On the relevance of carnosine and carnosinase for the development of diabetic nephropathy
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CHAPTER 9

SUMMARY AND GENERAL DISCUSSION
Diabetic nephropathy (DN) is becoming worldwide a leading cause for end stage renal disease (ESRD). This is on the one hand due to an increased incidence and prevalence for diabetes and on the other to an increased life expectancy of diabetic patients as a consequence of improved clinical care. Nonetheless, DN is associated with a high mortality rate, which is primarily related to an increased risk for coronary heart disease and stroke (3, 10).

DN is caused by a complex interplay of metabolic and hemodynamic disturbances that are evoked by chronic hyperglycemia. Clinically, the first measurable sign of incipient DN is microalbuminuria. At this stage irreversible structural changes have already occurred in the kidney. As renal function deteriorates in time, patients develop proteinuria and finally enter the stage of overt DN. Histology of DN reveals a massive mesangial matrix expansion, thickening of the glomerular basement membrane (GBM) and a concomitant loss of heparan sulphate proteoglycan in the mesangial matrix and the GBM.

Hypertension is one of the main features of DN and is present in most patients. During the last decade it has become evident that elevated blood pressure is a major determinant of deterioration of renal function. Although the prognosis of DN is greatly improved by adequate treatment of hypertension using angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor antagonists, treatment as such does not eliminate the susceptibility to develop DN (4, 5).

There is an unmet clinical demand for a better risk stratification of diabetic patients and for novel therapeutic modalities to prevent the onset of diabetic renal disease. Family and epidemiologic-based studies have convincingly demonstrated that susceptibility to develop DN likely involves the genetic make-up of the individual diabetic patient (33, 34). Identification of susceptibility genes and understanding their relation to DN is a key to a better risk stratification and holds the promise for novel therapeutic strategies.

Up till now, various studies have looked genome-wide for chromosomal regions that harbour susceptibility genes for DN. Such genome scans do not require prior knowledge of the biology of the susceptibility gene as they solely describe linkage between disease and a particular chromosomal region. Once a chromosomal region of interest (ROI) has been identified, most of the researchers are using a hypothesis-driven approach to search for candidate genes. This implies that the ROI is screened for genes encoding proteins expected to be involved in the pathogenesis of DN. Identified candidate genes are subsequently examined for polymorphisms and tested for association in family-based and/or case-control studies. Almost 100 different genes have been postulated as candidate genes for DN by now;
however, results from different studies were not always consistent and even conflicting for some of these genes.

Recently, a genome-wide scan for linkage with overt proteinuria was performed in 18 Turkish families. Using parametric linkage analysis, strong evidence for linkage was found on chromosome 18q22.3 – q23 (40). The locus was mapped between the markers D18S43 and D18S50 and was supported by a highly significant logarithm of odds (LOD) score of 6.1. Linkage of diabetic nephropathy to 18q was confirmed by an affected sibpair analysis performed on Pima Indians and by a large sibpair analysis of African Americans (40, 41). In 2005 the region was narrowed down to a single gene, i.e. the CNDP1 gene, encoding serum carnosinase or CN-1 (44). Within the signal peptide of CN-1 there is a polymorphic hydrophobic stretch containing a variable number of leucine residues (4 to 8 leucines). Since DN is less frequently found in diabetic individuals that are homozygous for the CN-1 gene variant encoding a hydrophobic stretch of 5 leucine, i.e. (CTG)5, it is believed that the (CTG)5 allele is a recessive protective gene variant. In healthy individuals this genotype is associated with low CN-1 activity in serum. The association between the CNDP1 gene polymorphism and DN has also been demonstrated in European Americans with type 2 diabetes (42). It should be underscored that the association between the CNDP1 (CTG)n polymorphism and diabetic nephropathy is mainly found in type 2 diabetic patients (44). Other studies in type 1 diabetic patients could not confirm CNDP1 as susceptibility locus (241), although it might predict progression to ESRD in patients with DN (242). It has also been suggested that the association between the CNDP1 gene and diabetic nephropathy is sex specific and independent of susceptibility for type 2 diabetes (194). Why the CNDP1 polymorphism is associated with DN is at present not completely understood. The studies presented in this thesis were therefore conducted to seek a biological plausibility for the association by looking at the functional role of the (CTG)n repeat, and by studying the beneficial properties of carnosine, the natural substrate of CN-1.

CN-1 is a secreted enzyme produced by the liver and the brain (55). Once secreted into the serum, CN-1 rapidly degrades the endogenous dipeptide carnosine, which might explain why carnosine concentrations in serum are generally low. Several studies have indicated that carnosine can function as a scavenger for reactive oxygen radicals and can inhibit the formation of advanced glycosylation endproducts (AGE) (110). For these reasons it has been suggested that carnosine might be a protective factor in DN. In chapter 2, we illustrate that the length of the (CTG)n repeat influences the efficiency of CN-1 secretion. While CN-1 is poorly
secreted when the CN-1 signal peptide contains a 4 or 5 leucine long hydrophobic stretch, i.e. the (CTG)$_4$ or (CTG)$_5$ CNDP1 gene variants, a more efficient secretion occurs when the CN-1 signal peptide contains 6 or more leucine repeats. Although the experiments have been carried out in transfected Cos-7 cells, they are in line with earlier findings in healthy human individuals where it was found that (CTG)$_3$ homozygous individuals have a lower serum CN-1 activity. Moreover, as demonstrated in chapter 6 (CTG)$_5$ homozygous individuals have a lower CN-1 protein concentration in serum, compared to individuals that carry a different genotype. The results of chapter 2 and 6 thus provide experimental evidence for the association between CNDP1 genotype and CN-1 activity (44) and suggest a functional role for the CNDP1 polymorphism.

Because signal peptides are of instrumental importance for protein allocation (243), it is conceivable that signal peptide polymorphisms can impair proper allocation. This might result in an unbalanced distribution of proteins, e.g. an excess or deficiency of critical enzymes, within the serum or other specific cellular compartments. The involvement of signal peptide polymorphisms and inappropriate protein allocation in the pathology of various diseases, such as Alzheimer disease and atherosclerosis, has been suggested (244-247). Hence, susceptibility to DN might equally be influenced by an increased secretion of CN-1 into the serum that is caused by a signal peptide polymorphism. Akin to mutations found for other trinucleotide repeats, the CTG repeat in the CNDP1 signal peptide may have increased by a dynamic gain-of-function mutation that causes an increased CN-1 secretion. Of note, CN-1 over-expression in diabetic db/db mice resulted in lower carnosine serum levels and more severe diabetic disease, reflected by worsened glucose metabolism (113).

Similar to CN-1, most human serum proteins are glycosylated. Changes in enzymatic glycosylation of serum proteins are well known to be involved in several diseases, such as microbial infections, inflammations, autoimmune diseases and tumors (248, 249). Moreover, it has been demonstrated that enzymatic glycosylation is influenced by hyperglycemia (24, 250). In chapter 3 we were able to show that apart from the (CTG)$_n$ polymorphism, N-glycosylation is essential for appropriate secretion and enzyme activity of CN-1. Deletion of all three N-glycosylation sites impairs CN-1 secretion efficiency, while CN-1 activity is already diminished when two sites are deleted. We also provide evidence that in CNDP1 transfected Cos-7 cells cultured in a high glucose milieu, the efficiency for N-glycosylation increases resulting in an increased secretion of CN-1. More importantly, CN-1 secretion was increased despite the fact that Cos-7 cells were transfected with the (CTG)$_3$ CNDP1 gene
variant, which, compared to other variants, is associated with a lower CN-1 secretion. This might indicate that in even in homozygous (CTG)$_5$ diabetic patients serum CN-1 concentrations could increase as a consequence of hyperglycemia. Indeed, we provide evidence in chapter 6 that homozygous (CTG)$_5$ diabetic patients have higher serum CN-1 protein levels and activity compared to genotype matched healthy controls. In addition, our data indicate that CN-1 protein expression in serum of homozygous (CTG)$_5$ diabetic patients seems to correlate with blood glucose levels, further supporting the relevance of blood glucose control for CN-1 secretion. Accordingly, poor blood glucose control in diabetic patients might result in an increased CN-1 secretion, even in the presence of the (CTG)$_5$ allele.

Independent of the relevance of CN-1 for diabetic complications, the data generated in chapter 3 and 6 propose a relationship between hyperglycemia and N-glycosylation/secretion of proteins. This might be a more general mechanism by which hyperglycemia changes protein profiles in a qualitative and quantitative fashion. In experimental diabetes, hyperglycemia induced changes in enzymatic glycosylation of proteins has already been demonstrated (24, 250). More recently, global changes in glycosylation of serum proteins have also been reported in type 1 diabetic patients. In fact, by analyzing the serum protein glycosylation pattern (251), the authors were able to tell whether the samples were obtained from diabetic or non-diabetic individuals. Although, the authors did not study individual serum proteins and did not study the impact of these changes with respect to chronic diabetic complications, they undoubtedly show that modifications in glycoprotein structures and their expression are common in serum of diabetic patients.

In chapter 4 we describe ELISA assays for the detection of CN-1 in serum samples in a highly reproducible manner. While the ATLAS-based assay correlates with CN-1 activity, this was not found for the RYSK-based assay. CN-1 seems to be present in different conformations as only a fraction of CN-1 detected with the ATLAS-based assay is also detected with the RYSK-based assay and detection of CN-1 with the RYSK-based assay is increased by addition of EDTA to serum or by denaturation of serum proteins. In chapter 5 we show that although CN-1 activity in serum is lower in children than adults, CN-1 protein concentrations are not remarkably different if measured in EDTA plasma with the RYSK173 ELISA. It should be emphasized however that there is no good correlation between CN-1 concentration and CN-1 activity when serum or plasma samples are measured with the RYSK173 ELISA. Nevertheless the RYSK173 ELISA showed qualitative differences between adults and
children in that serum of the majority of children were clearly positive. In children there was no significant difference in CN-1 concentrations between EDTA plasma and serum when measured with RYSK173.

RYSK173 is most likely not recognizing a conformational CN-1 epitope since addition of DTT to serum dramatically increased detection of CN-1 by this antibody. It is more likely that RYSK173 recognizes a linear epitope in vicinity of the metal ion binding residues within CN-1. The RYSK173 epitope might become masked upon binding of certain metal ions. Although addition of EDTA to serum increases detection of CN-1 in the RYSK173 ELISA, in adults the concentration of CN-1 in EDTA plasma is only approximately 10% of that detected in serum to which DTT was added. This might suggest that only a small proportion of metal ions within CN-1 can be chelated by EDTA. In RYSK173 positive sera, it might be that part of the CN-1 is either not occupied by metal ions, or alternatively, that part of the CN-1 is occupied by different metal ions that do not cause masking of the RYSK173 epitope. Evidence for ion dependent conformational changes in the CN-1 protein has been reported by Vistoli et al (173), Schoen et al (252) and Lenny et al (51). Their data underline the importance of metal ions, e.g. Magnesium or Cadmium, for CN-1 activity (54).

In general, allosteric modulation of enzymes enhances or inhibits substrate binding and thereby represents an important regulatory mechanism. Because CN-1 activity in children is low, it might be that CN-1 has an allosteric ligand, which is present in children but not in adults. At present it is unclear whether the different CN-1 conformations, albeit not completely understood at the structural level, contribute to susceptibility to develop DN or whether these conformations are relevant for CN-1 activity. With respect to the latter however, it should be emphasized that in RYSK173 positive sera, the amount of CN-1 detected by RYSK173 is less than 1 % of total CN-1, suggesting that its contribution to total CN-1 activity marginal.

In chapter 5 we present a competitive CN-1 inhibitor that is present in human serum, i.e. homocarnosine. Homocarnosine dose-dependently inhibits CN-1 activity towards carnosine as substrate. CN-1 activity for carnosine is 4-6 times higher in serum than in CSF, which is might be explained by a lower CN-1 expression, or alternatively by a higher homocarnosine concentrations in CSF. Homocarnosine may be a good candidate for therapeutic treatment of patients at risk for diabetic nephropathy, as it would be expected to reduce degradation of carnosine in serum. There is a great body of evidence that homocarnosine has neuroprotective
properties (164, 253). Because homocarnosine may serve as a repository for GABA production (254), supplementation with homocarnosine may consequently increase GABA levels. In fact, an analog of GABA, i.e. Gabapentin, is even used for the treatment of chronic pain caused by diabetic neuropathy (255). Treatment of diabetic patients with homocarnosine may therefore have a beneficial effect beyond its possible reno-protective properties. Yet, it remains to be evaluated whether the peptide or its degradation products exert toxic side effect.

In keeping with the fact that CN-1 activity might be influence by glycemic control, allosteric conformation and homocarnosine concentrations, it is clear that DN might develop even in the presence of a protective genotype. Therefore, genetic screening alone is not sufficient to identify patients at risk but should be accompanied by direct measurements of serum CN-1 activity or expression. Measurement of CN-1 activity is a protracted, interference-prone method that is difficult to standardize. For that reason, we established an ELISA system in chapter 4 to quantitatively determine the amount of CN-1 present in serum. CN1 concentrations can be assessed by the ATLAS ELISA and correlate with CN1 activity. The CN-1 ELISA provides the possibility to run large series and therefore could be the basis for risk stratification of diabetic patients. Measurement of serum CN-1 expression, enzyme inhibition or substrate supplementation could optimize current therapeutic approaches and disease management of diabetic patients.

To address the potential beneficial effect of carnosine on diabetic complications, we performed in vitro and in vivo experiments, described in chapter 7 and 8. In vitro studies demonstrate that carnosine inhibits hyperglycemia-induced collagen 6 (Col6) and fibronectin (FN) accumulation in human mesangial cells, both at the protein and mRNA level. Also the production of TGF-β was inhibited by carnosine. Interestingly, addition of exogenous TGF-β could not overcome the effect of carnosine on Col6 and FN expression, suggesting that inhibition of extracellular matrix accumulation was not mediated via inhibition of TGF-β production. Because carnosine also inhibits TGF-β mediated Smad2 phosphorylation, it seems that carnosine can modulate matrix accumulation in two ways. Firstly, inhibition of TGF-β production might result in an overall inhibition of matrix accumulation and secondly carnosine inhibits TGF-β induced matrix accumulation, most likely via inhibition of the ALK5 pathway.

In chapter 8 in vivo experiments are performed to address whether carnosine in-take in STZ induced diabetic rats influences biochemical changes and glomerular apoptosis in the kidney cortex. Although we did not find an anti-oxidative or anti-glycating function of carnosine in
our study, we could show that carnosine treatment inhibits the up-regulation of pro-apoptotic molecules in the diabetic kidney and prevents podocyte loss. Even though we cannot provide a general mechanism by which carnosine protects the diabetic kidney, carnosine seems to have at least cytoprotective and anti-fibrotic properties.

Overall, the results from chapter 7 and 8 suggest that carnosine might locally counterbalance glomerular cell loss and fibrosis, both of which cause progressive glomerular scarring that characteristic for diabetic glomerulosclerosis. Scarring is generally microscopically characterized by hypocellularity and excessive collagenation. The ability of carnosine to accelerate the production of scar tissue has already been described for skin wounds (256, 257). The apparent interference of carnosine with the TGF-β ALK-5 signaling on one hand and the failure of carnosine to counterbalance the diabetic biochemical dysbalance on the other hand, point out that the protective effect of carnosine is rather mediated by a direct than indirect mechanism. As indicated in chapter 1, many of the biological functions of carnosine have not yet been fully characterized. Although carnosine should be carefully evaluated for toxic side effects, the abundance of patents describing medical applications of carnosine attests its potential as therapeutic agents (258-262).

The availability of carnosine, either via ingestion or endogenously synthesized by carnosine synthase, is determined by CN-1 activity in serum. Gardener et al. have reported that ingested carnosine cannot be detected in human serum because of CN-1 activity, but can be recovered in urine. Carnosine recovery varied substantially between subjects and correlated negatively with CN-1 activity in plasma (70). At the time of Gardener’s publication, the relation between the CN-1 polymorphism and serum CN-1 activity was not known yet. The authors speculated that differences in physical activity of the studied subjects caused variation in CN-1 activity. It also needs to be discussed why individuals that have a higher CN-1 activity do not have per se a noticeable phenotype and why the CN-1 genotype is reno-protective in the setting of diabetes, but is not protective in coronary heart disease and is not associated with longevity (263). Interestingly, in chapter 8 we could show that carnosine is reduced in the diabetic kidney of rats compared to healthy rats. The diabetic environment might therefore reduce the amount of carnosine in the kidney and that might explain why carnosine levels subsequently become critical in diabetic individuals that have a high CN-1 activity. Nevertheless, unlike humans, rodents do not have a CN-1 in serum, but their cellular counterpart might also be up-regulated by hyperglycemia. Besides carnosine metabolism, carnosine clearance and excretion in the urine might be increased in diabetes. Profiling of carnosine in plasma and urine after
ingestion of the dipeptide by healthy and diabetic subjects could shed light on that issue and should therefore come into the focus of future studies.

It should be noted that a recently performed study did not find an association between the \textit{CNPDI} genotype and muscle carnosine content (264), but suggested that carnosine tissue levels are rather dependent on age, vegetarianism and gender. Because muscle tissue is an important side of carnosine synthesis and deposit, it is obvious that muscle tissue is not suitable to examine a general effect of CN-1 activity on tissue carnosine levels and does not exclude that CN-1 in serum determines carnosine levels in other tissues. It should also be emphasized that genotype dependent differences in CN-1 secretion on kidney carnosine levels remain to be investigated. Future studies should therefore focus on carnosine levels in the kidney and examine inter-individual differences with respect to genetic (\textit{CNDP1}) and environmental (hyperglycemia) factors. Yet, noninvasive measurement of kidney carnosine levels is difficult to achieve, and require the development of new robust methods.

In conclusion, the studies described in this thesis demonstrate that the \textit{CNDP1} (CTG)$_n$ polymorphism is a functional polymorphism which partly determines the expression of CN-1 in serum. Low serum CN-1 expression implicates that less carnosine is degraded and more carnosine is available to protection the diabetic kidney. Carnosine might avoid progressive glomerular scarring by inhibiting TGF-ß production and signaling through the ALK-5 pathway and prevents diabetes induced podocyte loss. It should be noted that carnosine levels are determined by three independent variables, i.e. carnosine up-take, carnosine synthesis and carnosine degradation. If carnosine is a protective factor in DN, susceptibility to develop DN is not entirely determined by the CN-1 genotype, but should also be influenced by diet, muscle content and physical activity. Moreover, independent of genotype the also environmental factors, e.g. hyperglycemia or homocarnosine concentrations, contributes to the susceptibility to develop DN, as they influence CN-1 secretion and activity, respectively. Risk stratification of diabetic patients has the potential for an improved clinical care of diabetic patients. Although the \textit{CNDP1} gene polymorphism and serum CN-1 quantification might help to meet this demand, other parameters such glycemic control should be implemented in such strategies. Further studies are required to address the importance of the carnosinase-carnosine system in DN, to elucidate why the \textit{CNDP1} gene polymorphism is not associated with DN in type 1 diabetic patients and to understand the partly conflicting studies on this association in type 2 diabetic patients (138).