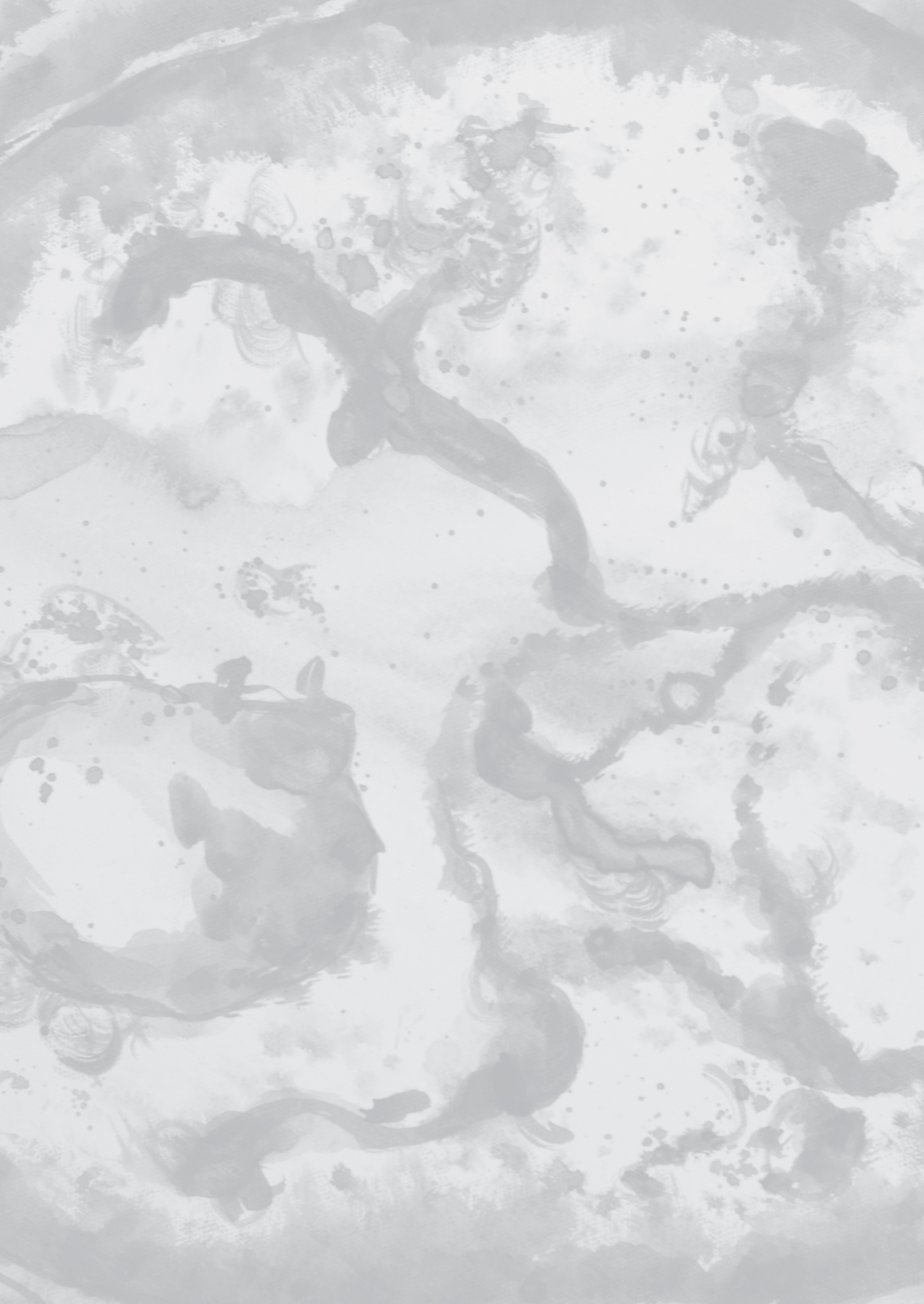
Diabetic nephropathy : from histological findings to clinical features

Klessens, C.Q.F.



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].

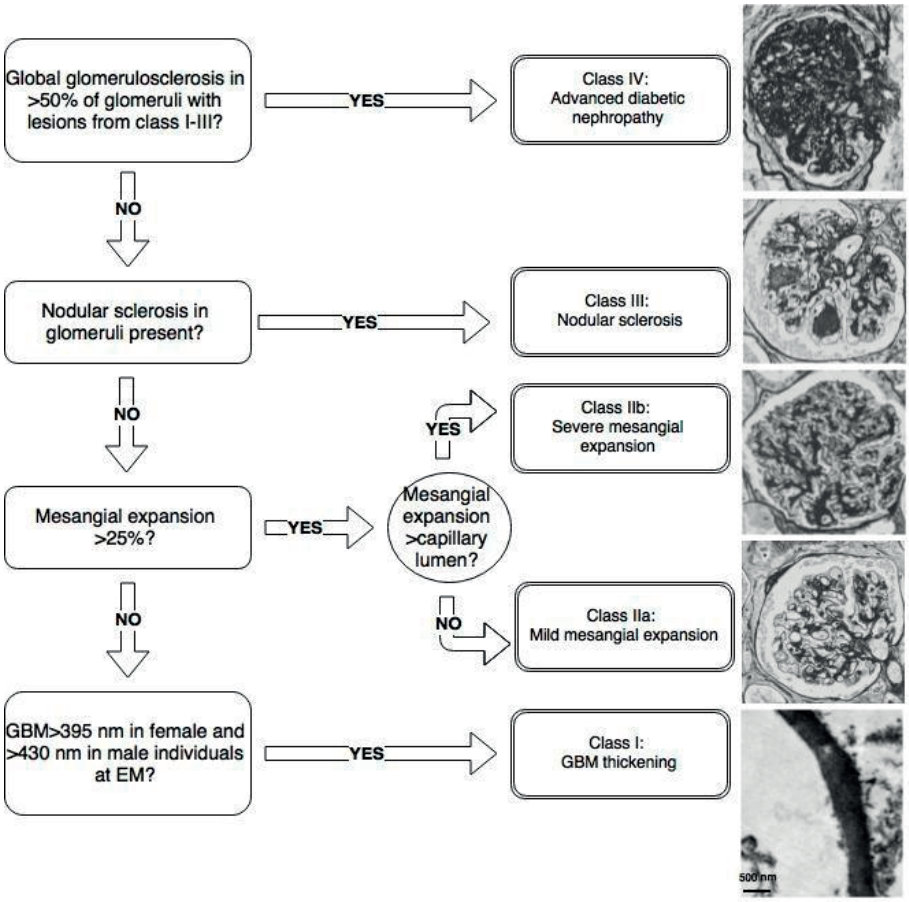
In past years, several validation studies investigated the clinical value of the pathologic classification system by associating the classification of diabetic nephropathy with renal outcome [24, 25, 30, 31]. Overall, these validation studies showed that there is a good association between the histopathological 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.

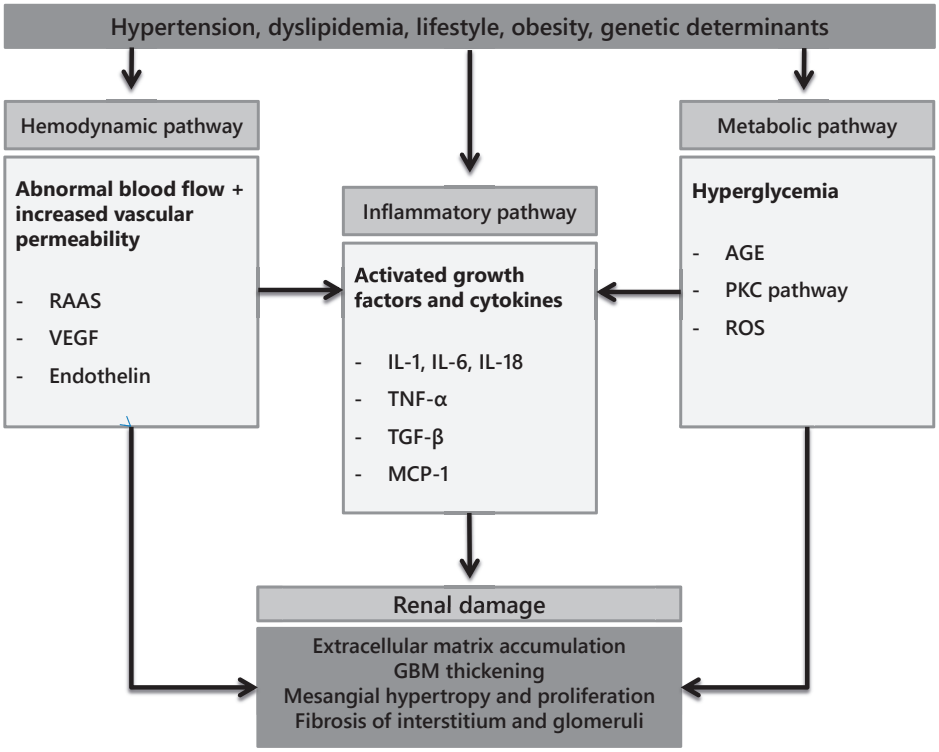
Regarding therapy response between the two types of diabetes, RAAS inhibition does not seem to prevent or delay the progression of renal damage in type 2 diabetes as effectively as it does in type 1 diabetes [46, 47]. The GISEN group hypothesized that the inhibition of RAAS is more effective in type 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.

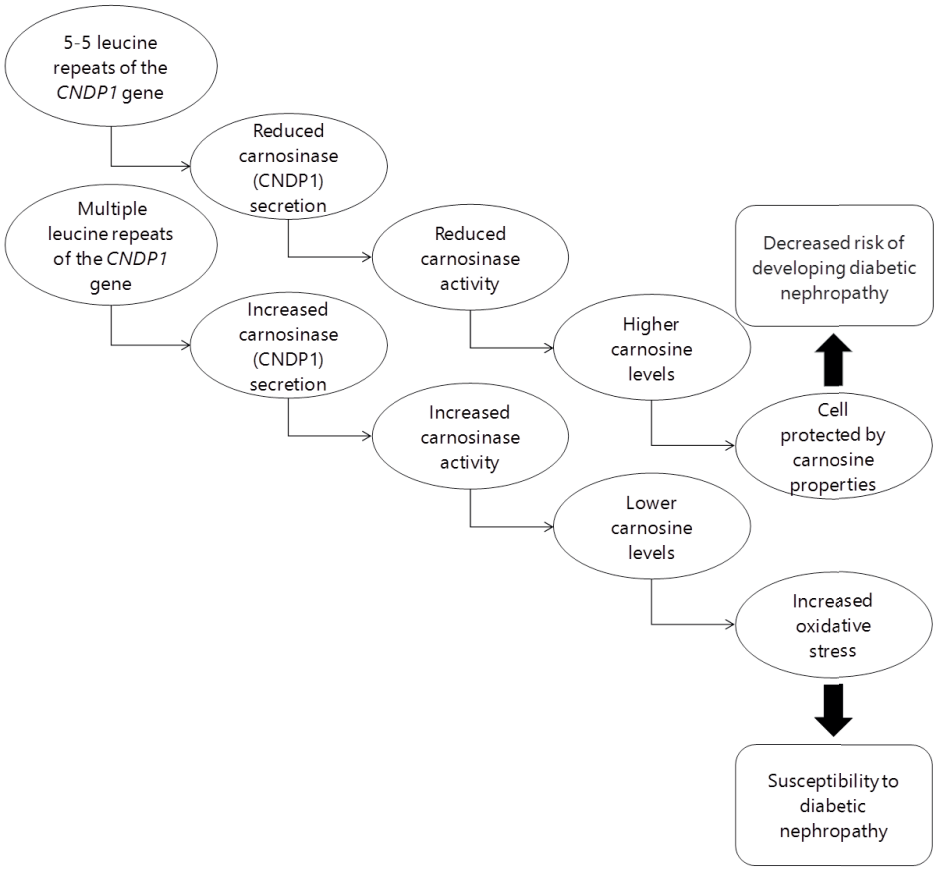
The first studies on genetic involvement in diabetic nephropathy were linkage analyses in family studies. Most of these family studies contained relatively small numbers of families, but they detected several consistent regions of linkage and revealed that there was variation in diabetic nephropathy 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.

REFERENCES

1. International Diabetes Federation. IDF Diabetes Atlas, Seventh Edition. 2015
2. Ali MK, Bullard KM, Gregg EW, et al. A cascade of care for diabetes in the United States: visualizing the gaps. *Ann Intern Med*, 2014; 161: 681-9
3. Andersen AR, Christiansen JS, Andersen JK, et al. Diabetic nephropathy in Type 1 (insulin-dependent) diabetes: an epidemiological study. *Diabetologia*, 1983; 25: 496-501
4. Chaturvedi N, Bandinelli S, Mangili R, et al. Microalbuminuria in type 1 diabetes: rates, risk factors and glycemic threshold. *Kidney Int*, 2001; 60: 219-27
5. Kunzelman CL, Knowler WC, Pettitt DJ, et al. Incidence of proteinuria in type 2 diabetes mellitus in the Pima Indians. *Kidney Int*, 1989; 35: 681-7
6. Afkarian M, Sachs MC, Kestenbaum B, et al. Kidney disease and increased mortality risk in type 2 diabetes. *J Am Soc Nephrol*, 2013; 24: 302-8
7. Cooper ME. Pathogenesis, prevention, and treatment of diabetic nephropathy. *Lancet*, 1998; 352: 213-9
8. Boron WF and Boulpaep EL. Medical Physiology. Second Edition. *Elsevier Health Sciences*
9. Remuzzi G, Schieppati A, and Ruggenenti P. Clinical practice. Nephropathy in patients with type 2 diabetes. *N Engl J Med*, 2002; 346: 1145-51
10. Pugliese G. Updating the natural history of diabetic nephropathy. *Acta Diabetol*, 2014; 51: 905-15
11. Gesualdo L and Di Paolo S. Renal lesions in patients with type 2 diabetes: a puzzle waiting to be solved. *Nephrol Dial Transplant*, 2015; 30: 155-7
12. Ponchiardi C, Mauer M, and Najafian B. Temporal profile of diabetic nephropathy pathologic changes. *Curr Diab Rep*, 2013; 13: 592-9
13. Alsaad KO and Herzenberg AM. Distinguishing diabetic nephropathy from other causes of glomerulosclerosis: an update. *J Clin Pathol*, 2007; 60: 18-26
14. Wada T, Shimizu M, Yokoyama H, et al. Nodular lesions and mesangiolysis in diabetic nephropathy. *Clin Exp Nephrol*, 2013; 17: 3-9
15. Stout LC, Kumar S, and Whorton EB. Focal mesangiolysis and the pathogenesis of the Kimmelstiel-Wilson nodule. *Hum Pathol*, 1993; 24: 77-89
16. Makino H, Shikata K, Kushiro M, et al. Roles of advanced glycation end-products in the progression of diabetic nephropathy. *Nephrol Dial Transplant*, 1996; 11 Suppl 5: 76-80
17. Yasuda T, Imai H, Nakamoto Y, et al. Significance of fibrils in the formation of the Kimmelstiel-Wilson nodule. *Virchows Arch Pathol Anat Histopathol*, 1992; 421: 297-303
18. Steffes MW, Osterby R, Chavers B, et al. Mesangial expansion as a central mechanism for loss of kidney function in diabetic patients. *Diabetes*, 1989; 38: 1077-81
19. Schwartz MM, Lewis EJ, Leonard-Martin T, et al. Renal pathology patterns in type II diabetes mellitus: relationship with retinopathy. The Collaborative Study Group. *Nephrol Dial Transplant*, 1998; 13: 2547-52
20. Sandison A, Newbold KM, and Howie AJ. Evidence for unique distribution of Kimmelstiel-Wilson nodules in glomeruli. *Diabetes*, 1992; 41: 952-5

21. Saito Y, Kida H, Takeda S, et al. Mesangiolysis in diabetic glomeruli: its role in the formation of nodular lesions. *Kidney Int*, 1988; 34: 389-96

22. Fioretto P and Mauer M. Histopathology of diabetic nephropathy. *Seminars in Nephrology*, 2007; 27: 195-207

23. Tonolo G and Cherchi S. Tubulointerstitial disease in diabetic nephropathy. *Int J Nephrol Renovasc Dis*, 2014; 7: 107-15

24. Okada T, Nagao T, Matsumoto H, et al. Histological predictors for renal prognosis in diabetic nephropathy in diabetes mellitus type 2 patients with overt proteinuria. *Nephrology (Carlton)*, 2012; 17: 68-75

25. Mise K, Hoshino J, Ubara Y, et al. Renal prognosis a long time after renal biopsy on patients with diabetic nephropathy. *Nephrol Dial Transplant*, 2014; 29: 109-18

26. Stout LC, Kumar S, and Whorton EB. Insudative lesions--their pathogenesis and association with glomerular obsolescence in diabetes: a dynamic hypothesis based on single views of advancing human diabetic nephropathy. *Hum Pathol*, 1994; 25: 1213-27

27. Fogo AB, Cohen AH, Jennette JC, et al. Fundamentals of Renal Pathology. 2007

28. Haas M, Rastaldi MP, and Fervenza FC. Histologic classification of glomerular diseases: clinicopathologic correlations, limitations exposed by validation studies, and suggestions for modification. *Kidney Int*, 2014; 86: 648

29. Tervaert TW, Mooyaart AL, Amann K, et al. Pathologic classification of diabetic nephropathy. *J Am Soc Nephrol*, 2010; 21: 556-63

30. Oh SW, Kim S, Na KY, et al. Clinical implications of pathologic diagnosis and classification for diabetic nephropathy. *Diabetes Res Clin Pract*, 2012; 97: 418-24

31. An Y, Xu F, Le W, et al. Renal histologic changes and the outcome in patients with diabetic nephropathy. *Nephrol Dial Transplant*, 2015; 30: 257-66

32. Thomas MC, Macisaac RJ, Jerums G, et al. Nonalbuminuric renal impairment in type 2 diabetic patients and in the general population (National evaluation of the frequency of renal impairment cO-existing with NIDDM [NEFRON] 11). *Diabetes Care*, 2009; 32: 1497-502

33. Magee GM, Bilous RW, Cardwell CR, et al. Is hyperfiltration associated with the future risk of developing diabetic nephropathy? A meta-analysis. *Diabetologia*, 2009; 52: 691-7

34. Mogensen CE, Christensen CK, and Vittinghus E. The stages in diabetic renal disease. With emphasis on the stage of incipient diabetic nephropathy. *Diabetes*, 1983; 32 Suppl 2: 64-78

35. Reutens AT and Atkins RC. Epidemiology of diabetic nephropathy. *Contrib Nephrol*, 2011; 170: 1-7

36. Wild S, Roglic G, Green A, et al. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*, 2004; 27: 1047-53

37. Kramer HJ, Nguyen QD, Curhan G, et al. Renal insufficiency in the absence of albuminuria and retinopathy among adults with type 2 diabetes mellitus. *JAMA*, 2003; 289: 3273-7

38. Retnakaran R, Cull CA, Thorne KI, et al. Risk factors for renal dysfunction in type 2 diabetes: U.K. Prospective Diabetes Study 74. *Diabetes*, 2006; 55: 1832-9

39. MacIsaac RJ, Panagiotopoulos S, McNeil KJ, et al. Is nonalbuminuric renal insufficiency in type 2 diabetes related to an increase in intrarenal vascular disease? *Diabetes Care*, 2006; 29: 1560-6

40. Kramer CK, Leitao CB, Pinto LC, et al. Clinical and laboratory profile of patients with type 2 diabetes with low glomerular filtration rate and normoalbuminuria. *Diabetes Care*, 2007; 30: 1998-2000
41. Fioretto P, Caramori ML, and Mauer M. The kidney in diabetes: dynamic pathways of injury and repair. The Camillo Golgi Lecture 2007. *Diabetologia*, 2008; 51: 1347-55
42. White KE and Bilous RW. Type 2 diabetic patients with nephropathy show structural-functional relationships that are similar to type 1 disease. *J Am Soc Nephrol*, 2000; 11: 1667-73
43. Olsen S. Identification of non-diabetic glomerular disease in renal biopsies from diabetics-a dilemma. *Nephrol Dial Transplant*, 1999; 14: 1846-9
44. Hasslacher C. Diabetic nephropathy: structural-functional relationships. *Contrib Nephrol*, 1989; 73: 24-8
45. Biesenbach G, Janko O, and Zazgornik J. Similar rate of progression in the predialysis phase in type I and type II diabetes mellitus. *Nephrol Dial Transplant*, 1994; 9: 1097-102
46. The GISEN Group. Randomised placebo-controlled trial of effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric, non-diabetic nephropathy. The GISEN Group (Gruppo Italiano di Studi Epidemiologici in Nefrologia). *Lancet*, 1997; 349: 1857-63
47. Schieppati A and Remuzzi G. The June 2003 Barry M. Brenner Comgan lecture. The future of renoprotection: frustration and promises. *Kidney Int*, 2003; 64: 1947-55
48. Ruggenenti P, Perna A, Benini R, et al. In chronic nephropathies prolonged ACE inhibition can induce remission: dynamics of time-dependent changes in GFR. Investigators of the GISEN Group. Gruppo Italiano Studi Epidemiologici in Nefrologia. *J Am Soc Nephrol*, 1999; 10: 997-1006
49. Ruggenenti P, Lauria G, Iliev IP, et al. Effects of manidipine and delapril in hypertensive patients with type 2 diabetes mellitus: the delapril and manidipine for nephroprotection in diabetes (DEMAND) randomized clinical trial. *Hypertension*, 2011; 58: 776-83
50. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature*, 2001; 414: 813-820
51. Wada J and Makino H. Inflammation and the pathogenesis of diabetic nephropathy. *Clin Sci*, 2013; 124: 139-52
52. Kim Y, Wie SH, Chang UI, et al. Comparison of the clinical characteristics of diabetic and non-diabetic women with community-acquired acute pyelonephritis: a multicenter study. *J Infect*, 2014; 69: 244-51
53. Toth-Manikowski S and Atta MG. Diabetic Kidney Disease: Pathophysiology and Therapeutic Targets. *J Diabetes Res*, 2015; 2015: 697010
54. Lim A. Diabetic nephropathy - complications and treatment. *Int J Nephrol Renovasc Dis*, 2014; 7: 361-81
55. Brownlee M. The pathobiology of diabetic complications - A unifying mechanism. *Diabetes*, 2005; 54: 1615-1625
56. Domingueti CP, Dusse LM, Carvalho M, et al. Diabetes mellitus: The linkage between oxidative stress, inflammation, hypercoagulability and vascular complications. *J Diabetes Complications*, 2016; 30: 738-45
57. Raptis AE and Viberti G. Pathogenesis of diabetic nephropathy. *Exp Clin Endocrinol Diabetes*, 2001; 109 Suppl 2: S424-37

58. Svensson M, Sundkvist G, Arnqvist HJ, et al. Signs of nephropathy may occur early in young adults with diabetes despite modern diabetes management: results from the nationwide population-based Diabetes Incidence Study in Sweden (DISS). *Diabetes Care*, 2003; 26: 2903-9

59. Ricardo SD, van Goor H, and Eddy AA. Macrophage diversity in renal injury and repair. *J Clin Invest*, 2008; 118: 3522-30

60. Anders HJ and Ryu M. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. *Kidney Int*, 2011; 80: 915-25

61. Haller H, Bertram A, Nadrowitz F, et al. Monocyte chemoattractant protein-1 and the kidney. *Curr Opin Nephrol Hypertens*, 2016; 25: 42-9

62. Navarro-Gonzalez JF and Mora-Fernandez C. Inflammatory pathways. *Contrib Nephrol*, 2011; 170: 113-23

63. Wada T, Furuichi K, Sakai N, et al. Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions of human diabetic nephropathy. *Kidney Int*, 2000; 58: 1492-9

64. Seok SJ, Lee ES, Kim GT, et al. Blockade of CCL2/CCR2 signalling ameliorates diabetic nephropathy in db/db mice. *Nephrol Dial Transplant*, 2013; 28: 1700-10

65. Duran-Salgado MB and Rubio-Guerra AF. Diabetic nephropathy and inflammation. *World J Diabetes*, 2014; 5: 393-8

66. Mosser DM and Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*, 2008; 8: 958-69

67. Tsalamandris C, Allen TJ, Gilbert RE, et al. Progressive decline in renal function in diabetic patients with and without albuminuria. *Diabetes*, 1994; 43: 649-55

68. Caramori ML, Fioretto P, and Mauer M. Low glomerular filtration rate in normoalbuminuric type 1 diabetic patients: an indicator of more advanced glomerular lesions. *Diabetes*, 2003; 52: 1036-40

69. Ekinci EI, Jerums G, Skene A, et al. Renal structure in normoalbuminuric and albuminuric patients with type 2 diabetes and impaired renal function. *Diabetes Care*, 2013; 36: 3620-6

70. Gilbert RE and Cooper ME. The tubulointerstitium in progressive diabetic kidney disease: more than an aftermath of glomerular injury? *Kidney Int*, 1999; 56: 1627-37

71. Taft JL, Nolan CJ, Yeung SP, et al. Clinical and histological correlations of decline in renal function in diabetic patients with proteinuria. *Diabetes*, 1994; 43: 1046-51

72. Pettitt DJ, Saad MF, Bennett PH, et al. Familial predisposition to renal disease in two generations of Pima Indians with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia*, 1990; 33: 438-43

73. Seaquist ER, Goetz FC, Rich S, et al. Familial clustering of diabetic kidney disease. Evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med*, 1989; 320: 1161-5

74. Vardarli I, Baier LJ, Hanson RL, et al. Gene for susceptibility to diabetic nephropathy in type 2 diabetes maps to 18q22.3-23. *Kidney Int*, 2002; 62: 2176-83

75. Placha G, Poznik GD, Dunn J, et al. A genome-wide linkage scan for genes controlling variation in renal function estimated by serum cystatin C levels in extended families with type 2 diabetes. *Diabetes*, 2006; 55: 3358-65

76. Bowden DW, Colicigno CJ, Langefeld CD, et al. A genome scan for diabetic nephropathy in African Americans. *Kidney Int*, 2004; 66: 1517-26

77. Imperatore G, Hanson RL, Pettitt DJ, et al. Sib-pair linkage analysis for susceptibility genes for microvascular complications among Pima Indians with type 2 diabetes. Pima Diabetes Genes Group. *Diabetes*, 1998; 47: 821-30

78. Freedman BI, Hicks PJ, Sale MM, et al. A leucine repeat in the carnosinase gene CNDP1 is associated with diabetic end-stage renal disease in European Americans. *Nephrol Dial Transplant*, 2007; 22: 1131-5

79. Janssen B, Hohenadel D, Brinkkoetter P, et al. Carnosine as a protective factor in diabetic nephropathy: association with a leucine repeat of the carnosinase gene CNDP1. *Diabetes*, 2005; 54: 2320-7

80. Tregouet DA, Groop PH, McGinn S, et al. G/T substitution in intron 1 of the UNC13B gene is associated with increased risk of nephropathy in patients with type 1 diabetes. *Diabetes*, 2008; 57: 2843-50

81. Wanic K, Placha G, Dunn J, et al. Exclusion of polymorphisms in carnosinase genes (CNDP1 and CNDP2) as a cause of diabetic nephropathy in type 1 diabetes: results of large case-control and follow-up studies. *Diabetes*, 2008; 57: 2547-51

82. Ng DP, Tai BC, and Lim XL. Is the presence of retinopathy of practical value in defining cases of diabetic nephropathy in genetic association studies? The experience with the ACE insertion/deletion polymorphism in 53 studies comprising 17,791 subjects. *Diabetes*, 2008; 57: 2541-6

83. Akai Y, Sato H, Ozaki H, et al. Association of transforming growth factor-beta1 T29C polymorphism with the progression of diabetic nephropathy. *Am J Kidney Dis*, 2001; 38: S182-5

84. Ahluwalia TS, Khullar M, Ahuja M, et al. Common variants of inflammatory cytokine genes are associated with risk of nephropathy in type 2 diabetes among Asian Indians. *PLoS One*, 2009; 4: e5168

85. Conserva F, Gesualdo L, and Papale M. A Systems Biology Overview on Human Diabetic Nephropathy: From Genetic Susceptibility to Post-Transcriptional and Post-Translational Modifications. *J Diabetes Res*, 2016

86. Mooyaart AL, van Valkengoed IG, Shaw PK, et al. Lower frequency of the 5/5 homozygous CNDP1 genotype in South Asian Surinamese. *Diabetes Res Clin Pract*, 2009; 85: 272-8

87. McDonough CW, Hicks PJ, Lu L, et al. The influence of carnosinase gene polymorphisms on diabetic nephropathy risk in African-Americans. *Hum Genet*, 2009; 126: 265-75

88. Babizhayev MA, Lankin VZ, Savel'Yeva EL, et al. Diabetes mellitus: novel insights, analysis and interpretation of pathophysiology and complications management with imidazole-containing peptidomimetic antioxidants. *Recent Pat Drug Deliv Formul*, 2013; 7: 216-56

89. Boldyrev AA. Does carnosine possess direct antioxidant activity? *Int J Biochem*, 1993; 25: 1101-7

90. Mozdzan M, Szemraj J, Rysz J, et al. Antioxidant properties of carnosine re-evaluated with oxidizing systems involving iron and copper ions. *Basic Clin Pharmacol Toxicol*, 2005; 96: 352-60

91. Dursun N, Taskin E, and Ozturk F. Protection against adriamycin-induced cardiomyopathy by carnosine in rats: role of endogenous antioxidants. *Biol Trace Elem Res*, 2011; 143: 412-24

92. Ma J, Xiong JY, Hou WW, et al. Protective effect of carnosine on subcortical ischemic vascular dementia in mice. *CNS Neurosci Ther*, 2012; 18: 745-53

93. Baba SP, Hoetker JD, Merchant M, et al. Role of aldose reductase in the metabolism and detoxification of carnosine-acrolein conjugates. *J Biol Chem*, 2013; 288: 28163-79

94. Negre-Salvayre A, Coatrieux C, Ingueneau C, et al. Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. *Br J Pharmacol*, 2008; 153: 6-20

95. Vistoli G, Orioli M, Pedretti A, et al. Design, synthesis, and evaluation of carnosine derivatives as selective and efficient sequestering agents of cytotoxic reactive carbonyl species. *Chem Med Chem*, 2009; 4: 967-75

96. Alhamdani MS, Al-Azzawie HF, and Abbas FK. Decreased formation of advanced glycation end-products in peritoneal fluid by carnosine and related peptides. *Perit Dial Int*, 2007; 27: 86-9

97. Alhamdani MS, Al-Kassir AH, Abbas FK, et al. Antiglycation and antioxidant effect of carnosine against glucose degradation products in peritoneal mesothelial cells. *Nephron Clin Pract*, 2007; 107: c26-34

98. Koppel H, Riedl E, Braunagel M, et al. L-carnosine inhibits high-glucose-mediated matrix accumulation in human mesangial cells by interfering with TGF-beta production and signalling. *Nephrol Dial Transplant*, 2011; 26: 3852-8

99. Kurdi A, De Meyer GR, and Martinet W. Potential therapeutic effects of mTOR inhibition in atherosclerosis. *Br J Clin Pharmacol*, 2016; 82: 1267-1279

100. Hou WC, Chen HJ, and Lin YH. Antioxidant peptides with Angiotensin converting enzyme inhibitory activities and applications for Angiotensin converting enzyme purification. *J Agric Food Chem*, 2003; 51: 1706-9

101. Nakagawa K, Ueno A, and Nishikawa Y. Interactions between carnosine and captopril on free radical scavenging activity and angiotensin-converting enzyme activity in vitro. *Yakugaku Zasshi*, 2006; 126: 37-42

102. Tsai SJ, Kuo WW, Liu WH, et al. Antioxidative and anti-inflammatory protection from carnosine in the striatum of MPTP-treated mice. *J Agric Food Chem*, 2010; 58: 11510-6

103. Lee YT, Hsu CC, Lin MH, et al. Histidine and carnosine delay diabetic deterioration in mice and protect human low density lipoprotein against oxidation and glycation. *Eur J Pharmacol*, 2005; 513: 145-50

104. Yan SL, Wu ST, Yin MC, et al. Protective effects from carnosine and histidine on acetaminophen-induced liver injury. *J Food Sci*, 2009; 74: H259-65

105. Boldyrev AA, Aldini G, and Derave W. Physiology and pathophysiology of carnosine. *Physiol Rev*, 2013; 93: 1803-45

106. Kohen R, Yamamoto Y, Cundy KC, et al. Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. *Proc Natl Acad Sci U S A*, 1988; 85: 3175-9

107. Aldini G, Facino RM, Beretta G, et al. Carnosine and related dipeptides as quenchers of reactive carbonyl species: from structural studies to therapeutic perspectives. *Biofactors*, 2005; 24: 77-87

108. Tanida M, Niijima A, Fukuda Y, et al. Dose-dependent effects of L-carnosine on the renal sympathetic nerve and blood pressure in urethane-anesthetized rats. *Am J Physiol Regul Integr Comp Physiol*, 2005; 288: R447-55

109. Veiga-da-Cunha M, Chevalier N, Stroobant V, et al. Metabolite proofreading in carnosine and homocarnosine synthesis: molecular identification of PM20D2 as beta-alanyl-lysine dipeptidase. *J Biol Chem*, 2014; 289: 19726-36

110. Jessen H. Taurine and beta-alanine transport in an established human kidney cell line derived from the proximal tubule. *Biochim Biophys Acta*, 1994; 1194: 44-52

111. Jessen H and Sheikh MI. Renal transport of taurine in luminal membrane vesicles from rabbit proximal tubule. *Biochim Biophys Acta*, 1991; 1064: 189-98

112. Teufel M, Saudek V, Ledig JP, et al. Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J Biol Chem*, 2003; 278: 6521-31

113. Forsberg EA, Botusan IR, Wang J, et al. Carnosine decreases IGFBP1 production in db/db mice through suppression of HIF-1. *J Endocrinol*, 2015; 225: 159-67

114. Sauerhofer S, Yuan G, Braun GS, et al. L-carnosine, a substrate of carnosinase-1, influences glucose metabolism. *Diabetes*, 2007; 56: 2425-32

115. Vistoli G, De Maddis D, Cipak A, et al. Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation. *Free Radic Res*, 2013; 47 Suppl 1: 3-27

116. Aldini G, Orioli M, Rossoni G, et al. The carbonyl scavenger carnosine ameliorates dyslipidaemia and renal function in Zucker obese rats. *J Cell Mol Med*, 2011; 15: 1339-54

117. Jia H, Qi X, Fang S, et al. Carnosine inhibits high glucose-induced mesangial cell proliferation through mediating cell cycle progression. *Regul Pept*, 2009; 154: 69-76

118. Riedl E, Pfister F, Braunagel M, et al. Carnosine prevents apoptosis of glomerular cells and podocyte loss in STZ diabetic rats. *Cell Physiol Biochem*, 2011; 28: 279-88

119. Peters V, Schmitt CP, Zschocke J, et al. Carnosine treatment largely prevents alterations of renal carnosine metabolism in diabetic mice. *Amino Acids*, 2012; 42: 2411-6

120. Peters V, Riedl E, Braunagel M, et al. Carnosine treatment in combination with ACE inhibition in diabetic rats. *Regul Pept*, 2014

121. Kamei J, Ohsawa M, Miyata S, et al. Preventive effect of L-carnosine on changes in the thermal nociceptive threshold in streptozotocin-induced diabetic mice. *Eur J Pharmacol*, 2008; 600: 83-6

122. Derave W, Ozdemir MS, Harris RC, et al. beta-Alanine supplementation augments muscle carnosine content and attenuates fatigue during repeated isokinetic contraction bouts in trained sprinters. *J Appl Physiol (1985)*, 2007; 103: 1736-43

123. de Courten B, Jakubova M, de Courten MP, et al. Effects of carnosine supplementation on glucose metabolism: Pilot clinical trial. *Obesity*, 2016; 24: 1027-34