Diabetic nephropathy : pathology, genetics and carnosine metabolism

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Summary and general discussion

Diabetic nephropathy is a severe complication of both type 1 and type 2 diabetes. In this thesis, several aspects of diabetic kidney disease relating histopathology, genetics and carnosine metabolism, were investigated.

In **chapter 2** we developed a histopathologic classification scheme for diabetic nephropathy in type 1 and type 2 diabetes. The classification system is based on glomerular lesions, consisting of four classes and reflecting the natural course of diabetic nephropathy. Class I "*Glomerular Basement Membrane Thickening (GBM)*" consists of isolated glomerular basement membrane thickening and only mild, non-specific changes by light microscopy that do not meet the criteria of classes II through IV. Class II "*Mesangial expansion, mild or severe*" is characterized by glomeruli with mild or severe mesangial expansion but without nodular sclerosis (Kimmelstiel-Wilson lesions) or global glomerulosclerosis in more than 50% of glomeruli. A biopsy is classified in class III "*Nodular sclerosis (Kimmelstiel-Wilson lesions)*" if at least one glomerulus with nodular sclerotic lesion (Kimmelstiel-Wilson lesion) is present without changes as described in class IV. Class IV "*Advanced diabetic glomerulosclerosis*" is characterized by more than 50% global glomerulosclerosis with clinical or pathological evidence that the sclerosis is attributable to diabetic nephropathy. We chose a classification scheme based on glomerular lesions because these seem to reflect the natural cause of progressive diabetic nephropathy (1). Furthermore, glomerular lesions are easy to recognize, which is also reflected in the good interobserver agreement of this classification scheme.

Interstitial lesions also contribute to the decline in renal function in diabetic nephropathy, and may be independent factors in the progression of diabetic nephropathy (2). Many studies have shown that the severity of chronic interstitial and glomerular lesions are closely associated (3-6). In our classification scheme a separate evaluation was developed for interstitial and vascular lesions.

A classification system for histopathological lesions in diabetic nephropathy that can be used in both type 1 and type 2 diabetes was proposed, as it is now generally recognized that there is substantial overlap between these two types with respect to histological lesions and renal complications (2;6). Various studies report that the proportion of non-diabetic nephropathies is higher in type 2 diabetic nephropathy (7;8). However, studies which investigated protocol biopsies, did not show such a high proportion of other kidney diseases. Most likely this is due to the fact that in some clinical practices, there is a policy only to perform a renal biopsy to exclude causes of renal disease characterized by proteinuria other than diabetic nephropathy, therefore

selecting for a high proportion of other kidney diseases. The classification system proposed in chapter 2 is meant for diabetic nephropathy only, but it can also serve to classify diabetic nephropathy when it is complicated by a superimposed other disease.

An important question for every histological classification system is whether it is predictive of clinical outcome. The classification scheme proposed in chapter 2 can be used to evaluate protocol biopsies. It would be an interesting undertaking to set up a prospective study, preferably including protocol biopsies of patients with both type 1 and type 2 diabetes, with clearly defined clinical endpoints. Archived renal biopsies with diabetic nephropathy performed for many different clinical indications, at many different time points during the course of the disease and clinical follow-up, were not always readily available. With the increasing demand for classifying the severity of diabetic nephropathy, we advocate a study with protocolized biopsies as previously mentioned. Furthermore, the reproducibility of the classification scheme should be thoroughly investigated, to clarify if it is indeed suitable for clinical practice.

Chapter 3 is focused on the genetic component of diabetic kidney disease. Many genes have been investigated in diabetic nephropathy, but it is unclear which genetic variants are reproducibly associated with diabetic nephropathy. For this study, all genetic variants which were associated with diabetic nephropathy and replicated in an independent dataset were selected from the literature. All subsequent studies of these reproduced variants were sought and were combined in a random-effects meta-analysis. In this study, we found a set of 21 genetic variants which were associated with advanced diabetic nephropathy. Three additional variants were associated in specific subgroups; type of diabetes, stage of diabetic nephropathy and ethnic group. Variants in or near *ACE, AKR1B1* (2 variants)*, APOC1, APOE, EPO, NOS3* (2 variants)*, HSPG2, VEGFA, FRMD3* (2 variants)*, CARS* (2 variants)*, UNC13B, 'CPVL and CHN2',* and *GREM1* (as well as four variants not near to known genes) were associated with diabetic nephropathy and *CCR5* (Asians), *ELMO1* (Asians) and *CNDP1* (type 2 diabetes) in a subgroup. These results support roles for angiotensin converting enzyme, aldose reductase, apolipoproteins C1 and E, oxidative stress with carnosine, nitric oxide and engulfment and cell motility protein 1 as possible key players. Furthermore, it suggests a role for inflammation with a key role for chemokine receptor 5, angiogenesis with a role for vascular endothelial growth factor and erythropoietin, glomerular filtration barrier defects in heparan sulphate proteoglycans, apoptosis through unc-13 homolog B and cell growth and differentiation mediated by gremlin 1 in the pathogenesis of diabetic

nephropathy. Functional studies remain to be performed to establish the precise role of these variants and pathways. Genetic variants initially identified using a genome-wide association approach in and near the *FRMD3*, *CARS*, *ELMO1* and *'CPVL and CHN2'* were detected. Despite their value in locating the vicinity of genomic variants that may be causing diabetic nephropathy, these variants are unlikely to be causative variants themselves (9). A first step in narrowing a genome wide signal to potentially causative variants is to study all the known SNPs which are inherited with the SNP found in the genome wide association scan and investigate if one of these variants has a stronger association with diabetic nephropathy. The functional effect of this variant can then be studied. However, the association can also be explained by rare variants and then extensive sequencing is indicated.

It should also be acknowledged that by selecting only genetic variants that were associated with diabetic nephropathy and where independent replication was available, genetic variants with smaller effect sizes may have been missed. An effect that may have proven significant using pooled analyses. However, by selecting only those genetic variants reproducibly associated with diabetic nephropathy, we reduced the chances of describing false positive associations.

Furthermore, diabetic nephropathy can be prevented or at least delayed by early treatment with angiotensin converting enzyme inhibitors and angiotensin receptor blockers (10). This might result in diabetes patients who are genetically susceptible to diabetic nephropathy but now enter the control group due to successful treatment. This leads to an underestimation of the estimated effect of the genetic variant in relation to diabetic nephropathy.

Chapters 4, 5 and, 6 are focused on one genetic variant associated with diabetic nephropathy, the *CNDP1* gene. Janssen *et al.* (11) found that the 5-5 homozygous genotype of the *CNDP1* gene is associated with a reduced risk of developing diabetic nephropathy. This finding was confirmed in a large study conducted by Freedman *et al.* in European American patients and end-stage diabetic nephropathy (12). The presence of more than 5 leucine repeats has been shown to lead to higher serum carnosinase secretion (13) and more serum carnosinase activity (11). Serum carnosinase degrades carnosine. Carnosine has been shown to delay senescence in cultured human fibroblasts and temporarily reverse the senescence phenotype (14;15). Carnosine is further known to scavenge reactive oxygen species, degrade advanced glycation end products (AGE) (16), inhibit angiotensin converting enzyme (ACE) (17), and reduce the TGF-β induced

synthesis of extracellular matrix components (11). All these properties have protective effects in a diabetic environment.

Chapter 4 reports the 5-5 homozygous *CNDP1* genotype frequency in three ethnic groups in the Netherlands; South Asian Surinamese, African Surinamese and White Dutch. We found that the frequency of the protective genotype for diabetic nephropathy, 5-5 homozygous genotype, was significantly lower in South Asian Surinamese compared to white Dutch and African Surinamese. This finding was confirmed in an independent South Asian Surinamese sample. This low frequency of the 5-5 homozygous genotype found in South Asian Surinamese is likely to be associated with the higher occurrence of diabetic nephropathy in South Asian populations (18-20). This is further supported by the finding that carnosinase activity increases with the amount of leucine repeats among South Asian Surinamese, similar to Caucasians (11). Finally we showed that carnosinase was expressed in kidney, supporting a role for carnosine in the kidney.

Although a direct link between the *CNDP1* gene and diabetic nephropathy still needs to be assessed, this study suggests that the high diabetic nephropathy occurrence in South Asian Surinamese can be partially attributed to the lower frequency of the 5-5 homozygous genotype in this population.

In **chapter 5,** we showed a sex-specific effect of the *CNDP1* genotype in relation to diabetic nephropathy, suggesting that the 5-5 homozygous genotype is only protective in women. The frequency of the 5-5 homozygous genotype was determined in three independent diabetic nephropathy groups. These groups were compared with two control groups; diabetes patients with a low risk of ever developing diabetic nephropathy and a sample from the general population. The former diabetic control group is generally used in the literature, and investigates the relevance of the gene to disease etiology. Compared with this diabetic control group, the 5-5 homozygous genotype frequency was significantly lower in women with diabetic nephropathy in all three cohorts, but not in men. The population control group serves to estimate the genotype risk for the population, showing that women with the 5-5 homozygous genotype have a 2-fold reduced risk of ever developing diabetic nephropathy. Furthermore, this study reports similar frequencies in type 2 diabetes patients and the general population, showing that this association is independent of a genetic susceptibility for type 2 diabetes. Although the mechanism behind the sex-specificity still needs to be elucidated, this study indicates that the association between the *CNDP1* gene and diabetic nephropathy is gender specific.

Carnosine is protective against oxidative stress and hemodynamic damage and this is not confined to diabetic nephropathy. In **chapter 6** the relation between other glomerular diseases and the *CNDP1* gene was investigated. Our results suggest that a higher number of leucine repeats in the *CNDP1* genotype is associated with a faster progression to end stage renal disease in patients with reduced kidney function arising from chronic glomerular inflammatory renal diseases and in patients with renal vascular disease. In line with these results, the mortality risk was increased in chronic glomerulonephritis patients with a higher repeat number, which was also associated with higher serum carnosinase levels. Our data show a correlation between the leucine repeat distribution of the *CNDP1* gene and renal vascular disease; however, there was no relationship with survival. As predicted, patients who developed end stage renal disease because of either polycystic kidney disease or tubulointerstitial nephritis had a *CNDP1* genotype distribution similar to that of healthy controls. These findings support the hypothesis that the high leucine repeat number in *CNDP1* may contribute to microvascular damage. Although this finding has to be replicated in an independent cohort, this study suggests that there is also a role for carnosine in other glomerular diseases.

In **chapter 7** we investigated determinants of muscular carnosine levels. As high carnosine levels are thought to be advantageous, it is important to understand the underlying factors. We studied the relation between muscular carnosine levels and serum carnosinase activity, *CNDP1* genotype, age, vegetarian diet and muscle fiber type. Serum carnosinase activity did reduce the carnosine content, but possibly due to the small number of investigated subjects no relation was shown with the *CNDP1* genotype. Carnosine content was found to decline with age, which could not be explained by the age-related increase in carnosinase activity. Meat-restriction for 8 weeks had no effect on carnosine levels, but in (long-term) vegetarians lower muscular carnosine levels were observed. There was no linear relationship with muscular fiber type and carnosine levels.

In addition, the relation with gender in the carnosine metabolism was studied. Women showed higher carnosinase activity than men and men had higher carnosine levels. This is in line with the idea that from an evolutionary point of view carnosine levels are more beneficial to men. This can be explained by the greater muscular activity needed for males to hunt for food, since carnosine is a buffer for the substances derived from lactic acid that are produced under anaerobic conditions. However, no relation between testosterone and carnosine was found in this study.

Future perspectives

The relation between diabetic nephropathy due to type 2 diabetes and the leucine repeat in exon 2 of the *CNDP1* gene seems to be established. Interesting would be to speculate on what this finding can eventually mean for the diabetic nephropathy patient. The possible relevance of the *CNDP1* genotype in diabetic nephropathy can be roughly divided in two groups; genotypic screening for diabetes patients to be able to predict the risk of developing diabetic nephropathy and novel biological insight in the etiology and pathogenesis of diabetic nephropathy, potentially leading to new therapies (21).

For screening of individuals with an increased risk of disease, the measurement of area under the curve (AUC) of the receiver operating characteristic curve (ROC) is often used. An AUC of 1 is perfect prediction and an AUC of 0.5 is similar to tossing a coin, and therefore not predictive. It has been suggested that an AUC above 0.75 can be considered of predictive value. For pre-symptomatic diagnosis of the general population the AUC should be > 0.99 (22). The *CNDP1* genotype could be used in a genotypic risk score, together with other associated variants related to diabetic nephropathy due to type 2 diabetes. In diabetic nephropathy no such genotypic scores have been tested in contrast to screening for type 2 diabetes. In type 2 diabetes, 18 genetic variants have been established and these genetic variants when combined have shown to reach an AUC of 0.6 in predicting type 2 diabetes onset. This is low when compared to traditional risk factors as only BMI, age and gender which have shown to have an AUC of 0.78 and risk scores, such as the QDS score (a scoring algorithm based on traditional risk factors without the need for laboratory tests) reaches an AUC of 0.85 in predicting type 2 diabetes onset (23). In a different study, the addition of genetic risk factors to clinical risk showed only a slight increase of the AUC (from 0.74 to 0.75), which seems to increase with duration of follow up (24). This indicates that genetic personalized medicine in type 2 diabetes will not easily be achievable in the near future. However, in diabetic nephropathy this still needs to be investigated.

Novel biological insight can, in the most fortunate case, lead to new therapeutic targets. The association between diabetic nephropathy and *CNDP1* (carnosinase) gene, would suggest a role for this enzyme and its substrate (carnosine) in diabetic nephropathy. It was shown that the genotype leading to the lowest carnosinase activity, leaving more carnosine, was associated with a reduced risk of diabetic nephropathy.

This suggests that a possible new therapeutic target for diabetic nephropathy would be increasing carnosine level in diabetes patients. This could be done by supplementing carnosine, reducing the degradation of carnosine by inhibiting carnosinase, or increasing carnosine production by carnosine synthase.

Supplementing carnosine would be an interesting treatment for patients with diabetic nephropathy. Carnosine has been known for its many protective capacities in a diabetic environment and is well tolerated. Carnosine and related compounds have already been used as therapies for cataract (25;26) and gastric ulcers (27). In diabetic mice treated with angiotensin converting enzyme inhibitors, adding carnosine to the diet led to a significant reduction in proteinuria compared to diabetic mice treated with only angiotensin converting enzyme inhibitors (28). In humans, carnosine seems to be hydrolyzed within 2 hours after carnosine is absorbed by the intestine and enters the bloodstream (29). However, when Parkinson patients were treated with carnosine in addition to dopamine (L-dopa), they had a decreased level of protein carbonyls in their blood plasma and increased levels of red cell superoxide dismutase compared to patients treated with only dopamine (L-dopa) (30). This suggests that supplementation with carnosine has a biochemical effect after oral intake even though carnosine is hydrolyzed quickly in serum. This means that carnosine supplementation could theoretically also be of therapeutic value in diabetic nephropathy.

An inhibitor of carnosinase activity is homocarnosine (31). In the literature, no studies have been performed in which patients were treated with homocarnosine. Although we would expect that homocarnosine treatment could reduce the serum degradation of carnosine, high concentrations of homocarnosine might lead to high concentrations of its degradation products gamma-aminobutyric acid (GABA) and histidine. Histidine can be easily converted into histamine and the impact on autonomic nerve activity would be unpredictable. It might result in further progression of renal insufficiency, since progressive renal disease has been shown to be associated with increased nerve activity (31). Future studies could also be focused on the regulation of the carnosinase gene, finding substances which downregulate the expression of *CNDP1*.

Recently the gene coding for carnosine synthase has been identified as a ATP-grasp domain containing protein 1 (*ATPGD1*) (32). The identification of this gene is an essential step in the understanding of carnosine production. It would for example be interesting to investigate in which organs this gene is expressed, and especially if it is expressed in the kidney; if so, in which cells of the kidney. Furthermore, the creation

of knockout mice or mice in which carnosine synthase is overexpressed, could help define the role of carnosine as a buffer, antioxidant, antiglycator and neurotransmitter, of which evidence now mostly comes from *in vitro* studies. It would be interesting to investigate whether overexpressing carnosine synthase in a diabetic kidney disease model, would lead to a milder phenotype of diabetic nephropathy. Finally, it would be interesting to investigate which substances upregulate carnosine synthase, leading to higher carnosine production and a possible therapeutic potential.

Most simple and safe would be to investigate carnosine treatment in diabetic nephropathy, before considering other substances. However, before starting treatment with carnosine, it might be advisable to first understand more about the mechanisms of carnosine metabolism. In chapter 5 of this thesis, a sex-specific effect of the relation between diabetic nephropathy and the *CNDP1* genotype was found. It would be interesting and important to understand the mechanism behind this sex-specific effect. A sex-specific effect has long been described in renal disease (33). In non diabetic progressive chronic kidney disease, female sex is thought to be protective. However, this advantage seems to be lost in diabetic nephropathy. The protective effect in non diabetic renal disease has been ascribed to the effect of estrogens (34). Female diabetes patients seem to have lower estrogens levels (35) and that may explain the loss of female protection in diabetic nephropathy. However, the relationship between sex and diabetic nephropathy seems to be more complicated. Several risk factors have been described to be involved in men while others are present in women (36-38). Of these risk factors, several genetic risk factors (37;39;40) have been documented to be either only involved in women or in men, but these findings have not yet been replicated. In this study, we detected an association in only women and could independently replicate this finding, providing evidence for a sex-specific component in the genetics of diabetic kidney disease.

With an increasing number of leucine repeats (6 or 7 instead of 5) in exon 2 of the *CNDP1* gene, carnosinase activity increases (11). The 5-5 homozygous genotype leads to lower carnosinase activity than that seen in the other genotypes (11), leaving more carnosine free to protect the kidney from oxidative stress. Since men have higher carnosine levels in their muscle tissue and women have slightly higher serum carnosinase levels (41), differences in carnosinase activity due to the different *CNDP1* polymorphisms may have a stronger impact in women.

Another explanation for the sex-specific effect found in this study is that the association between the *CNDP1* gene and diabetic nephropathy is lost in men due to selective survival by cardiovascular disease. As carnosine has shown to be protective against oxidative stress and hemodynamic damage (11;16;42), this might also explain its role in cardiovascular death in diabetic nephropathy patients. Men with diabetic nephropathy due to type 2 diabetes have a higher risk for cardiovascular disease than female diabetic nephropathy patients due to type 2 diabetes (43), therefore this might be more prominent in men. In this thesis we found that men with a diabetes duration < 10 years and the 5-5 homozygous genotype have a significantly lower mortality risk due to cardiovascular disease than patients with more than 10 leucine repeats in the *CNDP1* genotype. We found no difference in cardiovascular death between the different genotypes in women. This finding needs to be replicated, before drawing definite conclusions.

The two hypotheses are clearly different. According to the first hypothesis, carnosine seems to be mainly beneficial in women and suggests that future research should only focus on investigating carnosine metabolism in female diabetic nephropathy patients. The second hypothesis suggests that carnosine might also be protective in macrovascular complications of diabetes and that the sex-specific effect is in fact due to different survival between the sexes. Therefore, it would be helpful to understand the mechanism behind this sex-specific effect for future therapeutic studies.

In summary, the role for the *CNDP1* genotype in the prediction for diabetic nephropathy due to type 2 diabetes is probably limited. On the other hand, the potential new additional treatment for type 2 diabetes patients with carnosine to either prevent or slow down the progression of diabetic nephropathy should be investigated. Furthermore, it is essential to understand the mechanism behind the sex-specific effect of the association between the *CNDP1* genotype and diabetic nephropathy.

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