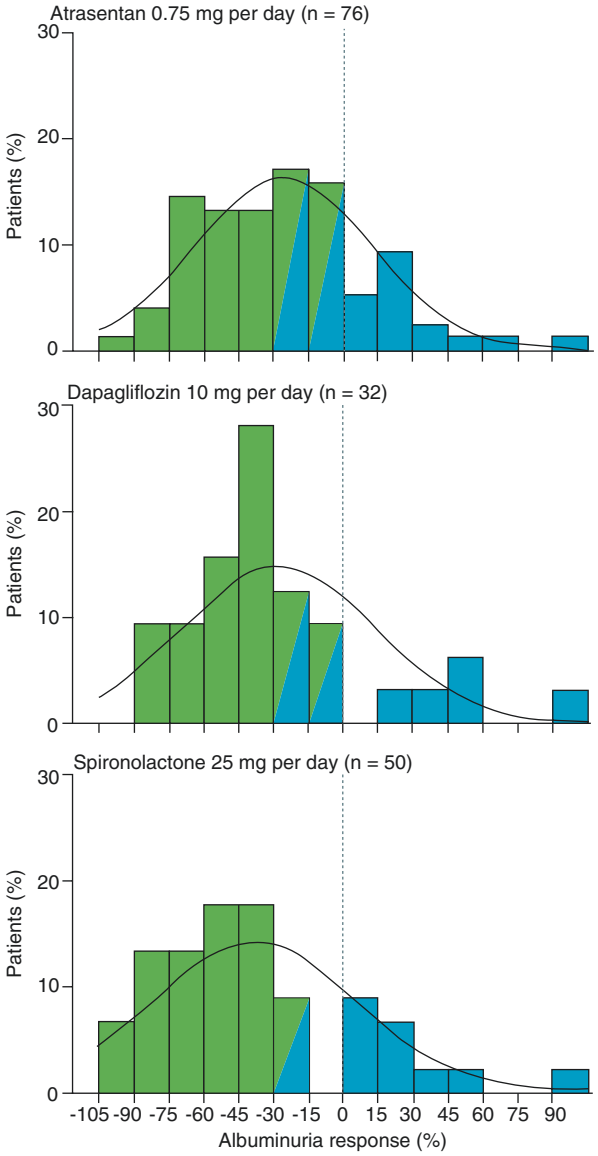

Fig. 29.3 Variable short-term response in albuminuria to drugs from three different drug classes. (*Adapted from:* de Zeeuw et al. [129]). Atrasentan was given for 12 weeks; dapagliflozin was given for 6 weeks; and the mineralocorticoid receptor antagonist spironolactone was given for 12 weeks. The dotted line denotes no response. Blue bars show patients with increase in albuminuria, blue and green bars show patients with a mild decrease in albuminuria not likely to be associated with protection from renal outcomes, and green bars show patients with albuminuria reduction likely to be associated with improved renal outcome

between individuals is also called *interindividual drug response variability*. For example, the Aliskiren Trial in Type 2 Diabetes Using Cardio-Renal Endpoints (ALTITUDE) trial did not show a beneficial effect of the direct renin inhibitor aliskiren on renal outcomes in the overall type 2 diabetic population. Yet, in the subgroup of patients with a reduction in albuminuria of more than 30% during aliskiren therapy – 37% of the overall population – a 55% lower risk was documented compared to those without a reduction in albuminuria during treatment [121]. In other trials, subgroups of patients could be identified who did not tolerate the drug of interest and were actually at a higher risk of a cardiovascular event. In the Bardoxolone Methyl Evaluation in patients with chronic kidney disease and type 2 diabetes mellitus: the Occurrence of Renal Events (BEACON) trial, patients with a brain natriuretic peptide (BNP) > 200 pg/ml or previous heart failure and randomized to bardoxolone methyl were at highest risk of heart failure [122].

A second important finding from the past negative trials is that a single drug affects many more processes than the one intended (i.e., a blood pressure-lowering drug may also affect other parameters including albuminuria, glucose, cholesterol, and serum potassium). Some of these short-term effects may be beneficial for renal and CV outcomes, such as a reduction in blood pressure, albuminuria, or uric acid [123, 124]. Yet, there are also other effects, such as an increase in potassium, which may increase renal and CV risk (Fig. 29.4) [56]. These short-term drug effects appear to vary within an individual, so-called intraindividual drug response variability. This means that in one patient, blood pressure and albuminuria may fall, whereas in another patient (treated with the same drug), blood pressure rises and albuminuria falls. The balance between the responses of these multiple parameters within an individual determines the ultimate renal and CV outcome of the patient. Thus, there is a large individual variability in short-term biomarker responses to a drug, and the sum of all these individual responses determines the ultimate renal and CV outcome.

In the context of the large *interindividual* and *intraindividual* variation in multiple renal and CV risk markers, one should monitor all risk marker changes following treatment initiation in order to optimally predict the renal and CV drug response. This means that algorithms should be developed and validated that translate a drug response on multiple renal and CV risk markers in the short term (up to a few months) into a predicted response on renal or CV outcomes in the subsequent years. Such an algorithm has been recently developed and validated. The so-called multiple Parameter Response Efficacy (PRE) score was originally developed in patients with DN within the RENAAL trial. Integrating the short-term drug effects on multiple risk markers in a PRE score provided a better prediction of the long-term drug effect on renal outcome than any change in single markers [125]. Several subsequent validation studies demonstrated that the PRE score accurately predicted long-term renal and/or CV outcome for direct renin inhibitors and a thiazolidinedione [126, 127]. Integrating changes in multiple risk markers in response to a drug thus provides a better estimate than changes in single risk markers to predict drug efficacy. This notion is substantiated by another study which showed that at an individual level, taking into account all drug-induced changes in response to the ARB

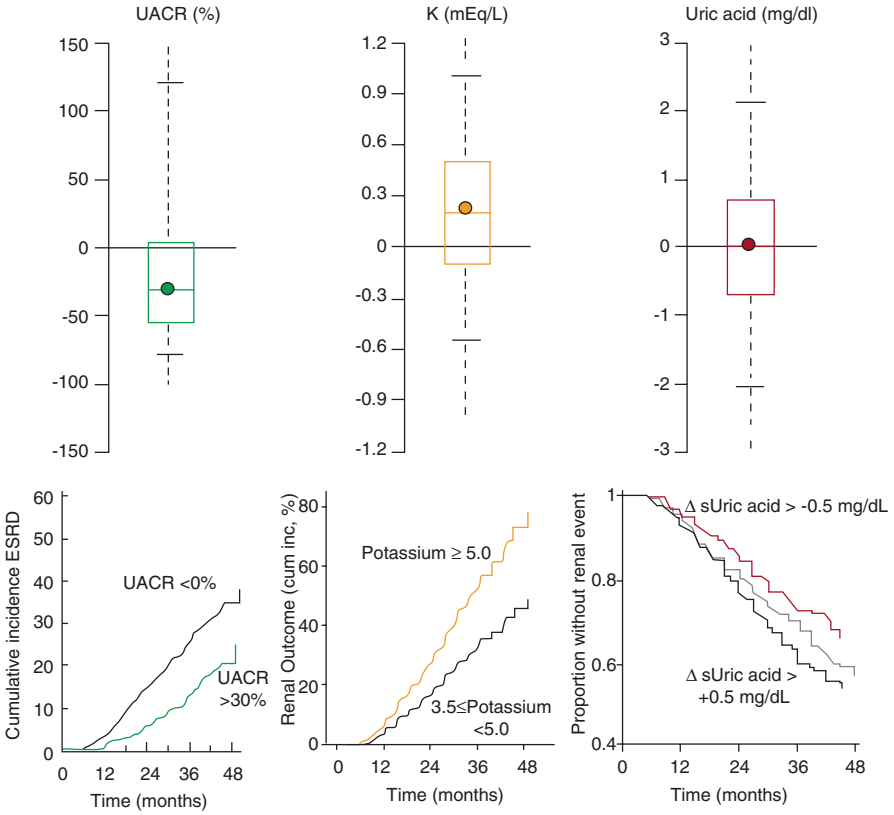

Fig. 29.4 Interindividual drug response variability in response to losartan. Losartan has variable effects on albuminuria (UACR), potassium (K), and uric acid. Reductions in albuminuria and uric acid were independently associated with lower renal risk, whereas rise in potassium was independently associated with higher renal risk. Results from post hoc analyses of the RENAAL trial

losartan, will provide a better prediction of who will benefit from ARB treatment in terms of renal and CV protection [128]. Whether implementing the PRE score in clinical practice leads to more renal and CV protection needs to be established in the future.

These proposed steps from a one size fits all to a personalized treatment approach require a mind-shift from many stakeholders. For instance, the pharmaceutical industry should change their business models and focus on more targeted patient groups, regulatory agencies should develop models to assess efficacy and safety and market drugs for specific targeted patient populations, and healthcare providers have to develop new guidelines and implement personalized medicine in clinical practice. Above all, patient organizations should be involved in all of these processes in order to ensure that patients are the ultimate beneficiaries.

Despite adequate lifestyle interventions, metabolic control, and the use of ACEi and ARBs, the residual renal and CV risk in patients with DN remains very high.

As outlined in this chapter, various drugs that may reduce renal and CV morbidity and mortality are currently in clinical development. These drugs may improve the cumbersome prognosis of patients with DN if they are properly tested in confirmatory clinical trials. The failure of late-stage clinical trials in the past 15 years has highlighted the need for adapted clinical trial designs that preselect patients based on their individual drug response, thus personalized medicine. The concept of personalized medicine is only in its infancy in DN clinical trials, and the implementation of personalized treatment approaches into drug development and clinical practice will require the united efforts from different stakeholders.

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