Chapter 7

Summary and General discussion

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7.3 Nederlandse Samenvatting
Chapter 7

7.1 Summary

Up till now, diabetic nephropathy (DN) accounts for approximately 40% of end stage renal disease (ESRD) worldwide. This is mainly due to the increasing incidence of diabetes, as well as the longer life expectancy of diabetic patients [1]. Patients with DN have a higher mortality risk, which is explained by an increased incidence of cardiovascular events and stroke [2]. Although intensive glycemic therapy delays the onset or progression of diabetic nephropathy (DN) in its early stages [3], controversy remains as to whether intensive therapy slows the progression of established DN [4, 5]. In addition, severe hypoglycemia has been associated with intensive glycemic therapy [6, 7], raising safety concerns that may be of particular relevance for patients with decreased kidney function. Improvement of diabetes management is therefore still warranted.

Angiotensin converting enzyme (ACE) inhibitors and angiotensin-2 receptor blockers (ARB) are the mainstay of first-line therapy for DN because of their anti-proteinuric effect. In addition, ACE inhibitors reduce the risk of acute myocardial infarction, cardiovascular events and all-cause mortality [8]. However, since ACEI do not completely block the production of Angiotensin II [9] and the current therapeutic modality is incomplete, there is still much room for improvement. One of the options could be the intervention in the carnosinase-carnosine system. Based on the postulated association between a polymorphism in the CNDP1 gene, the studies performed in this thesis are meant to provide a better understanding of the carnosinase-carnosine system and to evaluate if this system might be a target for the treatment of DN.

We have previously reported that CN-1 may exist in different conformations depending on whether the dinuclear Zn centers are occupied by divalent metal ions. In Chapter 2 we characterized the epitope of monoclonal antibody RYSK173, which
recognizes CN-1 in the presence of EDTA or after denaturation, to further substantiate the findings that not all CN-1 homodimers are complexed with Zn. Indeed we demonstrate by making use of myc-tagged CN-1 fragments and overlapping peptides that the RYSK173 epitope contains both Zn centers of the catalytic domain of CN-1. Although recognition of recombinant rCN-1 by RYSK173 strongly declined when it was spiked into human serum as compared to PBS or fetal calf serum, neither addition of Zn$^{2+}$ nor Cu$^{2+}$ impaired binding of RYSK173 to rCN-1. While in serum of healthy controls RYSK173 recognized less than 0.5% of total CN-1, in patients with ESRD this was significantly higher. The CN-1 RYSK173 proportion seems to be increased in ESRD patients, possibly due to deficiency in zinc and other trace elements [10]. In contrast, the RYSK173 proportion was decreased in post-dialysis as compared to pre-dialysis serum and was paralleled by a concomitant increase in serum Zn$^{2+}$ and Cu$^{2+}$ concentrations.

While some studies have shown that the CNDP1 gene is associated with DN in T2DM patients, others have claimed that this association is sex specific and not present in all ethnicities. In Chapter 3 we assessed if the frequency of the protective CNDP1 genotype, i.e. the (CTG)$_5$ homozygous genotype, remains gender specific when the diagnosis of DN is based on clinical inclusion criteria (CIC-DN) or when the diagnosis is based on biopsy material (biopsy proven DN (BP-DN)) only. It also assessed if the frequency of the protective genotype is changed with time on dialysis. To this end, 145 T2DM patients without DN (no-DN), 110 T2DM patients with CIC-DN, 30 T2DM with BP-DN, 22 patients with biopsy proven non-diabetic renal disease (BP-NDRD) and additional 85 uremic patients on hemodialysis were studied. Overall frequencies of the (CTG)$_5$ homozygous genotype in the different groups were 36% (no-DN), 38% (CIC-DN), 17% (BP-DN) and 32% (BP-NDRD) (p<0.05 for no-DN vs. BP-DN and for BP-DN vs. BP-NDRD). Gender stratification revealed a lower
frequency of the protective genotype in the female CIC-DN as compared to the no-
DN group (38% vs. 31%, no-DN vs. CIC-DN), yet this difference was only statistical
significant in female for the comparison with the BP-DN group (38% vs. 0%, no-DN
vs. BP-DN, p<0.05). No evidence for a significantly decreased frequency of the
protective genotype was found in male T2DM patients. The proportion of (CTG)₅
homozygous patients on hemodialysis (HD) increased with time on dialysis from
26%, 39%, to 48% for time on dialysis of: <18 months; 18-120 months; >120 months
respectively. Our study confirms that the association between the CNNDP1 genotype
and DN is most likely gender specific and clearly pronounced in BP-DN. It also
suggests that (CTG)₅ homozygous patients may have a survival advantage when
hemodialysis is required. Yet, it remains to be addressed why the frequency of
(CTG)₅ homozygous patients is increased with time on dialysis.

Even though T2DM patients without DN are more frequently homozygous for (CTG)₅
allele, a significant number of (CTG)₅ homozygous T2DM patients still develop DN.
Why this occurs is at present unknown. In Chapter 4 we therefore tested the
hypothesis that CNNDP1 (CTG)₅ homozygous T2DM patients with nephropathy have
higher serum CN-1 activities and concentrations as compared to those without
nephropathy. From a total of 272 T2DM patients (with DN n=127, without DN n=145)
92 patients (with DN: n=45, without DN: n=47) were homozygous for the (CTG)₅
allele. CNNDP1 (CTG)₅ homozygous T2DM patients with DN had significantly lower
CN-1 concentrations (30.4 ± 18.3 vs 51.2 ± 17.6 µg/ml, p<0.05) and activity (1.25 ±
0.5 vs 2.53 ± 1.1 µmol/ml/h, p<0.05) than those without nephropathy. Univariate
analysis confirmed significant lower CN-1 concentrations and activity in all T2DM
patients with DN. In multivariate regression analyses, estimated renal function
(eGFR) and to a lesser extent genotype were significantly associated with serum CN-
1 concentrations (95% CI of regression coefficients: eGFR: 0.10 – 1.94 (p=0.001);
genotype: -0.05 – 5.79 (p=0.055)). In a separate small group (n=12) of subsequently recruited patients with micro- or macro-albuminuria (range 39-836 ng/ml), carnosinasuria was detected, correlated inversely with serum albumin (r: 0.90) and positively with albuminuria (r: 0.83). Immuno-histology on sections of kidney biopsies retrieved from our archives suggested that CN-1 expression in proximal tubules is increased in patients with proteinuria as compared to healthy controls (controls: 0.014 ± 0.021, patients with proteinuria: 0.102 ± 0.130). Hence, our data suggest that serum CN-1 concentrations in T2DM patients with DN might be decreased as a consequence of carnosinasuria.

Iron has been suggested to affect the clinical course of type 2 diabetes (T2DM) as accompanying increased intracellular iron accumulation may provide an alternative source for reactive oxygen species (ROS). Although carnosine has proven its therapeutic efficacy in rodent models of T2DM, little is known about its efficacy to protect cells from iron toxicity. In Chapter 5 we sought to assess whether high glucose (HG) exposure makes cultured human umbilical vein endothelial cells (HUVEC) and renal proximal tubular epithelial cells (PTEC) more susceptible to metal induced toxicity and if this is ameliorated by L-carnosine. Cell viability was neither impaired under HG conditions nor did HG increase susceptibility to FeCl$_3$. HG did not change the expression of divalent metal transporter 1 (DMT-1), ferroportin (IREG), transferrin receptor protein 1 (TFRC)). Irrespective of glucose concentrations L-carnosine prevented toxicity in a dose dependent manner, only if it was present during the FeCl$_3$ challenge. Our study indicates that iron induced cytotoxicity is not enhanced under HG conditions. L-carnosine displayed a strong protective effect, most likely by chelation of iron.

In Chapter 6 we examined the effect of carnosine treatment in vivo in BTBR (Black and Tan, BRachiuric) ob/ob mice, which develop a phenotype that closely resembles
advanced human DN. BTBR \textit{ob/ob} mice were supplemented for 18 weeks with L-carnosine (4 mM) in drinking water. Treatment of BTBR \textit{ob/ob} mice with carnosine reduced plasma glucose and HbA1c levels, probably caused by elevated insulin secretion. Also, albuminuria and kidney weights were reduced in carnosine-treated mice. In the treated mice less glomerular hypertrophy was observed and the molecular composition of the expanded mesangial matrix was modified, while hyperglycemia-induced reduction of glomerular podocytes remained unaffected. Our data suggest that treatment with carnosine is able to improve glucose metabolism, kidney function and pathology in BTBR \textit{ob/ob} mice.
7.2 General discussion and future perspectives

DN is histologically characterized by mesangial expansion, glomerular basement membrane thickening, nodular sclerosis (Kimmelstiel-Wilson), and advanced diabetic glomerulosclerosis [11]. Renal biopsy is thus a golden standard for diagnosis of DN, but only performed in a minority of T2DM patients. DN is diagnosed in these patients mainly on the basis of clinical criteria, e.g. persistent macro-albuminuria in at least 2 independent measurements (albumin excretion rate > 300 mg/d or > 200 mg/l or ACR (albumin/creatinine ratio) > 300 mg/g). Along with albuminuria glomerular filtration rate (GFR) also gradually declines as DN progresses. Thus the combination of albuminuria and estimated (e)GFR is commonly used to predict DN according to the Kidney Disease Outcomes Quality Initiative (KDOQI) (shown in Tab. 1) [12]. It is however worthwhile to emphasize that up to 25% - 50% diabetic patients may develop renal diseases other than DN [13-15].

**Tab. 1 DN diagnosis (based on KDOQI guideline [12]).**

<table>
<thead>
<tr>
<th>GFR (ml/min)</th>
<th>CKD stage</th>
<th>Albuminuria</th>
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<td>&gt;60</td>
<td>1 + 2</td>
<td>At risk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Possible DN</td>
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<tr>
<td></td>
<td></td>
<td>DN</td>
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<tr>
<td>30-60</td>
<td>3</td>
<td>Unlikely DN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Possible DN</td>
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<td></td>
<td></td>
<td>DN</td>
</tr>
<tr>
<td>&lt;30</td>
<td>4 + 5</td>
<td>Unlikely DN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unlikely DN</td>
</tr>
<tr>
<td></td>
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<td>DN</td>
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Genetic studies that use clinical inclusion criteria (CIC) for group allocation therefore may wrongly assign patients to the DN group, which partly could underlie different outcomes on susceptibility for a particular genetic trait in different studies. Although our study described in Chapter 3 includes only a small number of biopsy proven DN
(BP-DN) patients, the results are compatible with previous studies that suggest the association of \textit{CNDP1} and DN is sex specific. Recent efforts for classification of DN have suggested 5 different stages on the basis of histo-pathological findings: i.e. mild or nonspecific light microscopic changes and electronic-microscope-proven GBM thickening (stage I), mild mesangial expansion (stage IIa), severe mesangial expansion (stage IIb), nodular sclerosis (Kimmelstiel–Wilson lesion, stage III) and advanced diabetic glomerulosclerosis (stage IV). At present it is not clear if the genotype distribution (i.e. the relative proportion of $(\text{CTG})_5$ homozygous individuals) is changed with severity of the classification. It might be that $(\text{CTG})_5$ homozygous individuals may progress to the early stages (I, IIa and IIb) but not to the more severe ones (stage III and IV). Clearly this requires further studies using renal biopsies from diabetic patients. These studies could also help to address whether in male diabetic patients there is no influence of the $(\text{CTG})_5$ genotype at all or whether it is just more pronounced in female as suggested in our small sample size. An interesting finding in Chapter 3 was the observation that the proportion of homozygous $(\text{CTG})_5$ T2DM patients increased with time on dialysis, while gender distribution remained equal. Because the annual mortality rate for patients on maintenance hemodialysis (HD) are several times higher than those of the general population [16], “enrichment” for homozygous $(\text{CTG})_5$ T2DM patients in the group of patients with a long history of HD may indicate a survival benefit for such patients. These findings are to some extent unexpected since in a recent prospective cohort study on T2DM patients Alkhalaf et al suggested that homozygous $(\text{CTG})_5$ female T2DM patients have higher risk for cardiovascular mortality as compared to males [17]. Since sudden cardiac death is the single most common form of death in dialysis patients, accounting for 20% to 30% of all deaths [18, 19], it would be expected that the proportion of homozygous $(\text{CTG})_5$ female patients would be less in the group of patients with a long history.
(>120 months) of HD. It should be emphasized however that out of the 27 individuals with a long history of dialysis only 9 patients had T2DM, yet, the majority of which were carrying the (CTG)\textsubscript{5} genotype. Clearly the small group size of this cross-sectional study impedes drawing firm conclusions and warrants further prospective analysis to test if the (CTG)\textsubscript{5} genotype indeed exerts a survival benefit in HD patients.

The cause of DN is so far not fully understood. Risk factors involved in DN progression include genetic make-up, blood pressure, glycemic control, the extent of proteinuria and AGEs formation. Although homozygous (CTG)\textsubscript{5} CN-1 T2DM patients (particularly females) may have a relative protection to develop DN, there is still a significant number of such patients that progress to DN. Interestingly, serum CN-1 concentrations, activity and conformation in (CTG)\textsubscript{5} homozygous T2DM with DN are significantly lower as compared to those without DN. This might be explained by the relative large proportion of dialysis patients in the group of T2DM with DN, as time on dialysis negatively correlates with CN-1 activity and concentrations. As demonstrated in Chapter 2, the RYSK173 monoclonal antibody recognized an epitope on CN-1 in the vicinity of Zn\textsuperscript{2+} centers. We also show that the relative proportion of CN-1 recognized by RYSK173 is higher in post- as compared to pre-dialyses serum samples, which is paralleled by an increased concentration of divalent metal ions. Based on this, and the finding that in the T2DM patients with DN a large number was on HD, it was expected that the relative proportion would be larger in this group as compared to the group without DN. In a separate small group of patients with micro- or macro-albuminuria we observed a significant correlation between albuminuria and carnosinasuria, raising the possibility that the low serum CN-1 concentrations in T2DM patient with DN might be a consequence of CN-1 loss via the urine. It should also be emphasized that HD patients may suffer from protein energy wasting, which
explains the reduced BMI in patients with poor renal function, and therefore may not sufficiently replenish CN-1 in serum. It might be that CN-1 is partly reabsorbed from the urine in the proximal tubules resulting in an increased CN-1 staining in proximal tubules. Yet, formal proof for reabsorption is lacking and thus the increase tubular staining could also be a consequence of de novo CN-1 production in the kidney. To assess the role of serum CN-1 and carnosinasuria on renal function deterioration, we have currently collected urine and serum samples of approximately 2835 Caucasian T2DM patients (DIAbetes COhoRtE (DIACORE) study). The study design of DIACORE study was described in detail by Dörhöfer et al. [20]. These patients will be followed up for approximately 10 years. Although most of these patients (approximately 66%) are normo-albuminuric patients with good eGFR, an ad hoc analysis suggested that the prevalence of carnosinasuria is increased in normo-albuminuric patients with poor eGFR. The extent of carnosinasuria was increased in the macro-albuminuric patients (approximately 34%) and showed a significant correlation with albuminuria (unpublished data, study is ongoing).

The salutary effects of carnosine have already been demonstrated in a large variety of in vivo and in vitro experiments [21-24]. Our findings in Chapter 5 and Chapter 6 are in line with the protective properties of carnosine in the setting of diabetes. Although the efficacy of carnosine to protect cells against iron mediated toxicity in vitro and its beneficial effect on albuminuria in vivo has clearly been shown it remains to be assessed if such protection may also be observed in human T2DM patients. The in vivo efficacy of carnosine could be overestimated since mice do not express CN-1 in serum. We therefore have generated CN-1 transgenic mice in a diabetic susceptible genetic background. These mice will enable us to 1) assess the influence of serum CN-1 on hyperglycemia and albuminuria 2) assess if carnosine feeding is still effective in these animals and 3) assess if carnosinase is an attractive target in
the treatment of DN. Although most of these studies are currently on the way, preliminary results already suggest that 1) hyperglycemia and albuminuria are much more severe in the CN-1 transgenic mice, 2) carnosine feeding seems to reduce hyperglycemia but not albuminuria in these mice, 3) renal carnosine concentration are significantly reduced in transgenic mice and can only marginally be increased by carnosine supplementation. In cooperation with Sanofi we also have identified a CN-1 inhibitor, which strongly inhibits CN-1 activity in our transgenic mice over a period of 24 hrs when applied subcutaneously. The influence of this inhibitor on hyperglycemia and albuminuria will be tested in our model by repeated administration.

In conclusion, our studies indicate that the carnosinase-carnosine system may play a significant role in either hyperglycemia and or the renal complications hereof. In human the salutary effects of carnosine might be lost as a consequence of CN-1 activity. Yet, the preliminary findings in CN-1 transgenic mice are promising, and may help to address the question as to whether this system is an attractive target in the treatment of DN in human.
7.3 Nederlandse Samenvatting

Door de toegenomen incidentie van diabetes, voornamelijk type 2, is ook de incidentie van de verschillende micro- en macrovasculaire complicaties t.g.v. diabetes in de afgelopen decenial fors toegenomen. Eén van deze complicaties is diabetische nefropathie (DN) hetgeen zich bij circa een derde van diabetes patienten ontwikkeld. Er zijn sterke aanwijzingen dat naast omgevingsfactoren zoals bloeddruk en bloedglukose instelling ook genetisch factoren bijdragen aan de gevoeligheid om DN te ontwikkelen. Een polymorphism in het gen van serum carnosinase, ook wel CN-1 genoemd, is één van deze genetische factoren die mogelijk in betrekking staan met DN. CN-1 is een enzym dat de dipeptiden carnosine, homocarnosine en anserine als substraat herkent. Deze substraten hebben als gemeenschappelijk kenmerk dat ze alle L-histidine als aminozuur bevatten in combinatie met β-alanine (carnosine) of gamma-aminobutyraat zuur (GABA) (homocarnosine). In het geval van anserine, bestaat de dipeptide uit β-alanine en een gemethylereerde vorm van histidine. Histidine bevattende dipeptiden worden bepaalde eigenschappen toegeschreven die met name bij hyperglycaeme patienten mogelijk gunstig kunnen werken op eventuele micro- en macrovasculaire complicaties.

De belangstelling voor CN-1 is primair ontstaan door genetische studies die op een samenhang tussen een polymorphism in het CN-1 gen en DN duiden. Op grond van een signifikante verschuiving in de verdeling van genotypen tussen type 2 diabetes patienten met of zonder DN werd gepostuleerd dat het zogenaamde Mannheim-allel (CNDP1 (CTG)5) een recessief allele is en in beperkte mate bescherming biedt tegen DN. Hoewel een associatie in meerdere studies is aangetoond, lijkt het er op dat de deze afhankelijk is van etniciteit, het type van diabetes (alleen bij type 2) en vooral voor vrouwen geldt. De studies beschreven in
dit proefschrift hebben enerzijds getracht de associatie tussen CN-1 en DN beter te begrijpen en anderzijds de vraag of carnosine bescherming bieden kan tegen DN te beantwoorden.

De belangrijkste uitkomsten van dit onderzoek zijn: 1) De associatie tussen CN-1 en DN in type 2 diabetes patiënten lijkt inderdaad sterker te zijn bij vrouwen als bij mannen. 2) Naast genotype lijkt ook de nierfunctie een onafhankelijk variabele te zijn die de concentratie van carnosinase in serum bepaald. 3) Carnosinase is aanwezig in urine van patiënten met proteinurie, maar dit is niet specifiek voor DN. De expressie van carnosinase in nierweefsel neemt toe bij patiënten met proteinurie hetgeen mogelijk een gevolg is van reabsorptie uit urine of de novo synthese van CN-1 in de proximale tubuli van de nier. 4) Hyperglycaemie verandert de toxische werking van ijzer niet. Zowel onder normo als ook hyperglycaeme condities beschermt carnosine endotheel- en nierepitheel cellen tegen de toxische werking van ijzer. 5) Het toevoegen van carnosine aan drinkwater verbetert de hyperglycaemie en proteinurie bij muizen die spontaan type 2 diabetes ontwikkelen.
Chapter 7

Reference


