Diabetic nephropathy : from histological findings to clinical features

Klessens, C.Q.F.

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from histological findings to clinical features

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Het moment van de ontdekking ligt daar waar het thuis hoort in de eeuwigheid

mijn impressie op basis van 'De ontdekking van de hemel, Harry Mulisch'

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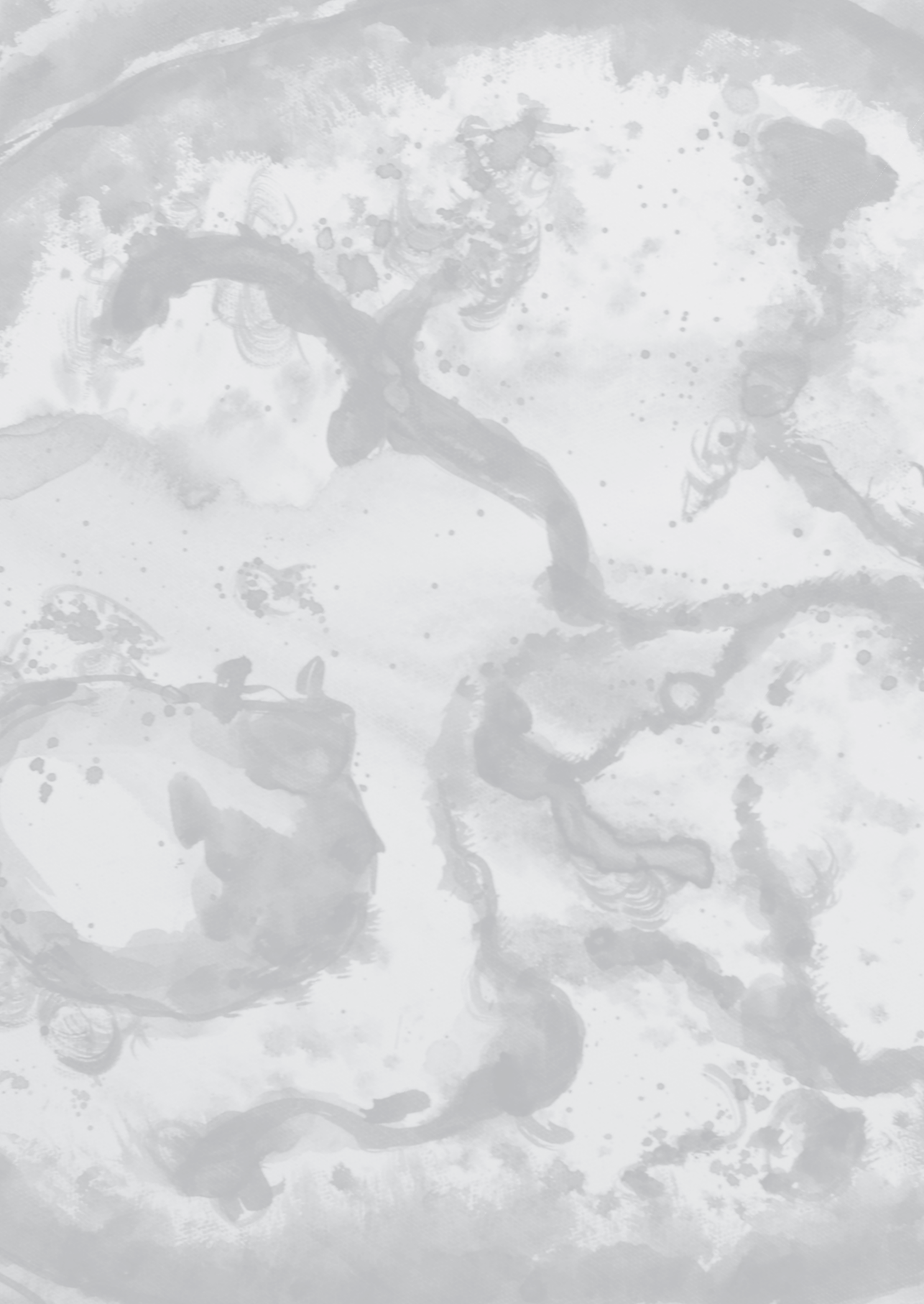


General introduction

1

PROLOGUE: 'DIABETES, A RISING ENDEMIC PROBLEM'

According to the International Diabetes Federation 415 million people suffered from diabetes in 2015 and the prevalence is expected to rise to 642 million people in 2040. In Europe, there are currently 59.8 million patients with diabetes and these numbers are expected to increase to 71.1 million patients in 2040. The increased global burden of this disease is mainly driven by changes in lifestyle; especially the rise in number of cases with obesity is thought have major impact on the increase of type 2 diabetes [1].

Interestingly, a cross-sectional study in the United States reported that three out of ten adults were unaware that they suffered from type 2 diabetes, which was diagnosed by measuring fasting plasma glucose or HbA1c levels. This study reveals a large population of underdiagnosed patients and suggests that the described prevalence of diabetes is probably underestimated [2]. Likewise, the International Diabetes Federation provides data that close to half of all patients with diabetes (46.5%) are undiagnosed; this means that approximately 193 million patients are currently unaware that they have diabetes. Additionally, the International Diabetes Federation stated that another 318 million adults suffer from impaired glucose tolerance, a condition which is associated with a higher risk of developing diabetes. The global mortality due to diabetes is 5.0 million deaths per year [1]. Although the mortality rates vary between countries, it suggests that every 6 seconds a person dies from diabetes or the complications of diabetes [1].

The 35% increase in the number of patients with diabetes in 2040 will inevitably lead to an increase in healthcare costs of diabetes and its related complications. The International Diabetes Federation reports that currently the total annual health expenditure for diabetes is estimated to be 673 billion US dollar and by 2040 is expected to rise to 802 billion US dollar; in Europe the costs in 2015 were 156 billion US dollar and will rise to 174 billion US dollar in 2040. This means that these costs will comprise 12% of global health expenditure in 2040 [1].

The renal complication of diabetes, diabetic nephropathy, develops in approximately 20-40% of patients with diabetes and is one of the major causes of end-stage renal disease [3-5]. Furthermore, patients with diabetic nephropathy have more risk of mortality due to cardiovascular complications [6]. Patients with end-stage renal disease require renal replacement therapy such as dialysis or renal transplantation. In the Netherlands, treatment of end-stage renal disease by dialysis and transplantation costs annually between 80.000 Euro and 120.000 Euro. According to the Nierstichting (the Dutch Kidney Foundation) these treatments are the most expensive therapies which are covered by the Dutch healthcare system.

The rising numbers and high costs indicate that diabetes and its renal complication are an endemic problem with major consequences not only for the individual patient but also for the general population and healthcare systems. At this moment, it is difficult to inhibit the development of diabetic nephropathy and the progression to end-stage renal disease. We believe that a better understanding of the pathogenesis of diabetic nephropathy may help to create more non-invasive diagnostic tools, decrease the progression of diabetic nephropathy, create novel therapy regimens and will eventually decrease the number of patients suffering from diabetic nephropathy.

In this thesis, we focused on histological lesions of diabetic nephropathy related to clinical parameters, inflammatory markers and a genetic component.
The introduction of this thesis consists of four parts: part one is an introduction on diabetes and the kidney. The second part introduces diabetic nephropathy, including histology and the pathologic classification, clinical features and pathogenesis. The third part presents the genetic aspect in diabetic nephropathy, the *CNDP1* gene and its substrate carnosine and part four provides the outline of the chapters of this thesis.

PART I DIABETES AND THE KIDNEY

Diabetes

Diabetes is a metabolic disorder, characterized by hyperglycemia. The two most common types of diabetes are type 1 diabetes and type 2 diabetes. The other, less common types of diabetes are gestational diabetes, maturity onset diabetes of the young (MODY) and mitochondrial diabetes.
Type 1 diabetes is the result of autoimmune destruction of the β-cells of the pancreas, resulting in an absolute insulin deficiency. 5-10% of the diabetic patients in Europe suffer from type 1 diabetes. The remaining 90-95% of the patients with diabetes in Europe suffer from type 2 diabetes with relative insulin deficiency and insulin resistance. Type 2 diabetes has an extraordinary heterogeneity, because it originates from a complex network of genetics, cellular pathways and multiple environmental factors. The complications of diabetes can generally be divided into macrovascular and microvascular, and these complications may develop in various organs. The macrovascular complications are mainly restricted to cardiovascular diseases. Microvascular complications mainly appear in the kidneys (nephropathy), eyes (retinopathy), peripheral lower extremities (diabetic foot) and nerves (neuropathy). In this thesis we will focus on the microvascular complication of diabetes in the kidneys: diabetic nephropathy.

Since type 1 and type 2 diabetes originate from different pathogenic mechanisms, the treatment for type 1 and type 2 diabetes differ. Type 1 diabetes is primary treated with insulin. For type 2 diabetes there are several therapy options available. Metformin, an oral anti-glycemic agent, is well-established and the most effective therapy and therefore it is the primary treatment for patients with type 2 diabetes. Next to the therapies which control the glucose levels, blood pressure control, regulation of the lipid spectrum and lifestyle intervention such as diet restrictions, stimulation of exercise and even bariatric surgery are part of the therapy strategy to regulate type 2 diabetes [7].

The Kidney

An introduction to the physiology and anatomy of the kidney will help improve the understanding of the pathological manifestations in the kidney. Renal blood supply originates from the abdominal aorta and requires 20% of the cardiac output. The high blood flow is necessary to generate ultrafiltrate in the glomeruli. The functional unit of the kidney is the nephron and each kidney consists of 800.000 to 1.2 million nephrons. A nephron consists of a glomerulus with an attached tubule. The glomerulus is a structure of several lobules of capillary loops, from which the plasma filtrate originates. In the glomerulus four important cell types can be found: mesangial cells, endothelial cells, visceral epithelial cells (podocytes) and parietal epithelial cells. Tubules are epithelial structures with many subdivisions and are surrounded by thin connective tissue, the interstitium, and branches of the renal arteries. The afferent arterioles branch off into a spherical bag of capillary loops, the glomerulus, and exit via the efferent arterioles. In the glomerulus the blood is filtered from the vascular system into the tubular system over the glomerular basement membrane. The glomerular basement membrane in adults is on average 300-350 nm thick. In different parts of the tubular system the filtrated blood is concentrated to pre-urine by reabsorption of essential molecules. The fine tuning of NaCl and water excretion is performed by the distal tubules and collecting duct system. The kidneys have three main functions; first, the filtration of blood by removing metabolic products and toxins and excreting them through the urine. Second, the regulation of the homeostasis by electrolyte balance and acid-basis balance (pH). Third, the kidneys' endocrine function, as the kidneys produce or activate hormones which are involved in the erythrogenesis, calcium metabolism and the regulation of blood pressure and blood flow [8]. The disturbance of these functions in diabetes or diabetic nephropathy will not be further addressed, as this thesis will focus on the histopathological damage in the kidneys.

PART II DIABETIC NEPHROPATHY; HISTOLOGY, PATHOLOGIC CLASSIFICATION, CLINICAL FEATURES AND PATHOGENESIS

Diabetic nephropathy

Diabetic nephropathy develops in approximately 20-40% of patients with diabetes [3-5] and the diagnosis is usually based on clinical manifestations by the presence of persisting microalbuminuria (urinary albumin excretion of 30-300mg/day) or proteinuria (>200 µg/min or 300 mg/day) and/or decline of renal function (measured by glomerular filtration rate) [9]. The clinical presentation of diabetic patients with renal complications may vary [10, 11]. A renal biopsy is still considered to be the golden standard to prove that the clinical manifestations are caused by renal damage attributable to diabetic nephropathy. Currently, the therapy of diabetic nephropathy patients will not be altered by the pattern of histological findings in the renal biopsy, such as mesangial expansion or nodular sclerosis. Consequently, there is currently no indication to perform standard renal biopsies when patients with diabetes have clinical presentations of this renal complication.

Histopathology

Diabetic nephropathy is characterized by structural and functional changes and can be observed in a renal biopsy. Renal histological lesions are divided in glomerular, tubulointerstitial and vascular damage. The structural pathological lesions in the glomeruli observed by electron microscopy are diffuse thickening of the glomerular basement membrane and accumulation of extracellular matrix primarily of the lamina densa. The lesions observed by light microscopy are mesangial expansion, nodular sclerosis and global glomerulosclerosis [12].

Nodular sclerosis is thought to be the most typical hallmark of diabetic nephropathy but it is not pathognomonic; these nodules are also known as Kimmelstiel-Wilson nodules [13]. Nodular sclerosis consists of areas of marked mesangial expansion forming rounds and mesangial zones with palisading mesangial nuclei; however their exact pathogenesis remains incompletely understood [14-17]. Usually, nodular sclerosis is concomitant with moderate to severe diffuse mesangial expansion, although occasionally, nodules are found in cases with mild diffuse mesangial expansion. It is hypothesized that nodular sclerosis results from a different pathogenic pathway compared to more widespread mesangial expansion [18, 19]. Consequently, the distribution patterns as well as the formation of nodular sclerosis in the glomerulus have been discussed by Ponchiardi *et al*. among others [12, 20]. Already in 1998 Schwartz *et al*.[19] questioned why some patients with type 2 diabetes do not develop nodular sclerosis as there is no

difference in their clinical manifestations compared to patients with nodular sclerosis; they concluded that nodular sclerosis has a different pathogenesis compared to mesangial expansion. Another question remains whether there are more types of nodules. Some nodules seem to have a laminated structure, indicating that mesangiolysis might be a precursor to their formation. In other nodules a more dense formation is observed, which is thought to be the result of recanalization of the capillaries which eventually forms nodules with layered structures [14, 21]. The appearance of these various forms suggests that also the development of nodular sclerosis may be the result of different factors [12], although further investigation is needed to confirm these hypotheses.

Interstitial and vascular lesions

Concomitantly to the glomerular lesions of diabetic nephropathy, changes are observed in the interstitium and tubules such as interstitial fibrosis and tubular atrophy; as well as vascular changes, like hyalinosis and arteriosclerosis. These tubulointerstitial and vascular lesions are often related to other factors that are present in patients with diabetes such as obesity, ageing, hypertension, metabolic syndrome and atherosclerosis and are therefore not thought to be specific lesions for diabetic nephropathy [22, 23], although the vascular and interstitial lesions in diabetic nephropathy can help to determine the severity of the renal disease. Besides, validation studies have shown that interstitial fibrosis and tubular atrophy as well as vascular lesions have prognostic value in diabetic nephropathy [24, 25]. For this reason, it is necessary that these lesions are taken into consideration while evaluating renal tissue specimens of patients with diabetic nephropathy.

Generally, interstitial fibrosis is considered to be the best histological marker for correlation with GFR in glomerular diseases [12]. In diabetic nephropathy and most other kidney diseases interstitial fibrosis and tubular atrophy is associated with a decreased number of peritubular capillaries, perhaps by decreased delivery of oxygen and nutrients to the interstitial and tubular epithelial cells [12].

Regarding the vascular changes, arteriolar hyalinosis of the afferent and efferent arterioles is characteristic but not restricted to diabetic nephropathy. Moreover, hyalinosis as a result of a hypertensive state affects the afferent but not the efferent arterioles [26]; however during histological evaluation of a renal biopsy it is often difficult to distinguish these two arterioles. Next to hyalinosis of peripheral arteries, hyalinosis can also be observed in the glomerulus in two characteristic patterns, capsular drops and hyaline caps, also known as fibrin caps. Capsular drops are spherical accumulations of hyaline material adjacent to or within the Bowman's capsule and hyaline caps are attached to the capillary lumen. These glomerular hyaline changes are frequently ob-

served in diabetic nephropathy cases but they can also occur as a result of other renal manifestations [27].

Pathologic classification of diabetic nephropathy

Histological lesions of several renal diseases are divided into classification systems, which classify specific lesions of the renal disease. These pathologic classification systems were developed to provide better communication between pathologists and clinicians and they can serve to implement diagnostic information with prognostic indications for several renal diseases [28].

In 2010, Tervaert *et al*. [29] proposed a pathologic classification system under the auspices of the Renal Pathology Society, which tried to create international uniformity in classifying diabetic nephropathy that could be used as a communication tool and was suitable for clinical practice. This classification system can be used to classify diabetic nephropathy with or without a co-existing renal disorder. It is primarily based on glomerular lesions whereas interstitial and vascular lesions are scored separately. The glomerular lesions are categorized into four classes (Figure 1). Class I is characterized by glomerular basement thickening. Class II is characterized by mesangial expansion and is subdivided; Class IIa with mild mesangial expansion (mild mesangial expansion in >25% of the observed mesangium) and Class IIb with severe mesangial expansion (severe mesangial expansion in >25% of the observed mesangium). Class III is characterized by at least one convincing nodule/Kimmelstiel-Wilson lesion. Finally, Class IV is characterized by more than 50% of global glomerulosclerosis in the glomeruli. As discussed above the interstitial and vascular parameters are of value to determine the severity of the renal damage, therefore these parameters are scored next to the glomerular damage in this pathologic classification. Interstitial fibrosis and tubular atrophy (IFTA) and interstitial inflammation are scored on a semi-quantitative scale. A score of 0 is assigned when no IFTA is present, a score of 1 is assigned when less than 25% IFTA is present, a score of 2 is assigned when in 25% to 50% of the interstitium IFTA is present, and finally, a score of 3 is assigned when at least 50% IFTA is present. Regarding interstitial inflammation, a score of 0 is assigned if interstitial infiltrates are absent, 1 if they only occur around atrophic tubules, and 2 if the inflammatory infiltrate is also in other areas than around atrophic tubules [29]. Regarding vascular lesions, the presence of hyalinosis and the amount of arteriosclerosis are scored. Arteriolar hyalinosis is scored 0 when it was absent; is scored 1, if at least one arteriole with hyalinosis is present and 2, if more than one arteriole with hyalinosis is observed in the entire biopsy. Arteriosclerosis is scored as follows: a score of 0 for no intimal thickening, 1 for intimal thickening less than the thickness of the media, and 2 for intimal thickening more than the thickness of the media [29].

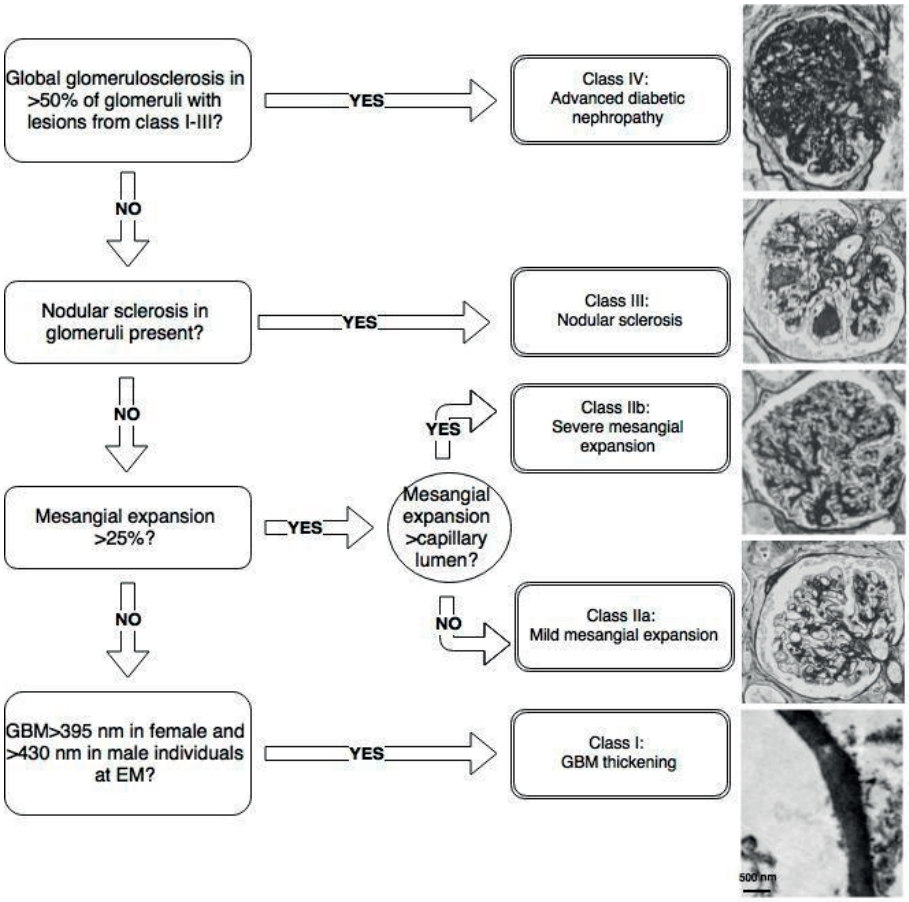
In past years, several validation studies investigated the clinical value of the pathologic classification system by associating the classification of diabetic nephropathy with renal outcome [24, 25, 30, 31]. Overall, these validation studies showed that there is a good association between the histopathological classes and renal prognosis. Nevertheless, these studies showed certain discrepancies that could be explained by several factors, such as the use of different inclusion criteria, the different power of the validation studies and the use of the classification in absence of class I or combining of class I and II.

This pathologic scoring system for diabetic nephropathy focusses on different histological lesions that can occur in diabetic nephropathy. Furthermore, multiple validation studies showed that the classification correlates with renal outcome [24, 25, 30, 31]; therefore this classification seems to be a suitable classification to classify histopathological lesions of diabetic nephropathy and can be used as a diagnostic and communication tool in clinical and research settings. Due to the reported discrepancies between the performed validation studies, it would be useful to update and/or re-evaluate definitions of the classification to optimize the communication and increase the diagnostic value. In this thesis, we used two methods to analyze the pathologic classification. In a research setting, we evaluated this classification via a meta-analysis and in clinical practice we created a survey to determine whether issues arose while using the pathologic classification of diabetic nephropathy.

Clinical features

In 1983, Mogensen *et al.* [32] described that the natural history of diabetic nephropathy consists of five stages. In the first stage, the glomeruli become hypertrophic, which leads to higher glomerular filtration rate and renal enlargement. It was been shown that this stage gives an increased risk to develop more advanced diabetic nephropathy, However, the first stage is still reversible [33]. The second stage is called 'silent nephropathy' and is characterized by intermittent periods of microalbuminuria. The majority of patients will remain in this stage for their entire lives; only one-third will progress to stage three [10]. Stage three is called 'incipient nephropathy' with persistent microalbuminuria. Stage four is called 'overt nephropathy' and is characterized by macroalbuminuria, decline in GFR and elevated blood pressure. Finally, the GFR loss progresses to end stage renal disease, which characterizes the fifth stage. Patients with end stage renal disease require renal replacement therapy, such as dialysis or kidney transplantation [34].

Figure 1. Flowchart of pathologic classification of diabetic nephropathy, adjusted from Tervaert *et al.* 2010



It is necessary to bear in mind that the original study on the natural history of diabetic nephropathy was mainly based on patients with type 1 diabetes [34]; nowadays most patients with diabetes suffer from type 2 diabetes [10]. It is known that in type 2 diabetes lifestyle, obesity, hypertension and ageing are major risk factors. These risk factors could explain why the clinical presentation of diabetic nephropathy may sometimes differ from what Mogensen initiated. The changes in diabetic therapy regimens of blood pressure control and other oral diabetic medication over the years may also influence the sequence of the five stages of diabetic nephropathy described by Mogensen [10]. More specific, the belief that microalbuminuria progresses to macroalbuminuria [35] and that loss of GFR only starts when macroalbuminuria is present may not necessarily be the order in which these stages occur [36]. This may explain why Kramer *et al.* [37] reported that between 30-50% of type 2 diabetes patients have chronic kidney disease

without proteinuria [37, 38]. It has been postulated that the absence of proteinuria resulted from atubular glomeruli, renal microvascular atherosclerotic disease and analgesics [39, 40]. Furthermore, large long-term clinical trials have demonstrated that improved blood glucose and blood pressure control slow down the progression of diabetic nephropathy [41].

Therapy regimens for diabetic nephropathy

The primary therapeutic regimen to lower the protein excretion and slow the rate of the progression of diabetic nephropathy are agents that interrupt the renin-angiotensin aldosterone system (RAAS) such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists. It is also important to maintain strict glycemic and blood pressure control in patients with diabetic nephropathy. Because the renoprotective agents are effective in both hypertensive and normotensive context, RAAS inhibitors are part of the anti-hypertensive therapies of patients with diabetes. Consequently, many patients without renal complications are already treated with RAAS inhibitors, which are then used as an anti-hypertensive drug. This treatment may therefore camouflage the clinical manifestations of potential renal structural damage. It is also beneficial for patients with diabetic nephropathy to control their lipid spectrum by lipid lowering medication. Better lipid spectrum control is likely to improve cardiovascular outcome. Additionally, dietary protein and/or salt restriction and adequate daily exercise seem to be beneficial in patients with diabetic nephropathy [7]. Currently, there is no known treatment that can reverse renal damage or cure diabetic nephropathy. Since the interruption of the RAAS system is the general treatment of all patients with diabetic nephropathy, there is no clinical indication to perform a renal biopsy in patients with clinically diagnosed diabetic nephropathy.

Type 1 and 2 diabetic nephropathy

The duration in which renal complications develop may differ between type 1 and type 2 diabetes. Still the morphological changes observed in the kidney seem to be similar and undistinguishable [42-45], but the majority of studies on renal structural changes have been performed in type 1 diabetes.

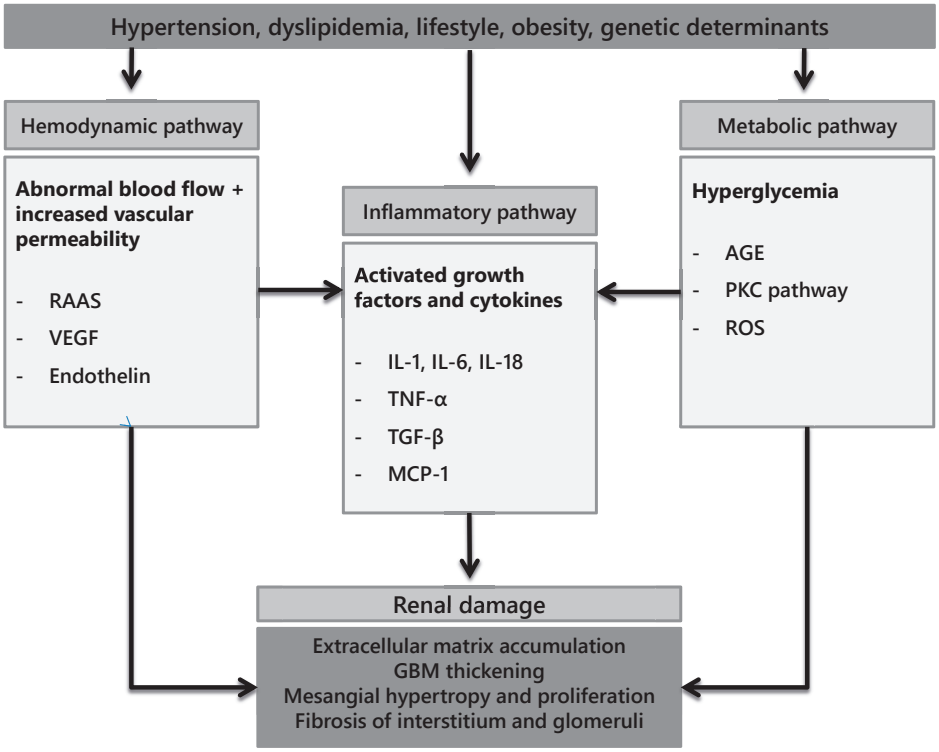
Regarding therapy response between the two types of diabetes, RAAS inhibition does not seem to prevent or delay the progression of renal damage in type 2 diabetes as effectively as it does in type 1 diabetes [46, 47]. The GISEN group hypothesized that the inhibition of RAAS is more effective in type 1 diabetes, since the primary manifestation of type 1 diabetes is proteinuria and consequently RAAS inhibition may slow down the progression of diabetic nephropathy [46, 48]. In type 2 diabetes loss of renal function can occur before or in absence of proteinuria, since several pathways seem to be in-

volved in the loss of renal function [49]. It is possible that type 2 diabetes patients have more benefit from a multiple intervention approach, because in this type of diabetes several risk factors, such as obesity, hypertension, and dyslipidemia are involved. These risk factors seem to decrease when there are adequate lifestyle changes in combination with oral diabetic medication; consequently these lifestyle interventions may be beneficial to decrease the development and progression of diabetic nephropathy.

Pathogenesis of diabetic nephropathy

The pathogenesis of diabetic nephropathy results from exposure of the kidneys to an altered internal milieu, which triggers multiple pathways [7, 50]. The pathogenesis of diabetic nephropathy is schematically described in Figure 2. Hyperglycemia is thought to be the major driving force upstream of these pathways. Downstream of these pathways, chronic inflammation and inflammatory markers are induced by hyperglycemia. Together with insulin resistance or deficiency and other risk factors of diabetes such as hypertension, dyslipidemia, obesity and genetic determinants, renal diabetic complications will develop [51].

Figure 2. Pathways involved in the development of diabetic nephropathy



Hemodynamic and metabolic pathways

Abnormalities in blood flow and increased vascular permeability are the main hemodynamic changes that occur in diabetes. These hemodynamic changes lead to activation of the renin-angiotensin aldosterone system (RAAS), which increases angiotensin II levels and activate vasoconstriction of the efferent arterioles. Elevated levels of angiotensin II are associated with increased albuminuria and nephropathy. The imbalance of the afferent and efferent arteriolar resistance results in increased glomerular hydrostatic pressure and leads to hyperfiltration [52, 53].

Hemodynamic fluctuations may also lead to activation of several other vasoconstrictors, such as an increase of vascular growth endothelial factors (VEGF) and an increase of endothelin-1 [54]. VEGF is a regulator of angiogenesis and regulates the preservation of endothelial cells. Endothelin-1 mimics the RAAS in several physiological functions; it is a vasoconstrictor, it plays a role in hypertension, in endothelial dysfunction, in inflammation and in fibrosis. In the kidneys, endothelin-1 activates a signaling cascade leading to mesangial hypertrophy and proliferation. Furthermore, it stimulates extracellular matrix production [54]. These changes are observed during the histological evaluation of renal tissue of patients with diabetic nephropathy.

Hyperglycemia also triggers several metabolic reactions. The hyperglycemic superoxide formation by the mitochondria is thought to be the common initiating factor [55]. The glycation reaction forms a chain of chemical reactions that result in irreversibly damaged proteins or lipids. These damaged lipids and proteins are known as advanced glycation end products (AGE). AGEs and their precursors damage cells via three mechanisms. Extracellularly, there is an abnormal interaction between AGE-modified matrix components and matrix components of other cells and receptors, like integrins. Intracellularly, there are the modified proteins with altered functions, like the activation of protein kinase C (PKC). The activation of PKC intervenes with the synthesis of nitric oxide (NO) and it increases oxidative stress. Oxidative stress increases both vascular permeability and contractility along with the synthesis of extracellular matrix and thickening of basement membrane. The inflammatory response is also activated by PKC through activation of cytokines and adhesion molecules. Lastly, there is production of reactive oxygen species (ROS) due to binding of AGE-modified plasma proteins to AGE receptors on endothelial cells, mesangial cells and macrophages [56].

Inflammatory pathway

Initially, it was thought that diabetic nephropathy resulted from metabolic and hemodynamic interactions [7, 57]. Over the past decades increased knowledge on cellular pathways showed that all features of the metabolic and hemodynamic changes will

inevitably lead to response reactions which increase inflammatory markers and cytokines in serum and (damaged) tissue. This chronic activation of the immune system and low-grade inflammatory state, especially in patients with type 2 diabetes, seem to influence the development of microvascular complications. Nowadays, potential new therapy regimens for type 2 diabetes are focusing on targeting the inflammatory pathway in diabetic nephropathy [58].

As a general immune response, macrophages can differentiate into more specific phenotypes according to the type of tissue damage. Macrophage phenotypes can be categorized as M1 macrophages, the classically activated macrophages, and M2 macrophages, the alternatively activated and anti-inflammatory macrophages [59]. M1 macrophages are activated via the classical immune pathways by tumor necrosis factor (TNF) cytokines and interferon-γ and M2 macrophages are activated after exposure to Th2-type cytokines [59]. M2 macrophages are intended to promote the regeneration and healing of tissue by creating an anti-inflammatory environment. Factors such as interleukin (IL)-10 and transforming growth factor (TGF)-β are released by M2 macrophages to decrease remodeling substantially. In literature, there is an ongoing debate whether dividing macrophages into M1 and M2 is correct [60], since it remains unknown whether macrophages remain in their primary activated state or whether they respond and modify based on their stimulus reaction. This modification may be stimulated by chemokines, chemoattractants and/or adhesion molecules that attract monocytes to the injury site or by secondary molecular signals within the microenvironment of the damaged tissue [59].

In the pathogenesis of diabetic nephropathy, inflammatory cytokines such as IL-1, IL-6, IL-18 and TNF are involved and are synthesized by a variety of inflammatory cells including macrophages [51]. Additionally, human and experimental studies showed that the monocyte chemoattractant proteins (MCP-1) are increased in diabetes. MCP-1 is a chemokine produced by many cells, including endothelial and epithelial cells. Binding of MCP-1 to its receptor, C-C motif chemokine receptor 2 (CCR2), stimulates the release of monocytes from bone marrow and activates the migration and translocation of monocytes and macrophages [61]. In experimental models with diabetic nephropathy, depletion of MCP-1 attenuated glomerular damage and proteinuria. These studies also showed that MCP-1 is involved in the influx of macrophages in the glomerulus [62-64]. These findings and the physiological reaction of macrophages on tissue damage indicate that the combination of immunologic and inflammatory mechanisms might play a pivotal role in the presentation, development and the progression of type 2 diabetes [65, 66]. To get more insight in the influence of the inflammatory pathway on diabetic nephropathy, we investigated the number of macrophages and their phenotypes in

humans with type 2 diabetes in this thesis. The results of this study may be useful in future research on potential anti-inflammatory therapies for diabetic nephropathy.

Clinical parameters and histological changes

Microalbuminuria is considered to be the first clinical sign of diabetic nephropathy. However, studies demonstrated that reduction of GFR may precede or occur separately from the development of albuminuria in some patients [32, 37, 67, 68]. Patients with diabetes in whom loss of renal function occurs separately from increased urinary albumin excretion are described as non-albuminuric or normoalbuminuric patients in several studies [10, 39]. Moreover, several studies reported that there was histological damage present in the renal tissue of these normoalbuminuric patients [68, 69]. For instance, Caramori *et al*. [68] reported that in renal biopsies of patients with type 1 diabetes who were normoalbuminuric, increased glomerular basement membrane (GBM) width and increased mesangial fractional volume [Vv(Mes/glom)] were observed, which they defined as advanced diabetic lesions. Moreover, Ekinci *et al*. [69] also showed that in renal biopsies of normoalbuminuric patients with type 2 diabetes, who did not use RAAS inhibition for 4-6 weeks, diabetic glomerular changes were observed. These observed glomerular changes were thickening of the glomerular basement membrane, mesangial expansion and nodular sclerosis. Their study showed that renal lesions can develop in the absence of albuminuria but in the presence of renal function decline, although they mentioned that these renal changes are less common in normoalbuminuric patients compared to patients with micro- or macroalbuminuria [69]. They stated that these results may be caused by the multifactorial pathogenesis of type 2 diabetes, in which ageing, hypertension and vascular diseases contribute to the development of microvascular complications. Another explanation could be that interstitial damage is a co-determinant of loss of renal function [70, 71], suggesting that the clinical manifestations of diabetic nephropathy, such as loss of renal function and albuminuria, are not only the result of glomerular damage. In this thesis we determined the prevalence of glomerular, interstitial and vascular damage in patients with type 1 and type 2 diabetes according to the pathologic classification of diabetic nephropathy. Additionally, we investigated whether these histological lesions could be associated with the clinical manifestations of these patients.

PART III GENETIC COMPONENT IN DIABETIC NEPHROPATHY, CNDP1 AND CARNOSINE

Genes in diabetic nephropathy

Multiple studies showed that certain families have a high risk of developing diabetic nephropathy [72, 73]. Additionally, it has been shown that the prevalence of diabetic nephropathy varies significantly between different ethnicities [72]. These differences are thought to be the result of a genetic component that seems to be involved in the development and progression of diabetic nephropathy. Genetic variation can be present in different forms in the human genome; it ranges from single nucleotide polymorphisms (SNPs) to structural and chromosomal rearrangements.

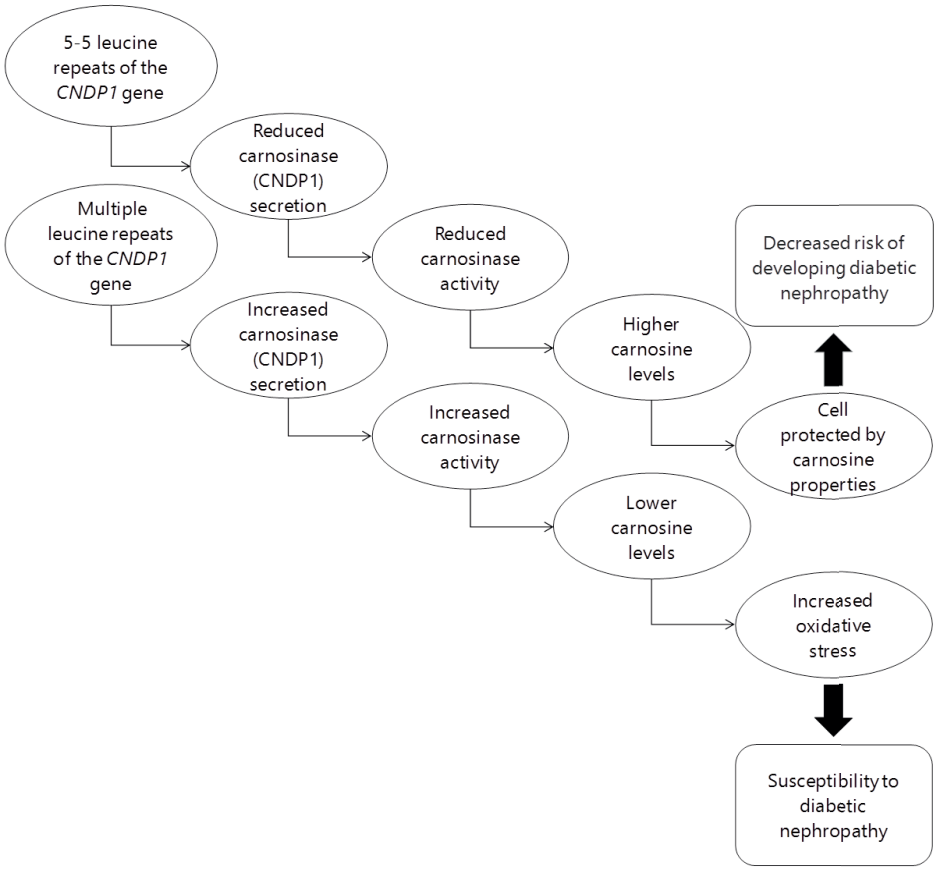
The first studies on genetic involvement in diabetic nephropathy were linkage analyses in family studies. Most of these family studies contained relatively small numbers of families, but they detected several consistent regions of linkage and revealed that there was variation in diabetic nephropathy between ethnicities [74-77]. Furthermore, genome-wide linkage scans of both type 1 and type 2 diabetic nephropathy were published and showed that the genetic susceptibility to develop complications differed between type 1 and type 2 diabetes [78-81]. Since the linkage studies were underpowered to assign the susceptibility of one specific gene, genetic association studies with candidate genes were created for further investigation. These studies reported the involvement of specific genes from different pathways in diabetic nephropathy, such as ACE [82], TGB-β [83], inflammatory cytokines [84]. The results of these studies are to some extent inconsistent, probably due to different study designs and the use of a broad definition of diabetic nephropathy which varied from microalbuminuria to biopsy proven diabetic nephropathy. However, the combination of both approaches, a family study together with a genetic association study, resulted in the successful finding of a genetic involvement in diabetic nephropathy, the *CNDP1* genotype.

CNDP1

It has been reported that the genetic variation of the carnosinase-1 gene (*CNDP1)* is one of the most consistent observations in the genetic field of diabetic nephropathy [85]. In 2005, Janssen *et al*. [79] found an association with diabetic nephropathy and the *CNDP1* gene in large study with Turkish families who were not treated for the disease. Since then, various studies reported that the shortest allele of the trinucleotide repeat in exon 2 of the *CNDP1* gene, 5-5 homozygous *CNDP1*, is associated with a reduced susceptibility for developing diabetic nephropathy and this association has repeatedly been confirmed in several ethnic groups, i.e. Caucasians, Afro-Americans and Asians

[78, 86, 87]. It is thought that the 5-5 homozygous *CNDP1* patients have lower serum concentrations of carnosinase-1 compared to patients with multiple leucine repeats of *CNDP1* gene. Serum carnosinase-1 degrades carnosine into peptides [78, 79]. Therefore, patients with 5-5 leucine repeats of the *CNDP1* gene will probably have more free carnosine in the circulation and tissue due to the lower concentration and activity of serum carnosinase-1 [79, 86]. Figure 3 illustrates the hypothesis of the *CNDP1* genotype related to diabetic nephropathy.

Figure 3. Hypothesis of the *CNDP1* gene related to diabetic nephropathy



Carnosine

Carnosine (β-alanine-L-histidine) and anserine (β-alanine-L-methyl histidine) are histidine-containing dipeptides. Carnosine has several protective properties; it plays a role as an antioxidant [88-90] by acting as a free radical scavenger [91, 92], as well as scavenging carbonyls [93-95]. Several animal and human studies reported the anti-glycation effects of carnosine [96, 97]. Furthermore, it has been suggested that carnosine influences insulin signaling pathways [98, 99]. Additionally, carnosine inhibits advanced glycation end products [96, 97], and it can function as an angiotensin-converting enzyme inhibitor [100, 101]. Finally, several mice studies reported that oral supplementation of carnosine lowered inflammatory cytokines, implying that carnosine may have anti-inflammatory effects [102-104]. Interestingly, all of these properties of carnosine seem to have a role in the development of diabetes and diabetic nephropathy. Carnosine is the best-characterized dipeptide of these two and therefore most studies focus on carnosine [105], but anserine seems to have similar effects. Anserine also has antioxidant properties [106] and it is a carbonyl scavenger [107]. Moreover, anserine seems to affect the renal sympathetic nerve activity and blood pressure [108].

In humans, these dipeptides are not incorporated into proteins, but they are stored in high concentrations in various tissues, including muscles, liver, kidney, pancreas, retina and myocardium. Carnosine is synthesized by the enzyme carnosine synthase [109], of which β-alanine is the rate limiting amino acid. There is uptake of β-alanine via the taurine transporter into cells in order to be synthesized into carnosine or anserine depending on the subsequent intracellular storage by carnosine synthase [110, 111]. Carnosine is degraded by carnosinase-1 in the circulation. Serum carnosinase-1 is synthesized and secreted by the liver [112].

The protective features of carnosine together with the association of the *CNDP1* gene and diabetic nephropathy suggest that the carnosine metabolism may play a role in the development and/or progression of diabetic nephropathy (Figure 3) and therefore carnosine may be of therapeutic value for patients with diabetic nephropathy. Based on data obtained from experimental studies, supplementation with carnosine might not only be beneficial for patients with diabetic nephropathy but it may also be used to prevent and treat cardiometabolic disease as well diabetes [103, 107, 113, 114]. Although these rodents studies showed promising results, it is necessary to bear in mind that in humans the actions of carnosine may be influenced by the presence of serum carnosinase, which hydrolyzes carnosine but is absent in rodents [115].

The results of diabetic experimental studies suggested that carnosine supplementation increased insulin secretion, reduced insulin resistance, reduced plasma glucose and

reduced markers of advanced glycation and chronic inflammation. More specifically, Forsberg *et al.* [113] reported that in db/db mice insulin levels increased and blood glucose decreased after 4 weeks of oral carnosine supplementation. Moreover, Sauerhofer *et al*. [114] reported that there was a delay in the development of type 2 diabetes in db/db mice due to the preservation of insulin secretion and an increased β-cell mass in the pancreas after 24 weeks of oral administration with carnosine. In obese Zucker rats, Aldini *et al*. [116] reported that after 24 weeks with oral carnosine supplementation there was an improvement of insulin resistance. In these obese rats, chronic carnosine administration also reduced cardiovascular risk factors such as dyslipidemia and hypertension, probably via a direct carbonyl quenching mechanism [116]. In another study with diabetic Balb/cA mice, reduced cholesterol and triglyceride levels were observed in heart and liver. Furthermore, decreased oxidation levels of lipids and glucose together with a suppressed glycation of HDL were found in these mice [103]. Lee *et al*. [103] also found increased insulin, decreased plasma glucose levels, fibronectin levels and inflammatory markers in diabetic Balb/cA mice which were supplemented with carnosine [103]. Additionally, the renoprotective properties of carnosine have been investigated in several experimental studies [103, 114, 117-121]. Specifically, Riedl *et al*. [118] showed that in streptozotocin-induced diabetic rats carnosine prevented glomerular cells from undergoing apoptosis and podocyte loss by inhibiting pro-apoptotic signaling. In diabetic db/db mice as well as in Balb/cA mice it was demonstrated that carnosine decreased the proliferation of mesangial cells [103, 114, 117, 121]. Interestingly, the study of Aldini *et al*. [116] reported that the renal function of obese Zucker rats improved after 24 weeks of oral carnosine supplementation. Similarly, Peters *et al*. [119] showed that in db/db mice proteinuria and renal vascular permeability was reduced by carnosine supplementation. These animal studies indicate that dietary supplementation with carnosine may be beneficial for diabetic nephropathy and diabetes. Carnosine is already available as an over-the-counter food additive, since it is used by athletes and it is relatively cheap [122]. Importantly, carnosine supplementation does not seem to have any significant side effects [123]. However, randomized clinical trials with strict controls need to be developed to investigate whether the effects of carnosine are similar in patients with diabetes and diabetic nephropathy compared to the studies with experimental models. As a start, in this thesis we investigated the carnosine metabolism in a physiological and pathological environment in human kidneys. Furthermore, we determined whether the *CNDP1* genotype is associated with glomerular histological lesions of diabetic nephropathy.

PART IV THESIS OUTLINE

This thesis comprises five chapters, in which diabetic nephropathy and the pathologic classification of diabetic nephropathy are the central themes. Several studies of this thesis used histological and clinical data obtained from an autopsy cohort of patients with diabetes. The renal tissue of this autopsy cohort provided us with the opportunity to investigate more than 100 glomeruli per case and gave us the opportunity to challenge several hypotheses in a unique setup.
The histological lesions in the renal tissue of our diabetic autopsy cohort were scored according to the pathologic classification of diabetic nephropathy proposed by the Renal Pathology Society in 2010. In **chapter two** we determined the prevalence of diabetic nephropathy in this cohort and the distribution over the histopathological classes. Our cohort of autopsy cases enabled us to observe the manifestations of diabetes in the kidney at various stages of the disease.

Currently, there is an increased focus on inflammatory therapy regimens in type 2 diabetes. The knowledge on infiltrating macrophages in humans with diabetic nephropathy is relatively limited, however. In **chapter three** we determined the amounts and types of macrophages which are present in renal tissue of patients with type 2 diabetes and histologically proven diabetic nephropathy. Furthermore, we associated these findings to the pathologic classification of diabetic nephropathy. Finally, the results of the amount and type of macrophages were correlated to clinical parameters.

Several schemes to classify histological lesions of diabetic nephropathy have been created but most of them were insufficient to be used for clinical practice. The most recent classification of diabetic nephropathy proposed by the Renal Pathology Society has been used in diagnostic as well as multiple research settings. To investigate whether this classification provides a proper communication tool between researchers and clinicians, and whether it has a prognostic value, we designed the study described in **chapter four**. This study gives an overview of the current validation studies of the pathologic classification of diabetic nephropathy. The prognostic value of this classification was analyzed in a meta-analysis. Additionally, a reproducibility study was performed to evaluate whether there are unclear definitions or issues within the classification. The combination of the meta-analysis and reproducibility study will provide an accurate overview of the current use of the pathologic classification system. Besides, it may help to redefine definitions and provide suggestions to update the pathologic classification of diabetic nephropathy in the near future.

Multiple studies showed that there is an association between the 5-5 polymorphism of the *CNDP1* gene and the susceptibility to develop diabetic nephropathy in patients with type 2 diabetes. All these studies used a clinical diagnosis of diabetic nephropathy to determine the possible association with the *CNDP1* genotype. We were able to determine the *CNDP1* gene of most of the patients from the diabetic autopsy cohort; therefore we were able to investigate this genetic association in cases with histologically proven diabetic nephropathy. The aim of **chapter five** was to determine whether the 5-5 *CNDP1* polymorphism is associated with histologically proven diabetic nephropathy and whether this polymorphism may influence the development of specific glomerular lesions of diabetic nephropathy.

Several experimental studies showed the beneficial effects of carnosine in diabetes and diabetic nephropathy. Carnosine has several functions which are altered in diabetes; additionally the association with the *CNDP1* gene and diabetic nephropathy suggests that carnosine might play a role in diabetic nephropathy. In **chapter six** we investigated whether carnosine or other histidine containing dipeptides, as well as carnosine synthase and carnosinase are present in the human kidney, which would suggest that the kidney has its own carnosine metabolism. Additionally, we investigated whether these enzymes change in patients with diabetic nephropathy, since it may be that the enzymes of the carnosine metabolism are reallocated to different parts of the nephron. Therefore, we scored the intensity of a CNDP1 protein staining in different parts of the tubules of renal biopsies from patients with diabetic nephropathy. These results were compared to the intensity scoring of the CNDP1 protein of control cases without diabetic nephropathy.

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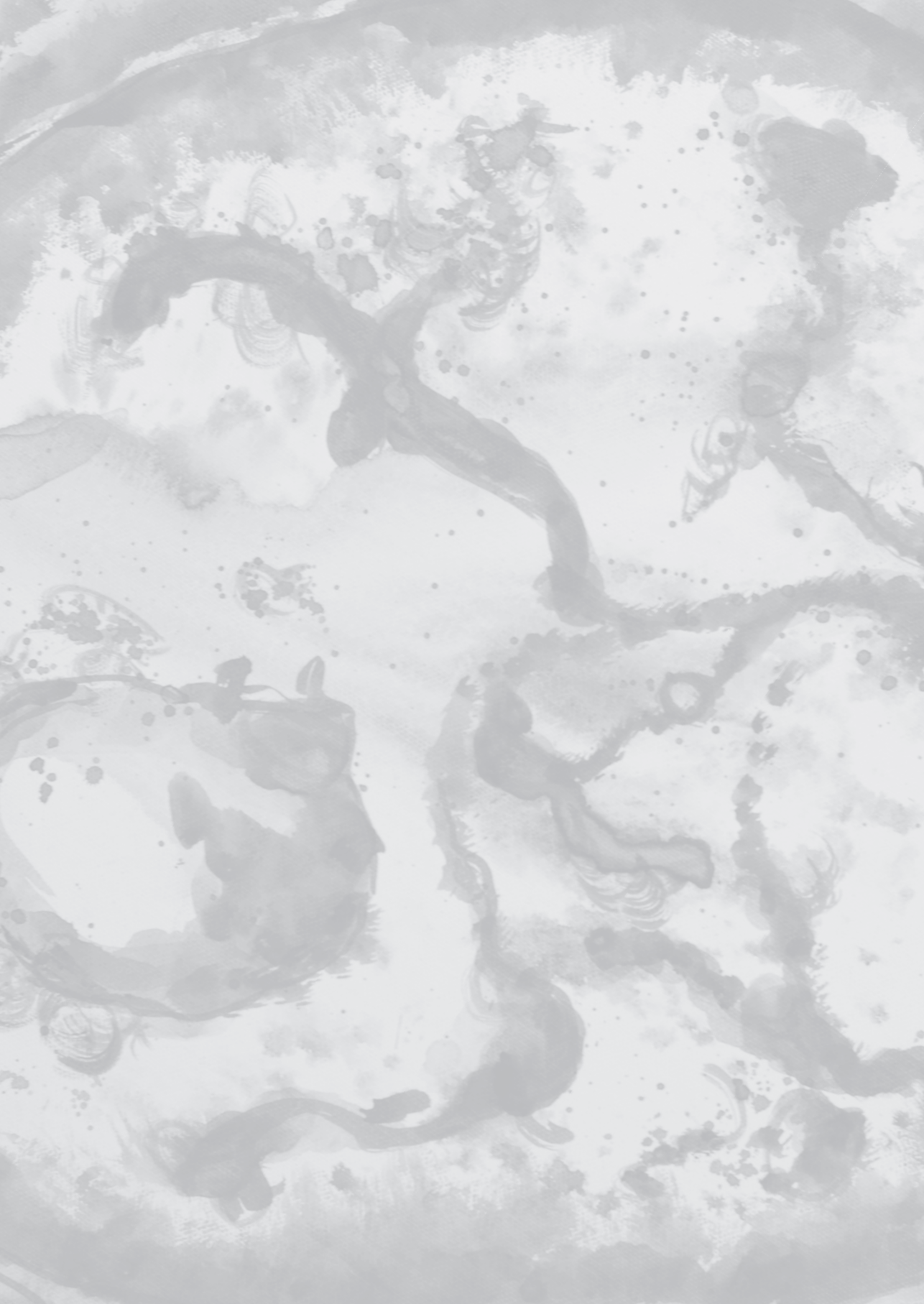

An autopsy study suggests that diabetic nephropathy is underdiagnosed

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ABSTRACT

The reported prevalence of diabetic nephropathy (DN) among diabetes patients varies widely. Most studies use the presence of microalbuminuria for clinical onset of DN in absence of a histopathological evaluation. In this autopsy study, we collected and analyzed data from a cohort of patients with type 1 and 2 diabetes. We determined the prevalence of histologically proven DN in patients with or without clinical manifestations of renal disease. We also examined the distribution among histopathological classes with respect to clinical parameters.

Renal tissue specimens from autopsies and clinical data were collected retrospectively from 168 patients with diabetes. The histopathological classification for DN was scored as well as interstitial and vascular parameters.

In this cohort, 106 of 168 patients had histopathological changes in the kidney characteristic of DN. Twenty of the 106 histologically proven DN cases did not present with DN associated clinical manifestations within their lifetime. Glomerular and interstitial lesions were associated with renal function, but not with proteinuria.

In this study, the prevalence of histologically proven DN was higher than previously appreciated, and we found a relatively high proportion of DN that was clinically underdiagnosed yet histologically proven, suggesting that DN lesions may develop before the onset of clinical findings. We also found that underdiagnosed DN may encompass all histopathological classes except the sclerotic class.

INTRODUCTION

Diabetic nephropathy (DN) is one of the leading causes of end-stage renal disease [1-3] that develops in approximately 10-30% of patients with diabetes mellitus. The reported prevalence of DN depends upon the type of diabetes, the duration of diabetes, and ethnicity. DN presents approximately ten years after the onset of type 1 diabetes (T1D) [4]; in contrast, the time of onset of DN among patients with type 2 diabetes (T2D) is highly variable [5]. The prevalence of clinically diagnosed DN in T1D varies between 5-20% [6-8] and in T2D between 25%-35%, based on microalbuminuria or proteinuria [5, 7]. Data on renal pathology in patients with DN is relatively limited, as a renal biopsy is performed only in cases in which the renal disease's manifestations cannot be explained sufficiently by the presence of clinically suspected DN [9, 10]. Relatively few studies on DN confirmed clinical manifestations of DN by renal biopsy [11-14].

It is generally considered that the clinical onset of DN is characterized by microalbuminuria. However, some studies suggest that a reduction in glomerular filtration rate (GFR) may precede the development of microalbuminuria [15-18]. Relatively little is known about the amount and severity of histological lesions in the kidney prior to the clinical onset of DN. Thickening of the glomerular basement membrane and mesangial changes were described in 1985 by Mauer *et al.* as the earliest histological manifestations of DN [19]. A recent study with normoalbuminuric T1D patients showed that greater glomerular basement membrane width is an independent predictor for progression to DN [20]. Nodular sclerosis is often encountered in patients with substantial proteinuria. Interestingly, there is evidence that to some extent, lesions of DN in T1D may be reversible [21, 22].

A histopathological classification for DN was launched in 2010 [23]. The original study included a substudy on interobserver agreement amongst pathologists, showing good agreement for the evaluation of the classes. Over the years, several clinical validation studies appeared [11, 13, 14], of which the most recent study [11] showed that the severity of glomerular and interstitial lesions is significantly associated with renal outcomes in patients with DN. In a smaller study, interstitial lesions—but not glomerular lesions—were determined to be a significant predictor of renal prognosis [14]. Studies which include diabetic patients who underwent a renal biopsy may be subject of selection bias regarding the moment in time at which the biopsy was taken [13, 24]. To avoid such a selection bias, we obtained tissue samples from autopsies rather than from biopsy samples.

In this autopsy study, we collected and analyzed data from a unique cohort of patients with T1D or T2D, and we determined the prevalence of histologically proven DN in patients with or without clinical manifestations of renal disease. Virtually none of the included patients underwent a renal biopsy during their lifetime. The aim of this study was to investigate the prevalence of histopathologically proven DN in patients with diabetes with or without clinical signs of DN, and its distribution over histopathological classes. Furthermore, we investigated which clinical parameters were related to the histopathological classes in patients with and without clinical manifestations of DN.

METHODS

Patients were included retrospectively from autopsies performed in 1984 through 2004 via the database of the pathology archives at Leiden University Medical Center for autopsy material. The primary inclusion criteria were the presence of either T1D or T2D in patients who were over the age of 18 years at the time of autopsy. We initially included 204 patients via our search in Delphic. We excluded 11 patients because their medical history revealed that they received a pancreas and/or kidney transplant. In nine cases, renal tissue blocks were not available, and these patients were excluded as well. Renal autopsy tissues from the remaining 184 cases were prepared for light microscopy; 16 of these samples were excluded due to poor tissue quality. Thus, 168 patients were included in the clinical histopathological analysis.

Clinical data

The clinical information was obtained via the medical records available at Leiden University Medical Center and via the patients' general practitioners. Approval was obtained from the medical ethics committee of Leiden University Medical Center to obtain relevant clinical data from the patients' practitioners for at least one year prior to the patient's death (the response rate from the practitioners was 80%). The following laboratory parameters were included: serum creatinine, eGFR (calculated using the MDRD formula), microalbuminuria (30-300 mg/L), proteinuria (>300 mg/L) via 24-hour urine or dipstick test, systolic and diastolic blood pressure, serum hemoglobin, serum cholesterol, and serum glycated hemoglobin. These data were collected retrospectively from the period starting one year before the patient's death. Data that reflected a stable representation of the serum and/or urine levels were included. Data were excluded if clearly affected by an unstable clinical condition. The decision to exclude data was made in consultation with an experienced nephrologist. We also obtained data regarding co-morbidities, duration of diabetes, medication history, hypertension, smoking, and diabetic complications. The causes of death were obtained from the autopsy reports.

Diagnosis of DN

The presence or absence of clinical DN was determined via the medical records of each patient. DN was considered present when the 24-hour urine was positive for more than 30mg/L albuminuria and/or dipstick was positive between + and ++++ in a stable period of diabetes during the year before death.

Absence of clinical diagnosis of DN

Absence of clinical DN (absence of albuminuria) was determined via the medical records of each patient. Clinical DN was considered absent when the urine dipstick tests were reported as negative or trace and/or when the 24-hour urine contained less than 30mg/L albuminuria in a stable period of diabetes during the year before death. In some cases the data was too limited to either confirm or rule out a clinical diagnosis of DN. These cases were registered as missing and were not used in the analysis.

Matched control group

A non-diabetic control group (N=40), which was matched to the underdiagnosed DN group (N=20 patients) with respect to gender, age, hypertension, and smoking habit was created to distinguish histological changes of underdiagnosed DN from age-related pathology. Each underdiagnosed DN patient had two matched control subjects for which clinical data was collected. The renal tissue specimens from the 40 control subjects were stained with hematoxylin and eosin (H&E), Periodic-acid Schiff (PAS), and silver in order to determine whether this group contained histological lesions.

Histopathology

Renal tissue was fixed in 10% buffered formalin and embedded in paraffin. Slices were cut at 1-µm and 3-µm thickness and stained with H&E, PAS, and silver stain (for the 1-µm thick sections).

Renal tissue specimens containing ≥100 glomeruli were scored by two investigators who were blinded with respect to the patients' clinical data. Glomerular lesions, interstitial lesions, and vascular lesions were scored in accordance with the established histopathological classification for DN [23]. Patients without histological lesions in our study were designated as class 0 DN. In addition, the following glomerular lesions were noted and scored as either present or absent: FSGS, cholesterol emboli, any other glomerular lesions, capsular drops, and hyalinosis of the glomerular vascular pole.

We evaluated all 45 cases with class III DN in order to determine the percentage of mesangial sclerotic nodules in each case. The percentage of mesangial sclerotic lesions was defined as the percent of glomeruli with at least one nodule in the total number of

non-sclerotic glomeruli in the tissue specimen. Additionally, we evaluated the percentage (and range) of mesangial expansion in the underdiagnosed cases. We determined the amount of mesangial expansion in either mild (class IIa) or severe (class IIb) DN in 100 glomeruli per case.

Transmission electron microscopy

Formalin-fixed, paraffin-embedded tissue blocks were reprocessed for electron microscopy. Two areas containing open glomeruli were identified on the corresponding microscopic slide, and 2-mm punches were made within these areas in the corresponding paraffin block. The paraffin was melted overnight in a 70°C oven, and the tissue was deparaffinized. After the paraffin was removed, the tissue was washed for 20 min in 0.1 M sodium cacodylate buffer (pH 7.4) containing 3% sucrose (w/v), post-fixed for 90 min in 2% osmium tetroxide diluted 1:1 with 2% potassium ferrocyanide, and dehydrated in ethanol at 70, 80, and 90% (1 hour each), followed by two one-hour incubations in 100% ethanol. The tissue was then transferred to propylene oxide for 10 min, embedded in epon, and polymerized in a 70°C oven for 30 hours. The epon blocks were trimmed, 1-µm thick survey sections were stained with toluidine blue solution, and ultrathin (100-nm thickness) sections of at least one glomerulus were cut using a Leica Ultracut UCT ultra microtome. The ultrathin sections were collected on copper grids. The sections were contrast-stained with uranyl acetate (7 min) and Reynold's lead citrate (7 min) (both from Sigma) and examined using a JEOL JEM-1011 electron microscope operating at 60 kV. Images were digitized using a MegaView III camera, and the glomerular basement membrane was measured using the Soft Imaging Solutions program (Olympus).

For all cases in our study we had to use paraffin tissue. Because reprocessing of paraffin tissue for electron microscopy causes artefactual glomerular basement membrane thinning, an additional 34% was added to the glomerular basement membrane width as recommended in a previous study of Nasr *et al*. [25], who showed that the mean reduction in glomerular basement membrane thickness in paraffin-embedded material was 34% in DN. Taking this calculation into account, the cut-off levels described by Tervaert *et al.* [23] could then be used in our study.

Statistical analysis

The SPSS statistical software package, version 20.0 (IBM, Armonk, NY) was used for all statistical analyses. Statistical differences between groups were analyzed by ANOVA and a univariate general linear model with polynomial contrast for linear trend. Differences with a p-value <0.05 were considered statistically significant. The histopathological

data were analyzed using the chi-square test. The data in the tables are presented as a percentage or as the standard error of the mean (SEM).

Ethics

All tissue samples were coded and then handled and analyzed anonymously in accordance with the Declaration of Helsinki.

RESULTS

The baseline characteristics of the 168 included patients are summarized in Table 1. The cohort contained 17 patients with T1D and 127 patients with T2D; in 24 cases, the type of diabetes was unclear. The mean age of the 168 patients was 69 years, and 55% of the cohort was male. The histopathological examination revealed lesions that were consistent with DN in 106 patients. For 21 patients, the clinical data were insufficient for determining whether they had received a clinical diagnosis of DN. In 65 of 106 patients, their clinical diagnosis of DN was in accordance with the histological lesions, i.e. a clinical diagnosis of DN had been made before death and lesions consistent with DN were found at autopsy. In 20 of the 106 patients with histological signs of DN at autopsy, no clinical diagnosis of DN was made prior to death, as neither microalbuminuria nor proteinuria had been observed during these patients' lifetime. The 20 underdiagnosed DN patients had multiple negative dipsticks and/or no albuminuria (<30mg/24h) found in the 24h urine in the year before death (Table 2). Of these 20 patients, the histopathological classification revealed that 7 patients had class I DN, 5 patients had class IIa DN, 3 patients had class IIb DN, and 5 patients had class III DN (Figure 1). Table 3 summarizes the characteristics of patients with diagnosed and underdiagnosed DN, as well as the matched control group. The decades of death varied among the underdiagnosed patients (one patient before 1990, 15 patients between 1990-2000 and four patients between 2000-2004), so the care of diabetes did not seem to influence this phenomenon. Eight patients were diagnosed clinically as having DN; however, no lesions consistent with DN were found in their renal tissues at autopsy.

Table 1. Baseline characteristics of the cohort

Baseline characteristic	Percentage or mean (SEM)
Gender (% males)	54.8
Age, years	69.3 (0.96)
T1D (%)	10.1
Duration of diabetes, years	13.69 (1.27)
eGFR, ml/min/1.73 m^2	53.14 (2.85)
Microalbuminuria or proteinuria (%)	49.4
Creatinine serum, µmol/L	136.21 (11.23)
Hb, mmol/L	7.12 (0.13)
HbA1c, (% units)	8.2 (0.82)
Cholesterol, mmol/L	4.97 (0.24)
Death by CV event (%)	47.6
Systolic pressure, mmHg	134 (2.63)
Diastolic pressure, mmHg	75.6 (1.30)

SEM, standard error of the mean; T1D, type 1 diabetes; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; HbA1c, glycated hemoglobin; CV, cardiovascular

Table 2. Clinical diagnosis of 20 underdiagnosed patients

	DN class	Method for absence of albuminuria	Stage of CKD	ACE inhibition	Hypertension	History of smoking
1	1	24h urine	3	Yes	Yes	No
2	1	Dipstick tests	2	Missing	Yes	Not described
3	1	Dipstick tests	1	No	Yes	Yes
4	1	Dipstick tests	1	No	Yes	No
5	1	24h urine	1	No	No	No
6	1	24h urine	1	No	Yes	Not described
7	1	Dipstick tests and 24h urine	2	Missing	No	Yes
8	2a	Dipstick tests	3	No	Yes	Not described
9	2a	Dipstick tests	2	No	Yes	Yes
10	2a	Dipstick tests		No	Yes	Yes
11	2a	Dipstick tests	3	No	No	Not described
12	2a	Dipstick tests	3	No	No	Yes
13	2b	Dipstick tests	4	Yes	Yes	Not described
14	2b	Dipstick tests	1	No	No	Yes
15	2b	Dipstick tests	3	Yes	Yes	No
16	3	Dipstick tests	3	Missing	Missing	Not described
17	3	Dipstick tests	3	No	No	No
18	3	Dipstick tests and 24h urine	3	No	No	Yes
19	3	Dipstick tests	3	No	No	Yes
20	3	Dipstick tests	Missing	Missing	Missing	Not described

DN class, histopathological classification of diabetic nephropathy; Stage of CKD, GFR estimated by KDIGO guidelines; Dipstick tests were reported as negative or trace, 24h urine was <30mg/L albuminuria

Table 3. Characteristics of patients with diagnosed and underdiagnosed DN

Baseline characteristic	Diagnosed DN (N=86)	Underdiagnosed DN (N=20)	*p*-value*
Gender (% males)	53.4	65	0.327
Age, years	69.8	65	0.043
T1D (%)	12	10.5	0.853
Duration, years	13.4	14	0.594
eGFR, ml/min/1.73 m^2	50.6	69.2	0.381
Microalbuminuria or proteinuria (%)	60.8	0.00	<0.0001
Creatinine serum, µmol/L	168	121	0.523
Hb, mmol/L	7.1	6.8	0.634
HbA1c, (% units)	9.2	7.3	0.351
Cholesterol, mmol/L	5.1	4.3	0.405
Death by CV event (%)	48	45	0.832
Systolic blood pressure, mmHg	136	129	0.196
Diastolic blood pressure, mmHg	76	73	0.745
Anti-hypertensive medication (%)	53.1	56.3	0.773
ACE inhibitor or ARB (%)	26.8	18.8	0.494

SEM, standard error of the mean; T1D, type 1 diabetes; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; HbA1c, glycated hemoglobin; CV, cardiovascular; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker. Measured using the chi-square test

Histopathological lesions

Lesions consistent with DN were found in 63% of the tissue specimens (106/168). Twenty-two of the samples had class I DN, which is characterized by a thickened glomerular basement membrane as determined by electron microscopy. The glomerular basement membrane width can change due to hypertension, but in this cohort there was no significant difference between patients with and without hypertension between class I and class 0 (p=0.904). Therefore, it was unlikely that increased glomerular basement membrane width was caused by hypertension. Thirty-three samples had class II DN, characterized by mesangial expansion; 21 of these samples were class IIa, and 12 samples were class IIb. Forty-five samples had class III DN, characterized by the presence of nodular sclerosis. The remaining six samples had lesions that were consistent with class IV DN, characterized by more than 50% of glomeruli with global sclerosis. We found that the percentage and range of glomeruli with mesangial sclerotic nodules was significantly lower in the underdiagnosed group compared to the diagnosed group. Overall, the mean percentage of nodules in the 45 cases with class III DN was 22.9% (range: 2.6-67.6%). In the diagnosed and underdiagnosed groups, the mean percentage of nodular sclerosis was 24.4% (range: 5.6-67.6%) and 10.9% (range: 3.0-26.2%), respectively (p=0.031).

In addition, we examined mesangial expansion in the underdiagnosed group. The five patients with class IIa DN had mesangial expansion in 40, 51, 51, 57, and 60% of glomeruli (mean: 51.8%). The three patients with class IIb DN had mesangial sclerosis in 92, 93, and 100% of glomeruli (mean: 95%).

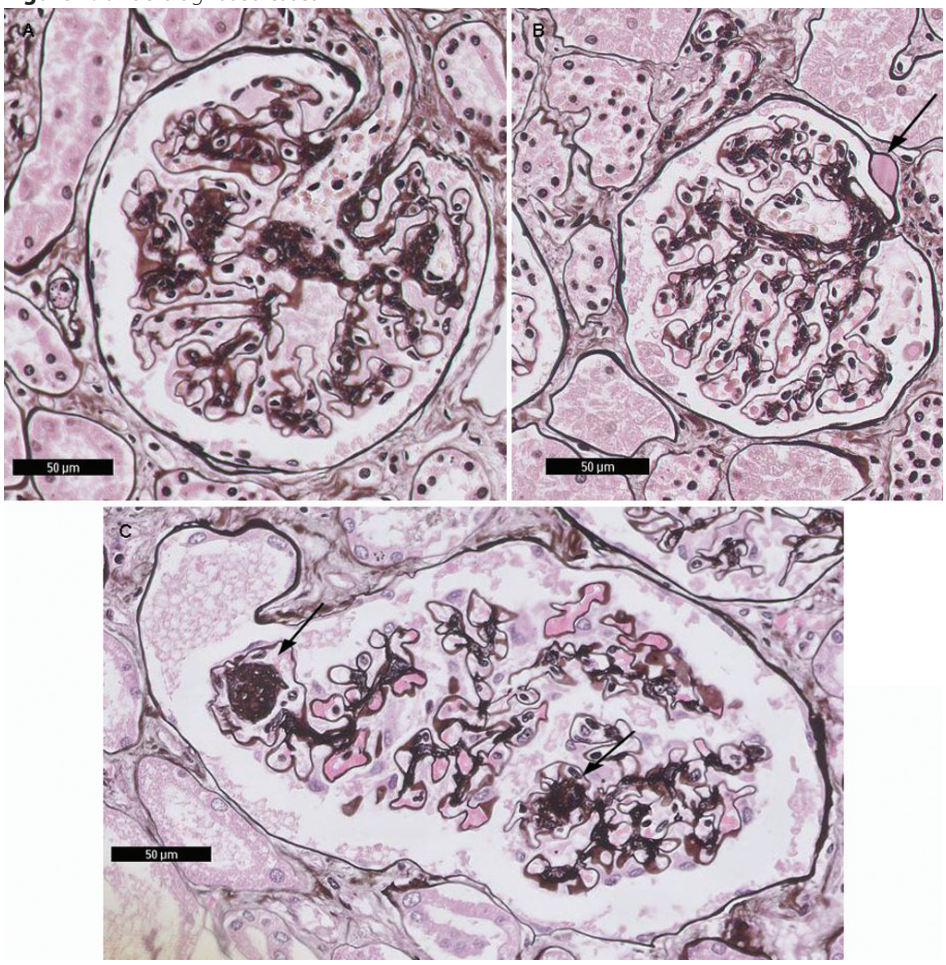
Glomerular lesions are associated with other histopathological lesions in DN, including interstitial fibrosis and tubular atrophy (IFTA) (p<0.001) and lesions like arteriosclerosis, hyalinosis, capsular drops and glomerular hyalinosis (p=0.238, p<0.001, p=0.004, p<0.001, respectively). IFTA, arteriosclerotic lesions in the arterioles, and hyalinosis were more prevalent among the patients with histologically proven DN than among the patients who had no DN lesions (i.e., class 0 DN) (p=0.007, p=0.017, and p<0.001, respectively). There were significantly more IFTA, arteriosclerotic lesions and hyalinosis with more severe DN (p<0.001 for both arteriosclerotic lesions and hyalinosis). In contrast, no significant correlation was found between the degree of arteriosclerosis and the histopathological class of DN (p=0.238). The number of capsular drops and glomerular hyalinosis increased significantly with the increasing severity of DN (p=0.004 and p<0.001, respectively). The underdiagnosed group contained significantly more capsular drops compared to the diagnosed group (p<0.001) (Figure 1 B, Supplementary Table1).

DN was absent in the control group. The amount of IFTA, arteriosclerosis, and hyalinosis of interstitial arterioles did not differ between the underdiagnosed DN patients

and controls (Supplementary Table 2). Hyalinosis of the glomerular vascular pole was sporadically present in 10 of the forty controls, but occurred significantly more often in the underdiagnosed DN group where it was present in 15 of the 20 cases ($p<0.0001$).

Focal segmental glomerulosclerosis not otherwise specified (FSGS, NOS) was present in 15 cases; 13 of these 15 patients had evidence of DN at autopsy. Six patients had cholesterol emboli; three of these six patients also had histologically proven DN. Two patients had mild glomerulonephritis suggestive of post-infectious glomerulonephritis, but no histologically proven DN, and three patients had pyelonephritis; two of these three patients had histologically proven DN.

Figure 1. Underdiagnosed cases



A: class II DN, **B**: capsular drop (arrow), **C**: class III DN (arrows indicate nodular sclerosis), Scale bars = 50 µm

Clinical data

The eGFR (estimated GFR) values and the presence of microalbuminuria, proteinuria, or other diabetic complications did not differ between the patients with T1D and the patients with T2D. A significant association between diabetes duration and DN class was found (p=0.035). Specifically, a significant difference was found between class 0 and class III patients (p=0.004) and between class IIa and class III patients (p=0.008). However, no linear trend over the classes was identified (p=0.44). Also the severity of IFTA correlated with the duration of diabetes (p=0.02). The eGFR values decreased significantly as DN class increased (p=0.006) Also, when the eGFR was staged by KDIGO CKD guidelines, there was a linear trend (p=0.001), and eGFR was linearly correlated with DN class (p<0.001). The eGFR values were inversely correlated with IFTA (p=0.001) and arteriosclerosis (p=0.002), but not with arteriolar hyalinosis. No significant correlation was found between microalbuminuria and/or proteinuria and the presence of DN (p=0.150). eGFR staged by KDIGO guidelines correlated significantly with albuminuria in a linear trend (p=0.001). Microalbuminuria and/or proteinuria was correlated linearly with IFTA (p<0.001). In addition, IFTA was inversely correlated with hemoglobin levels (p<0.001).

The medical records revealed that 29 out of 113 patients had received therapy with an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin receptor blocker. These 29 patients were distributed equally between the patients with and without clinical manifestations of DN (p=0.494). Data were sparse with respect to diabetic retinopathy; diabetic retinopathy was specifically noted as present in 14 patients and absent in one patient. All 14 patients, in whom diabetic retinopathy was recorded, had histologically proven DN (two had class IIa, ten had class III, and two had class IV); only one of these 14 patients was clinically underdiagnosed with DN. One patient with a clinically documented absence of both DN and diabetic retinopathy had histological evidence of class III DN at autopsy. Finally, cardiovascular-related death showed a significant linear trend with DN class (p=0.008), but not with IFTA (p=0.130).

DISCUSSION

In our diabetic cohort of 168 patients, histopathological changes attributable to DN were present in the kidney samples of 106 patients. In 20 of 106 histologically proven DN patients, clinical manifestations associated with DN had been absent during their life. This may indicate that renal lesions consistent with DN may develop before the onset of clinical abnormalities. Our data also show that an underdiagnosed DN may encompass all classes except the sclerotic class, as in the 20 patients with histopathologically proven DN who had no signs of DN during their lives, classes I, II and III were

diagnosed in respectively 7, 8, and 5 patients. Of note, next to the absence of albuminuria in several tests in 10 cases the absence of clinical DN was explicitly stated in their medical record. In this study, microalbuminuria or proteinuria was not associated with the presence of histologically proven DN.

Interestingly, the presence of microalbuminuria and/or proteinuria was correlated with IFTA, but not with the severity of DN. These findings might be explained by the capacity of healthy tubular epithelium to reabsorb proteins from the glomerular filtrate, thereby disguising glomerular protein leakage. Only after interstitial damage has occurred, the resulting loss of reabsorption capacity causes the onset of proteinuria, explaining why severe glomerular damage consistent with DN can occur prior to the onset of microalbuminuria or proteinuria [26].

In our cohort, the incidence of non-DN–related renal disease was relatively low. Based on light microscopy, 26 patients (15.5%) had lesions consistent with other renal abnormalities. The reported prevalence of non-diabetic renal disease detected on renal biopsy was previously reported up to 79% [13, 24, 27, 28]. However, this relatively high prevalence may have been influenced by selection bias with respect to the reasons for performing the renal biopsy.

Our cohort of autopsy cases enabled us to observe the manifestations of diabetes in the kidney by histology at various stages of the disease, irrespective of the clinical manifestations present in the patient prior to death. We can only compare our study results with those of clinical validation studies with respect to the clinical data at the time of tissue sampling, because per definition, our study lacks a clinical follow-up. Previous clinical validation studies yielded conflicting results with respect to the correlation between glomerular classes, interstitial lesions, and clinical parameters [11-14]. The strong association found in our study between eGFR and the classes of DN as well as between DN classes and interstitial and vascular lesions are in line with the previous studies by An *et al*. [11] and Oh *et al*. [13], who observed similar associations. In our retrospective study, diabetic retinopathy was not a clinical indicator for the presence of DN. However, data regarding the specific presence or absence of diabetic retinopathy were restricted to only 15 patients. Recently, Zhang *et al*. [29] investigated the clinical characteristics and predictive factors of subclinical DN in T2D patients and found that these patients had increased renal size, abnormal levels of tubular injury markers, high blood pressure, and abnormal circadian rhythm. In our retrospective study, data on these parameters were too limited for further evaluation.

In our study, we had the huge benefit of being able to study at least 100 glomeruli per patient, which are approximately ten-fold more glomeruli than usually available in a renal biopsy sample. In a substantial percentage of cases, we observed that explicit lesions such as nodular sclerosis were either relatively sparse or concentrated in specific areas of tissue. Thus, it is conceivable that these histopathological patterns are partly responsible for the underrepresentation of DN in renal biopsy studies. Given the large amount of tissue available, we were not only certain about the presence or absence of DN, but also in case of DN of its distribution over the four classes and the severity of interstitial lesions.

Despite the advantages discussed above with respect to our unique cohort, our study also has some limitations that merit discussion. The cohort may suffer from selection bias, because all patients included underwent an autopsy whereas not all deceased patients in the Netherlands are autopsied and those who are were mostly hospitalized before death. Our samples were taken from autopsies performed in 1984 through 2004; although we attempted to retrieve all of the clinical information available for each patient, some of the data were incomplete, and some information was missing due to the fact that medical records were sometimes destroyed after 15 years. The observed correlations between clinical and pathological parameters are based on a population of mainly Caucasians with DN. Therefore, one should be cautious with extrapolating these results to other populations. Nodular lesions similar to those seen in class III DN may occur in patients with hypertension and a history of smoking but without diabetes (typically elderly males) [30]. It seems unlikely that our results were influenced by this entity because only 2 patients in the underdiagnosed group were smokers and they did not have hypertension. Moreover, the subjects in the matched control group did not reveal histological changes characteristic of DN.

In conclusion, we found a high proportion of clinically underdiagnosed yet histologically proven DN. The potential clinical benefit of identifying this underdiagnosed group of patients remains to be determined. For example, the ability to diagnose DN in an early stage might enable clinicians to begin a specific therapeutic regimen that could slow disease progression and may ultimately prevent the onset of end-stage renal disease. Clinical studies should be designed in order to investigate whether initiating such therapeutic regimens in an early phase of DN can affect the course of renal complications associated with diabetes. Equally important is the need to develop diagnostic tools that could be used to accurately detect currently underdiagnosed patients.

DISCLOSURE

All authors declare that they have no conflict of interest.

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SUPPLEMENTARY TABLES

Supplementary Table 1. Comparison between histological parameters of diagnosed and underdiagnosed DN patients

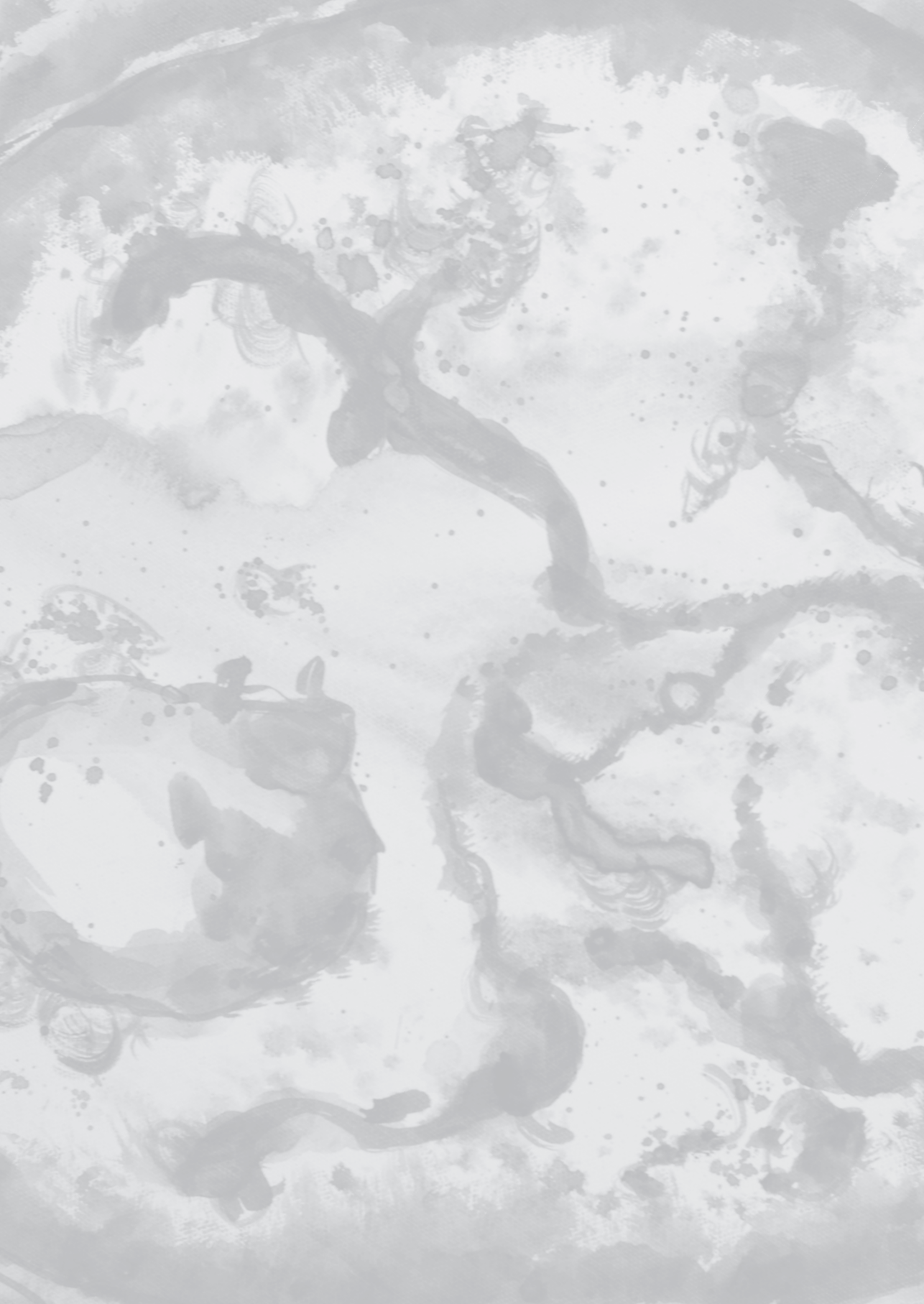
	Underdiagnosed DN (N=20)	Diagnosed DN (N=86)	*p*-value (χ^2 test)
DN classification (I/II/III/IV)	(7/8/5/0)	(15/25/40/6)	<0.0001
Global glomerulosclerosis (mean, %) †	9.95 (19.8)	17.03 (19.8)	0.89
IFTA (0/1/2/3)	(4/11/3/2)	(12/49/12/13)	0.87
Arteriosclerosis (0/1/2/3)	(2/11/5/2)	(6/45/20/15)	0.85
Hyalinosis (0/1/2/3)	(6/12/2/0)	(22/30/19/15)	0.06
Glomerular hyalinosis of the vascular pole (present/absent)	(15/5)	(12/74)	0.23
Capsular drops (present/absent)	(11/9)	(12/74)	<0.0001

†, *p*-value based on Student's t-test

Supplementary Table 2. Matched parameters and histological parameters of underdiagnosed DN patients and controls

		Underdiagnosed DN (N=20)	Controls (N=40)*	*p*-value (χ^2 test)
Matched parameters	Gender (male)	13/20	26/40	1.000
	Age (year)	65.45	65.28	0.504
	Hypertension absent/present	44.4/55.56	52.6/47.4	0.567
	Smoking history (yes/no/missing)	40/25/35	27.5/32.5/40	0.608
Histological parameters	Global glomerulo sclerosis (mean, %) (SD) (range) †	8.25 (11.5) (1-40%)	6.25 (6.3) (0-25%)	0.445
	IFTA (0/1/2/3) (%)	(20/55/15/10)	(40/47.5/12.5/0)	0.121
	Arteriosclerosis (0/1/2/3) (%)	(10/55/25/10)	(22.5/65/12.5/0)	0.086
	Hyalinosis (0/1/2) (%)	(30/60/10)	(52.5/30/17.5)	0.082
	Glomerular hyalinosis of the glomerular vascular pole (absent/present) (%)	(25/75)	(75/25)	<0.001

Controls were matched for gender, age, hypertension, and smoking habit; †, p-value based on Student's t-test



Macrophages in diabetic nephropathy in patients with type 2 diabetes

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ABSTRACT

Inflammation plays a role in the development of diabetic nephropathy (DN) in type 2 diabetics. Although macrophages have been found in experimental models of DN, little is known regarding the presence of macrophages in patients with DN. Therefore, we investigated the presence and phenotype of glomerular and interstitial macrophages in relation to clinical and histopathological parameters in patients with DN.

Renal autopsy samples were obtained from eighty-eight type 2 diabetic patients with histologically proven DN and stained for CD68 and CD163 as general and M2/anti-inflammatory markers of macrophages. Renal damage was scored based on histopathological classification of DN. Control renal autopsy samples were obtained from patients without renal abnormalities and from diabetic patients without DN. Positive cells per glomerulus were counted. Interstitial macrophages were counted semi-quantitatively.

Macrophages were present in all groups. In the DN group, the mean number of CD68+ cells/glomerulus and CD163+ cells/glomerulus was 4.2 (range 0-19) and 2.1 (range 0-14.47), respectively. The distribution was similar between all histopathological classes. Glomerular CD163+ macrophages were positively associated with DN class, interstitial fibrosis and tubular atrophy, and glomerulosclerosis. Interstitial CD68+ macrophages were correlated with GFR stage and albuminuria.

Our results demonstrate that macrophages are present in the glomeruli and interstitium of type 2 diabetic patients with DN and of controls. Although patients and controls had similar numbers of glomerular macrophages, glomerular anti-inflammatory CD163+ macrophages were associated with pathological lesions in DN. Taken together with the correlation between interstitial macrophages and IFTA, DN class, and renal function, this finding suggests that macrophages may play a role in DN progression. Therefore, targeting macrophages may be a promising new therapy for inhibiting the progression of DN.

INTRODUCTION

The global prevalence of type 2 diabetes (T2D) is increasing rapidly [1]. A severe complication associated with T2D is the development of diabetic nephropathy (DN), a major cause of end-stage renal disease [2]. Despite current therapy, many patients with DN progress to renal failure [3]. Increasing our understanding of the pathophysiology that underlies DN can help to develop therapeutic regimens for inhibiting the development and/or progression of DN. Traditionally, DN was believed to result from interactions between hemodynamic factors and metabolic factors [4, 5]. Recent studies, however, found that inflammatory mediators play an important role in the early stages of the disease [6]. Thus, newly emerging treatment options focus on inhibiting this inflammatory pathway [7].

Experimental studies have shown that the inflammatory response that precedes and helps promote insulin resistance in diabetes depends largely upon the accumulation of macrophages in tissue [8]. Furthermore, experimental models of DN have shown that DN is accompanied by an influx of macrophages in response to an upregulation of monocyte chemotactic protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) [8, 9]. In these models, inhibiting the influx of macrophages reduces albuminuria, reduces glomerular damage, and slows the progression of renal disease [10, 11]. The influx of macrophages has also been observed in other chronic kidney diseases, including anti-GBM nephritis and ANCA-associated pauci-immune crescentic glomerulonephritis [12, 13]. Animal models of these renal diseases revealed that renal injury can be mitigated by depleting macrophages and/or by disrupting macrophage recruitment [12]. Nevertheless, whether these macrophages affect the course of the disease or merely represent a response to injury remains unknown [10, 14]. In addition, infiltrating macrophages can differentiate into distinct phenotypes in response to the microenvironment, thereby expressing pro-inflammatory, anti-inflammatory, or profibrotic cytokines [8, 15, 16].

In patients with DN, MCP-1 is upregulated in renal tissue, and MCP-1 levels are increased in the urine, suggesting that an influx of macrophages plays a pathogenic role in the development of proteinuria, in glomerular damage, and in the progression of renal disease in humans [17-20]. However, relatively little is known about the presence or phenotype of macrophages in the kidneys of patients with diabetes. To date, only one study investigated the presence of macrophages in patients with DN; this relatively small study found macrophages in the glomeruli and interstitium of diabetic patients but did not investigate the phenotype of these macrophages [21].

The aim of this study was to investigate the presence and phenotype of macrophages in renal autopsy samples obtained from a cohort of patients with histologically proven DN. In addition, we examined the association between these macrophages and both histopathological and clinical parameters. Renal damage was scored in accordance with the histopathological classification of DN, which is linked to renal outcome [22-24].

METHODS

In this retrospective autopsy study, renal autopsy tissue specimens were obtained from patients with type 2 diabetes. The samples were retrieved from the pathology archives at Leiden University Medical Center. A total of 88 patients were included retrospectively from autopsies performed in 1984 through 2004 via the database of the Department of Pathology for autopsy material. The primary inclusion criterion was the presence of T2D in patients who were over the age of 18 years at the time of death. We also used two control groups. One control group consisted of renal autopsy material obtained from non-diabetic patients with no other renal abnormalities but with other comorbidities, including hypertension, heart failure, or atherosclerosis (N=5). The second control group consisted of diabetic patients with no histological evidence of DN (N=18).

We classified renal damage in the diabetic patients using the histopathological classification of DN, which is based on defined glomerular damage [25]. This classification system was used to investigate whether the presence of renal macrophages was associated with glomerular damage. The presence of CD68+ and CD163+ cells was used to indicate total macrophages and M2 (anti-inflammatory) macrophages, respectively [16].

Clinical data

Clinical data were obtained from the medical records available at Leiden University Medical Center and from the patients' general practitioners. Approval to obtain relevant clinical data from the patients' practitioners was obtained from the medical ethics committee of Leiden University Medical Center. The following laboratory results were obtained: serum creatinine; eGFR (calculated using the MDRD formula); microalbuminuria (i.e., 30-300 mg/L) or macroalbuminuria (i.e., >300 mg/L), measured via 24-hour urine or dipstick tests; systolic and diastolic blood pressure; serum hemoglobin, serum cholesterol; and serum HbA1c. GFR was staged in accordance with the stages of chronic kidney disease established by the KDIGO (Kidney Disease Improving Global Outcomes) guidelines. Dipstick tests were interpreted and staged as follows: absent when negative or trace; microalbuminuria when 1+ or 2+ (30-300 mg/L); or macroalbuminuria when 3+ or 4+ (>300 mg/L). These data were collected retrospectively from the period start-

ing one year before the patient's death. Data that reflected a stable representation of the patient's serum and/or urine levels were included. We also obtained data regarding comorbidity, duration of T2D, medication history, hypertension, smoking history, and diabetic complications such as retinopathy, cardiomyopathy, polyneuropathy, and diabetic foot ulcers. For each patient, the cause of death was obtained from the autopsy report.

Histopathology

Renal tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at 1-µm and 3-µm thickness and stained with hematoxylin and eosin (HE), periodic-acid Schiff (PAS), and silver stain (for the 1-µm thick sections).

Renal tissue specimens containing ≥100 glomeruli were scored by two investigators who were blinded with respect to the patients' clinical data. Glomerular lesions, interstitial lesions, and vascular lesions were scored in accordance with the established histopathological classification for DN [25]. Specifically, a score of 0 was given if interstitial fibrosis and tubular atrophy (IFTA) was not present in the cortex. A score of 1 was given if less than 25% IFTA was present. A score of 2 was given when at least 25% but less than 50% IFTA was present. Finally, a score of 3 was assigned when at least 50% IFTA was present [25]. In addition, the following glomerular lesions were noted and scored as either present or absent: focal segmental glomerulosclerosis (FSGS), cholesterol emboli, any other glomerular lesions, capsular drops, and hyalinosis of the glomerular vascular pole.

Immunohistochemistry

To detect and characterize the macrophages presence in the kidney samples of DN patients and controls, immunohistochemical staining was performed on sequential slides using monoclonal mouse antibodies against human CD68 (1:2000, DakoCytomation, Glostrup, Denmark), a general macrophage marker, and monoclonal mouse antibodies against human CD163 (1:10, Abcam, Cambridge, UK), an anti-inflammatory macrophage marker (M2) [16]. As a control for CD68+ and CD163+ cells, we used a monoclonal antibody against CD45 (1:800, DakoCytomation, Glostrup, Denmark), a pan-leukocyte marker. Paraffin-embedded kidney samples were sectioned and then deparaffinized. Antigen retrieval was performed using Tris-EDTA buffer, pH 9.0 (for CD45 and CD68 immunostaining) or citrate buffer, pH 6.0 (for CD163 immunostaining). After blocking endogenous peroxidase, the sections were incubated in the relevant primary antibody for 1 hour. As a negative control, mouse IgG1 negative control fraction (DakoCytomation) was used at the same concentration as the respective primary antibody. Followed by antibody detections using the DAKO Envision+ System and was visualized

using diaminobenzidine as the chromogen. Finally, the sections were counterstained with hematoxylin and coverslipped.

Immunohistochemistry analysis

The immunostained sections were scanned in an IntelliSite Pathology Ultra-Fast Scanner (Philips Healthcare, Eindhoven, the Netherlands). Using a Philips Image Viewer, the number of macrophages was counted in 50 glomeruli per section. Only CD68+ and CD163+ cells with a visible nucleus and well-defined cytoplasm were counted as positive in the glomeruli, similar to previous studies [26, 27]. The mean number of macrophages in the glomeruli was determined by dividing the total number of macrophages by 50. The investigators were blinded with respect to DN class and clinical parameters and scored the number of CD68+ and CD163+ cells per glomerulus. Interstitial macrophages were quantified in a separate session.

Interstitial CD68+ macrophages in the cortex were counted in a total of ten high-power fields (20x) and graded semi-quantitatively on a 0-3 scale. Grade 0 corresponded to macrophages in <10% of the interstitium; grade 1 corresponded to macrophages in 10-30% of the interstitium; grade 2 corresponded to macrophages in 30-50% of the interstitium; and grade 3 corresponded to macrophages in >50% of the interstitium [28].

Statistical analysis

The SPSS statistical software package, version 20.0 (IBM, Armonk, NY) was used for all statistical analyses. Statistical differences between groups were analyzed using the Jonckheere-Terpstra test for trends, the Kruskal-Wallis test, or the Mann-Whitney *U* test. Correlations for non-normally distributed data were evaluated by calculating Spearman's rank correlation (rho). Regression models (linear and ordinal) were used to investigate the influences of independent variables. Kappa score (k) was determined using the method of Landis and Koch and was used to quantify intraobserver and interobserver agreement of interstitial macrophage scoring [28]. Differences with a p-value ≤ 0.05 were considered statistically significant. The data in the tables are presented as either a percentage or the mean value (± SEM).

Ethics

All tissue samples were coded and then handled and analyzed anonymously in accordance with the Declaration of Helsinki.

RESULTS

The clinical characteristics of the 88 patients with type 2 diabetes (T2D) and histologically proven DN are summarized in Table 1. Mean age was 71 years, and 53.4% were male. The mean duration of T2D was 12.7 years. Kidney samples obtained from these patients were classified according to the histopathological classification of DN [25]. Nineteen patients had class I DN, which is characterized by a thickened glomerular basement membrane. Twenty-nine patients had class II DN, which is characterized by mesangial expansion; 18 of these patients had class IIa DN, and 11 patients had class IIb DN. Thirty-four patients had class III DN, which is characterized by the presence of nodular sclerosis. Finally, the remaining six patients had class IV DN, which is characterized by global sclerosis in more than 50% of glomeruli. The five control samples obtained from non-diabetic subjects had no other renal abnormalities, and the 18 control samples obtained from diabetic patients without DN had no histological lesions characteristic of DN based on light microscopy and electron microscopy. In the control group (N=23; 57% male), mean age was 69 years, and mean blood pressure was 131/76 mmHg. The eighteen diabetic controls had a mean duration of diabetes of 8 years. With respect to renal damage, no glomerular damage was observed in the renal tissue specimens of the control group. However, some damage was observed in the interstitium and vessels, and this damage was probably age-related (e.g., atherosclerosis, ischemia, etc.). Neither IFTA nor the number of globally sclerotic glomeruli differed significantly between the diabetic control group and the DN cases (p=0.441 and 0.474, respectively).

Table 1. Clinical characteristics of the study group with histologically proven DN

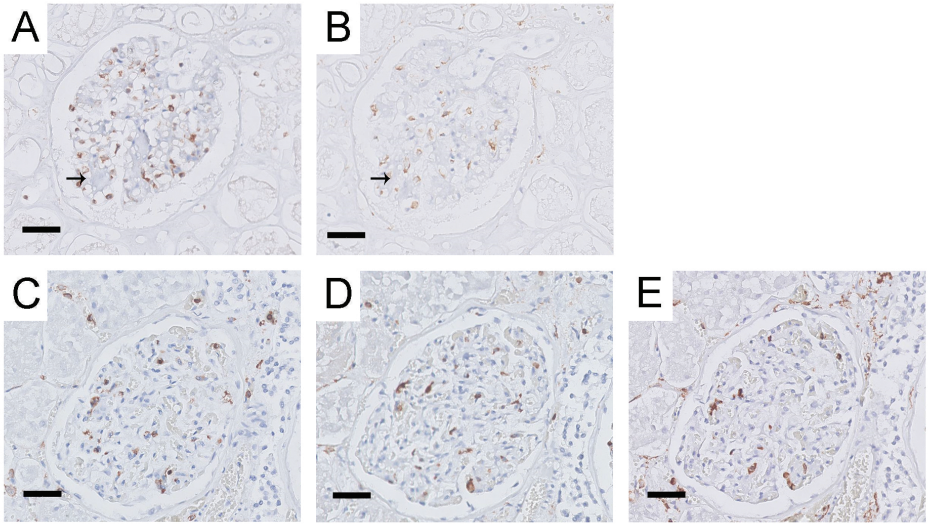
Clinical characteristics (N=88)	
Gender (% male)	47/88 (53.4)
Age (years)	70.6 ± 10.8
Duration of T2D (years)	12.7 ± 1.286
eGFR (ml/min/1.73m^2)	48.98 ± 3.696
Serum creatinine (µmol/L)	163 ± 11.826
HbA1c (% units)	8.56 ± 0.45
Proteinuria present[a]	36/55 (40.9%)
Systolic blood pressure, mmHg	136 ± 3.54
Diastolic blood pressure, mmHg	76.7 ± 1.55
Hb, (mmol/L)	7.0 ± 0.178
Death by CV event (%)	53.4
Ante mortem sepsis and/or renal insufficiency, N (%)	26 (29.5)

[a] Data regarding proteinuria were available for 55 patients only. Except where indicated otherwise, data are expressed as mean ± SEM. T2D, type 2 diabetes; Hb, hemoglobin; CV event, cardiovascular event

Glomerular macrophages and histological and clinical findings

In the group of 88 patients with T2D-associated DN, we counted the number of cells that stained positive for CD68 (a general marker of macrophages and monocytes) and CD163 (a scavenger receptor that is used as a marker of M2 or anti-inflammatory macrophages) in not globally sclerotic glomeruli. In all four histopathological classes of DN, both CD68+ and CD163+ macrophages were present in the glomeruli (Figure 1). When all four classes of DN were pooled, the mean number of CD68+ cells was 4.2 cells per glomerulus (median: 2.9; range: 0-19), and the mean number of CD163+ cells was 2.1 cells per glomerulus (median: 1.63; range: 0-14.74); the distribution of the mean number of cells/glomerulus in each sample is shown in Figure 2A. Thus, in each sample, the ratio of CD163+/CD68+ cells was approximately 1:2, indicating that 50% of the macrophages had a M2 phenotype. CD68+ macrophages were also present in the glomeruli of the samples obtained from the non-diabetic and diabetic control groups, with 5.5 cells per glomerulus (range: 0.9-15) and 4.5 cells per glomerulus (range: 2.3-6.8), respectively (Figure 2 B). In a subgroup of nine patients with DN, we stained for CD45 in sequential sections in order to confirm that the CD68+ cells were infiltrating leukocytes; CD68+ cells co-localized with CD45+ cells, confirming that the CD68+ cells were indeed inflammatory cells (Figure 1 C-E).

Figure 1. Accumulation of macrophages in the glomeruli of patients with type 2 diabetes and histologically proven diabetic nephropathy



A and **B:** Example immunostained renal sections obtained from a patient with class III DN. Sequential sections for CD68+ cells (**A**) and CD163+ cells (**B**) of a class III DN case with nodular sclerosis (arrow). **C-E:** Example immunostained renal sections obtained from a patient with class I DN. Sequential sections for CD45+ cells (as a control marker for infiltrating leucocytes) (**C**), CD68+ cells (**D**) and CD163+ cells (**E**)

No associations were found between the number of glomerular CD68+ cells and DN class (p=0.63), interstitial fibrosis and tubular atrophy (IFTA) (p=0.09), or global glomerulosclerosis (p=0.16) in the cohort of diabetics with DN, and similarly no association was found between the number of CD68+ cells and IFTA (p=0.68) or global glomerulosclerosis (p=0.46) in the diabetic without DN control group. In contrast, glomerular CD163+ cells were positively correlated with DN class (p=0.03), interstitial fibrosis and tubular atrophy (p <0.001), and global glomerulosclerosis (p=0.05). Renal function was inversely associated with glomerular CD68+ cells (p=0.017), but not with CD163+ cells (p=0.399). Finally, neither glomerular CD68+ cells nor glomerular CD163+ cells were associated with the presence of albuminuria (p=0.23, p=0.49, respectively) or with any other clinical parameters, including T2D duration (p=0.59, p=0.29, respectively), HbA1c (p=0.53, p=0.60, respectively), and cholesterol (p=0.60, p=0.45, respectively). Therapeutic regimens, including the use of RAAS blockers or oral diabetic medication, had no significant effect on the type or number of glomerular macrophages in our cohort. However, the use of RAAS blockers was correlated with interstitial macrophages (p=0.046) and

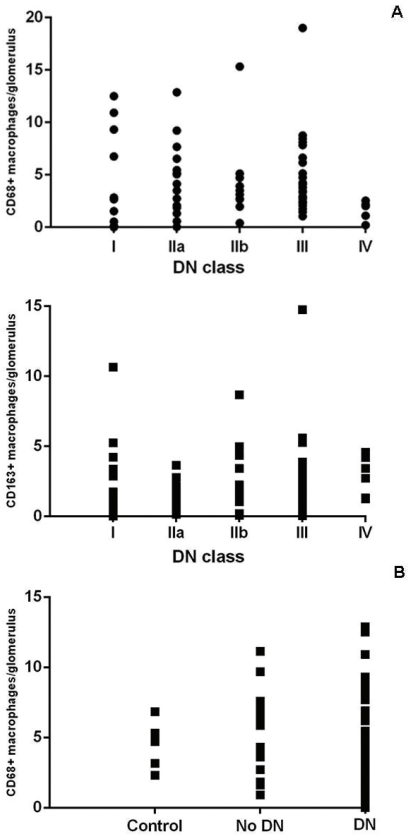


Figure 2. Glomerular macrophages in all four DN classes and control groups

Autopsy renal samples were sectioned and immunostained for CD68 or CD163, after which CD68+ and CD163+ cells were counted in 50 not globally sclerotic glomeruli per section. **A**) Distribution of the average number of CD68+ cells (upper plot) and CD163+ cells (lower plot) per glomerulus (N=88 patients). **B**) Average number of CD68+ cells per glomerulus in control non-diabetic subjects with no other renal abnormalities (N=5), diabetic patients without histologically proven DN ("No DN"; N=18), and type 2 diabetic patients with histologically proven DN (N=88). In each plot, each symbol represents an individual patient.

GFR (p=0.028). To correlate pathological lesions (e.g., IFTA, FSGS, cholesterol embolus, hyalinosis, and/or global glomerular sclerosis) with hypertension, we divided the cases into two groups containing hypertensive cases and non-hypertensive cases. Only the number of globally sclerotic glomeruli differed significantly between these two groups (p=0.004); however, these glomeruli were already excluded from further analysis.

Interstitial macrophages and histological and clinical findings

Next, we counted interstitial CD68+ macrophages using a semi-quantitative method. The semi-quantitative analysis of interstitial macrophages had substantial interobserver and intraobserver agreement (k=0.66 and 0.74, respectively). Our analysis revealed a significant positive correlation between the presence of these cells and the histopathological class of DN (p=0.026). The histological parameters (i.e., DN class, IFTA, and global glomerulosclerosis) correlated positively with interstitial CD68+ macrophages in the DN patient cohort (Table 2). With respect to clinical parameters, interstitial macrophages were significantly correlated with GFR stage, serum creatinine, and albuminuria (Table 3). In the diabetic control group, interstitial CD68+ macrophages were correlated with IFTA, but were not correlated with global glomerulosclerosis (Table 2). The multiple regression model showed that sepsis did not predict type or the number of glomerular CD68+ and CD163+ cells in patients with similar class of DN (p=0.995 and p=0.304, respectively). The multiple regression model showed that interstitial macrophages were associated with sepsis in patients with similar amount of IFTA (p=0.018). However, the correlations between interstitial macrophages and clinical parameters remained significant when cases with sepsis were excluded (Supplementary Table 1). Next, we divided the cause of death into cardiovascular (53.4% of cases), inflammatory (33%), and other (13.6%). No significant difference was found between the causes of death with respect to the number of macrophages in the glomeruli or interstitium (mean glomerular CD68+ cells, p=0.650; mean glomerular CD163+ cells, p=0.115; interstitial macrophages p=0.580).

Table 2. Correlation between histology and interstitial CD68+ macrophages

	T2D patients with DN (N=88)		Diabetic controls[a] (N=18)	
	rho[b]	*p*-value	rho[b]	*p*-value
DN class	0.236	0.03*	NA	NA
IFTA	0.494	<0.0001*	0.650	0.01*
Global sclerosis	0.444	<0.0001*	0.423	0.13

[a] Patients with diabetes but without DN (based on light and electron microscopy). [b] Spearman correlation coefficient. * Statistically significant ($p \leq 0.05$). DN class, histopathological DN classification; IFTA, interstitial fibrosis and tubular atrophy; NA, not applicable

Table 3. Correlation between clinical parameters and interstitial CD68+ macrophages

	rho[a]	*p*-value
GFR stage	0.302	0.009*
Serum creatinine, µmol/L	0.317	0.006*
Presence of albuminuria	0.292	0.03*
Microalbuminuria and Macroalbuminuria	0.293	0.03*
HbA1c (% units)	-0.266	0.14
Diabetes duration	-0.051	0.72
RAAS blockers	0.255	0.046*
Oral diabetic medication	-0.129	0.32

[a] Spearman correlation coefficient. * Statistically significant ($p \leq 0.05$). GFR stage, estimated glomerular filtration rate based on the KDIGO CKD guidelines; HbA1c, glycated hemoglobin; RAAS blockers, renin-angiotensin-aldosterone system blockers; NS, not significant, might be due to insufficient data

DISCUSSION

This study demonstrates the presence of both glomerular and interstitial macrophages in all four histopathological classes of DN in a relatively large cohort of T2D patients with histologically proven DN. Similarly, macrophages were also observed in control subjects. Interestingly, a subset of macrophages (specifically, anti-inflammatory CD163+ cells) was also seen in patients from all four histopathological classes of DN. The presence of glomerular CD163+ cells was positively associated with DN class, IFTA, and global sclerosis. Therefore, we speculate that the function of infiltrating macrophages becomes increasingly anti-inflammatory when the histopathological parameters become more severe. Based on our results, we cannot conclude whether the presence of macrophages is a reaction to or a mediator of renal damage. A higher number of interstitial CD68+ macrophages was correlated with higher histopathological class, increased IFTA, increased global glomerulosclerosis, decreased eGFR, and the presence of albuminuria.

The functional role of macrophages in the development of DN is not completely understood. Nevertheless, our results show the presence of macrophages with different phenotypes in the glomeruli of patients with DN. Interestingly, macrophages were present in the glomeruli of all three groups, including diabetic patients with DN, diabetic patients without histologically proven DN, and control subjects with no histological renal abnormalities. However, the patients in the two control groups had comorbid conditions, including hypertension, heart failure, and/or atherosclerosis, and these conditions could have played a role in the presence of macrophages in their renal samples; nevertheless, these groups were suitable controls, as these comorbid

conditions were also present among our cohort of patients with DN. These findings are consistent with the findings of Nguyen *et al.* [21], who reported the presence of macrophages in a small cohort of patients with diabetes as well as in non-diabetic controls. Moreover, Nguyen *et al.* found no difference between diabetic patients and controls with respect to glomerular CD68+ macrophages. Nguyen *et al.* also found a correlation between both glomerular and interstitial macrophages and progression to renal failure. However, no additional analysis was performed between subsets of macrophages. In contrast, our study, which was based on a large autopsy cohort containing more than 50 glomeruli per case, revealed that anti-inflammatory CD163+ macrophages were present in patients from all DN classes, including class I and class IIa.

The histopathological classification of DN has not been proposed as a model for progressive diabetic damage; rather, it has been offered as a description of the various histological lesions that can present in patients with DN. Several studies reported a robust association between the DN classification and renal outcome [22-24]. Moreover, Mauer *et al.* studied patients with type 1 DN and found a correlation between thickening of the glomerular basement membrane, the index of mesangial expansion, and albuminuria [29, 30]. In contrast, in our study of patients with type 2 diabetes and histologically proven DN, no correlation between thickening of the glomerular basement membrane (i.e., class 1 DN) and albuminuria was found. Moreover, we found no correlation between albuminuria and mesangial expansion (i.e., class II DN). This discrepancy may be explained by an increased heterogeneity of lesions among patients with T2D [31] due to the increased prevalence of additional damaging factors in T2D, including hypertension, aging, atherosclerosis, and dyslipidemia [32]. Because the influx of macrophages was similar in all four DN classes, inflammation may play a role in all diabetes-related lesions in T2D.

Despite the relatively low number of human studies performed to date [21, 33], compelling evidence obtained using experimental models of DN supports the accumulation of macrophages. For example, in both streptozotocin-induced and db/db mice, the accumulation of renal macrophages has been associated with the progression of glomerular and tubular damage [9, 14]. In the early stages of streptozotocin-induced DN, depleting macrophages with irradiation inhibits both the development of glomerular hypertrophy and the production of collagen IV [34]. In addition, a recent study found that M2-like (i.e., anti-inflammatory) macrophages can facilitate renal repair by increasing the expression of enzymes involved in matrix degradation [35].

The interstitial accumulation of macrophages is believed to positively or negatively affect the progression of renal disease [16, 35]. In several renal diseases, the accumulation

of these cells is closely correlated with progressive renal failure [36-38]. In addition, interstitial macrophage accumulation is an adverse prognostic finding in DN and correlates closely with the progression of renal insufficiency [21]. Interestingly, reducing the number of interstitial macrophages in experimental models decreases both tubulointerstitial injury and interstitial fibrosis [39]. In our patient cohort, the interstitial macrophages correlated significantly with decrease in renal function, suggesting that the influx of interstitial macrophages is associated with the progression of DN.

Given the consistent finding of macrophage involvement in both experimental models and human studies, we hypothesize that the influx of macrophages plays a role in the inflammatory pathway underlying DN. However, because no correlation was found between the number of glomerular macrophages and clinical findings or histological damage, their numbers cannot fully account for the difference in damage. Thus, glomerular macrophages may simply be innocent bystanders in the kidney. Alternatively, the expression profile of the macrophages—rather than their absolute numbers—may mediate glomerular damage. For example, in several renal diseases the differentiation of macrophages into pro-inflammatory, anti-inflammatory, or profibrotic cells can influence inflammation [40].

Our study has a few limitations that warrant discussion. First, because we used autopsy material, the presence of interstitial macrophages could have been influenced by ante mortem comorbidity. However, we believe this is unlikely, as interstitial macrophages were significantly correlated with clinical parameters measured long before death. In addition, no significant differences were observed between the cause of death and either glomerular or interstitial macrophages. Second, some clinical data were not available for some patients, thereby limiting our power with respect to measuring correlations between groups for some clinical parameters (e.g., albuminuria).

Despite the abovementioned limitations, we believe that this study has high clinical relevance, as emerging therapeutic approaches focus on inhibiting the inflammatory pathway as a mean to prevent renal failure in DN. Although, currently available treatments can slow the progression of diabetic complications, still many patients develop end-stage renal disease. Recently, two clinical trials revealed that treating T2D patients with an MCP-1 inhibitor or an inhibitor of its receptor (C-C chemokine receptor type 2, CCR2) attenuated proteinuria [41, 42]. This finding underscores the important role that inflammation plays in DN, and it highlights the potential of anti-inflammatory therapy. Therefore, obtaining insight into the presence and the various phenotypes of macrophages in the kidneys of T2D patients is essential for optimizing this therapeutic approach.

In conclusion, this study showed that macrophages were present in both the glomeruli and interstitium of a clinically heterogeneous cohort of patients with type 2 diabetes, regardless of the stage of DN. Despite the lack of difference in the number of glomerular macrophages between patients and controls, the number of glomerular CD163+ macrophages (M2-like, i.e., anti-inflammatory) is associated with nodular sclerosis, global glomerulosclerosis, and interstitial fibrosis. Together with the correlation between the number of interstitial macrophages and IFTA, DN class, and renal function, this finding suggests that the number of macrophages may play a role in the progression of DN. Although future studies are needed to determine the precise role that macrophages play in DN, macrophages may serve as an effective therapeutic target for inhibiting the progression of renal damage in patients with DN.

DISCLOSURES

None.

ACKNOWLEDGEMENTS

This study was supported by a Junior Postdoc (Project code: 14OKG06) and Kolff Student Research Grant (Project code: 14OKK12) from the Dutch Kidney Foundation. CQFK and DHTIJ collected and analyzed data. MZ and CQFK carried out experiments. DHTIJ, CQFK and IMB analyzed histological data. RW helped designing and performed statistical analyses together with CQFK. RW, JAB, TJR, IMB, and DHTIJ were involved in the study design. All authors were involved in writing the paper and had final approval of the submitted and published versions. The authors have no conflict of interest.

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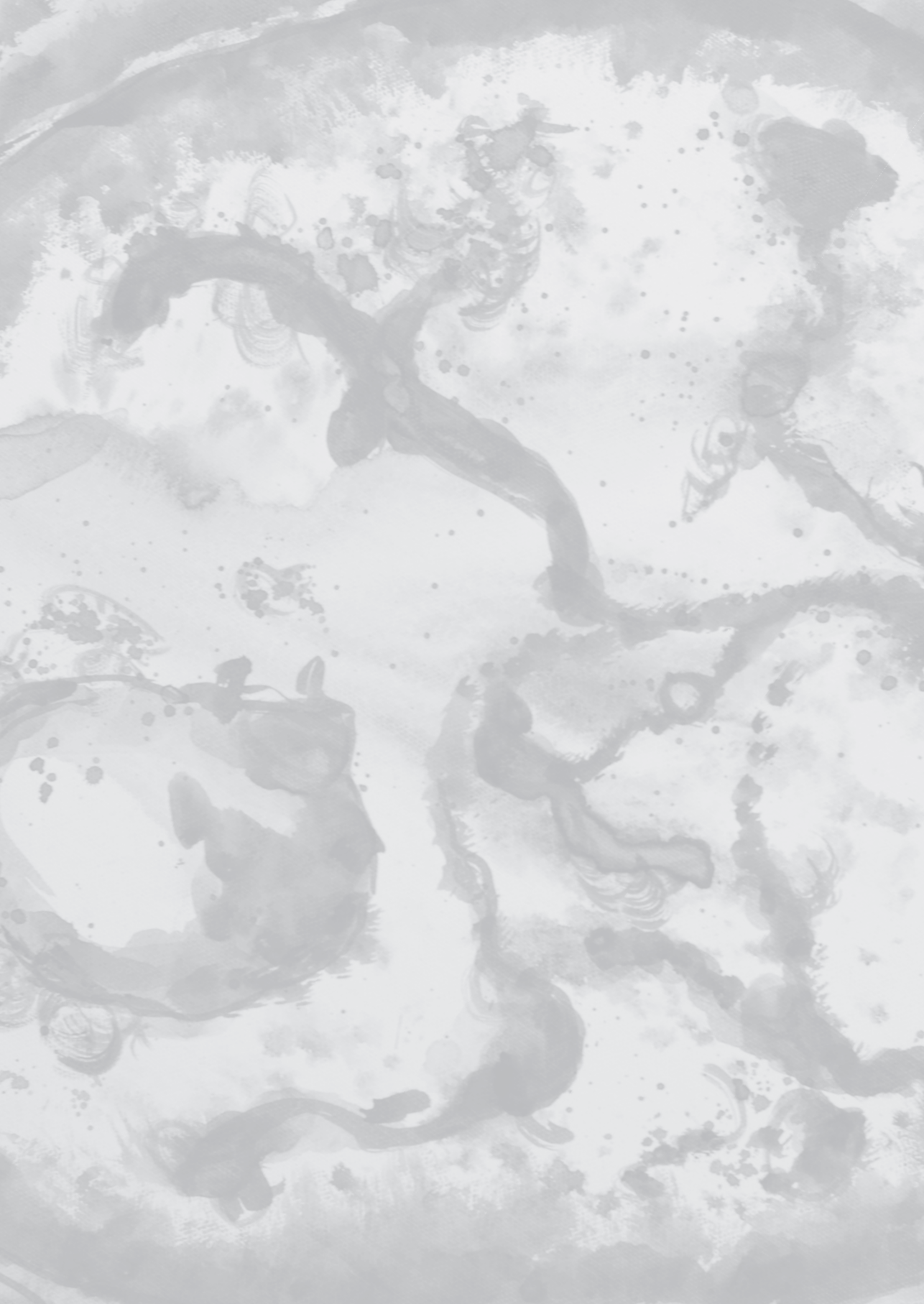
SUPPLEMENTARY TABLES

Supplementary Table 1. Correlation between clinical parameters and interstitial CD68+ macrophages in patients without sepsis

	rho[a]	*p*-value
GFR stage	0.303	0.035*
Serum creatinine	0.265	0.068
Presence of albuminuria	0.487	0.003*
Microalbuminuria and Macroalbuminuria	0.497	0.002*
HbA1c (% units)	-0.147	0.524
Diabetes duration	-0.034	0.848
RAAS blockers	0.358	0.023*
Oral diabetic medication	0.047	0.775

[a] Spearman correlation coefficient. Statistically significant ($p \leq 0.05$)

GFR stage, estimated glomerular filtration rate based on the KDIGO CKD guidelines; HbA1c, glycated hemoglobin; RAAS blockers, renin-angiotensin-aldosterone system blockers



Pathologic classification of diabetic nephropathy; reproducibility and validation

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ABSTRACT

In 2010, a pathological classification for diabetic nephropathy (DN) was developed. Most validation studies of this classification showed a significant association with renal outcome, but either compared different combinations of classes with outcome or had insufficient power. An adequate reproducibility study of this classification system is missing. Therefore, we performed a reproducibility study together with a meta-analysis study for all validation studies to better estimate the prognostic role of the different DN classes.

In order to asses agreement for reproducibility, a DVD with 13 digitized biopsies of DN patients was sent to all members of the Renal Pathology Society. The interobserver agreement was determined by intraclass correlation values (ICC). Additionally, data was extracted from the validation studies and a meta-analysis for the different DN classes was performed.

The ICC for DN class was 0.74. Additional parameters had the following ICCs: IFTA: 0.72; interstitial inflammation: 0.47; arteriolar hyalinosis: 0.41 and arteriosclerosis: 0.43. ICCs of all pathologists did not differ substantially from ICCs of pathologists with a high level of expertise. The meta-analysis showed an increased risk for a poor renal outcome for each of the classes IIb, III and IV compared to class IIa ($p<0.0001$).

This study shows that the DN classification is suitable for clinical practice. Our meta-analysis data show a good relation with renal prognosis over the classes of DN as currently defined. Furthermore, the DN classification has relatively good reproducibility although improvements could be made by fine-tuning definitions.

INTRODUCTION

Pathological classification systems exist for several renal diseases such as lupus nephritis [1], IgA nephropathy [2] and ANCA-associated glomerulonephritis [3]. In 2010, a classification system was developed for diabetic nephropathy (DN) [4]. This classification is primarily based on glomerular damage whereas interstitial and vascular lesions are scored separately. The classification system can be used for DN in both type 1 and type 2 diabetes mellitus patients.

In general, classification systems are created to provide better communication between pathologists and clinicians, and the possibility to link diagnostic information together with prognostic indication in order to guide therapeutic decision making. By means of subsequent studies focusing on the clinical validation and interobserver agreement of classification systems, the systems are continuously modified and improved.

For the pathological classification of DN, several validation studies of the DN classes showed an association with renal outcome. However, all these studies combined classes, and moreover, combined them differently to have adequate power to find an association with outcome [5-9]. The reproducibility of the DN classification was investigated in the original article and one validation study, but these reproducibility studies were relatively small [4, 8].

Therefore, the aim of the present study was to perform an adequate reproducibility study by means of a survey of members of the Renal Pathology Society (RPS). Based on the data obtained, we here provide suggestions for clarifications of some of the definitions. Furthermore, we performed a meta-analysis study for all validation studies published to date, to adequately estimate the prognostic role of the different DN classes.

METHODS

Reproducibility study; Case selection and survey

For this study 13 biopsies of DN patients were selected from the archives at Leiden University Medical Center. The renal damage within these cases was exclusively attributable to DN. The representative cases contained the full spectrum of lesions which can be found in DN. For each case high quality Periodic Acid-Schiff (PAS) and silver stains were provided. The biopsies were handled, coded and anonymized according to the Dutch National Ethical guidelines.

In order to assess interobserver agreement, slides were digitalized and saved as an HTML-document on a DVD. This DVD was sent to all 360 members of the RPS with an invitation to participate in the project. Additionally, an Excel response sheet with a flowchart of the classification, instructions, and the original article of the classification were provided on the DVD [4]. The participants were asked to add remarks when difficulties occurred while scoring. The participating pathologists were requested to assess their level of expertise by which they were divided into the following categories: all pathologists; the subgroup of pathologists with a high level of expertise; the subgroup of pathologists with low or moderate expertise and those who did not mention their level of expertise by self-assessment.

Pathological classification system of DN

All cases were categorized based on the pathological classification of DN as previously described. For the exact details of the DN classification, we refer to the original paper [4]. In brief, the classification encompasses 4 classes: class I, glomerular basement thickening by electron microscopy without specific light microscopic changes; class IIa, mild mesangial expansion (mild mesangial expansion in >25% of the observed mesangium); class IIb, severe mesangial expansion (severe mesangial expansion in >25% of the observed mesangium); class III, at least one lesion with nodular sclerosis; class IV global glomerulosclerosis in > 50% of glomeruli.

Interstitial fibrosis and tubular atrophy (IFTA) and interstitial inflammation are scored on a semiquantitative scale. Arteriolar hyalinosis is scored 0 when it was absent; 1, if at least one arteriole with hyalinosis is present and 2, if more than one arteriole with hyalinosis is observed in the entire biopsy. Arteriosclerosis is scored as follows: 0 for no intimal thickening, 1 for intimal thickening less than the thickness of the media, and 2 for intimal thickening more than the thickness of the media.

Eligibility criteria, data selection and extraction of validation studies

To provide an overview of clinical outcome studies in relation to the pathological classification of DN, all articles citing the original manuscript were collected via a search on Web of Science and Google Scholar. To ensure maximum sensitivity, no limits or filters were used in the searches. Language restrictions were not included in the initial search. This search was performed by a trained librarian in April 2016. Two observers (CK and LV) independently reviewed all studies to include all validation studies of the pathological classification of DN, which associated DN class with renal outcome. Furthermore, an expert of the field (IB) was consulted to ensure that all validation studies investigating DN class were included. Included were studies investigating type 1 and 2 diabetes correlating DN class with renal outcome. Renal outcome was defined as end stage renal disease (ESRD) or doubling of se-

rum creatinine. Studies were excluded which only investigated interstitial lesions or which used the same cohort in relation to different clinical outcome parameters. In the latter case, we included the first published validation study in our meta-analysis. Data were extracted if possible. Preferably hazard ratios were extracted with confidence intervals, otherwise these data were extrapolated from Kaplan-Meier curves or data on absolute risks.

Statistical analysis

For all the parameters in this study a reliability analysis was conducted by calculating the intraclass correlation coefficient (ICC) (0=no agreement, 1=perfect agreement). In this calculation, the given answers were compared between participants rather than comparing the answers with a 'gold standard'. ICCs were calculated using a mixed model to estimate the variance components of the ICC. An ICC of >0.75 was considered to show excellent reproducibility, ICC of 0.4 to 0.75 to indicate fair to good reproducibility, and ICC of < 0.4 to indicate poor reproducibility [10]. These analyses were performed using SPSS statistics 20.0 (IBM, Armonk, NY). For the meta-analysis of the validation study a generic-invariance method was used in a random effects model and analyses were performed in ReviewManager (RevMan) version 5.3. Preferably hazard ratios from the individual studies were used with class IIa as a reference group. If these were not available, relative risks and standard errors were calculated with the available data. Heterogeneity within the studies was estimated by the I^2, which is the percentage of the total variation across studies due to heterogeneity rather than chance. An I^2 of 25%, 50% or 75% was considered low, moderate or high, respectively.

RESULTS

Reproducibility study

A total of 13 biopsies with lesions attributable to DN were scored by 77 pathologists from 28 different countries, of which 38 (49%) had a self-assessed high level of expertise, 19 (25%) had a moderate level, 3 (4%) had a low level and 21 (22%) did not mention their level of expertise. The response rate of the reproducibility study was 21.4% (77/360).

Table 1. Intraclass correlation (ICC) of pathologic classification of DN

ICC score	All pathologists	Pathologists with high expertise level
DN class	0.74	0.76
IFTA	0.72	0.73
Interstitial inflammation	0.47	0.57
Arteriolar Hyalinosis	0.41	0.43
Arteriosclerosis	0.43	0.44

ICCs of all pathologists did not differ substantially from ICCs of pathologists with a high level of expertise

Table 1 gives an overview of intraclass correlations (ICC) of the lesions scored by all participating pathologists and the ICC of a subgroup of pathologists with a high level of expertise. ICCs of all pathologists did not differ substantially from ICCs of pathologists with a high level of expertise The ICC score for glomerular lesions (i.e. DN class) amongst all pathologists was 0.74; amongst pathologists with high expertise it was 0.76. The best concordance was found in class III and IV; the least concordance was found in class I and II, and in the subdivision of class II.

IFTA had an ICC score of 0.72. Severe IFTA cases had more concordance compared to cases with mild IFTA involvement. Regarding the vascular lesions, arteriolar hyalinosis had an overall reproducibility of 0.41. Arteriosclerosis had an overall interobserver agreement of 0.43. The participating members of the RPS could provide separate remarks in addition to the scoring system. Table 2 provides an overview of certain remarks, followed by our recommendations to clarify these definitions.

Meta-analysis on validation studies

The initial search found 258 studies, of which 240 studies were excluded because these were case reports, reviews, or studies which used the classification system in an experimental setting, but were not validation studies. Finally, 12 studies were regarded as possible validation studies, however, some only investigated IFTA with renal outcome or the same cohort was used in more than one study. Therefore, in the end, 4 validation studies were included in the meta-analysis (Figure 1). The basic characteristics of these studies are provided in Table 3. In the study of Mise *et al.* and Okada *et al.* data including the hazard ratios with confidence interval (of which the standard error could be calculated) were available. In the study by Okada *et al.* no data for class I were available. In the study by Oh *et al.* the described absolute risks were used to calculate an relative risk and standard error. In the study by An *et al.* there was a hazard ratio and confidence interval available for all classes together. The hazard ratio was extrapolated for all independent classes from this hazard ratio and the standard error was calculated from the relative risk which was extrapolated from the Kaplan-Meier curves.

Table 2. Recommendations survey for DN classification

		Remarks	Recommendations
Glomerular lesions	*DN class*	Dividing class I and II: definition and cut-off points criticized as difficult	Define more straightforward definitions for mesangial alterations and examine these in future validation studies.
		Class III: only one nodule in cases with overall mild mesangial expansion	The formation of nodular sclerosis may be a specific trait of some patients with DN, who are not yet more distinctly defined Therefore, recognition of one nodule seems appropriate to designate a specific class of DN. Results from the meta-analysis indicate specific outcome for this class.
Interstitial lesions	*IFTA*	IFTA is a good predictor for renal outcome, but can be the result of other renal diseases	Take the severity of IFTA into account and specifically note in all biopsy reports
	Interstitial inflammation	The relatively low ICC of interstitial inflammation	Clarification of this definition: only score inflammation in areas without IFTA. Determine the relative effects of interstitial inflammation in non-scarred areas versus total interstitial inflammation on the prognosis of DN in further studies.
Vascular lesions	*Arteriolar hyalinosis*	Unclear in which vessel type arteriolar hyalinosis needs to be scored	Just identify and mention hyalinosis if present; most of the hyalinosis will occur in arterioles.
	Arteriosclerosis	What is the definition of a large vessel	Eliminate the vessel size from the definition, and to focus only on the presence of intimal thickening/fibrosis in vessels which are larger than arterioles

Based on the remarks obtained from the reproducibility study, recommendations of each parameter were proposed to improve the use of the DN classification

Figure 2 shows the results of our meta-analyses of the validation studies. The validation studies resulted in a pooled hazard ratio of class I versus IIa of 0.49 (95% C.I. 0.13-1.90, p=0.30). The pooled hazard ratio of class IIa versus class IIb was 2.96 (95% C.I. 1.82-6.05, p<0.00001), showing an increased risk for developing a poor renal outcome in patients with class IIb compared to IIa. For class IIa versus class III a significant difference also was seen (p<0.00001) with a pooled hazard ratio of 5.26 (95% C.I. 2.75-10.04, p<0.00001), showing that patients with class III have an increased risk of a poor renal outcome compared to class IIa. Finally, class IV versus IIa showed a poorer renal prognosis for class IV, hazard ratio 11.23 (95% C.I. 4.56-27.68, p<0.00001). There is low to moderate heterogeneity between the groups (class IIa vs I, I^2= 0%, class IIa vs IIb, I^2= 0%, class IIa vs III, I^2= 49% and class IIa vs IV, I^2= 67%).

Figure 1. Flowchart illustrating how the validation studies of the DN classification were selected for the meta-analysis

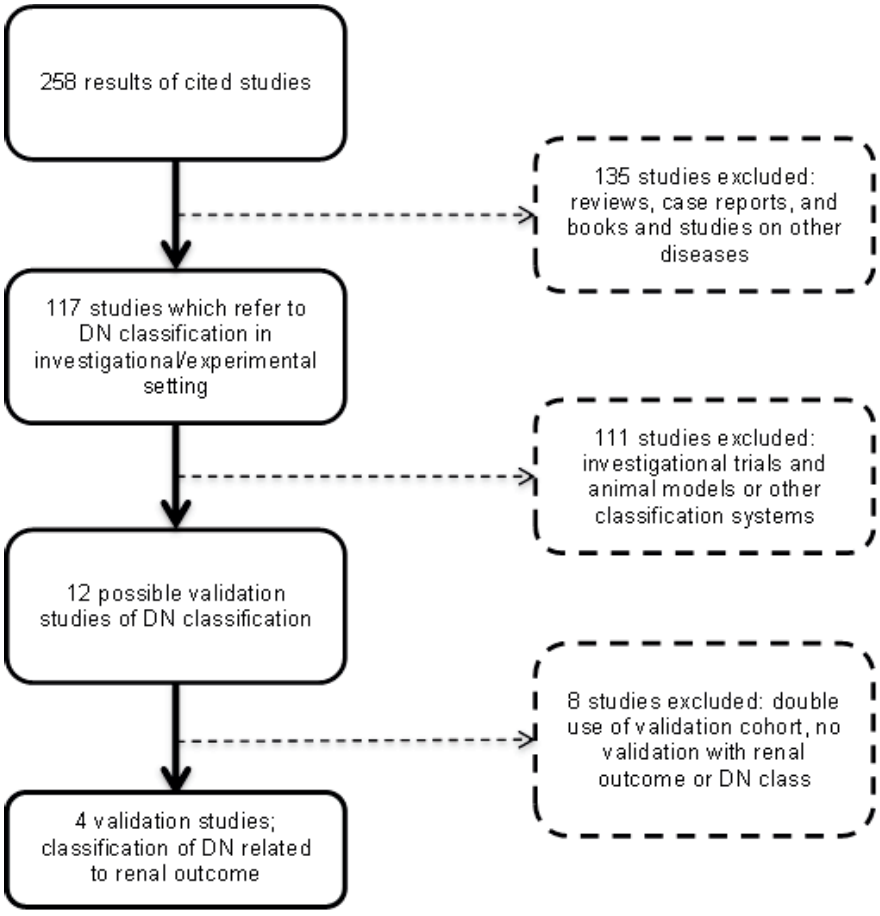
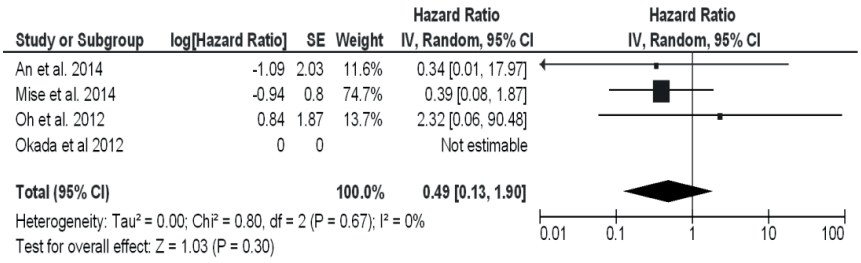


Table 3. Overview of validation studies using the histopathological classification of DN

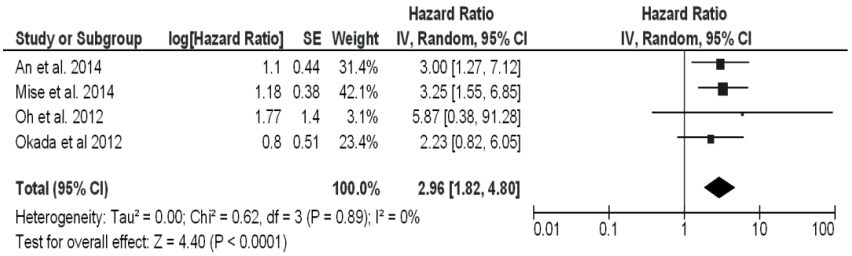
Study	Diabetes type	Number of patients	Ethnicity	Patient characteristics	Follow up	Renal outcome
Okada *et al.* 2011	Type 2 diabetes	N= 69	Japanese	Patients with overt proteinuria and biopsy-confirmed diabetic nephropathy with mesangial expansion	Mean and median follow-up duration was 59 +/-41 and 52 months (range 6 – 180 months)	Chronic dialysis or doubling of serum creatinine
Oh *et al.* 2012	Type 2 diabetes	N= 126	Korean	50 patients with pure DN, 65 with non diabetic renal disease and 11 mixed. Only 50 pure DN patients scored following the DN classification	Follow-up during 69.2 +/- 35.2 (0.4–137.6) months after renal biopsy to detect ESRD	End-stage renal disease
Mise *et al.* 2014	Diabetes mellitus	N= 205	Japanese	Patients with renal biopsy and inclusion study criteria (eGFR>10mL/min/1.73m^2, biopsy>10 glomeruli)	The mean follow-up period was 62.9 ± 68.3 months	Renal death defined as dialysis by end-stage renal disease
An *et al.* 2014	Type 2 diabetes	N= 396	Chinese	Patients with biopsy proven DN	At least one year follow up	Renal outcome defined as progression to end-stage renal disease or doubling of serum creatinine

Figure 2. Meta-analysis of DN classes of the performed validation studies at 80 month follow-up

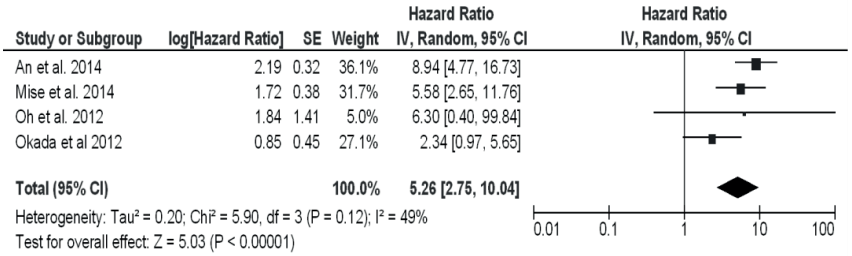
IIa v I



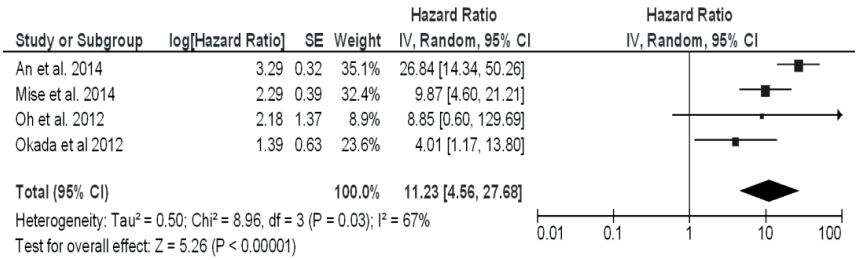
IIa vs IIb



IIa vs III



IIa vs IV



DISCUSSION

In 2010, we launched the histopathological classification for diabetic nephropathy, which since then has been used in multiple research and diagnostic settings. In general practice, nephropathologists worldwide make use of the classification, but fine-tuning of some of the definitions of lesions may be appropriate. Therefore, a survey was launched through the RPS to obtain insight into interobserver agreement amongst nephropathologists worldwide, and issues related to day-to-day practical issues. With regards to definitions, we here summarize the comments from RPS members who joined in our survey. Whereas reproducibility was proven to be sufficient for classes III and IV, disagreement among observers was noticed for class I, IIa and IIb. Although many studies citing the classification are present in the literature, a careful investigation showed that actually only a limited number of validation studies had been conducted, i.e. those with a specific aim to validate the classes of the DN classification. All these studies came from Asia. We also performed a meta-analysis of these studies, showing that the DN classification has a correlation with renal outcome for most classes using class IIa as a reference, which underscores the clinical usefulness of the classification system. For class I versus IIa there was no significant difference found in this meta-analysis. This is likely explained by the fact that the available studies were underpowered for this comparison as only a small number of patients were investigated with only few events (especially in class I).

Because the meta-analysis showed a good association with renal outcome for most classes, it seems appropriate to maintain the subdivision as previously described. However, more straightforward definitions for mesangial alterations to distinguish between class I, IIa and IIb are called for, but need to be examined in future validation studies using modifications of the original definitions that would be tested for both interobserver agreement and correlation with clinical outcomes. For the moment, no clear-cut suggestions came out of the survey for an intermediate solution.

With respect to classes III and IV, there was good interobserver agreement in the recognition of these classes and comments from participants were few. There was a concern from some participants whether the presence of one nodule was sufficient to classify a sample as class III DN, especially in cases with overall mild mesangial expansion. We postulate that the formation of nodular sclerosis may be a specific trait of some patients with DN, who are not yet more distinctly defined [11]. Therefore, recognition of one nodule still seems appropriate to designate a specific class of DN (i.e. class III), and results from the meta-analysis indicate a specific outcome for this class by its current definition.

Our reproducibility study showed a high ICC for IFTA, similar to findings in the original Oxford IgA nephropathy study [2]. A common remark from survey participants was that IFTA could have been the result of other renal diseases. The point about IFTA being a good predictor for renal outcome despite its nonspecific appearance in virtually all renal diseases has been frequently raised. IFTA is not a primary parameter of the DN classification, but several validation studies on IFTA showed that IFTA has impact on renal prognosis in diabetic nephropathy [5, 7-9, 12]. Because of the prognostic value of IFTA it is certainly useful to take the severity of IFTA into account during evaluation of the biopsy, and the amount of IFTA should be specifically noted in all renal biopsy reports in cases of DN. The ICC of interstitial inflammation was remarkably low. This could be the result of lack of clarity in the definition of whether or not to score inflammation in areas with or without IFTA. In concordance with other guidelines for scoring inflammation in renal diseases, it would seem appropriate also in DN only to score inflammation in areas without IFTA. Future studies need to clarify the definition of interstitial inflammation and should determine the relative effects of interstitial inflammation in non-scarred areas versus total interstitial inflammation on the prognosis of diabetic nephropathy.

According to the original manuscript of the DN classification, arteriosclerosis needs to be scored in large vessels. However, the definition of a large vessel was perceived as unclear. A straightforward and simple solution would be to eliminate the vessel size from the definition, and to focus only on the presence of intimal thickening/fibrosis in vessels which are larger than arterioles. In addition, the vessel type and/or size in which arteriolar hyalinosis needs to be scored was also not evident. A straightforward solution here would be just to identify and mention hyalinosis if present – given that the lesion is relatively easy to recognize in a PAS-staining – and most of the hyalinosis lesions will occur in arterioles.

In the present study we reflect on problematic issues of the DN classification by using a two-way approach, namely through a survey of renal pathologists from the RPS and by reviewing the literature by means of a meta-analysis of validation studies so far performed. Each of these routes has its own limitations. The survey approach may have suffered from a bias in response due to factors outside our control, because RPS members could become involved in this part of the study by their own initiative. Nevertheless, participants in this study on diabetic nephropathy appeared to reflect the RPS membership relatively well with regards to the participants' distribution among different countries and with respect to the range of experience among our participants. During the evaluation of the validation studies included in our meta-analysis, we observed some limitations of these studies that merit discussion. The performed validation studies had different study designs and all glomerular classes were not included in

all studies. For example, Okada *et al.* did not include class I in their study [7]. Some of the studies, especially by Oh *et al.* and Okada *et al.* were very small. Furthermore, all studies were performed in Asian populations, and therefore it could be debated if these data can be fully extrapolated to other populations.

This study shows that the pathologic classification of DN has relatively good reproducibility but improvements can be made by fine-tuning definitions. Our meta-analysis data showed a good relation with renal prognosis over the classes of DN as currently defined. On the basis of results from a two-way approach into reproducibility and prognostic value of the classification, we have listed the current issues with recommendations here. In an international workgroup on DN we are currently working on the modifications of the DN classification.

ACKNOWLEDGEMENTS

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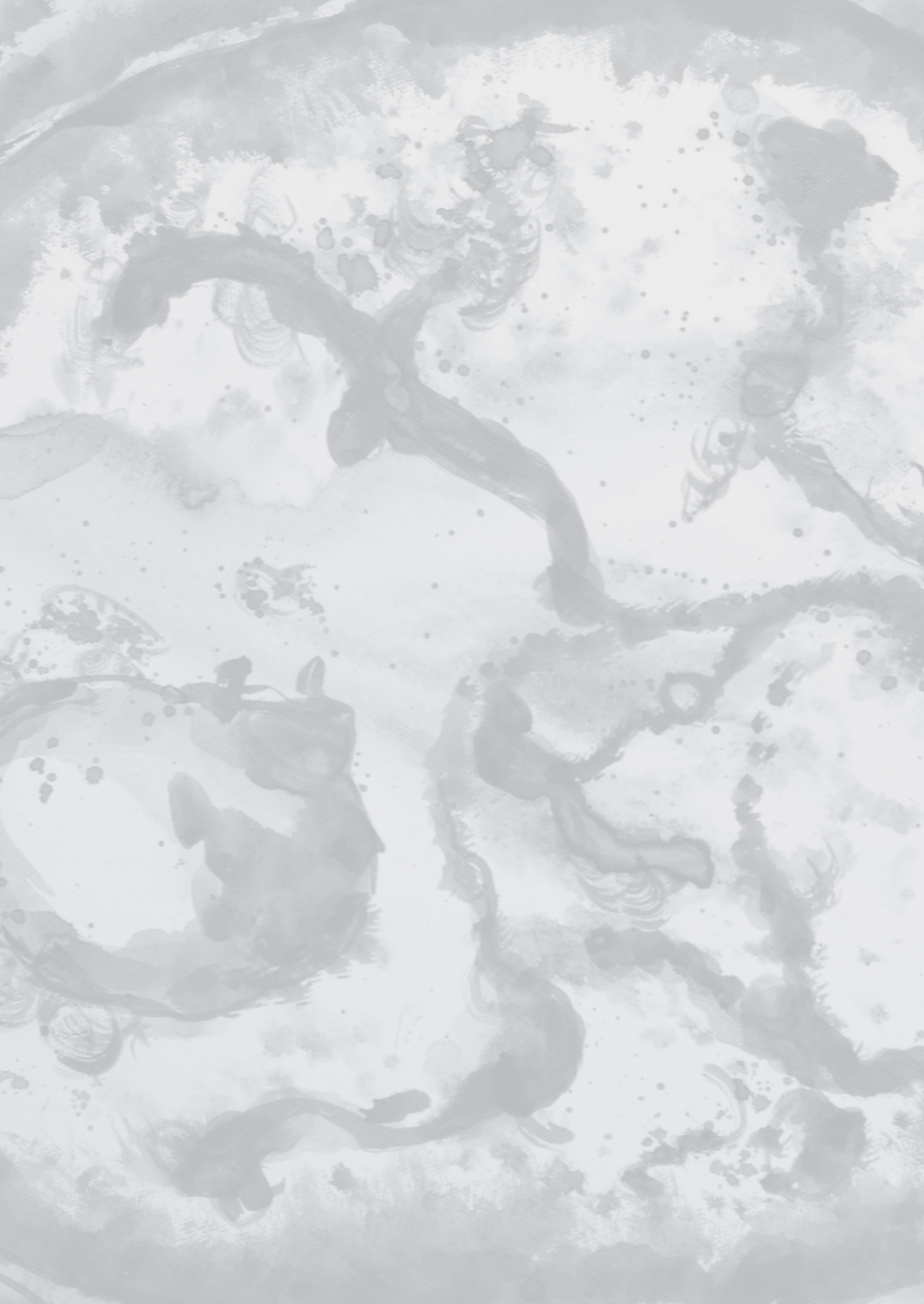

Histologically proven diabetic nephropathy is associated with a leucine repeat of the *CNDP1* gene

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Submitted

ABSTRACT

The 5-5 homozygous *CNDP1* genotype is associated with a reduced risk for diabetic nephropathy (DN) in patients with type 2 diabetes, based on studies relying on clinically diagnosed DN. The present study investigates whether this association can be confirmed in diabetic patients with histologically proven DN.

Renal autopsy tissue specimens of 204 diabetic patients from Leiden University Medical Center were used to determine the glomerular lesions according to the pathological classification of DN. The *CNDP1* genotype was determined from DNA isolated from spleen and/or liver paraffin-embedded material of these cases.

136 samples were included in the study. 63% of the renal tissue specimens had glomerular lesions conform the pathological DN classification. The frequency of 5-5 leucine repeats *CNDP1* was significantly different between patients with and without histologically proven DN (p=0.031). Furthermore, the 5-5 leucine repeats of *CNPD1* gene was associated with the presence of nodular sclerosis (p=0.013).

This study shows that there is an association between the *CNDP1* gene and DN in histologically proven DN, which confirms the findings of previous studies based on patients with a clinical diagnosis of DN. The *CNDP1* gene may play a role in the development of nodular sclerosis. The direct link between the *CNDP1* genotype and the development of DN remains to be elucidated. However, our results indicate that the *CNDP1* genotype could serve as a genetic biomarker to identify diabetic patients with high risk profiles of DN.

INTRODUCTION

Several studies have shown a reduced susceptibility for the development of diabetic nephropathy (DN) in patients with type 2 diabetes of Caucasian origin and homozygosity for the five leucine repeats of the carnosinase (*CNDP1)* gene compared to diabetic patients with six to eight leucine repeats of the *CNDP1* gene [1-3]. In these studies, DN was diagnosed on the basis of clinical findings; none of these studies performed renal biopsies to confirm the diagnosis of DN by histology. The presence of more than five leucine repeats of *CNDP1* has been shown to be associated with higher levels and activity of serum carnosinase [3, 4]. Serum carnosinase degrades carnosine and other histidine-containing dipeptides. Carnosine has multiple beneficial properties: it is a reactive oxygen scavenger [5], a natural angiotensin converting enzyme (ACE) inhibitor [6], it degrades advanced glycation end products (AGEs) [7], it reduces the synthesis of extracellular matrix components, and it reduces transforming growth factor (TGF)-β in renal cells [1]. These factors have in common that they are disturbed in DN. Therefore, Freedman *et al.* [2] suggested that carnosine and the *CNDP1* gene play a role in the susceptibility to DN in type 2 diabetes.

Glomerular lesions attributable to DN can be classified into four groups according to the pathological classification proposed by Tervaert *et al.* [8]. The exact pathogenesis of these diabetic glomerular lesions remains incompletely understood, especially the development of nodular sclerosis [9], which is scored as Class III in the pathological classification. It has been reported that nodular sclerosis may have a different pathogenesis from more widespread mesangial expansion [9]. However, little is known about the development and variable structures of these nodules [10, 11]. Schwartz *et al.* [11] questioned why some patients with DN develop nodules whereas others do not, especially given that their clinical manifestations are undistinguishable. It might be that a genetic determinant influences the development of nodular sclerosis. Makino *et al.* [12] showed that AGEs, which can be degraded by carnosine, play a role in the development of nodules through impairment of assembly of matrix proteins.

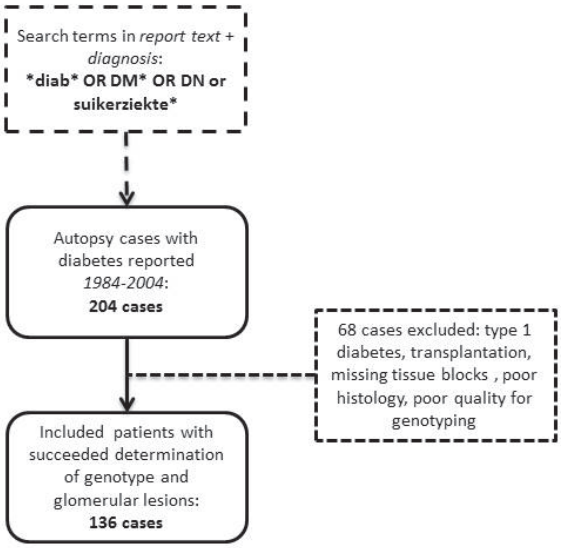
The aim of the current study is to investigate the association between *CNDP1* polymorphism and DN in type 2 diabetes patients with histologically proven DN. In addition, we investigate whether there is an association between *CNDP1* polymorphism and the occurrence of nodular sclerosis.

METHODS

Study design and methods

Patients with diabetes were included retrospectively after a search in the database of the pathology archives from autopsies performed at Leiden University Medical Center between 1984 and 2004. The primary inclusion criteria were the presence of type 2 diabetes in patients who were older than 18 years at the time of autopsy and who had not had a renal transplantation. All samples were handled according to the code of conduct of responsible use. Cases from who the tissue blocks were missing or with poor renal histology due to autolysis were excluded from the study (Figure 1). We collected the paraffin-embedded renal tissue blocks for histological evaluation of the glomerular lesions. Paraffin-embedded tissue blocks from spleen and/or liver were collected for DNA isolation to perform *CNDP1* genotyping of the included patients.

Figure 1. Flowchart of search strategy



The search terms were entered in the database of the pathology archives. The inclusion of patients was based on two subsequent searches to increase the power of the study population

Renal tissue evaluation

Renal tissue was cut at 1- and 3-µm thickness and stained with haematoxylin and eosin, periodic acid–Schiff, and silver stain (3-µm, 3- µm and 1-µm thick sections respectively). Glomerular lesions were scored in renal tissue specimens from autopsy cases containing 100 or more glomeruli by two investigators who were blinded with respect to the genetic profile and clinical data of the patients. The glomerular changes were classified

according to the pathological classification of DN [8]. First, renal tissue specimens were observed by light microscopy. When no light microscopic changes were observed electron microscopic evaluation was performed to measure the glomerular basement membrane (GBM) thickness. Class I, based on electron microscopic measurements, is characterized by thickening of the GBM (GBM width >395nm in females and >430 nm in males). since reprocessing of paraffin tissue for electron microscopy causes artefactual GBM thinning, a correction adding 34 % was used, as described by Nasr *et al*. [13] who showed that this correction is needed to correct for the GBM width in paraffin embedded-material of DN. Taking this calculation into account, the cut off levels described in the histopathogical classification of Tervaert *et al.* [8] could then be used in our study. The remaining classes were evaluated by light microscopy; Class II is characterized by mesangial expansion, subdivided into Class IIa with mild mesangial expansion in >25% of the observed mesangium) and Class IIb with severe mesangial expansion (severe mesangial expansion in >25% of the observed mesangium; Class III is characterized by nodular sclerosis and Class IV by more than 50% global glomerulosclerosis.

CNDP1 genotyping

To determine the CNDP1 genotype, DNA was extracted from paraffin-embedded spleen and/or liver tissue with the automated TPS DNA extraction system (Siemens). A standard PCR protocol was performed with a 5'FAM-labelled forward primer (GC-GGGGAGGGTGAGGAGAAC) and a standard reverse primer (CCCTTCCAGGCTGCGTCC), as described elsewhere [1]. The denaturating, annealing and extension temperatures were 95°C, 60°C, and 72°C, respectively. Fragment analysis was performed on the ABI-3130 analyzer (Perkin Elmer) to determine the number of leucine repeats on each allele. Genemapper® Software was used to determine the CNDP1 polymorphisms and the results were independently analyzed by two investigators.

Statistical analysis

IBM SPSS Statistics, version 20.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses. The 5-5 homozygous *CNDP1* genotype was compared with six or more leucine repeats (5-6, 5-7, 6-6, 6-7, 7-7) of *CNDP1* genotype [3] in patients with and without histologically proven DN. Statistical differences between groups were analyzed using chi-square tests. Difference with a *p*-value less than 0.05 was considered statistically significant.

RESULTS

In this study 136 autopsy cases with type 2 diabetes were included. The baseline characteristics of the included patients are described in Table 1. The mean age was 71 years and 53% of the patients were male. The duration of diabetes was known from 67 patients and the mean duration was 11 years.

Table 1. Baseline characteristics of the included patients

Baseline characteristics (N=136)	
Gender (% male)	53
Age (years)	71 ± 10.6
Diabetes duration (N=67)	11 ± 9
5-5 leucine repeats *CNDP1* gene (%)	38

Data are expressed as mean ± standard deviation, except where indicated otherwise. Diabetes duration expressed in years

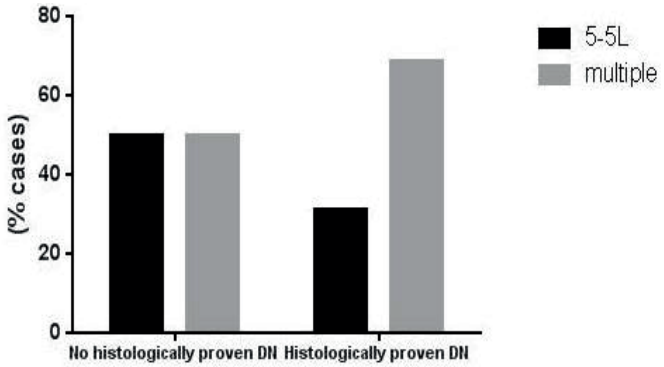
The histological evaluation of the glomeruli revealed that 63% (86/136) of the cases had histological changes according to the pathological classification of DN. In 50 renal tissue specimens no glomerular lesions were observed by light microscopy or electron microscopy and were designated as Class 0. Electron microscopy revealed Class I DN in 18 cases, characterized by thickening of the GBM. Light microscopic evaluation revealed that 26 samples had a class II DN, characterized by mesangial expansion; 16 cases had mild mesangial expansion, designated as Class IIa DN and 10 cases had severe mesangial expansion, designated as Class IIb DN. 36 samples had a class III DN, characterized by nodular sclerosis. The remaining 6 cases had a class IV DN, characterized by more than 50% of glomeruli with global sclerosis. Table 2 presents the distribution of histopathological glomerular lesions of DN between the 5-5 homozygous *CNDP1* and the *CNDP1* genotype with multiple leucine repeats. The frequency of 5-5 leucine repeats in the *CNDP1* gene was significantly lower in patients with histologically proven DN compared to patients without histologically proven DN (p=0.031) (Figure 2). However, no significant difference in the frequency of 5-5 leucine repeats of *CNDP1* was found between the different DN classes (p=0.148).

Table 2. *CNDP1* genotype distribution in histopathological lesions of DN

CNDP1 genotype distribution in histopathological lesions (N=136)			
	5-5 leucine repeats	Multiple leucine repeats	Total
Class 0	25 (50%)	25 (50%)	50 (100%)
Class I	8 (44.4%)	10 (66.6%)	18 (100%)
Class IIa	7 (43.8%)	9 (56.2%)	16 (100%)
Class IIb	3 (30%)	7 (70%)	10 (100%)
Class III	6 (16.7%)	30 (83.3%)	36 (100%)
Class IV	3 (50%)	3 (50%)	6 (100%)
Total	52 (38.2%)	84 (61.8%)	136 (100%)

Data show number of patients and percentages of *CNDP1* distribution per DN class

Figure 2. Distribution of *CNDP1* gene (5-5 leucine repeats and multiple leucine repeats) between histologically proven DN (p=0.031)



We also investigated whether *CNDP1* genotype was associated with the presence of nodular sclerosis (class III DN). Cases with nodular sclerosis (36 cases) were compared to all other cases with histologically proven DN, i.e. Class I, IIa, IIb and IV DN (50 cases), resulting in a significant difference in the presences of nodular sclerosis between cases with 5-5 homozygous *CNDP1* gene and multiple leucine repeats of the *CNDP1* gene (p=0.013). This indicates that cases with homozygosity for the 5-5 leucine repeat of the *CNDP1* gene are less susceptible to develop nodular sclerosis.

DISCUSSION

This study shows that homozygosity for the 5-5 leucine repeat of the *CNDP1* gene is associated with less susceptibility in developing DN in patients with histologically proven DN. This study, relying on histological findings, supports the results of previous studies

in which this genetic association with DN was found in patients with a clinical diagnosis of DN. Since we recently showed that not all cases with histologically proven DN have clinical manifestations [14], it is useful to determine if this genetic association can be found in histological proven DN. We also found an association between the *CNDP1* gene and the occurrence of nodular sclerosis, indicating that this gene may be involved in the development of nodular sclerosis in DN.

The direct effect of the *CNDP1* gene on the pathogenesis of DN remains to be elucidated. It has been suggested that homozygous 5-5 leucine repeats of the *CNDP1* gene result in a lower amount of serum carnosinase in patients, and thereby higher amounts of carnosine [3, 4]. Carnosine has several properties which are affected by diabetes, such as scavenging of reactive oxygen species [15-17], degradation of advanced glycation end-products (AGE) [18], inhibition of mesangial cell proliferation [19], inhibition in both podocytes and mesangial cells of TGF-β–mediated transcription of extracellular matrix proteins [20]. The ability of carnosine to moderate in the protein glycation and the inhibition of AGE formation together with powerful antiglycative and antioxidative effects [21] could indicate that high amounts of carnosine may protect the kidneys against oxidative damage, which plays a central role in the pathogenesis of DN [22].

Regarding nodular sclerosis, it is reported that AGEs also play a role in their development [23]. However, little is known about the development of these nodules and it is suggested that the development of these nodules results from another pathway than mesangial expansion [9, 11]. Although in this study nodular sclerosis occurred in a substantial amount of patients with homozygous 5-5 leucine repeats of *CNDP1* gene, the significant difference between patients with homozygous 5-5 leucine *CNDP1* and patients with multiple leucine repeats *CNDP1* suggests that the *CNDP1* gene may be involved in the development of nodules in diabetic nephropathy. Therefore, this genetic risk factor may help to solve the question of Schwartz *et al.* [11] why certain patients develop nodular sclerosis even when they are clinically undistinguishable from patients with mesangial expansion. More studies are needed to investigate the relationship between the *CNDP1* gene and nodular sclerosis. These additional studies might give insight in the pathogenesis of nodular sclerosis, on the differences in structure and the amount of nodules in patients with different clinical and/or other histological parameters, and could clarify if the *CNDP1* genotype is involved in the development of nodular sclerosis.

In literature, genetic associations in complex diseases such as DN have been plagued by inconsistencies [24]. The lack of reproducibility can often be ascribed to small sample sizes and false positive results [25]. The association with *CNDP1* gene and DN

was first reported in a large cohort that was not treated for the disease, and repeatedly confirmed in other cohorts of patients with type 2 diabetes of Caucasian and Asian ethnicities [2, 3]. The results were also supported by a meta-analysis in Caucasians with type 2 diabetes [26], indicating that the association with *CNDP1* and DN is reproducible.

In this autopsy study we were able to determine histological glomerular lesions attributable to DN in a large amount of glomeruli (>100 per case), although the study had some limitations that merit discussion. First, paraffin-embedded material was used to determine the *CNDP1* polymorphism and glomerular damage. Therefore, some cases needed to be excluded as the material was not sufficient for evaluation due to poor quality. Second, we focused only on the glomerular changes of DN but we did not investigate whether other histological parameters such as interstitial and/or vascular lesions are associated with the *CNDP1* genotype.

In conclusion, this study reports an association between histologically proven DN and the *CNDP1* gene, thereby confirming the results of previous genetic association studies of *CNDP1* and DN which were based on the clinical diagnosis of DN. Large-scale epidemiological studies underscore the need for more extensive characterization of kidney disease in individuals with diabetes to determine a more specific phenotype profile for DN [27]. Additionally, it might be that genetic biomarkers are beneficial to identify individuals risk profiles for DN before it becomes clinically apparent [27]. The direct link between *CNDP1* gene and DN, and nodular sclerosis still needs to be assessed, but our results indicate that the *CNDP1* gene may serve as a potential genetic biomarker to determine diabetic patients with high risk profiles to develop DN. Finally, intervention studies with carnosine supplementation could reveal whether *CNDP1* is of therapeutic value in patients with DN.

DISCLOSURE

All the authors declared no competing interests.

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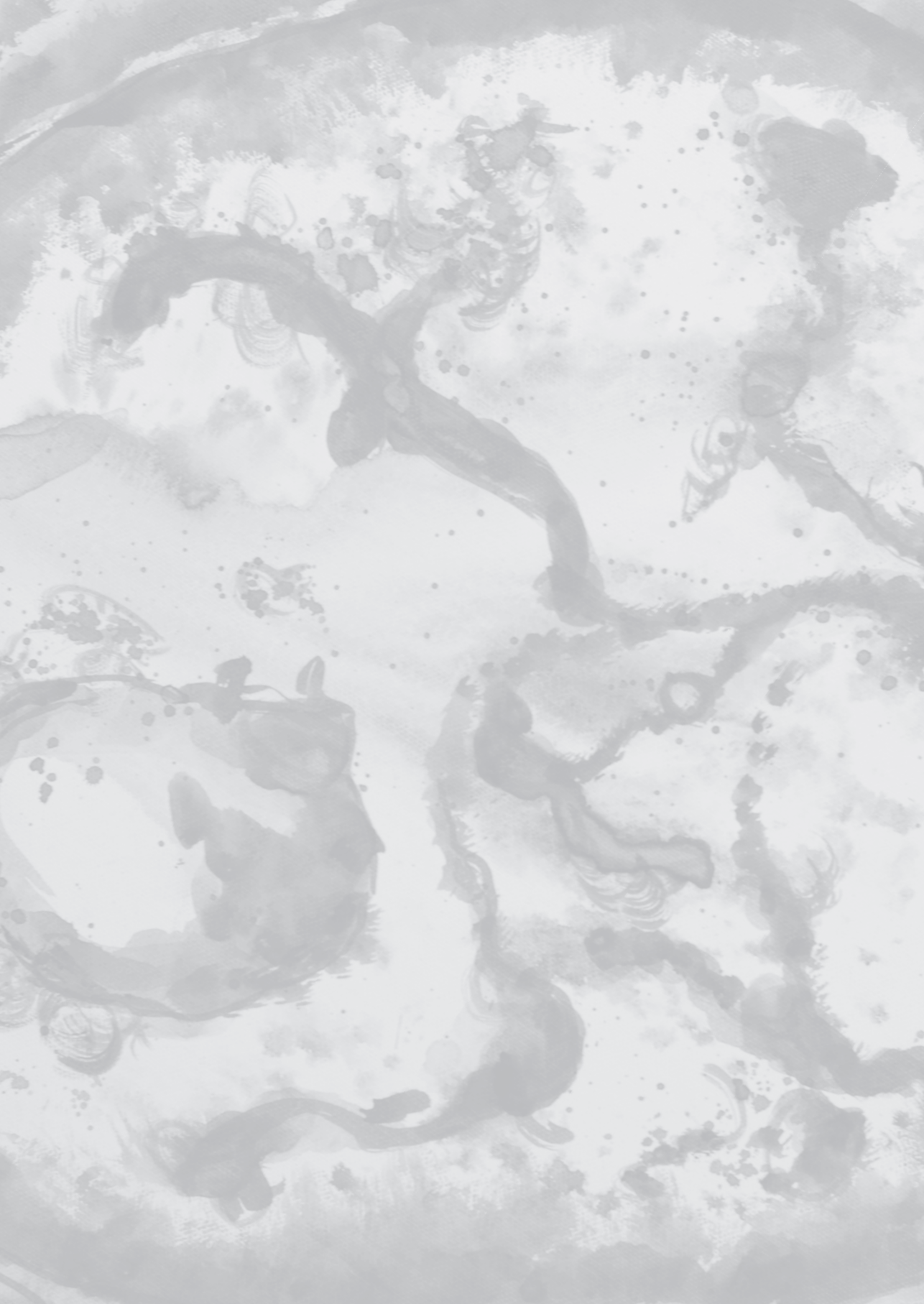

Intrinsic carnosine metabolism in the human kidney

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6

ABSTRACT

Histidine-containing dipeptides like carnosine and anserine have protective functions in both health and disease. Animal studies suggest that carnosine can be metabolized within the kidney. The goal of this study was to obtain evidence of carnosine metabolism in the human kidney and to provide insight with regards to diabetic nephropathy.

Expression, distribution, and localization of carnosinase-1 (CNDP1), carnosine synthase (CARNS), and taurine transporters (TauT) were measured in human kidneys. CNDP1 and CARNS activities were measured *in vitro*. CNDP1 and CARNS were located primarily in distal and proximal tubules, respectively. Specifically, CNDP1 levels were high in tubular cells and podocytes (20.3±3.4 and 15±3.2 ng/mg, respectively) and considerably lower in endothelial cells (0.5±0.1 ng/mg). CNDP1 expression was correlated with the degradation of carnosine and anserine (r=0.88 and 0.81, respectively). Anserine and carnosine were also detectable by HPLC in the renal cortex. Finally, TauT mRNA and protein were found in all renal epithelial cells. In diabetic patients, CNDP1 seemed to be reallocated to proximal tubules.

We report compelling evidence that the kidney has an intrinsic capacity to metabolize carnosine. Both CNDP1 and CARNS are expressed in glomeruli and tubular cells. Carnosine-synthesizing and carnosine-hydrolyzing enzymes are localized in distinct compartments in the nephron and increased CNDP1 levels suggest a higher CNDP1 activity in diabetic kidneys.

INTRODUCTION

Histidine-containing dipeptides such as carnosine (β-alanine-L-histidine) and anserine (β-alanine-L-methyl histidine) are stored in several tissues, with the highest concentrations occurring in skeletal muscle [1]. These dipeptides have several important protective functions. The best-characterized histidine-containing dipeptide is carnosine [2, 3], which plays many roles in maintaining health, including antioxidant activity [4-6] and the ability to scavenge carbonyls [7-9], inhibit glycation [10], and inhibit angiotensin-converting enzymes [11, 12]. Carnosine also has several neuroprotective roles [2, 13, 14]. Anserine has similar benefits, acting as an antioxidant [15] and carbonyl scavenger [16], as well as affecting renal sympathetic nerve activity and blood pressure [17]. Functional differences between anserine and carnosine have been reported. For example, anserine has higher anti-radical capacity than carnosine [18], lacks anti-crosslinking activity [19], and activates the uptake of calcium by mammalian mitochondria [20].

The naturally occurring amino acid β-alanine is the rate limiting amino acid in the biosynthesis of histidine-containing peptides. β-alanine is internalized by specific cells in order to synthesize carnosine for intracellar storage. Previous studies found that the taurine transporter (TauT), which is both sodium- and chloride-dependent, is responsible for the uptake of β-alanine in renal cells [21, 22].

Carnosine is synthesized by the enzyme carnosine synthase (CARNS), which is present in skeletal and heart muscle, as well as in certain regions in the brain [23]. The gene that encodes CARNS is *ATPGD1* [24], however, the expression and distribution of this enzyme are poorly understood [2].

In primates, carnosine is degraded predominantly by the enzyme carnosinase-1 (CNDP1), which is synthesized and secreted by the liver into the circulation; CNDP1 is encoded by the *CNDP1* gene [25]. In rodents, CNDP1 is absent in the circulation. CNDP1 is filtrated into the urine and reabsorbed into tubular cells, which express CNDP1 within their cytosolic compartment [25]. Two forms of carnosinase (CNDP) are expressed in primates: CNDP1, which is also called serum carnosinase, and CNDP2, which is also called tissue carnosinase or cytosolic nonspecific dipeptidase [26].

Given its ability to scavenge reactive oxygen species, carnosine might be beneficial with respect to diabetic nephropathy (DN) [27]. In animal models with diabetes the renal protective properties of carnosine have been described [28-33]. With respect to human patients, Jansen *et al*. [34] reported that a trinucleotide repeat in the *CNDP1* gene is associated with a differential susceptibility for developing DN in patients with

type 2 diabetes. The number of leucine repeats in the leader peptide of the pro-enzyme affects the efficiency of the enzyme secretion [35], thereby altering the effective concentration of this enzyme in the circulation [36].

Although the above-mentioned association with microvascular diabetic complications has been supported by several clinical studies, understanding the underlying mechanism requires experimental evidence. Thus, Sauerhöfer *et al.* [37] generated a transgenic mouse that overexpresses human *CNDP1* under the control of a liver-specific promoter. Giving these mice oral carnosine after induction of diabetes altered their glucose metabolism, but had no significant effect on the development or progression of DN, even though these transgenic mice express human CNDP1 in their serum. These diabetic mice have increased renal CNDP1 activity and reduced renal histidine dipeptide concentrations [28], and carnosine supplementation mitigates DN, reduces renal vasculopathy, normalizes vascular permeability [28], and improves wound-healing [29]. In rats with streptozotocin-induced diabetes, carnosine treatment prevents apoptosis of glomerular cells and podocyte loss [30, 31], decreases vascular damage [32], and decreases the oxidative damage associated with DN [33].

Based on these previously reported findings, we hypothesized that the human kidney is equipped with its own system for metabolizing carnosine. To provide a context for the findings obtained from rodent studies, and to test our hypothesis, we measured the expression level, enzyme activity, distribution, and storage of CNDP1, as well as CARNS, β-alanine uptake levels, and the distribution of TauT in the nephron, in human kidney tissues and in cultured renal cells. We also investigated whether carnosine metabolism differs in DN patients.

METHODS

In this study, we used human kidneys tissue obtained from healthy donors (Eurotransplant); the donor kidneys were unsuitable for transplantation due to technical reasons only; the tissue was de-identified. The organs were collected between 1995 and 2012. The renal cortex and isolated glomeruli were used to investigate the presence of components involved in carnosine metabolism in the kidney and the compartments of the renal cortex.

Antibodies

To examine the localization of the CNDP1 protein in human tissue samples, we generated a polyclonal anti-CNDP1 antibody. Two rabbits were immunized with a synthetic peptide corresponding to CNDP1, as described by Teufel *et al.* [25]. The serum was col-

lected and pre-adsorption with the synthetic peptide was used to confirm specificity (Supplementary Figure 1). The monoclonal anti-CARNS antibody was a generous gift from Prof. Frank L. Margolis (University of Maryland School of Medicine, Baltimore, MD); this antibody has been described previously [38, 39]. The specificity of the anti-CARNS antibody was confirmed by performing double-staining of COS-7 cells transfected with a His-tagged CARNS construct; the antibody showed co-localization with an anti-His antibody (Supplementary Figure 2). The rabbit anti-TauT antibody (raised against the C-terminal domain of the TauT protein, which is encoded by the *SLC6A6* gene) was obtained from Sigma-Aldrich (St. Louis, MO). For negative controls, the rabbit immunoglobulin fraction (solid-phase absorbed) and normal mouse serum (DakoCytomation, Glostrup, Denmark) were used at the same concentration as their respective primary antibody.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were used to detect the metabolic enzymes CARNS and CNDP1, the localization of histidine-containing dipeptides, and the TauT in the kidney. For CARNS and CNDP1 immunohistochemistry, the tissue sections were deparaffinized, and antigen retrieval was performed by incubating the sections with proteinase K (DakoCytomation) for 10 minutes at room temperature. Endogenous peroxidases were blocked with 0.125% H_2O_2 (v/v in distilled water) for 20 minutes. Immunohistochemistry using the anti-TauT antibody was performed as described above except, antigen retrieval was performed using citrate buffer. After antigen retrieval, the sections were incubated for 60 minutes with primary antibodies against CARNS, CNDP1, or the TauT. After washing with PBS, the sections were incubated with the following secondary antibodies: anti-mouse Envision (DakoCytomation) conjugated with HRP (for anti-CARNS) or anti-rabbit Envision (DakoCytomation) conjugated with HRP (for anti-CNDP1 and anti-TauT). HRP was visualized by incubation with DAB^+ substrate solution (DakoCytomation) for 10 minutes. The nuclei were counterstained with hematoxylin.

Double-label immunofluorescence was used to distinguish between the distal and proximal tubules. Tamm-Horsfall was used as a marker of distal tubules. Sections were incubated with anti-CARNS and anti-Tamm-Horsfall and sections were incubated with anti-CARNS and anti-CNDP1 for 60 minutes. The following secondary antibodies were used: Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 546 goat anti-mouse IgG, and Alexa Fluor 488 goat anti-rabbit IgG (all obtained from Life Technologies, Grand Island, NY). As a negative control, the primary antibodies were replaced with normal mouse serum (DakoCytomation) and rabbit immunoglobulin fraction (DakoCytomation) at the same concentration as their respective primary antibody (Supplementary Figure 3).

Cultured cells

Cultured cells from various compartments of the kidney were used to examine the cell-specific distribution of carnosine metabolic enzymes. SV40 immortalized human podocytes were used to measure mRNA levels. For measuring protein activity, we used conditionally immortalized mouse podocytes generated from the ImmortoMouse (Charles River, Wilmington, MA) [40]. Differentiation was induced by growing mouse podocytes ≥10 days on collagen type I (BD Biosciences, Bedford, MA) under permissive conditions at 33°C with interferon-γ (10 U/ml; Roche Diagnostics, Mannheim, Germany) or under non-permissive conditions at 37°C without interferon-γ. Podocytes and HK2 tubular epithelial cells (CRL-2190, American Type Culture Collection) were cultured in RPMI 1640 medium (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 10% FCS (Biochrom GmbH, Berlin, Germany) and penicillin/streptomycin (1% for HK2 cells and 2% for podocytes; Biochrom GmbH). Early-passage-number (passage number 8-15) human umbilical vein endothelial cells (HUVEC) were cultured in fetal calf serum containing endothelial cell growth supplement, epidermal growth factor, heparin, hydrocortisone, and 1% penicillin/streptomycin in accordance with the manufacturer's instructions (PromoCell GmbH, Heidelberg, Germany). All cells were cultured at 37°C in 5% CO_2 and harvested by adding 60 µl pre-lysis buffer containing 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 20 mM NaF, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA (all obtained from Sigma-Aldrich) (Complete Mini, Roche Diagnostics).

mRNA quantification by RT-PCR

mRNA was isolated from the whole human kidney cortex samples, isolated glomeruli, SV40 immortalized human podocytes, HUVEC cells, and cultured tubular epithelium (HK2) cells, after which *ATPGD1* (which encodes the CARNS protein) and *CNDP1* mRNA levels were quantified using RT-PCR. SYBR Green quantitative PCR was performed to quantify the levels of *ATPGD1* and *CNDP1* mRNA. All cDNA samples were amplified in duplicate. The following primers were used to amplify *ATPGD1* mRNA: forward, GAAGCTGGAGGAGGAGGAG; reverse, GTGGCCTATCACCCTGTGTC. The following primers were used to amplify *CNDP1* mRNA: forward, TTCAATCCGTCTAGTCCCTCACATG; reverse, TGCAATCCACGGGTGTAGTCC. The amplified mRNA levels were normalized to the expression levels of the housekeeping genes *GAPDH* and *HPRT* as described by Baelde *et al*. [41].

Carnosinase protein concentration

CNDP1 protein concentration was measured using a modified ELISA assay [42]. In brief, highly absorbent microtiter plates (Greiner Labortechnik, Frickenhausen, Germany) were coated with 100 µl goat polyclonal anti-human CNDP1 (10 µg/ml; R&D Systems, Wiesbaden, Germany); purified rabbit anti-CNDP1 IgG (Atlas, Abcam, Cambridge,

United Kingdom) was used to detect bound CNDP1. A biotinylated goat anti-rabbit IgG was added, followed by avidin-HRP. Deep-blue peroxidase (POD; Roche Diagnostics) was used for color development, and the plates were read immediately at 450 nm. Recombinant human CNDP1 (R&D Systems, Minneapolis, MN) was used as a standard; CNDP1 protein concentrations were measured in the linear part of the dilution curve. The sensitivity of the ELISA assays was approximately 15 ng/ml.

Anserine and carnosine concentrations

Anserine and carnosine concentrations were measured fluorometrically using high-performance liquid chromatography as previously described [43]. Frozen kidney tissue was homogenized in cold buffer containing 20 mM HEPES, 1 mM ethylene glycol-tetraacetic acid (EGTA), 210 mM mannitol and 70 mM sucrose per gram tissue, pH 7.2. The homogenate was centrifuged at 1,500 x g for 5 minutes at 4°C, and the supernatant was kept at -80° C until analysis. The kidney homogenate and the homogenized cells were diluted with sulfosalicylic acid in order to precipitate the proteins. After the samples were derivatized using carbazole-9-carbonyl chloride, they underwent liquid chromatography and quantification using fluorescence. The retention time of each component was determined by spiking the sample with purified L-carnosine or anserine. All samples were measured at least twice, and one sample was spiked with the standards to identify each analyte. The reliability of the method was 0.91.

Carnosinase and carnosine synthase activity

CNDP1 activity was assayed as described previously [25, 44]. In brief, the reaction was initiated by the addition of carnosine to cell homogenates at pH 7. The reaction was terminated at pre-determined intervals by adding 1% trichloroacetic acid. Liberated histidine was derivatized by adding *o*-phthalaldehyde, and fluorescence was read using a MicroTek plate reader (λ_{Ex}: 360 nm; λ_{Em}: 460 nm). To avoid nonspecific CNDP2 activity, Bestatin (Sigma-Aldrich, St. Louis, MO) was added to block the activity of CNDP2. Addition of Bestatin did not affect carnosine or anserine degradation, showing that CNDP2 was not active in our experiments. V_{max} values were obtained from at least three separate assays by fitting the Dixon plots using a linear regression program. The kinetic parameters were determined using various concentrations of substrates, and the data were fit using the Michaelis-Menten equation.

CARNS activity was determined by measuring the incorporation of radiolabeled β-alanine into carnosine [24]. In brief, the reaction was initiated by the addition of [3H]-alanine to cell homogenates. The cell homogenate was then separated by HPLC, and radioactive carnosine was measured using a scintillation counter (Beckman).

CNDP1 protein in DN patients

For the investigation of the role of carnosine metabolism in relation to renal disease, we used biopsies from patients with type 2 diabetes and DN (N=14) and compared them to healthy controls (N=7) [45]. The CNDP1 staining was scored by intensity degrees between 0 and 2.

Statistical analysis

A minimum of three independent experiments were performed in duplicate. All summary data are provided as mean ± SD. To compare ≥3 groups, a one-way analysis of variance was performed, followed by post-hoc analyses using Tukey's test. For the intensity analysis to compare diabetic patients with controls we used an independent Student t-test. All statistical analyses were performed using SPSS, version 20.0 (IBM, Armonk, NY).

Ethical considerations

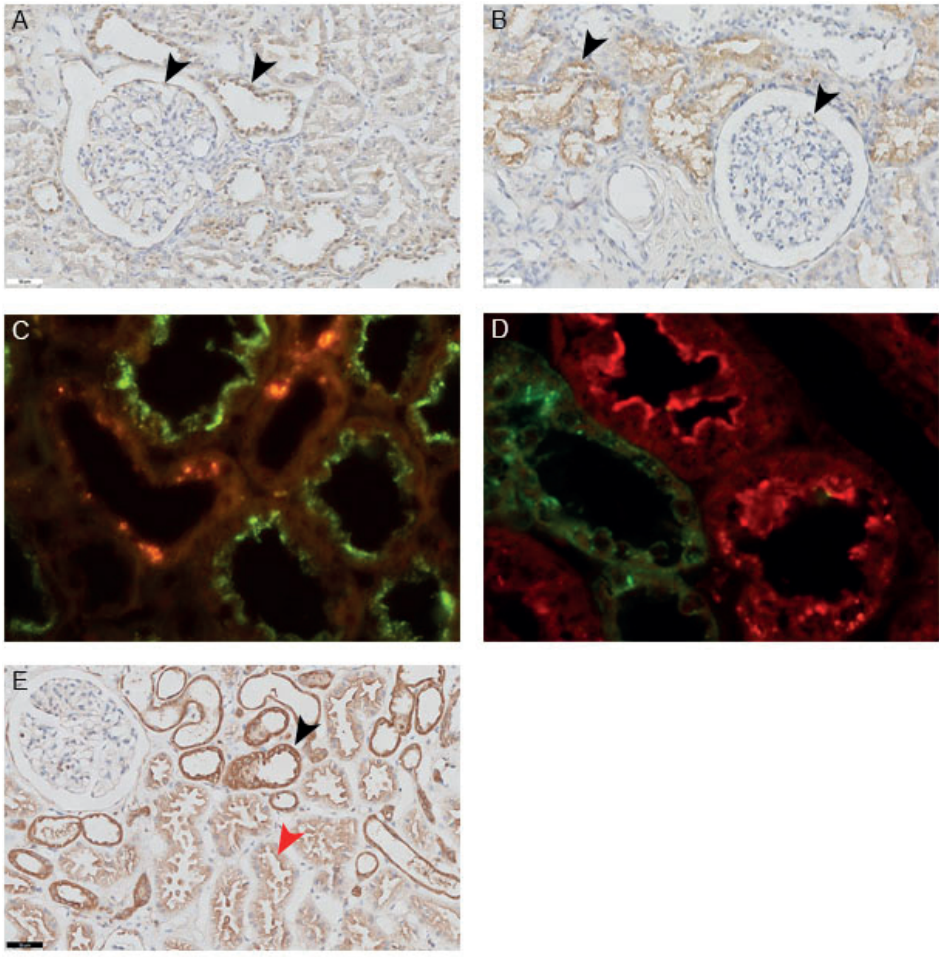
All tissue samples were coded, then handled and analyzed anonymously in accordance with the ethical principles stated in the Declaration of Helsinki.

RESULTS

CNDP1

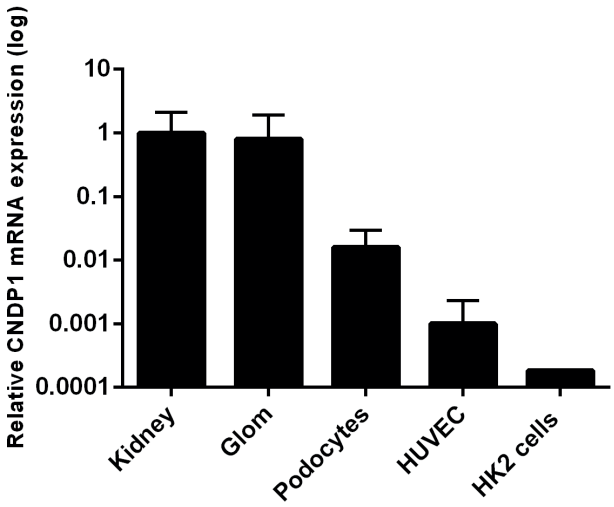
Immunohistochemistry showed that the CNDP1 protein is localized primarily in the distal tubules and in the glomeruli (Figure 1A). Next, we measured the mRNA levels, protein levels, and enzyme activity of CNDP1 in human kidney samples and cultured cells. The relative transcription levels were highest in the human kidney (1.00±1.12 relative units), glomeruli (0.802±1.1), whereas extremely low levels of *CNDP1* mRNA were detected in HUVEC cells (0.001±0.0013) and HK2 cells (0.000185) (Figure 2). In immortalized podocytes the relative transcription was also high (0.016±0.013). Consistent with this rank order of *CNDP1* expression, CNDP1 protein levels were high in immortalized podocytes (15±3.2 ng/mg protein) and low in HUVEC cells (0.5±0.1 ng/mg protein); interestingly, CNDP1 protein levels were high in HK2 cells (20.3±3.4 ng/mg protein). CNDP1 activity reflected high catabolic rates of carnosine and anserine in podocytes (2.8±1.7 and 2.9±1.5 nmol/mg/h, respectively) and tubular cells (2.6±0.2 and 3.9±0.4 nmol/mg/h, respectively) and low carnosine and anserine catabolic rates in HUVEC cells (1.3±0.4 and 0.05±0.08 nmol/mg/h, respectively) (Figure 3). Both, CNDP1 protein levels and CNDP1 enzyme activities were correlated to carnosine (r=0.88) and anserine (r=0.81) degradation.

Figure 1.



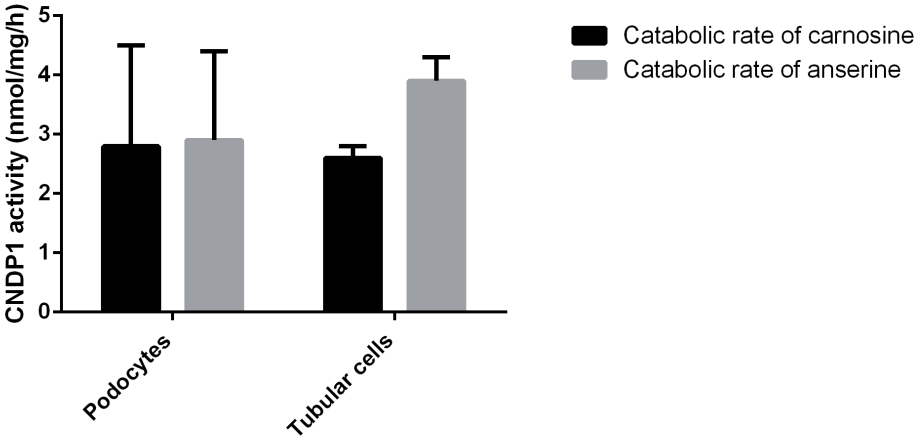
A: Immunohistochemistry showing the presence of CNDP1 in the glomeruli and distal tubules (arrows). **B**: Immunohistochemistry showing CARNS expression in proximal tubules and glomeruli (arrows). The nuclei were counterstained with hematoxylin. **C**: Immunofluorescence showing carnosinase (red) and carnosine synthase (green) in separate compartments in tubular cells. Immunohistochemistry showing CARNS expression in proximal tubules and glomeruli (arrows). **D:** Immunofluorescence showing non-overlapping expression of CARNS (red) in the proximal tubules and Tamm-Horsfall protein (green) in the distal tubules. **E:** Immunohistochemistry showing that the TauT is expressed in proximal tubules (red arrow) and distal tubules (black arrow) in human kidney samples. The highest proteins levels were present in the distal tubules. The nuclei were counterstained with hematoxylin

Figure 2.



CDNP1 mRNA was amplified from human kidney samples (1.00±1.12) (N=8), human glomeruli (Glom) (0.802±1.1) (N=8), immortalized human podocytes (0.016±0.0013) (N=2), human endothelial cells (HUVEC) (0.001±0.0013) (N=5), and human proximal tubular epithelial cells (HK2 cells) (0.000185) (N=1). All values were normalized to the mean value obtained from the human kidney samples. Note that the *y*-axis is plotted on a logarithmic scale, expressed as mean± SD of relative units

Figure 3.

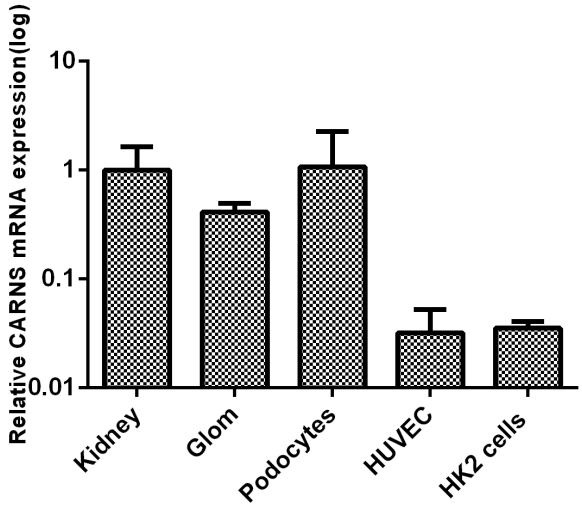


CNDP1 activity (in nmol/mg/h) was measured as the rate of degradation of carnosine (black) and anserine (grey) in mouse podocytes (2.8±1.7 and 2.9±1.5 nmol/mg/h, respectively) and human proximal tubular epithelial cells (HK2 cells) (1.3±0.4 and 0.05±0.08 nmol/mg/h, respectively), expressed as mean ± SD

CARNS

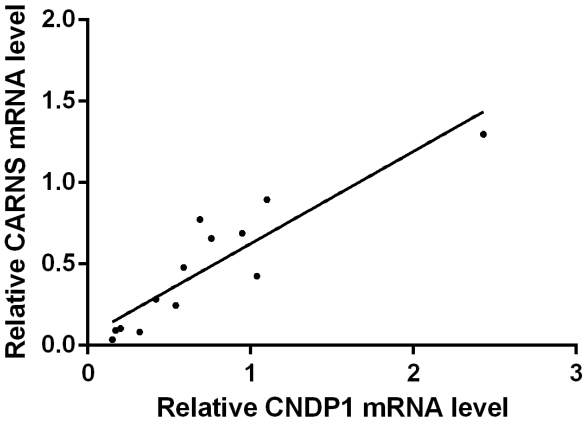
Immunohistochemistry revealed that CARNS protein was primarily localized close to the apical membrane of the proximal tubules, as well as in the glomeruli (albeit at low levels (Figure 1B)). Double-label immunofluorescence for CARNS and Tamm-Horsfall protein showed a lack of co-localization, indicating that CARNS is not present in distal tubules. Immunofluorescence revealed a lack of co-localization between CNDP1 and CARNS, as CARNS is localized primarily in proximal tubules (Figure 1C and 1D). CARNS mRNA was detected in human kidney samples, (1.00±0.63 relative units), glomeruli (0.4136±0.08), and tubular cells (0.035±0.005) (Figure 4). CARNS mRNA levels were also high in the immortalized podocyte cell line (1.07±0.15) but were low in the HK2 (0.035±0.005) and HUVEC (0.032±0.02) cells. To examine whether β-alanine affects CARNS activity in the cell lines, HK2 cells were treated with β-alanine. This treatment did not increase CARNS activity in the cells. In normal individual kidney samples, we found relatively high variation of CNDP1 and CARNS levels. Despite this variation, we found a strong positive correlation between *CNDP1* and *CARNS* mRNA levels in individual samples (r=0.81), suggesting that the expression levels of *CNDP1* and *CARNS* are controlled by a similar pathway (Figure 5).

Figure 4.



CARNS mRNA was amplified from human kidney samples (1.00±0.63) (N=8), glomeruli (Glom) (0.4136±0.08) (N=4), immortalized human podocytes (1.07±0.15) (N=2), human endothelial cells (HUVEC) (0.032±0.02) (N=5), and proximal tubular epithelial cells (HK2 cells) (0.035±0.005) (N=2). All values were normalized to the mean value obtained from the human kidney samples. Note that the *y*-axis is plotted on a logarithmic scale, expressed as mean ± SD of relative units

Figure 5.

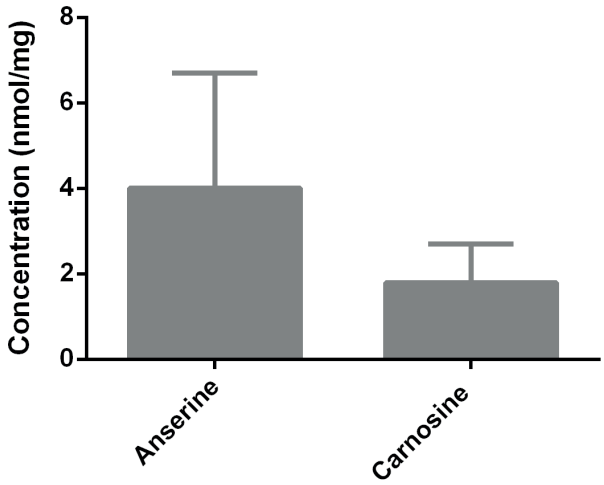


Relative *CARNS* mRNA level is plotted against the relative *CNDP1* mRNA level measured in human kidney samples. Each data point represents a separate sample. The solid line is a linear fit of the data r=0.81 (N=13)

Histidine-containing dipeptides

We next measured the concentrations of histidine-containing dipeptides using high performance liquid chromatography (HPLC).Repeated measurement of samples coming from three human controls resulted in six values with a mean of 4 nmol/mg anserine and a standard deviation of 2.7 [95%CI: 2.3-6.4] and a mean of 1.8 nmol/mg carnosine and a standard deviation of 0.9 [95%CI:1.1-2.8] (Figure 6). Thus, human renal tissue contains more anserine than carnosine. Anserine and carnosine were also present in the cultured podocytes but the concentration varied strongly with concentrations of 2.1-13.8 nmol anserine/mg [95%CI] and 1.7-8.1 nmol carnosine/mg protein [95%CI], whereas in tubular cells lower amount of carnosine could be detected (0.2 nmol/mg protein) and anserine was below detection limit.

Figure 6.



Concentrations of anserine (4±2.7) [95%CI: 2.3-6.4] and carnosine (1.8±0.9) [95%CI:1.1-2.8] in human kidney samples (N=3) measured using HPLC, expressed as mean ± SD

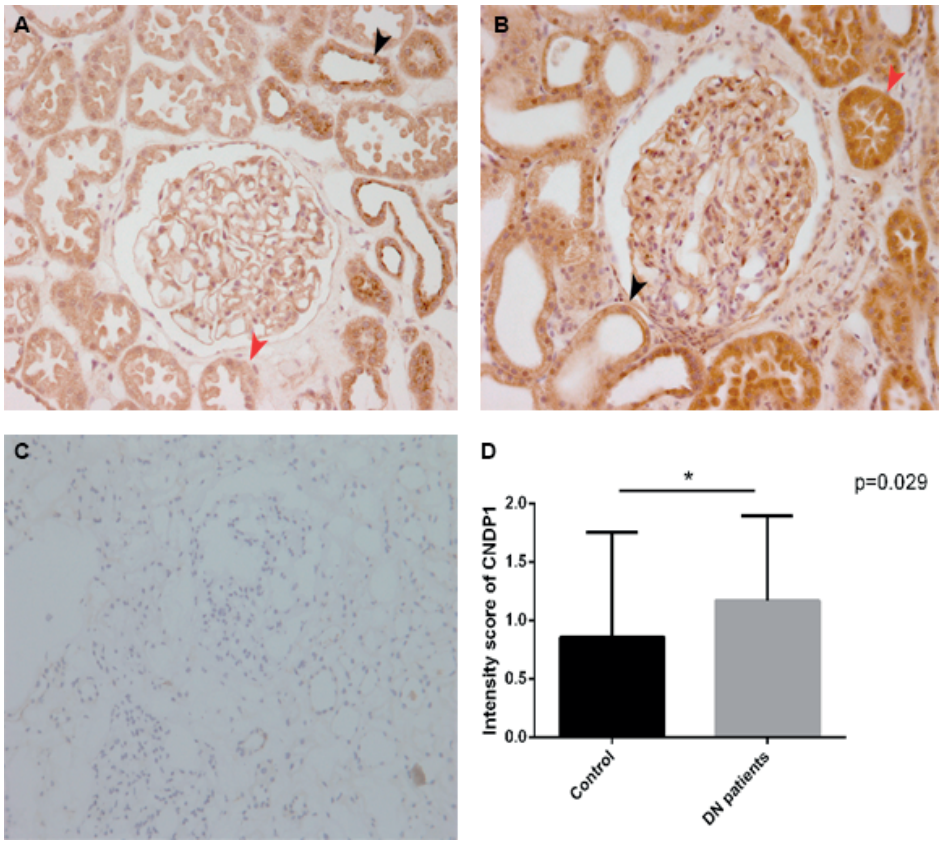
Taurine transporter

The TauT transports β-alanine into cells. Immunohistochemistry revealed that the TauT is present in glomerular cells and on the membranes of all renal tubules (Figure 1E). These data were supported by mRNA measurements (data not shown). Treating HK2 cells dose-dependently (0.1-5mM) with β-alanine for 24 and 48 hours had no effect on the expression of TauT (data not shown).

CNDP1 protein of DN patients

We also compared diabetic patients with DN (N=14) to controls (N=7) by scoring the intensity of CNDP1 immunostaining of renal tissue. In the tubules, we found a significant difference between DN patients and controls (Figure 7). The higher levels of CNDP1 in DN patients in the proximal tubules could indicate increased hydrolysis of carnosine and anserine. Moreover, CNDP1 might be accumulated in the proximal tubules as a result of reabsorption of CNDP1 caused by proteinuria in patients with DN.

Figure 7. CNDP1 in diabetic patient (N=14) and control (N=7)



Immunohistochemistry and instensity score *(p=0.029). It shows a reallocation of CNDP1 from distal to proximal tubules in diabetic patients with renal damage. **A**: Healthy control (0.857±0.8997), **B**: Diabetic nephropathy patients (1.171±0.726), **C**: Negative control, **D**: intensity staining difference; red arrow: proximal tubules, black arrow: distal tubules; expressed in mean ± SD

DISCUSSION

This study provides the first evidence that the human kidney has an intrinsic system for metabolizing carnosine. Also, we investigated the relation of CNDP1 protein to DN in humans. Combining several experimental approaches, we found that the proteins involved in carnosine metabolism are located in distinct compartments within the nephron. The presence of metabolizing enzymes and the presence of stored histidine-containing dipeptides supports our hypothesis of kidney-specific carnosine metabolism. The staining intensity of CNDP1 was significantly higher in the renal tubules of patients with DN, and immunohistochemistry revealed that CNDP1 was reallocated to the proximal tubules.

We compared the amount of carnosine in the kidney to that in the skeletal muscles fibers, which have the highest concentration of carnosine in the human body. The carnosine concentration determined in human muscles was compared to that in the human kidney. The renal concentrations of anserine (1.1-7.4 mmol/kg for anserine) almost reach the levels of the carnosine concentration of the skeletal muscles (7.2-30.7 mmol/kg dry muscle mass), suggesting that carnosine metabolism plays an important role in maintaining normal kidney function, consistent with the protective properties of carnosine in the muscle. The range of carnosine and anserine concentrations in cultured podocytes differed, and were probably based on the passage number of the cells and culturing conditions.

One of the kidney's primary functions is to remove and detoxify low-molecular weight compounds. Several studies reported a difference between the protective properties of anserine and carnosine [18-20]. For example, anserine has a higher anti-radical capacity and more antioxidant properties than carnosine; therefore, we hypothesize that anserine protects renal function against the detrimental effects of oxygen radicals.

The podocytes and proximal tubules provide the first line of defense after the fenestrated endothelium. We hypothesize that in these structures, CARNS is required to maintain sufficient anserine concentrations, thereby supporting their protective function. The exchange of protons with K^+ and Na^+ ions requires continuous low pH in the distal tubules. Because carnosine has high pH buffering capacity, carnosine must be removed from the distal tubules, thereby explaining the high concentration of CNDP1 in this renal compartment. Teufel *et al.*(1989) reported high levels of CNDP1 in the stomach epithelium, a site that also requires an extremely low pH.

Next, we found that the TauT is present in the membranes of all renal tubular cells. Taurine and β-alanine compete for binding to TauT [46]. In the proximal tubules, CARNS synthesizes carnosine from β-alanine and histidine. Therefore, TauT is believed to stimulate the internalization of β-alanine primarily in the proximal tubular epithelium. Because taurine is an osmolyte, the expression of TauT in the distal tubules is regulated by osmolar stress [47] (Figure 1E). The gene that encodes TauT is regulated by a complex interplay between transcription factors and response elements [47]. Over time, β-alanine can deplete taurine from tissues, including renal tissue, thereby upregulating the synthesis and activity of TauT. However, applying β-alanine to HK2 cells did not increase carnosine levels, nor did it appear to induce the expression of either CARNS or the TauT, possibly because the level of TauT protein in the membrane is static.

Although we found clear evidence of organ-specific carnosine metabolism in the kidney and found CNDP1 changes in DN conditions, this study had some limitations. First, HUVEC cells, podocytes, and HK2 cells were used to investigate the specific locations of the mRNA levels and enzyme activities in the glomeruli. It is possible that when these cell lines were created, they lost part of their original expression profile. Future studies could focus on knockout models with segment specific genetic manipulation.

Considering the physiological function of podocytes in glomerular homeostasis, the high levels of mRNA, proteins, and enzyme activities measured in immortalized podocytes suggest that carnosine metabolism plays a role in glomerular function. In endothelial cells, CNDP1 protein and activity levels were consistently low both *in vitro* and *in vivo*; therefore, endothelial cells likely play only a minor role in carnosine metabolism in the kidney. The levels of cell type–specific carnosine and anserine degradation by CNDP1 in renal cells were closely correlated with CNDP1; in other cells, factors such as allosteric conformation and substrate inhibition play a role [44, 48]. Interestingly, significant levels of anserine and carnosine were present in the human kidney tissue samples; in contrast, their levels were much lower in the cultured cells, possibly due to low CARNS activity, high CNDP1 activity, intracellular degradation, and/or high turnover.

Our results on DN patients are in line with several studies, which reported that carnosine might play a role in the pathogenesis of DN [34, 37]. In this respect, diabetic podocyte-specific conditional knockout mice should be developed; these mice would shed light on the role of CNDP1 and CARNS in the glomerulus. Future studies could focus on the regulation of transcription levels, uptake in tubular cells of CNDP1 in DN and investigate whether the histidine-containing dipeptide concentrations are decreased in diabetic state. Also, studies should focus on the role of β-alanine in renal health and disease. In addition, it would be extremely interesting to determine whether diabetes-related

oxidative stress changes carnosinase activity in the kidney. In this respect, manipulating renal carnosine metabolism may provide novel therapeutic options for treating DN.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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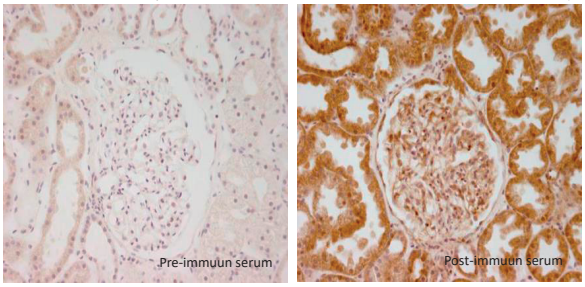
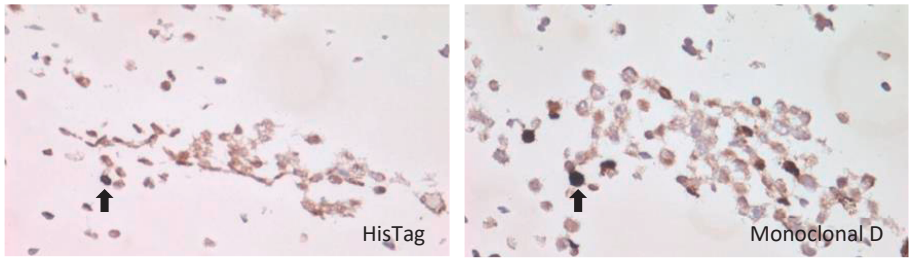
SUPPLEMENTARY FIGURES

Supplementary figure 1. Carnosinase-1 (CNDP1) antibody specificity



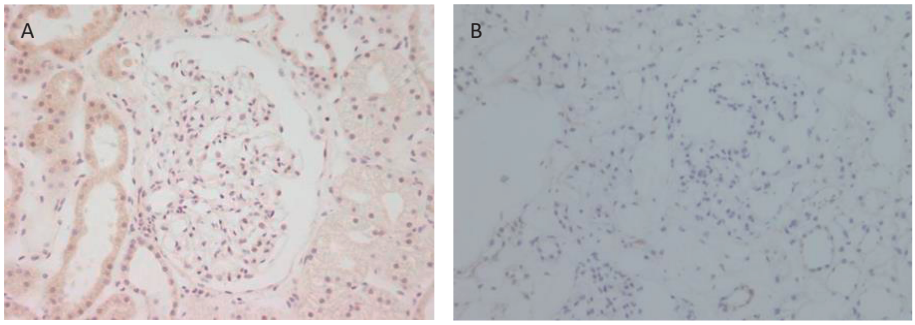
Serum from rabbits immunized by CNDP1 for peptide inhibition. Pre-immune serum is negative control and post-immune serum is positive control

Supplementary figure 2. Carnosine synthase (CARNS) (ATPGD1) antibody specificity

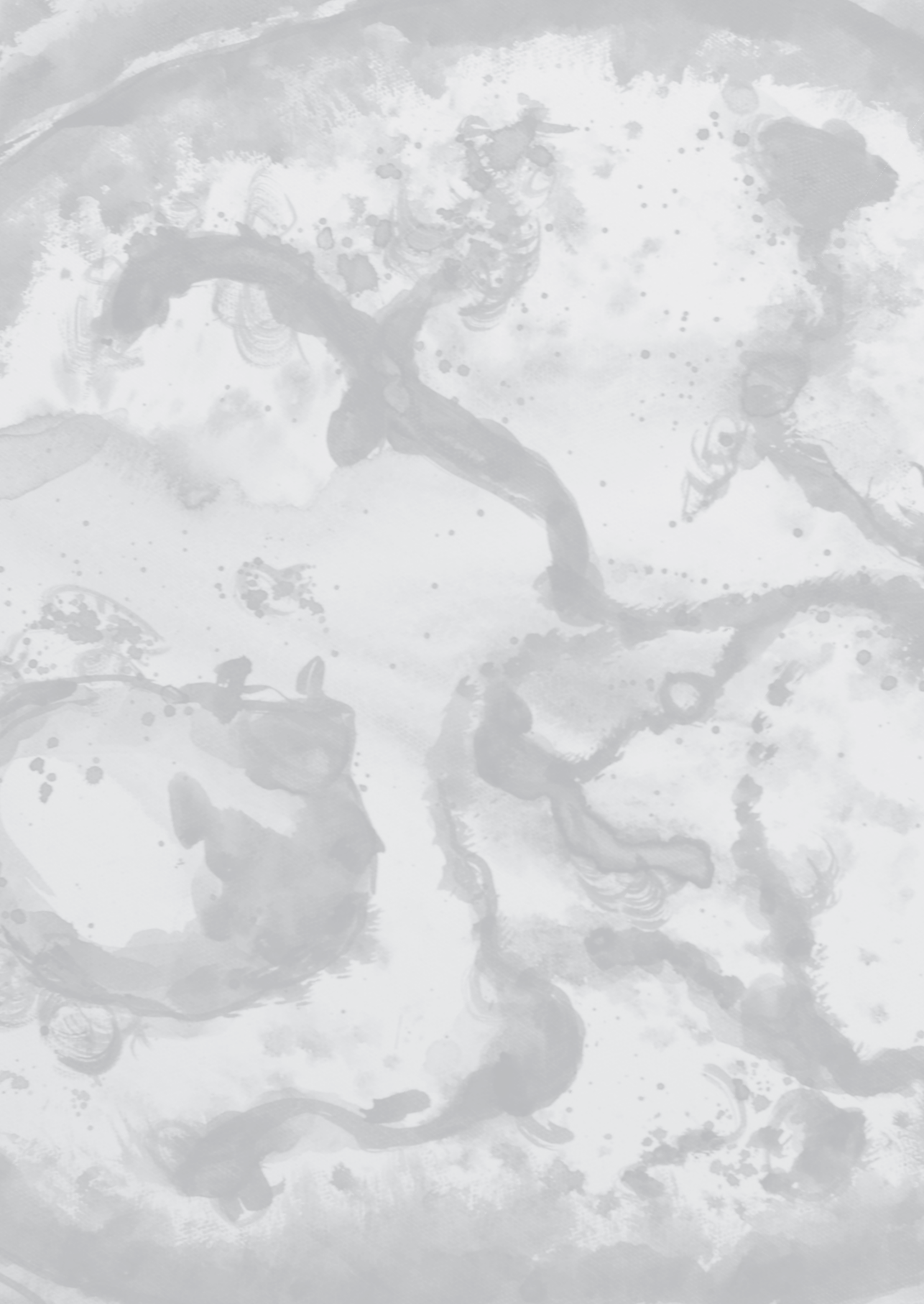


The antibody for CARNS was provided by the group of F. Margolis, who isolated the enzyme in a previous study (Margolis, 1987), before the gene for CARNS was discovered in 2010 by Drozak *et al.* By performing double staining with this antibody and anti-HisTag antibody on cryo sections of MNNG cells transfected with HisTag labelled CARNS protein. Arrows show that cells which were positive for polyhistidine also show a positive staining with the monoclonal antibody for carnosine synthase indication its specificity

Supplementary figure 3. Negative controls of CNDP1 and CARNS



A: negative control of CNDP1(pre-immune serum) **B:** negative control of CARNS (normal mouse serum and immunoglobulin fraction)



Summary, Discussion and Future Perspectives

7

Diabetic nephropathy is a severe complication of diabetes and is one of the leading causes of end-stage renal disease worldwide. In this thesis we investigated several aspects of diabetic nephropathy, including histological lesions scored according to the pathologic classification of diabetic nephropathy, inflammatory markers, the *CNDP1* gene and the carnosine metabolism.

The diabetic autopsy cohort of **chapter two** enabled us to observe histological manifestations of diabetes in the kidney at various stages of the disease. The renal damage was scored according to the pathologic classification of diabetic nephropathy. The use of autopsy material of 168 patients with diabetes gave us the opportunity to study at least 100 glomeruli per case, which excluded inadequate classification due to sampling errors. During the evaluation of the renal tissue specimens, we observed that the distribution pattern of histological lesions such as nodular and glomerular sclerosis may vary substantially, not only between patients but also within areas of the renal tissue of an individual patient.

Next, the histological lesions were associated to clinical parameters. We found a strong association between the histopathological classes of diabetic nephropathy and renal function as well as between these classes and interstitial and vascular lesions. These findings are in line with the results of the validation studies by An *et al.* [1] and Oh *et al.* [2]. Furthermore, in previous studies the incidence of non–diabetic-related renal disease was reported to be up to 79%; in our study this incidence was relatively low (15.5%). An explanation for this difference could be that the other studies used histological data of renal biopsies; these studies may suffer from a selection bias with respect to the reasons for performing the renal biopsy and this may be the reason for the higher amount of non-diabetic renal diseases in these studies [3-5].

The most interesting finding of our study was that neither microalbuminuria, nor proteinuria was associated with the presence of histological lesions in 20% of cases with diabetes. These patients were defined as patients underdiagnosed for diabetic nephropathy. The histological lesions of the underdiagnosed patients were generally not distinguishable from the cases with clinically diagnosed diabetic nephropathy, although the percentage of nodular sclerosis was significantly lower in the underdiagnosed patients. It may be that the presence of glomerular damage before the onset of albuminuria in the underdiagnosed patients is the result of the capacity of healthy tubular epithelium which is able to reabsorb proteins from the glomerular filtrate, thereby disguising glomerular protein leakage. In our study, the correlation between interstitial fibrosis and tubular atrophy (IFTA) and the presence of microalbuminuria and/or proteinuria suggest that in cases with more severe IFTA the reabsorption capac-

ity of the tubules is lost and therefore microalbuminuria becomes apparent in urinary samples.

In conclusion, the histological findings in the underdiagnosed patients indicate that renal lesions consistent with diabetic nephropathy may develop before the onset of clinical abnormalities. Yet, the clinical benefit to identify these underdiagnosed patients remains unknown and requires further investigation in future studies.

Our publication was accompanied by an editorial commentary in Kidney International [6]. In this commentary, Said and Nsar [6] stated that our results of glomerular and interstitial damage before albuminuria are in agreement with previous morphometric studies [7, 8]. Furthermore, they highlighted the advantage of the use of autopsy material, in which renal morphology can be analyzed regardless of the patients' clinical parameters; since most clinicohistological studies use material of renal biopsies, which are often performed when the patients' clinical features are unexplained and cannot be related to diabetic nephropathy. Finally, they suggested that there is need for a novel biomarker, which may facilitate an early diagnosis and guide the therapeutic regimens of diabetic nephropathy. They noticed that, although many studies are currently focusing on the development of novel non-invasive biomarkers, none of these potential biomarkers are sufficient at this moment and that validation of these markers in large cohorts is required before they may be used in clinical practice [9].

Emerging therapeutic approaches are focusing on targeting the inflammatory pathway to prevent end-stage renal disease in patients with type 2 diabetes and diabetic nephropathy. We believe that better knowledge of the influx and type of macrophages in renal tissue of patients with type 2 diabetes is essential to create optimal therapies to intervene in the inflammatory pathway.

The results of **chapter three** demonstrated that there is an influx of CD68+ and CD163+ macrophages in both glomeruli and interstitium in diabetic nephropathy. These macrophages were associated with renal damage, scored by the pathologic classification of diabetic nephropathy, and with clinical parameters. Additionally, we investigated whether therapeutic regimens, such as renin angiotensin aldosterone system (RAAS) blockers or oral diabetic medication had effect on the type or number of glomerular macrophages in our cohort, but no significant difference was found between patients with or without these therapies.

Interestingly, we found that anti-inflammatory CD163+ cells were present in the renal tissue of patients from all four histopathological classes of diabetic nephropathy. Be-

sides, the presence of glomerular CD163+ cells was positively associated with the class of diabetic nephropathy, IFTA and global glomerulosclerosis. Based on these findings, we speculate that the function of infiltrating macrophages becomes increasingly anti-inflammatory when the histopathological parameters become more severe.

We included two control groups in this study: one group consisted of five non-diabetic patients without other renal abnormalities but with comorbidities, including hypertension, heart failure or atherosclerosis. The other control group consisted of eighteen patients with diabetes but without histological evidence of diabetic nephropathy. In these control subjects, macrophages were observed as well. Due to the presence of macrophages in the renal tissue of both the cases with diabetic nephropathy and the control groups, it is not possible to conclude whether the presence of macrophages is a reaction to or a mediator of renal damage. Therefore, we hypothesized that the expression profile rather than the absolute numbers of these macrophages enhance renal damage.

Our findings of macrophage involvement in renal tissue are comparable to the results of several animal and human studies. More specifically, Nguyen *et al.* [10] reported the presence of macrophages in a relatively small cohort of diabetic patients. Similar to our findings, no significant difference was found between diabetic patients and controls with respect to glomerular CD68+ macrophages, but in their study they did not stratify for the different types of macrophages. On the other hand, they were able to associate their findings to clinical parameters of renal outcome and found a correlation between both glomerular and interstitial macrophages, and progression to renal failure [10].

In studies with experimental models for both type 1 and type 2 diabetes on inflammatory markers, Chow *et al.* [11, 12] showed in streptozotocin-induced and db/db mice respectively that the accumulation of renal macrophages was associated with the progression of glomerular and tubular damage. In conclusion, the results of chapter three along with the results of others suggest that the inflammatory pathway may be targeted by novel therapeutic regimens.

The histopathologic classification of diabetic nephropathy proposed by the Renal Pathology Society has been used in multiple research and diagnostic settings. In **chapter four,** we investigated whether fine-tuning of definitions of the classification might be appropriate. Combining two approaches to analyze the pathologic classification within this study gave insight into issues which may occur while scoring renal biopsies according to the pathologic classification of diabetic nephropathy in either clinical practice or in a research setting.

The first part, the reproducibility study, consisted of a survey in which the opinions from participants who are experienced in the field of renal pathology were obtained. In this survey, the reproducibility was proven to be sufficient for classes III and IV, but it revealed that there was some disagreement among observers for class I, IIa and IIb as well.

The second part, the overview of validation studies together with the meta-analysis, enabled us to determine the prognostic value of the classification. The meta-analysis revealed that there is a good association with renal outcome and the histopathological classes for all classes except for class I and class IIa. An explanation for the lack of significance between these two classes may be that the available validation studies were underpowered for this comparison as only a small number of patients and a limited amount of events could be investigated. The results of the reproducibility study and the meta-analysis were indicative of the clinical usefulness of the classification of diabetic nephropathy.

Based on the comments obtained from the reproducibility study suggestions to redefine the classification were proposed in the discussion part of chapter four. The participants of the survey suggested creating more straightforward definitions for mesangial alterations to distinguish between classes I, IIa and IIb; however, validation studies are required to determine the clinical value of newly proposed redefinitions.

A concern in the reproducibility study was whether the presence of one nodule was enough to classify a sample as class III. In chapter two we showed that the distribution pattern of histological lesions in renal tissue of patients with diabetes varies. Additionally, we believe that formation of nodular sclerosis may be a specific trait of some patients with diabetic nephropathy, who are not yet more distinctly defined. Therefore, we believe that the presence of one nodule is sufficient to classify a renal tissue specimen as class III. Another concern was about the relevance of IFTA within this scoring system. Since IFTA seems to have prognostic value for the renal outcome in diabetic nephropathy, some participants of the survey suggested that IFTA may be scored as a primary parameter in the classification. However, they also noticed that IFTA could have been caused by other renal diseases. Therefore, we recommended taking the severity of IFTA into account during evaluation of the biopsy, and to specifically note the amount of IFTA in all renal biopsy reports in cases of diabetic nephropathy, but not define it as a primary parameter in the pathologic classification. Next, the relation of IFTA with inflammation was re-evaluated, because in our survey a low intraclass correlation coefficient was observed for this parameter. The following clarification of this definition was given: only score inflammation in areas without IFTA. Furthermore,

future potential studies on inflammatory markers may determine the relative effects of interstitial inflammation.

Overall, the comments of the participating pathologists in the reproducibility study indicated that an update of the classification may be useful. When an updated version of the classification is proposed, it is necessary to create validation studies which investigate whether the newly proposed classification has similar or even better associations with renal outcome.

The number of 5-5 leucine repeats of the *CNDP1* gene has been associated with diabetic nephropathy in patients with type 2 diabetes in several studies [13-16]. However, all of these studies were based on the clinical diagnosis of diabetic nephropathy in the absence of renal biopsies. Since we were able to determine the *CNDP1* genotype in our autopsy cohort, we believed it would be interesting to determine whether the association could also be found in cases with histologically proven diabetic nephropathy.

In **chapter five**, we showed that 5-5 leucine repeats of the *CNDP1* gene are also associated with histologically proven diabetic nephropathy based on the glomerular damage scored according to the pathologic classification of diabetic nephropathy. Furthermore, we investigated whether the *CNDP1* gene could be associated with nodular sclerosis. Several studies have hypothesized that these nodules may develop from different pathways compared to severe mesangial expansion; although in the current literature, there is no evidence on what could cause the development of these lesions. Therefore, in this study we hypothesized that the *CNDP1* gene may be involved in the development of nodular sclerosis. Interestingly, we found an association between the *CNDP1* gene and the occurrence of nodular sclerosis. More research in larger cohorts and in validation studies is required to prove that this association is not a coincidence. Besides, we observed a variation in the amount and structure of the nodules during our histological evaluation. These different structures suggest that there even may be different sub-entities within nodular sclerosis. Further research is needed to obtain more insight in the pathogenesis of nodular sclerosis and may determine whether these nodules influence the prognosis or the progression of diabetic nephropathy.

The combination of several experimental approaches described in **chapter six** resulted in the evidence that the kidney has an intrinsic organ-specific metabolism for carnosine. In this study, we showed that the proteins involved in the synthesis, methylation and degradation of carnosine are located in different segments of the nephron. Furthermore, this study provided evidence that in cases with diabetic nephropathy the enzymes involved in the carnosine metabolism seem to be reallocated to different

parts of the nephron. This finding suggests that aberrant carnosine metabolism may be involved in diabetic nephropathy. The observed changes of the enzymes involved in the carnosine metabolism in diabetic nephropathy may be supported by the fact that several functions of carnosine can be altered in diabetes. Additionally, multiple studies have reported that carnosine has several renoprotective functions, such as it decreases proliferation of mesangial cells [17-20] and it can function as an ACE inhibitor [21, 22].

To get more insight in the change of the carnosine metabolism, future studies should investigate the reallocation of the proteins of carnosine metabolism in diabetic nephropathy in larger and more specified groups. Additionally, clinical trials in which patients with diabetic nephropathy are supplemented with oral carnosine or the rate limiting amino acid, β-alanine should be created to investigate whether carnosine may be of therapeutic value. Since it is hypothesized that 5-5 homozygous *CNDP1* patients have higher amounts of carnosine, it might be that the response on carnosine supplementation relies on the *CNDP1* genotype. Therefore, it is possible that patients with multiple leucine repeats, who probably have lower amounts of carnosine, will have more benefit from an intervention with carnosine.

FUTURE PERSPECTIVES

We believe that if there is a better understanding of the involved pathways of diabetic nephropathy and their interaction, more specific therapy regimens may be added to the treatment of diabetic nephropathy in the future.

Currently, the treatment of diabetes and its secondary complications consist of several therapeutic regimens including the normalization of glucose levels, the treatment of hypertension and the regulation of the lipid spectrum next to lifestyle interventions. Regarding diabetic nephropathy, the most effective and specific treatment is to influence the renin-angiotensin aldosterone system (RAAS) with medication such as angiotensin-converting enzyme (ACE) inhibitors [23]. This treatment regimen slows down the progression of diabetic nephropathy, however in many cases the progression to end-stage renal disease cannot be avoided.

In this thesis we provided evidence that the inflammatory pathway, the *CNDP1* gene and the carnosine metabolism may play a role in the development and/or progression of diabetic nephropathy. In connection with these results we will speculate on the future perspectives and potential novel therapy regimens of diabetic nephropathy.

Anti-inflammatory agents

In chapter three, we showed that there is an influx of infiltrating macrophages in the glomeruli and interstitium of patients with type 2 diabetes and diabetic nephropathy, suggesting that the inflammatory pathway may be targeted in these patients. Furthermore, multiple studies have shown that renal complications in patients with diabetes are triggered by inflammation [24]. These results have led to the hypothesis that anti-inflammatory therapies could protect the kidneys from inflammation.

Interestingly, approximately 30 years ago several studies reported the potential renoprotective effect of non-steroidal anti-inflammatory drugs (NSAIDs) in patients with proteinuria [25-27]. At that time, it was thought that the renoprotective effect of these NSAIDs was caused by reducing intra-glomerular pressure via hemodynamic effects. Due to the limitations of specific assays for inflammatory markers, it was not possible to challenge this hypothesis [25, 27]. Nowadays, with improved techniques and increased understanding on various inflammatory components which cause tissue damage in patients with diabetes, the hypotheses on inflammation in diabetic nephropathy can be challenged. Eventually, intervention of these inflammatory target sites may serve as potential therapeutic options to decrease proteinuria in diabetic nephropathy [24].

Several studies are already trying to target specific anti-inflammatory markers to decrease diabetic nephropathy. Two recent clinical trials investigated whether blockade of monocyte chemoattractant protein-1 (MCP-1) would ameliorate the pro-inflammatory state in diabetic nephropathy [28, 29]. It seems that the blockade of MCP-1 may preserve the number of podocytes by alleviation of the pro-inflammatory state, as the binding of MCP-1 to its receptor stimulates the inflammatory cascade by releasing monocytes and activating migration of monocytes and macrophages; MCP-1 inhibition may serve as a therapeutic option to block this reaction [29].

More specific, the first study of Menne *et al*. [29] showed that after treatment with a MCP-1 inhibitor for 12 weeks albuminuria decreased by 15%; however no significant difference was observed compared to the placebo intervention. The most interesting finding of this study was that the lowered albuminuria persisted for a long period after cessation of the MCP-1 medication.

The other study, which also focused on inhibition of the MCP-1 axis of the inflammatory pathway by de Zeeuw *et al*. [28] investigated the effect of different doses of a MCP-1 inhibitor in patients with type 2 diabetes and macroalbuminuria. The effect of a low dose (5mg/day) of this MCP-1 inhibitor was in line with the result of decrease in albuminuria reported by Menne *et al.* [29] (18%) and also persisted throughout 52 weeks follow-up.

Interestingly, in the patients, who received a dose that was twice as high (10mg/day) the long term effect disappeared during follow up.

The results of these studies provide evidence that blockade of MCP-1 might delay the progression of diabetic nephropathy. Both studies used albuminuria to determine the efficacy of these drugs, yet it remains unknown whether this is the right surrogate for these anti-inflammatory drugs. Heerspink and the Zeeuw suggested that in theory, it could be that anti-inflammatory agents may not decrease albuminuria but still serve as renoprotective agents [30]. This hypothesis is based on the fact that it has been shown that re-uptake of albumin in the tubules triggers toxic effects and inflammatory responses. This could mean that blocking pro-inflammatory pathways downstream of the albuminuria uptake may prevent loss of renal function without effecting albuminuria itself [30, 31]. Therefore, it may be that other more specific inflammatory markers are better surrogates to determine the efficacy of these anti-inflammatory drugs. Another question that should be kept in mind is that we do not know whether these anti-inflammatory agents affect the structural damage in the kidney; although the long lasting effects of the above mentioned studies suggest that these anti-inflammatory drugs may improve underlying structural renal damage. The mechanism behind these long lasting effect should be investigated in future studies.

Carnosine supplementation

The results of chapter five and six of this thesis on the *CNDP1* genotype and the carnosine metabolism, suggest that intervention of these pathways may serve as another therapeutic option to target diabetic nephropathy. Administration of oral carnosine supplementation, or supplementation with the rate limiting amino acid of carnosine, β-alanine, could be used to target this pathway.

Oral carnosine supplementation is an over-the-counter food additive and is frequently used by athletes [32, 33]. In the recent review of the physiological and therapeutic effects of carnosine, Baye *et al.* [34] reported that carnosine is well tolerated by humans and has no significant side effects. Furthermore, they reported that carnosine is already used as a therapy in cardiovascular diseases, neurodegenerative diseases, and mental health conditions [34]. Studies also reported that carnosine supplementation reduces cardiovascular risk factors such as dyslipidemia and hypertension [19, 20, 35-37].

The potential therapeutic role of carnosine related to diabetes and diabetic nephropathy has already been investigated by many others. Several experimental models showed that carnosine supplementation is beneficial in the prevention and treatment of type 2 diabetes and its complications. To our knowledge there are currently no studies which

investigated the potential benefit in patients with diabetes or diabetic nephropathy, but there are studies on effect of carnosine supplementation regarding glucose and insulin tolerance in non-diabetic patients.

Recently, a pilot study with carnosine supplementation by Courten *et al*. [38] showed in a cohort of nondiabetic obese patients, who received oral carnosine supplementation for 12 weeks, that there was a relative preservation of insulin sensitivity and secretion as well as normalization of glucose intolerance in the carnosine supplemented group compared to the placebo group. The results on the insulin and glucose levels are promising and indicate that carnosine supplementation may be beneficial in diabetes. Perhaps, patients with diabetic nephropathy will even have more profit from the carnosine intervention compared to patients without renal involvement due to the renoprotective features of carnosine.

We would like to speculate on the potential mechanism of action of carnosine in diabetic nephropathy. In chapter six, we found that the storage of carnosine-related enzymes in patients with diabetic nephropathy seems to be reallocated. It could be that there is an increase of oxidative stress within the kidney due to the reallocation of these proteins. These oxidative stress factors may induce the development of renal damage. Therefore, it might be that by the supplementation of carnosine the reallocation of these enzymes is preserved or restored, which will consequently lead to less oxidative stress factors in the kidney and will therefore inhibit the development of renal damage. In future studies, it is necessary to investigate the difference of the carnosine metabolism in patients with diabetic nephropathy and controls in larger groups with well-characterized clinical and histological data, since the number of patients with diabetic nephropathy, in which the primary results were obtained, were relatively small.

Finally, future investigations may reveal whether all patients will benefit from oral carnosine supplementation. It may be possible that the response on this intervention depends on the *CNDP1* gene. If this is the case, the *CNDP1* gene could serve as a genetic biomarker which determines the response rate on carnosine supplementation in patients with diabetes and diabetic nephropathy.

For both the anti-inflammatory as well as the carnosine supplementation, it would be very interesting to investigate whether these potential therapies only inhibit renal damage or whether these therapeutic agents actually reverse structural renal damage. The thought on the reverse of renal damage by therapeutic intervention originates from the study of Fioretto *et al*. [39], who investigated the renal structures of a small group of patients with type 1 diabetes after 10 years follow-up. These patients had

received a pancreas transplantation and were therefore normoglycemic. Fioretto *et al.* [39] reported that after ten years follow-up glomerular and interstitial lesions of diabetic nephropathy were reversible in these patients. They hypothesized that the substantial architectural remodeling in the kidneys is the result of long-term normalization of glucose and insulin levels. Based on these findings, it would be interesting to determine whether other treatments are also able to reverse the structural damage of diabetes in the kidneys.

Research methods for diabetic nephropathy

At this moment, diabetic nephropathy is diagnosed by clinical parameters including microalbuminuria, proteinuria and/or decline of renal function [23]. Still, a renal biopsy is the golden standard to establish that the renal damage is caused by diabetes. It is necessary to bear in mind that in clinical practice a renal biopsy is only performed when it might have therapeutic consequences i.e., mostly when another renal disease is suspected next to diabetic nephropathy, which may change the therapeutic regimen. Therefore, studies which use renal biopsy material of patients with diabetic nephropathy may not be representative for the structural damage of the general diabetic nephropathy population.

In this thesis we used renal tissue specimens of autopsy material of patients suffering from diabetes to evaluate the histological lesions. In autopsy material there is a decreased selection bias compared to a renal biopsy material regarding the moment in time at which the biopsy was performed; the commentary accompanied with our publication by Said and Nsar [6] underscored this benefit. Another benefit of autopsy material is that it is possible to observe a relatively large amount of renal tissue – at least one hundred glomeruli – compared to a renal biopsy, which approximately contains ten glomeruli. On the other hand, research with autopsy material has some limitations, since post-mortem, autolytic processes may influence certain histological and clinical markers. Still, the use of autopsy material is a non-invasive option to correlate and quantify histological findings to clinical features and is therefore useful to investigate the pathogenesis of renal damage in diseases such as diabetes. The beneficial aspects of autopsy material in the research on diabetic nephropathy suggest that autopsy material should be used more frequently for research purposes.

Another method to investigate the pathogenesis of diabetic nephropathy is to investigate the involved pathways in experimental models. Additionally, specific diabetic knockout models could be used to determine the pharmacological and renoprotective effects of potential therapies. This research method has also some limitations that merit discussion.

The use of experimental models of diabetic nephropathy have been constrained by the fact that most models fail to recapitulate important functional and structural features of the human renal disease [40]. The validation criteria for animal models regarding diabetic nephropathy are based on the clinical findings, including more than 50% decrease in renal function, more than ten-fold increase in albuminuria as well as histological findings, including thickening of the glomerular basement membrane, advanced mesangial matrix expansion, presence of nodular sclerosis, arteriolar hyalinosis and tubulointerstitial fibrosis [41]. The ideal experimental model would comprise all of these criteria; at this moment, none of the existing models entirely comply with those criteria [42], the problem is that the existing models fail to develop histological lesions such as nodular sclerosis and tubulointerstitial fibrosis in combination with progressive renal insufficiency [43]. Finally, it is necessary to question whether the pathophysiological pathways induced in the kidney of these experimental models reflect similar pathways observed in patients with diabetic nephropathy [40]. Currently, it is accepted that combining the results of several rodent models obtained from these available experimental models can be used to study diabetic nephropathy [43].

Inflammatory markers in the pathologic classification

Due to the pivotal role of inflammation in diabetic nephropathy it would be interesting to investigate whether inflammatory parameters can be added to the pathologic classification of diabetic nephropathy. Since we hypothesized in chapter three that probably the expression profile of the macrophages are equally or more important than their numbers, investigations regarding histological markers should focus on the different types of macrophages present in the glomeruli and interstitium of patients with diabetic nephropathy. Next, it would be necessary to evaluate whether these inflammatory markers predict the severity of the renal damage caused by inflammation and if they can be associated with clinical data. Clear cut-off points on the intensity of these stainings or a cumulative scoring system may help to create generally accepted histological inflammatory markers of diabetic nephropathy. The next step would be to associate these markers to the pathologic classification of diabetic nephropathy; eventually these markers might be added to this classification. Validation studies may be created to investigate whether these newly developed histological inflammatory stainings are associated to renal outcome. Finally, these markers can be standardized in research setting and may eventually be used in diagnostic setting during the evaluation of a renal biopsy of patients with diabetic nephropathy.

CONCLUSION

We highlighted several possible research methods for the investigation of diabetic nephropathy. Not one method seems to be sufficient to solve the complex puzzle of diabetic nephropathy. However, the combination of different study designs on the pathogenesis and the involved pathways of diabetic nephropathy together with clinical trials, which need to determine the effect of potential novel therapy options in patients with diabetic nephropathy, might help to decrease the development and the progression of diabetic nephropathy in the future.

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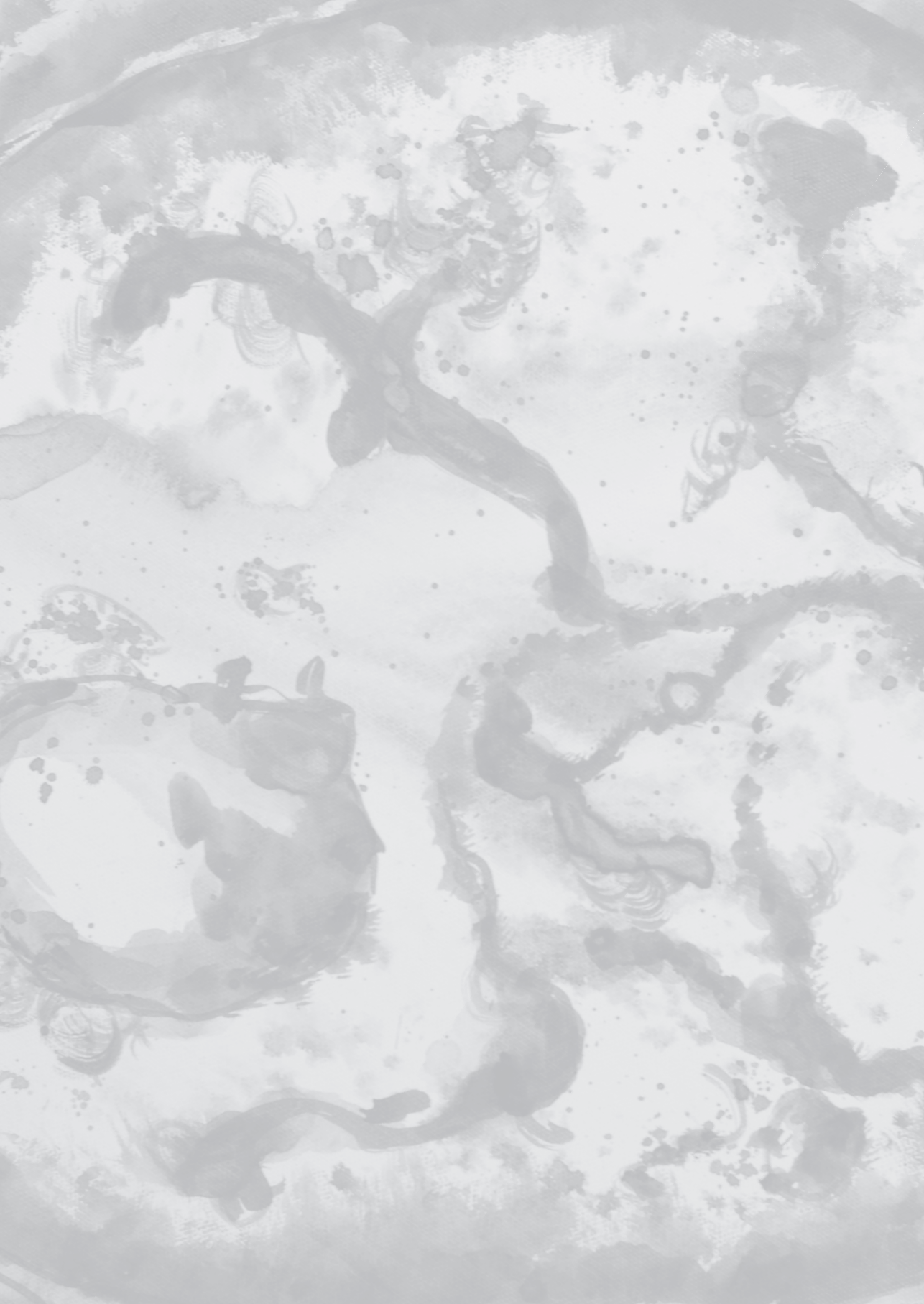


Nederlandse samenvatting

8

Diabetes mellitus - in de volksmond bekend als suikerziekte - is een ziekte waarbij er een (relatief) tekort is aan het hormoon insuline in het lichaam. Patiënten met diabetes hebben door dit tekort te hoge suiker (glucose) in hun bloed en urine. Insuline zorgt ervoor dat glucose in de cel wordt opgenomen alwaar het gebruikt wordt als brandstofbron.

De twee bekendste vormen van diabetes mellitus zijn type 1 en type 2 diabetes. Bij patiënten met type 1 diabetes zijn de cellen in de alvleesklier die insuline produceren door toedoen van een auto immuunreactie door het lichaam zelf vernietigd. Door de afwezigheid van insuline moeten deze patiënten zichzelf levenslang insuline toedienen om glucose op te kunnen nemen. Bij patiënten met type 2 diabetes produceert de alvleesklier nog wel insuline, hoewel vaak in een verminderde hoeveelheid. Bij deze patiënten is het probleem dat het weefsel de glucose, die in de bloedbaan circuleert, niet of in verminderde mate opneemt. Er is sprake van insulineresistentie, een verminderde gevoeligheid voor insuline. De behandeling van type 2 diabetes is gericht op het verlagen van de insulineresistentie enerzijds door de weefsels te stimuleren om glucose op te nemen en anderzijds door het stimuleren van de alvleesklier om meer insuline te produceren. Uiteindelijk kunnen patiënten met type 2 diabetes ook insuline behoeftig worden. Door middel van verschillende behandelopties wordt geprobeerd om goede, stabiele bloedsuikerspiegels in type 1 en type 2 diabetes te behouden. Hoge glucose- en insulinewaardes kunnen na verloop van tijd resulteren in schade aan de vaten en zenuwen van de ogen, voeten, hart en nieren. Dit proefschrift richt zich op de afwijkingen aangericht door diabetes mellitus in de nieren, het onderliggende mechanisme daarvan en het reactiepatroon van het lichaam hierop; wat in medisch jargon diabetische nefropathie wordt genoemd.

De diagnose diabetische nefropathie wordt gesteld aan de hand van aanwezigheid van teveel eiwitten in de urine (microalbuminurie) en verminderde nierfunctie gemeten in het bloed. Deze klinische diagnose kan worden bevestigd door middel van een nierbiopt. Na het nemen van het biopt wordt het verkregen nierweefsel onder de microscoop onderzocht. Hierbij wordt door middel van histologie (weefselleer) gekeken naar afwijkingen in de nier (pathologie). Deze nierschade aangericht door diabetes mellitus kan in verschillende structuren van de nier voorkomen, waaronder de zeeflichaampjes - structuren met zeer kleine bloedvaatjes die stoffen uit het bloed filteren (glomeruli), het omliggende bindweefsel (interstitium), het buizensysteem (tubuli) en de bloedvaten. In één nier zijn ongeveer één miljoen nefronen aanwezig. Een nefron is een glomerulus met bijbehorende tubuli.

Bij patiënten met diabetische nefropathie worden weinig nierbiopten afgenomen, zeker als er geen sprake is van een afwijkend klinisch beloop. De behandeling van diabetische nefropathie is gebaseerd op bevindingen in de kliniek en niet zozeer op de bevindingen in het nierbiopt.

Wanneer er wel een biopt wordt genomen, kan door middel van een histologisch classificatiesysteem voor diabetische nefropathie de nierschade aangericht door diabetes worden geclassificeerd. Een dergelijke classificatie is nuttig om de mate van de nierschade in kaart te brengen en eenduidig hierover te kunnen communiceren. Binnen de classificatie zijn de afwijkingen onderverdeeld in vier klassen. In eerste instantie worden afwijkingen ingedeeld aan de hand van de afwijkingen in de glomeruli. Daarnaast worden ook afwijkingen in de andere compartimenten van de nier, het interstitium en de bloedvaten, gescoord.

In klasse I is er sprake van een verdikking van de basaalmembraan in de glomerulus, het filtermembraan waarover stoffen passeren van het bloed naar de voorloper van urine. Klasse II wordt gescoord wanneer er toename is van het mesangium. Mesangium ondersteunt het vaatbed in de glomerulus. Deze klasse kan worden onderverdeeld in klasse IIa en IIb naar mate er sprake is van milde of ernstige toename van het mesangiale weefsel. Bij klasse III is er een nodulaire laesie (bolvormige afwijking) aanwezig in tenminste één glomerulus. Een dergelijk nodulus staat bekend als een Kimmelstiel-Wilson laesie en wordt vaak gezien bij diabetische nefropathie. Klasse IV diabetische nefropathie is het laatste stadium met glomerulosclerose, verbindweefseling van de gehele glomerulus, in meer dan 50% van de glomeruli in het nierbiopt.

In **hoofdstuk 2** werd gebruik gemaakt van nierweefsel verkregen bij obductie van een cohort van patiënten met diabetes die overleden zijn aan een willekeurige doodsoorzaak. Het voordeel van obductiemateriaal is dat een relatief groot oppervlakte, tenminste 100 glomeruli, van de nier bekeken kan worden onder de microscoop in vergelijking met een nier biopt, wat gemiddeld 10 glomeruli bevat.

In deze studie, zijn de geobserveerde afwijkingen in het nier obductie materiaal geclassificeerd volgens het histologische classificatiesysteem van diabetische nefropathie. Met deze studie hebben we geprobeerd meer inzicht te verkrijgen in de relatie tussen de histologische veranderingen en klinische gegevens van patiënten met diabetes met en zonder klinisch vastgestelde diabetische nefropathie. We vonden dat een substantieel deel (20%) van de patiënten met histologische nierschade geen eiwitverlies in de urine had tijdens hun leven. Dit suggereert dat er al afwijkingen door diabetes in de

nier aanwezig kunnen zijn voordat dit zichtbaar is in de urine van de patiënt. Het is niet bekend of het relevant is om deze patiënten eerder op te sporen en te behandelen, hiervoor is meer onderzoek nodig.

De meerderheid van de patiënten die niervervangende therapie, zoals dialyse of niertransplantatie, nodig hebben zijn diabetespatiënten. Dit komt mede doordat er op dit moment geen therapie is die diabetische nefropathie kan tegengaan, terugdringen, danwel genezen. Er is op dit moment veel onderzoek gaande naar potentiële behandelingen die kunnen bijdragen om de nierschade te verminderen. Een van de potentieel nieuwe behandelingen berust op een anti-inflammatoire therapie, een therapie die zich richt tegen ontstekingscellen.

Het is de afgelopen decennia duidelijk geworden dat er een ontstekingsreactie optreedt bij patiënten met type 2 diabetes; deze reactie lijkt ook invloed te hebben op de ontwikkeling van de nierschade. Er zijn in de literatuur weinig studies die gekeken hebben naar de aanwezigheid van ontstekingscellen in de nieren van patiënten met diabetes en diabetische nefropathie. Daarom hebben wij in **hoofdstuk 3** de aantallen en bepaalde typen ontstekingscellen in de glomeruli en het omliggende bindweefsel in patiënten met type 2 diabetes uit de onderzochte groep van hoofdstuk 2, het autopsie cohort, onderzocht. Deze resultaten zijn gerelateerd aan de histologische classificatie. Tevens is er gekeken of er een correlatie bestaat met klinische parameters. De hoeveelheid ontstekingscellen kon niet gerelateerd worden aan de verschillende histologische klassen. Het zou kunnen zijn dat het type van de ontstekingscellen van grotere invloed is op de nierschade dan de exacte hoeveelheid. Het is niet mogelijk in deze studie het exacte werkingsmechanisme van deze verschillende ontstekingscellen in diabetische nefropathie te onderzoeken. De aanwezigheid van de ontstekingscellen bij de patiënten met diabetische nefropathie in deze studie geeft wel aanwijzing voor de mogelijkheid om behandelingen te ontwikkelen die zich kunnen richten tegen ontstekingsprocessen in de nieren van patiënten met diabetische nefropathie.

In **hoofdstuk 4** hebben we de hierboven beschreven histologische classificatie van diabetische nefropathie onder de loep genomen en gekeken naar de reproduceerbaarheid en validiteit van het classificatiesysteem. In het eerste deel werden professionals gevraagd om biopten te scoren volgens het classificatiesysteem en daarnaast hun oordeel te geven over onduidelijkheden binnen de classificatie. In het tweede gedeelte zijn de validatiestudies, die beschreven zijn in de literatuur, in kaart gebracht en geanalyseerd. Er werden vier validatie studies gevonden, deze studies maakten alle vier gebruik van Aziatische populaties uit Japan, China en Korea. Via een meta-analyse is er gekeken naar de relatie tussen de verschillende klassen gerelateerd aan de overlevingsduur van

de nier. Een meta-analyse is een onderzoek dat resultaten uit eerder beschreven studies samen neemt om een preciezere uitspraak te doen over een bepaald fenomeen of theorie. Door de classificatie op deze twee manieren te onderzoeken, zijn er voorstellen gedaan om de histologische classificatie van diabetische nefropathie te verbeteren.

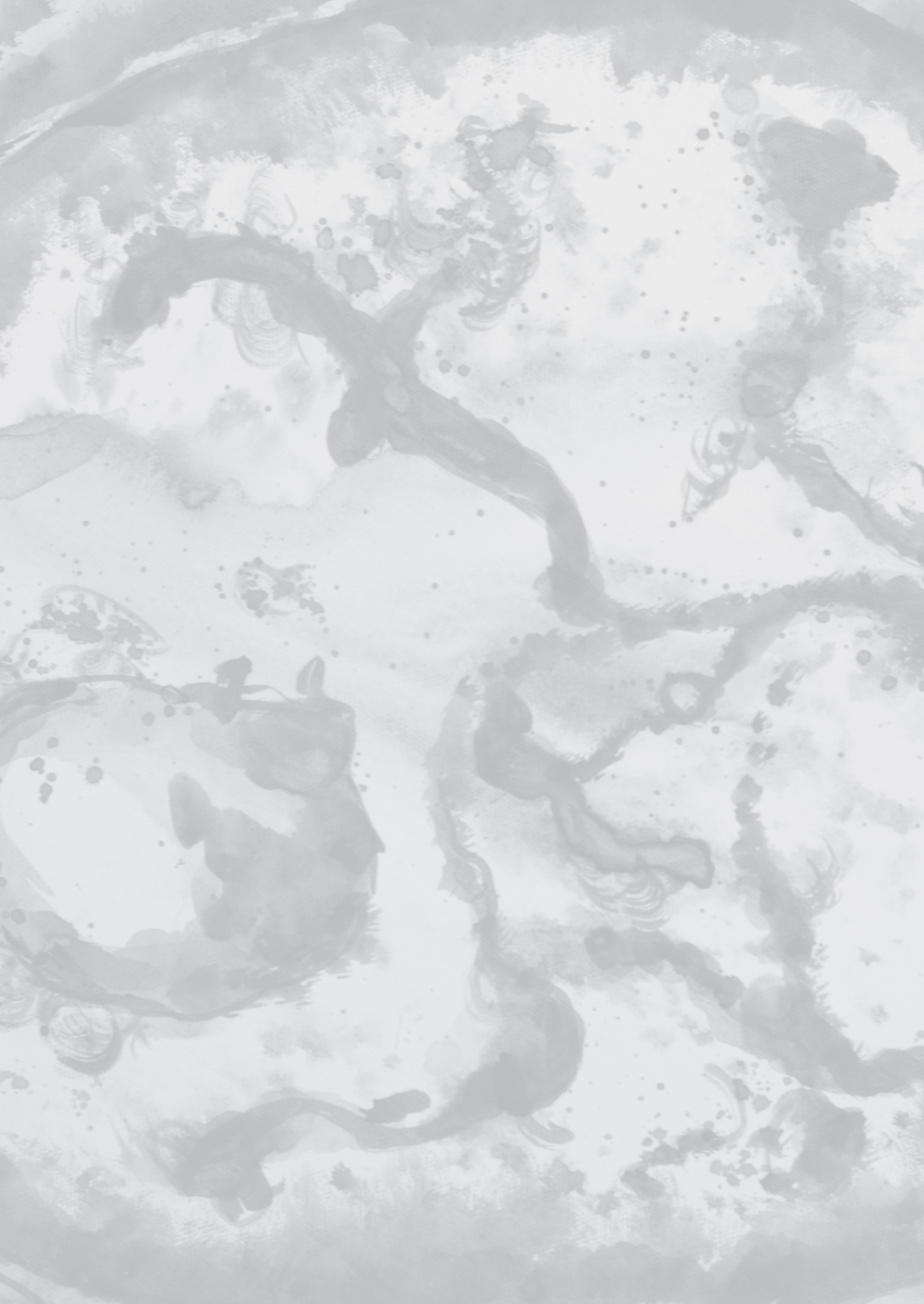
Uit wetenschappelijk onderzoek is gebleken dat er een genetische component meespeelt in de ontwikkeling van diabetische nefropathie; een van de genen die geassocieerd is met diabetische nefropathie, is het *CNDP1* gen. Het *CNDP1* gen codeert voor het enzym carnosinase. Dit is een enzym dat het eiwit carnosine afbreekt. Er wordt gedacht dat het 5-5 homozygote polymorfisme van het *CNDP1* gen leidt tot verminderde carnosinase uitscheiding en dus meer carnosine in de bloedbaan, vergeleken met de andere polymorfismen van het *CNDP1* gen. Carnosine heeft verschillende beschermende effecten in het lichaam; het is een antioxidant, het kan werken als pH buffer en het werkt potentieel bloeddrukverlagend. Daarnaast kan het zogenaamde 'advanced glycation end products' afbreken, afvalstoffen die ontstaan door te hoge bloedsuikers bij diabetes mellitus. In de nier kan carnosine de uitbreiding van mesangiale matrixeiwitten voorkomen. De mesangiale matrix is vaak verbreed bij diabetische nierziekte zoals beschreven bij klasse II van de classificatie.

Tot nu toe is het *CNDP1* gen alleen geassocieerd met diabetische nefropathie in type 2 diabetes uitgaande van patiënten waarbij diabetische nefropathie alleen klinisch gediagnosticeerd was, dus zonder histologische bevestiging middels een nierbiopt. In **hoofdstuk 5** hebben wij laten zien dat deze associatie ook aanwezig is bij patiënten met histologisch bewezen diabetische nefropathie. Tevens vonden we dat de nodulaire afwijkingen, de Kimmelstiel-Wilson noduli, die kenmerkend zijn voor diabetische nefropathie, minder voorkomen bij patiënten met het 5-5 homozygote polymorfisme van *CNDP1* ten opzichte van de andere polymorfismen. Op dit moment is er nog weinig bekend over de ontwikkeling van deze noduli en of deze invloed hebben op het beloop van de ziekte. Het zou kunnen zijn dat het *CNDP1* genotype betrokken is bij de ontwikkeling van deze laesies.

In **hoofdstuk 6** hebben we onderzocht of de nier een eigen carnosine metabolisme heeft. Dat wil zeggen dat zowel het eiwit carnosine als de afbrekende en synthetiserende enzymen, carnosinase en carnosine synthase, aanwezig zijn in verschillende compartimenten van de nier. Deze hypothese is met behulp van verschillende onderzoeken bevestigd. Ook vonden we dat deze enzymen verschoven binnen de verschillende compartimenten van de nier bij patiënten met diabetische nefropathie. Dit suggereert dat het carnosine metabolisme betrokken is bij de ontwikkeling danwel het beloop van de nierschade door diabetes mellitus.

Orale carnosine suppletie zou mogelijke een nieuwe therapie voor patiënten met diabetische nefropathie kunnen zijn. Op dit moment wordt carnosine veel gebruikt als voedingssupplement door sporters voor sneller herstel en vanwege het anti-oxidatieve effect. Het gebruik van carnosine lijkt weinig bijwerkingen te hebben. Meerdere dier-experiment studies, die carnosine in het drinkwater van de dieren toevoegden lieten zien dat de toevoeging van carnosine een positief effect had op de glucose en insuline spiegels. Het zou kunnen zijn dat hogere carnosine spiegels gunstig zijn voor patiënten met diabetische nefropathie. Toekomstige studies zouden tevens onderzoek kunnen doen naar de invloed van de verschillende polymorfismen van het *CNDP1* gen en de respons op de suppletie, wanneer patiënten carnosine als voedingssupplement zouden krijgen toegediend. Het is namelijk niet duidelijk of patiënten met het beschermende *CNDP1* polymorfisme minder gebaat zijn bij de orale toediening van carnosine dan patiënten met andere polymorfismen van het *CNDP1* gen.

De resultaten uit dit proefschrift bieden de mogelijkheid om in toekomstige studies te onderzoeken of anti-inflammatoire therapie of carnosine suppletie invloed hebben op de ontwikkeling en progressie van diabetische nefropathie, waarbij naast albuminurie ook naar andere potentiele markers voor diabetische nefropathie gekeken moet worden. Samenvattend biedt dit proefschrift nieuwe inzichten in de onderliggende mechanismes van diabetische nefropathie.



List of publications

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Dankwoord

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Céline Quirine Françoise Klessens is op zondag 26 april 1992 geboren in Rotterdam, waar zij opgroeide en in 2010 haar gymnasiumdiploma (Natuur & Gezondheid en Natuur & Techniek) aan het Erasmiaans Gymnasium behaalde. In datzelfde jaar begon Céline met de studie Geneeskunde aan de Universiteit Leiden en werd lid van L.S.V. Minerva. Haar ambitie voor een internationale carrière en interesse voor alternatieve geneeswijzen leidden ertoe dat zij in de zomer van haar tweede jaar (2012) het vak 'Traditional Chinese Medicine' aan de Universiteit van Hangzhou, China, volgde.

Tijdens haar bachelor Geneeskunde startte Céline met haar onderzoeksproject over diabetische nefropathie bij de afdeling Pathologie van het Leids Universitair Medisch Centrum. Daarnaast volgde zij de Honours Class 'Metabolic Disorders', waar metabole ziektes vanuit verschillende disciplines belicht werden. Hiermee verbreedde ze haar kennis over de omvang en maatschappelijke impact van dit probleem. Het volgen van deze Honours Class in combinatie met haar onderzoeksproject resulteerde erin dat de Universiteit Leiden haar opnieuw benaderde om te participeren aan het Honours College, dat zij gelijktijdig met haar bachelor diploma Geneeskunde in september 2013 afrondde.

Na haar bachelor diploma vertrok ze naar Calcutta, India, voor vrijwilligerswerk bij Future Hope, een non-profit organisatie (NGO), die kansarme straat kinderen een toekomst biedt. Voor Future Hope heeft Céline samen met anderen verschillende keren in Nederland een benefietdiner georganiseerd. Koken is namelijk een grote passie van haar. Toen ze terug kwam uit India eind 2013, is ze begonnen met fulltime promotieonderzoek op de afdeling Pathologie van het Leids Universitair Medisch Centrum (hoofd: prof. dr. V.T.H.B.M. Smit). In 2014 en 2015 ontving zij de Kolff student-onderzoekers beurs van de Nierstichting. Céline heeft meerdere keren de resultaten van haar onderzoek gepresenteerd op internationale congressen zoals de American Society of Nephrology Kidney Week in Atlanta in 2013, Philadelphia in 2014 en San Diego in 2015. Voor deze congressen ontving ze verscheidene beurzen van het Leids Universiteits Fonds (LUF), Tebu Bio en Stichting Preventie Diabetes. Tevens werden de resultaten van haar abstract in 2014 uitgelicht in een persbericht in de Verenigde Staten en later ook in Nederland. Voor het congres in San Diego in 2015 werd Céline verkozen om deel te nemen aan het 'Kidney Stars' programma van de American Society of Nephrology.

Naast haar medische, wetenschappelijke carrière, heeft Céline ook een 'business-mind'; zo is ze tijdens haar PhD werkzaam geweest als consultant bij 'De Kleine Consultant', dit is een NGO die door studenten wordt geleid en strategisch advies geeft aan bedrijven. A.T. Kearney, het consultancy kantoor dat de projecten van 'De Kleine Consultant' begeleid, heeft gevraagd of Céline een aantal maanden bij hen stage wilde komen lopen. Dit heeft zij met veel plezier gedaan in het voorjaar van 2016. In juni 2016 is Céline begonnen aan haar coschappen en zal in het najaar van 2018 haar artsexamen behalen.

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