Diabetic nephropathy : from histological findings to clinical features

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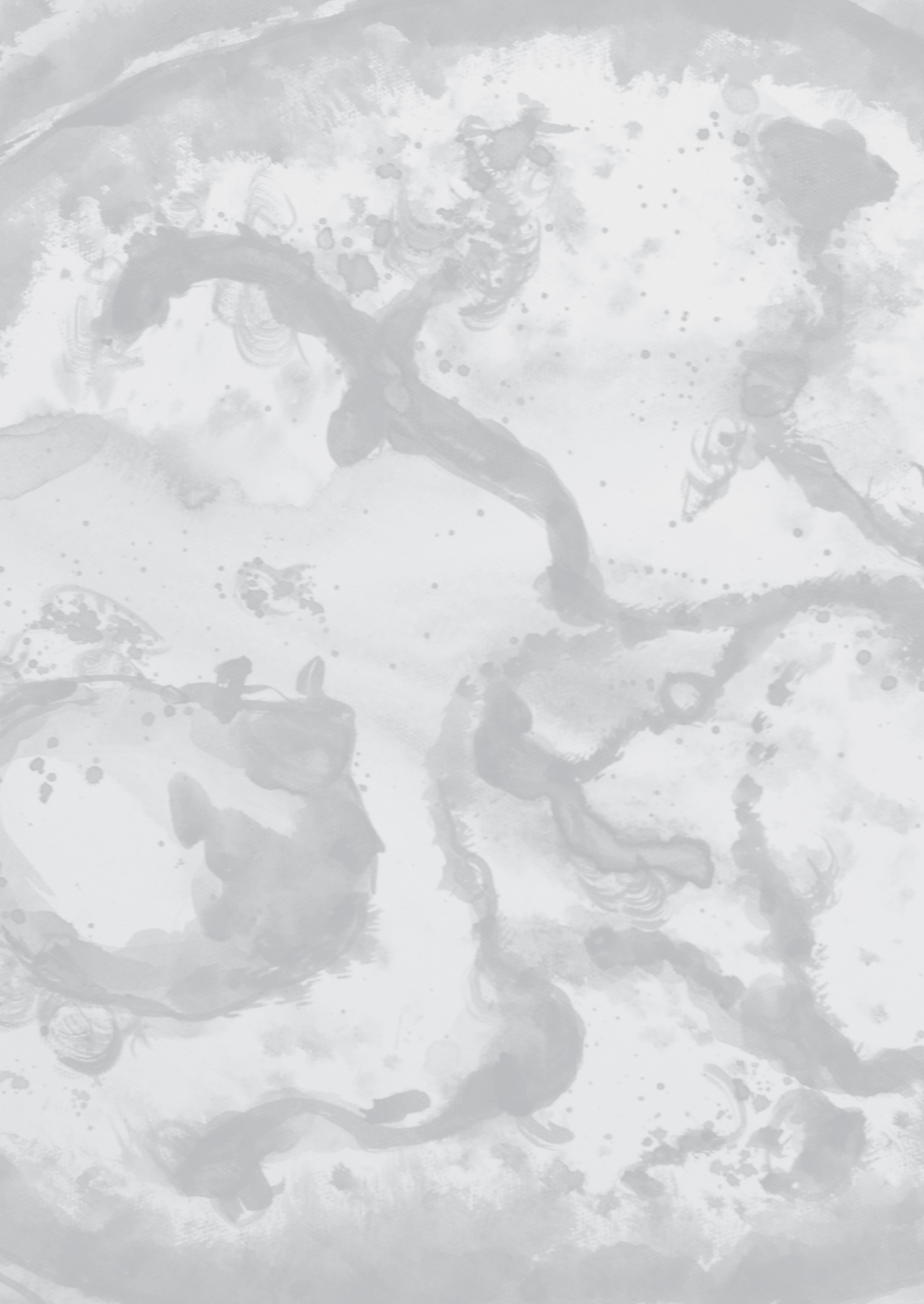

Summary, Discussion and Future Perspectives

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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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