

# Tubulointerstitial disease in diabetic nephropathy

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**Abstract:** Diabetes mellitus is the major cause of end stage renal disease (ESRD). We cannot predict which patient will be affected. ESRD patients suffer an extremely high mortality rate, due to a very high incidence of cardiovascular disease. Several randomized, prospective studies have been conducted to quantify the impact of strict glycemic control on morbidity and mortality, and have consistently demonstrated an association between strict glycemic control and a reduction in ESRD. Within the past 20 years, despite the implementation of treatments that were presumed to be renoprotective, diabetes mellitus has continued to rank as the leading cause of ESRD, which clearly indicates that we are still far from understanding the mechanisms involved in the initiation of ESRD. Progressive albuminuria has been considered as the sine qua non of diabetic nephropathy, but we know now that progression to diabetic nephropathy may well happen in the absence of initial microalbuminuria. The search for new biomarkers of early kidney damage has received increasing interest, since early identification of the pathways leading to kidney damage may allow us to adopt measures to prevent the development of ESRD. Most of these biomarkers are deeply influenced by environment, genetics, sex differences, and so on, making it extremely difficult to identify the ideal biomarker to target. At present, there are no new drugs that come close to providing the solutions we desire for our patients (ie, reducing complications). Even when used in combination with standard care, renal complications are, at best, only modestly reduced, at the considerable expense of additional pill burden and exposure to serious off-target effects. In this review, some of the hypothesized mechanisms of this heterogeneous disease will be considered, with particular attention to the tubule–interstitial compartment.

**Keywords:** TGF- $\beta$ 1, ESRD, Ox-LDL, diabetes, ESRD

## Introduction

Diabetes mellitus is the major cause of end stage renal disease (ESRD).<sup>1</sup> About 20% of patients with either type 1 diabetes (T1DM) or type 2 diabetes (T2DM) develop nephropathy after many years of diabetes, but we cannot predict which patient will be affected, and the pathophysiological mechanisms that are critically involved in triggering the development of ESRD are still unclear. Within the past 20 years, despite implementation of treatments that were presumed to be renoprotective, diabetes mellitus has continued to rank as the number one cause of ESRD.<sup>1</sup> Recently, it has become clear that diabetic nephropathy is a heterogeneous entity, conditioned by several factors, including diabetes type, genetics, social status, blood glucose and pressure levels, environment, sex, etc.

Much has been learned about the role of the vasculature and the glomerulus, including mesangial cells and podocytes, in the pathophysiology of the diabetic

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kidney.<sup>2,3</sup> It remains unclear whether the tubule has a pivotal role early in the initiation of diabetic nephropathy. Because of obvious space limitations, and the wide variety of the thematic involved, it will not be possible to provide a detailed, comprehensive review of all published work on this topic. However, in this review, we will try to outline the possibly pivotal role of the kidney tubule–interstitial milieu in diabetic nephropathy, and the difficulty of targeting adequate drug therapy.

## Differences between T1DM and T2DM

Renal disease in T2DM appears to be more heterogeneous than in T1DM. Usually, when proteinuria is found in T1DM, a well-defined constellation of renal structural abnormalities are expected to occur in parallel (ie, mesangial expansion, arteriolar hyalinosis, and tubulointerstitial changes).<sup>4–8</sup> In a classical view, proteinuria is almost always associated with diabetic retinopathy. This has been discussed recently by the EURODIAB IDDM Complications study,<sup>9</sup> which showed that diabetic retinopathy is not observed as frequently as previously believed in microalbuminuric T1DM patients, since it is strictly associated with increased circulating levels of von Willebrand factor, a marker of endothelial dysfunction. Indeed, if microalbuminuria antedates overt nephropathy in 80% of T1DM patients, this is the case in only 20%–30% of T2DM patients.<sup>10–12</sup> Fewer than 50% of T2DM patients with overt nephropathy have diabetic retinopathy (oculo–renal syndrome), despite having micro- and even macroalbuminuria. Heterogeneity of histological lesions has been reported in T2DM.<sup>13,14</sup> About 29% of patients have normal or near-normal renal structure; 29% have changes typical of diabetic nephropathy (as commonly seen in T1DM); and 42% have important tubulointerstitial fibrosis and/or arteriolar hyalinosis, with glomerular sclerosis and relatively trivial abnormal changes. This suggests that the tubule is important in the initiation of kidney disease, at least in some cases. Indeed, in this last group of T2DM patients, who show mainly tubulointerstitial fibrosis, an increase in kidney arteriolar resistance has been suggested.<sup>15,16</sup> High glucose exposition and hypertension play important roles in the development of kidney damage in both T1DM and in T2DM patients. However, a genetic component is also present (since family history of kidney disease is a strong predictor of renal functional decline).<sup>17,18</sup> Previously, we described a familial clustering of increased urinary albumin excretion (AER+), in T2DM families, with no association with hypertension.<sup>19</sup> Our data were in keeping with what has previously been described in Pima

Indian families; but, in Pima subjects, AER+ clustering was aggregately associated with hypertension – and hypertension preceding diabetes onset was a strong predictor of future renal damage.<sup>20–22</sup> These data illustrate the importance of genetic/familial factors in the development of kidney disease in diabetes, at least in T2DM subjects.

Ethnic and social factors are also important in the development of diabetic renal disease. Native Americans, Hispanics (especially Mexican Americans), and African Americans have much higher risks of developing ESRD than non-Hispanic whites with T2DM.<sup>23</sup> This might be the result of varying degrees of access to the health system.

## Role of tubule in diabetic nephropathy

While diabetic glomerulopathy is present in the early stages of diabetic nephropathy,<sup>24,25</sup> tubulointerstitial fibrosis is typical only of advanced disease. This often heralds the onset of progression towards ESRD,<sup>26,27</sup> after which the diabetic milieu, and the prolonged interaction of albuminuria (and other factors in the glomerular filtrate) with the tubular system, triggers renal oxidative stress and cortical interstitial inflammation, with resulting hypoxia and tubulointerstitial fibrosis, which determines, to a great extent, the progression of renal disease.<sup>4,28–30</sup> Tubular damage might well be the trigger of the disease. A recent study showed that regression of microalbuminuria in patients with T1DM is associated with lower levels of urinary tubular injury biomarkers (kidney injury molecule-1 and N-acetyl- $\beta$ -d-glucosaminidase), which is consistent with the notion that tubular dysfunction is a critical component of the early course of diabetic nephropathy.<sup>31</sup>

Poor glucose control, and the renin–angiotensin aldosterone system (RAS), are well established risk factors for the development of nephropathy, both in T1DM and T2DM patients.

When renal epithelial cells in culture are exposed to high glucose concentrations, they produce increased amounts of type I and type IV collagens,<sup>32</sup> leading to fibrosis. The profibrogenic cytokine transforming growth factor beta 1 (TGF- $\beta$ 1) has been implicated in the development of renal fibrosis in a number of kidney diseases; it is closely linked to the development and progression of diabetic nephropathy.<sup>33</sup> In murine mesangial cells in culture, increased levels of glucose stimulate the production of TGF- $\beta$ 1, which then acts, through autocrine mechanisms, to enhance collagen production.<sup>34</sup> The same mechanisms apply to human proximal tubule cells exposed in vitro to high glucose concentrations; the expression of high levels of TGF- $\beta$ 1, in the presence of

platelet-derived growth factor, leads to subsequent synthesis of type I and type IV collagens.<sup>35</sup>

The local RAS in kidney tissues clearly play a role in the pathophysiology of diabetic nephropathy.<sup>36</sup> The widespread use of RAS inhibitors, in order to reduce AER+ in diabetic patients, is indicative of the role of intrarenal RAS in the progression of diabetic nephropathy. It has been reported that levels of urinary angiotensinogen increase before glomerular injury in diabetic patients,<sup>37</sup> as well as in rodents,<sup>38</sup> which suggests that urinary angiotensinogen might be a biomarker of RAS activation in the tubule, in diabetes. RAS inhibitors ameliorate intrarenal RAS activation,<sup>39,40</sup> reduce tubular angiotensinogen and decrease tubular injury, with no increase in urinary  $\alpha$ 1-microglobulin (an established early marker of proximal tubule dysfunction in diabetic nephropathy).<sup>41</sup> Despite the widespread use of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, kidney function decrease in diabetic patients continues to have a significant prevalence. Angiotensin II is able to induce TGF- $\beta$ 1; all intracellular signaling activated by glucose entering the cell is able to increase transcription of this profibrogenic cytokine. TGF- $\beta$ 1 can influence the concentration of small, noncoding, single-stranded RNA molecules called microRNAs (miRNAs) in the tubular compartment, as well as at the glomerular level.<sup>42,43</sup> These miRNAs, which are synthesized from longer precursors, through a multistep maturation process, act as translational repressors of specific target mRNAs.<sup>44</sup> Animal models, using TGF- $\beta$ 1 antibodies, were able to slow the progression of diabetic nephropathy. Together with recent studies on miRNA in experimental diabetic nephropathy,<sup>45-47</sup> this opens the future possibility that TGF- $\beta$ 1 might become a candidate for developing new drugs to prevent or reduce the impact of kidney disease – in particular, tubular damage in diabetic patients. Although animal models would be extremely useful in this regard,<sup>48</sup> so far, unfortunately, real animal models that resemble tubular damage in induced diabetes are lacking.<sup>49</sup>

Unlike T1DM, wherein insulin deficiency is due to massive destruction of pancreatic beta cells, relative insulin deficiency in T2DM is secondary to insulin resistance, which is often associated with obesity. In the early stages of obesity, the action of insulin is preserved, or even enhanced, as far as action on lipogenesis and adipogenesis is concerned, particularly in liver and skeletal muscle. Ectopic fat accumulation damages metabolic responses to insulin in muscle and liver, and may lead to impaired insulin synthesis and secretion in pancreatic beta cells. At this stage, overt diabetes may occur,

lipogenesis and adipogenesis become stationary, and lipolysis is activated.<sup>50</sup> Obesity is related to progression of the rate of ESRD, independent of metabolic control of glucose, blood pressure, and baseline AER+, in T2DM subjects.<sup>12</sup> But, little information is available with regard to fat accumulation in the kidney before the overt development of diabetes in obese patients.

It has been proposed that glucose overload, at the kidney level, may contribute to an observed glycogen deposit not associated with evident accumulation of intrarenal fat.<sup>51</sup> In diabetic subjects, renal glucose uptake is inversely correlated with free fatty acid (FFA) uptake,<sup>51,52</sup> which suggests the existence of a renal glucose-FFA cycle. Even if the absolute uptake of FFA by the kidney is not increased in T2DM patients, studies using diabetic animals have shown that FFA, entrapped in the mitochondrial matrix, leads to production of mitochondrial reactive oxygen species (ROS), lipid peroxidation, and mitochondrial damage and dysfunction.<sup>51,53</sup> In renal tissue, as well as in the overall systemic vascular bed, the generation of oxidized lipids can modulate the formation of macrophage foam cells. Oxidized low-density lipid (LDL) (ox-LDL) encompasses various oxidation-specific neoepitopes, such as malondialdehyde-modified LDL, and the group of phosphorylcholine-headed, oxidized phospholipids. These neoepitopes are recognized both by adaptive, lymphocyte T cell-dependent, and by innate, T cell-independent mechanisms.<sup>53</sup> Scavenger receptors of macrophages play a central role in lipotoxicity in tubulointerstitial tissue. CXCL16 is one of the main receptors for the uptake of ox-LDL in podocytes; CD36 plays this role in tubular renal cells, as well as in macrophages.<sup>54</sup> Heme oxygenase is induced in epithelial cells following exposure to ox-LDL, and is accompanied by worsening of several indices of cytotoxicity, *in vitro*. The uncanny resemblance of the behavior of renal tubular epithelial cells to endothelial cells, after exposure to ox-LDL, both in terms of oxidant-mediated toxicity and expression of heme oxygenase, leads to speculation that the paradigmatic role of ox-LDL in atherogenesis may apply to tubular injury as well.<sup>55</sup> Cellular iron is assigned a central importance in the toxicity of ox-LDL in tubular cells.<sup>56</sup> In proteinuric renal diseases, abnormal amounts of iron are present in the kidney.<sup>57</sup> Pretreatment with an iron chelator blocks the cytotoxicity of ox-LDL and attenuates upregulation of heme oxygenase. The mechanisms for oxidation of LDL in proteinuric renal disease are not completely understood. LDL is vulnerable to oxidative attack in the urinary space, where it may be endocytosed by tubular cells. Alternatively, LDL may be oxidized within tubular cells, and in the microvascular

bed that surrounds tubulointerstitial tissue. This hypothesis is supported by the observation that tubular damage can be found at a stage at which loss of glomerular permselectivity does not yet permit LDL to enter the urinary space.<sup>56,57</sup> This argument has previously been discussed extensively.<sup>58</sup> Indeed, we have shown previously that treatment with statins is able to reverse urinary albumin excretion in T2DM patients;<sup>59</sup> a meta-analysis indicated that lipid reduction may preserve kidney function in diabetic patients.<sup>60</sup>

Given the understanding that the proximal tubule plays a vital role in the pathophysiology of the diabetic kidney, we are beginning to better understand the molecular basis of complex interactions between the diabetic milieu, proximal tubule, and tubulointerstitium. Tubular uptake of glucose is important in the detrimental renal effects of diabetes, as well as in glucose homeostasis. Inhibition of glucose reabsorption in the proximal tubule is a promising approach to lowering blood glucose levels. Glucose is taken into the proximal tubule primarily through two sodium-glucose transporter proteins. In the early part of the proximal tubule, glucose is taken up by a high-capacity, low-affinity sodium-glucose cotransporter SGLT2 (SLC5A2). In the later portions of the proximal tubule, glucose uptake occurs primarily through SGLT1 (SLC5A1), a high-affinity, low-capacity sodium-glucose cotransporter. mRNA levels of both SGLT1 and SGLT2 are increased in the renal cortex of obese Zucker rats,<sup>61</sup> being upregulated by high glucose levels. Proximal<sup>62</sup> and interstitial<sup>63</sup> cells respond to high levels of glucose, with production of profibrotic mediators before an increase in filtered albumin, or any measurable change in glomerular levels. In a streptozotocin rat model of diabetic nephropathy, insulin administration was able to reverse increased mRNA levels of proximal tubular procollagen alpha 1 before any measurable change at the glomerular level.<sup>64</sup> This unique early growth phenotype of the proximal tubule might be identified as a potential target for the prevention of early glomerular hyperfiltration and increased tubular reabsorption, as well as an early link to tubulointerstitial inflammation, fibrosis, oxidative stress, hypoxia, and renal failure in diabetes. Differences in the capacity of the tubule to respond to hyperglycemia might explain why some diabetic patients, in response to glucose overload, develop diabetic nephropathy, while others do not. The tubule's SGLT2 protein is upregulated in T2DM patients, increasing glucose reabsorption, resulting in a net renal glucose uptake. Thus, drugs that target this mechanism are promising in diabetes therapy. Although the long-term efficacy and safety of SGLT2 inhibitors remain under study, this class represents a novel therapeutic approach, with potential

for treatment of both type 2 and type 1 diabetes, as recently reviewed.<sup>65</sup> TGF- $\beta$ 1 regulates SGLT2 expression in response to high glucose in proximal tubular cells in culture. Empagliflozin, a SGLT2 inhibitor, reduces high glucose-induced inflammatory and fibrotic markers, by blocking glucose transport, without a compensatory increase in SGLT1 and glucose transporter 2 (GLUT2) expression.<sup>66</sup>

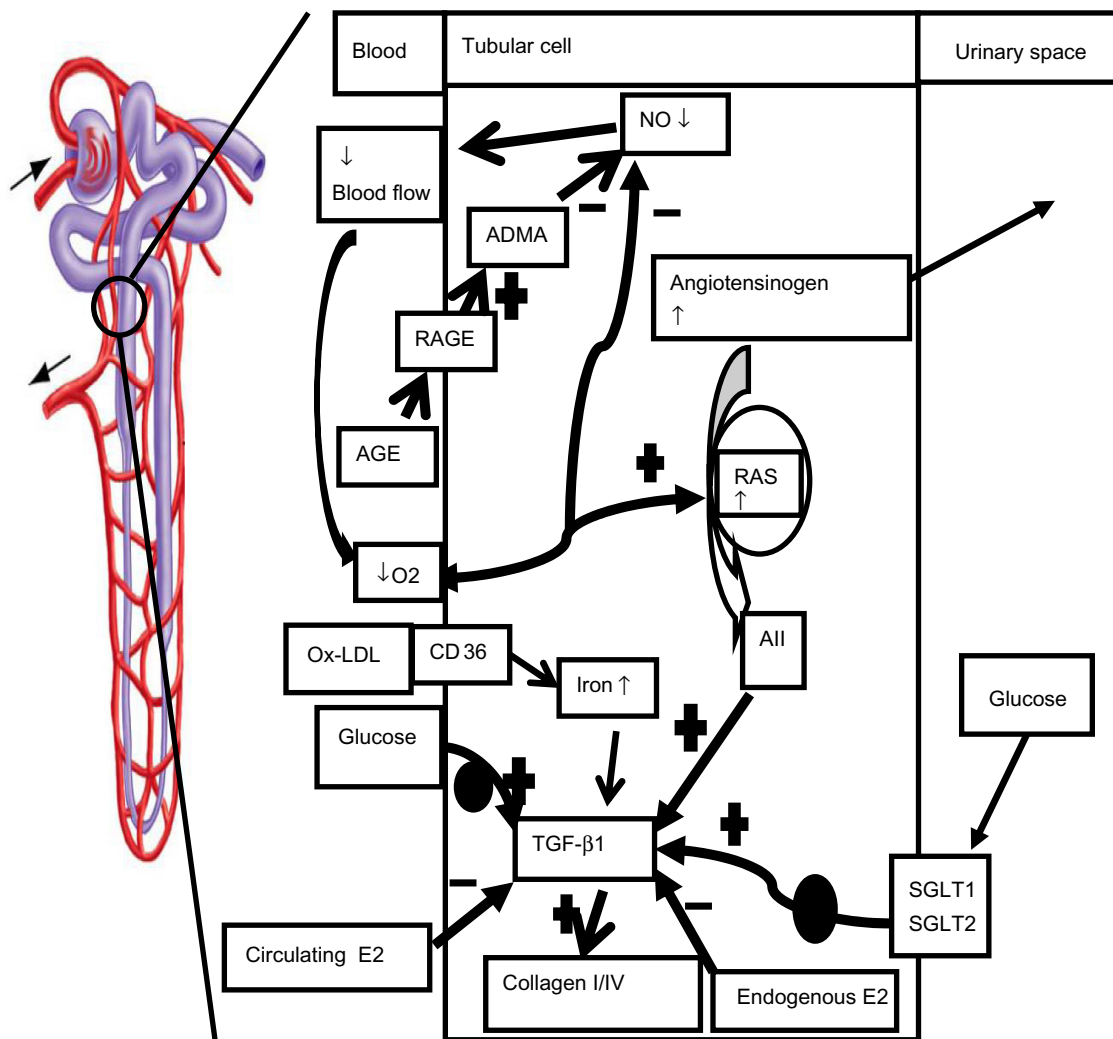
Other new drugs for diabetes therapy appear to have possible tubular-protective effects, besides glucose-lowering effects. The use of incretin-based therapies, such as dipeptidyl peptidase-4 (DPP-4) inhibitors and glucagon-like peptide-1 (GLP-1) analogs, is becoming widespread in T2DM patients. Besides optimization of glycemic control in a wider range of patients, these new classes of drugs have shown pleiotropic effects at the kidney level. GLP-1 stimulates glucose-dependent insulin secretion from pancreatic beta cells, and suppresses glucagon release from alpha cells, thereby improving glucose control. Besides its action on the pancreas, GLP-1 has direct effects on the heart, vessels, and kidney, mainly via the GLP-1 receptor. A GLP-1 receptor agonist, in streptozotocin-induced diabetic rats, is able to inhibit the advanced glycation end products and their generation of receptor-mediated asymmetric dimethylarginine (ADMA) (an endogenous inhibitor of nitric oxide synthase) – a mechanism that is involved in tubular dysfunction and diabetic nephropathy.<sup>67–69</sup> This effect is mediated by suppressing protein arginine methyltransferase-1, an enzyme that generates mainly ADMA in human proximal tubular cells, via inhibition of ROS generation, thereby protecting against the development and progression of diabetic nephropathy.<sup>70</sup> DPP-4 inhibitors act to reduce the degradation of incretins, such as GLP-1 and gastric inhibitory polypeptide. Other molecules that have proven renal and cardiovascular effects are DPP-4 substrates, such as brain natriuretic peptide/atrial natriuretic peptide, neuropeptide Y, peptide YY, and stromal cell-derived factor 1alpha. It is not surprising that these drugs might have different pleiotropic effects at the tubular level, as well as the vascular level. Through a mechanism yet not fully understood, the DPP-4 inhibitor vildagliptin was able to functionally protect the kidney in a rat model of unilateral renal ischemia. Reduced tubular necrosis observed was associated with antiapoptotic, immunological and antioxidative changes.<sup>71</sup> Studies have suggested possible direct renoprotective effects of DPP-4 inhibition that may go beyond its glucose-lowering potential.<sup>72,73</sup>

Another important factor that contributes to tubulointerstitial injury is chronic hypoxia. In animal models, renal hypoxia can be due to enhanced tubular oxygen consumption,

as shown *ex vivo* in cortical and medullar tubular cells of streptozotocin-diabetic rats.<sup>74</sup> Modification of vasoactive factors, such as nitric oxide and angiotensin II, may induce interstitial vascular rarefaction via postglomerular blood flow modifications, impairing peritubular blood flow and oxygen delivery to the tubules. This mechanism may be maintained and enhanced through the concurrent action of anemia, appearing in impaired renal function,<sup>75</sup> and through oxygen consumption in the remnant nephrons.<sup>76</sup> *In vitro* studies in human proximal tubular epithelial cells showed that hypoxia increased total collagen production,<sup>77</sup> with decreased collagen IV mRNA levels and increased collagen I mRNA levels, which suggested the induction of interstitial collagen. Although hypoxia did stimulate TGF- $\beta$ 1 production, it did not appear to mediate the profibrogenic stimulus of hypoxia.<sup>77,78</sup>

These findings link both hypoxia and oxidative stress to the tubulointerstitial accumulation of extracellular matrix, and fibrosis in the diabetic kidney.

Identification of the mechanisms leading to tubule–interstitial damage is complicated by the general lack of clinical attention to male–female differences, although the impact of sex on diabetic nephropathy has been reviewed previously.<sup>79–81</sup> In general, women show greater protection than men against the development and progression of renal disease. However, the advantage is less evident in diabetic nephropathy than in non-diabetic kidney diseases.<sup>82,83</sup> When type 1 diabetes is first diagnosed during adolescence, a sex-related difference is present between men and women for diabetic kidney disease, which suggests a role for sex hormones in mediating this difference.<sup>84</sup> A simple concept



**Figure 1** Proposed mechanisms of tubular dysfunction in diabetes mellitus

**Abbreviations:** NO, nitric oxide; ADMA, asymmetric dimethylarginine; AGE, advanced glycation end products; RAGE, advanced glycation end products receptor; OxLDL, oxidized low-density lipoprotein; CD36, receptors for the uptake of Ox-LDL in tubular renal cells; RAS, renin–angiotensin–aldosterone system; AII, angiotensin II; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; SGLT2, high-capacity, low-affinity sodium-glucose cotransporter; SGLT1, high-affinity, low-capacity sodium-glucose cotransporter; E2, 17 $\beta$ -estradiol.



arising from this observation is, regarding susceptibility to kidney disease, that  $17\beta$ -estradiol (E2) is good, and testosterone is bad. However, the underlying pathways by which E2 and testosterone confer their effects are complex. Under good metabolic control, diabetic women are more likely to develop diabetic nephropathy than diabetic men, whereas the situation is reversed under poor metabolic control. In females; protection from diabetic kidney disease via E2 occurs, in part, by regulation of TGF- $\beta$ 1. TGF- $\beta$ 1 serum levels are higher in men than in women;<sup>85</sup> rodent animal models with low E2 levels (both diabetic and non-diabetic) show increased renal expression of TGF- $\beta$ 1.<sup>86</sup> In streptozotocin-induced diabetic rats, E2 supplementation provides renoprotectivity by reducing extracellular matrix synthesis, and by increasing degradation and attenuating tubulointerstitial fibrosis.<sup>87</sup> E2 treatment has the same effects on TGF- $\beta$ 1 at the glomerular level in other rat models,<sup>88,89</sup> suggesting that the mechanism might be non-peculiar, or that it is not the first initiating event in tubular damage. There are several mechanisms proposed for E2's effects on TGF- $\beta$ 1 expression that have been exhaustively described.<sup>90</sup>

If E2 protects the kidney from diabetic damage by interfering with TGF- $\beta$ 1 signaling, an obvious extension of these studies would be to administer E2 to diabetic patients, to prevent or ameliorate diabetic kidney damage. Since exogenous estrogen's protection against the development and progression of cardiovascular disease has been questioned,<sup>91–94</sup> a clear answer to the possible protective role of E2 on kidney function should come from large-scale, randomized trials (which are not planned, so far). There are several theoretical limitations within the concept that E2 might be directly responsible for the observed sex difference in diabetic nephropathy. Firstly, diabetic men already have higher levels of E2 than non-diabetics.<sup>95</sup> In male diabetic rats, overexpression of kidney aromatase induces a higher rate of conversion of testosterone to E2, leading to higher intrarenal E2 levels.<sup>96</sup> Secondly, oral contraceptives that contain high doses of estrogens promote the risk of diabetic nephropathy, whereas lower-estrogen doses have no influence on renal function; thus, the effects of estrogens might be variable, depending on the dose.<sup>83</sup> Thirdly, the kidney is able to synthesize steroid hormones; local variation in the concentrations E2 and testosterone in the kidney could affect the abovementioned expression, and signaling of TGF- $\beta$ 1, independently of serum hormone concentrations.

Sexual hormones also significantly affect the RAS system; this might well be another way by which sexual

hormones can condition the development and progression of diabetic nephropathy differently in the two sexes.<sup>97–99</sup> The problem is far more complicated, since sex differences interact with the environment and genetic background. For example, the M235T polymorphism in the angiotensinogen gene increases the incidence of diabetic nephropathy in males but not in females, both in T2DM<sup>100</sup> and T1DM<sup>101</sup> patients.

## Conclusion

Rather than the orthodox view (that glomerular injury is primary in diabetic nephropathy, with the tubular injury secondary), it is evident that the kidney tubule plays a critical role in the genesis of diabetic nephropathy, as it is also that progression of diabetic nephropathy is best correlated with the degree of tubulointerstitial disease. There are many biomarkers of human tubular injury available at the moment. The main problem is that most of the biomarkers are still at an intermediate phenotype level, which is too distant from the gene level; therefore there is a high risk of influence by other gene products or the environment. Integrative analysis of the proteomic and transcriptomic features at different levels, among different categories of patients affected by diabetic nephropathy, could finally lead to the identification of new, early markers, closer to the gene level. Better identification of the first molecules involved in tubular damage might enable therapies to target the mitigation of initial injury to the proximal tubule, and the subsequent inflammation and fibrosis that lead to diabetic nephropathy. Therefore, additional effort is required to understand the wide network of biochemical pathways linking diabetes to eventual tubular damage. Despite current therapy that aims to improve glucose control and lower intraglomerular pressure by controlling systemic blood pressure, and even with inhibitors of the renin–angiotensin system, the prevalence of diabetic nephropathy continues to increase. Several pharmaceutical approaches in humans: modulating lipid metabolism and insulin action (ie, statins, metformin, peroxisome proliferator-activated receptor gamma, and nicotinamide adenine dinucleotide phosphate oxidase inhibitors), as well as chemokine expression and function, the new drug classes of incretins, and DPP-4 and SGLT2 inhibitors, might be helpful to understanding these issues, and may be more successful in slowing the progression of diabetic nephropathy, which is the single most important contributor to the epidemic of end stage kidney disease worldwide.

## Disclosure

The authors report no conflicts of interest in this work.

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