Chapter 1

General introduction

1.1 General introduction

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1.1 General introduction

1.1.1. Diabetes and diabetic nephropathy

Diabetes, clinically manifested as hyperglycemia, is a chronic syndrome which is either caused by insulin deficiency (type 1) or insulin resistance (type 2). The incidence of diabetes, particularly for type 2 diabetes, is increasing worldwide and is becoming a major problem in both developed and developing countries as diabetes associated healthcare expenditure is increasing in parallel. Diabetes is associated with hyperglycemia-specific microvascular complications; however, it also imparts a two- to fourfold risk for cardiovascular disease (CVD) [1]. It is the leading cause of end stage renal disease (ESRD) in the world. According to the latest figures from the International Diabetes Federation, 382 million people live with diabetes around the world (International Diabetes Federation (IDF) Diabetes Atlas. 6th edition, available online: http://www.idf.org/diabetesatlas), of which approximately one third will eventually develop chronic kidney disease [2]. Current therapies directed at delaying the progression of diabetic nephropathy (DN) include intensive glycemic and optimal blood pressure control, proteinuria and albuminuria reduction, interruption of the renin angiotensin-aldosterone system (RAAS) through the use of angiotensin converting enzyme inhibitors (ACEI) and angiotensin type-1 receptor blockers (ARB), along with dietary modification [3]. Although intensive glycemic therapy delays the onset or progression of DN in its early stages [4], controversy remains as to whether intensive therapy slows the progression of established DN [5, 6]. In addition, severe hypoglycemia has been associated with intensive glycemic therapy [7, 8], raising safety concerns that may be of particular relevance for patients with decreased kidney function. Improvement of diabetes management is therefore still warranted.

It has been suggested that in the course of DN the RAAS becomes dysregulated, leading to an increase of glomerular capillary pressure. Because ACEIs and ARBs
inhibit the production and the action of angiotensin II respectively, ACEI or ARB treatment will decrease the intra-glomerular pressure and thus can minimize progression of glomerular disease in the absence of glycemic control [9, 10]. A number of experimental animal studies and landmark clinical trials have clearly demonstrated the efficacy of the RAAS blockade in terms of reduced glomerulosclerosis and albumin creatinine ratio [11-14]. Yet renal protection provided by most of the current therapeutic modalities is incomplete, hence new promising renoprotective therapies are emerging, e.g. inhibition of the sodium glucose transporter 2 (SGLT2) [15, 16].

1.1.2 Pathophysiology of diabetic nephropathy

Permselectivity of the glomerular basement membrane (GBM) is based on both charge and size selectivity to assure that smaller negatively charged proteins, e.g. albumin, cannot pass the filtration barrier. Microalbuminuria (persistent albuminuria at levels of 30 - 300 mg/24 hours) or incipient nephropathy is the first clinical manifestation of DN. Heparan sulfate proteoglycans (HSPG) are abundantly present in the GBM, and may in part be responsible for the negative charge of the GBM. Hence, the loss of permselectivity of the GBM already in the early stage of DN might be a consequence of an altered HSPG expression [17-20], yet findings in the podocyte specific Ext 1 [21] and in the NDST1 [22] gene knock-outs do not support this assumption. In the early stage of DN there is an increase in glomerular filtration rate (GFR) also known as hyperfiltration. It is believed that at this stage, autoregulation of renal blood flow is impaired. Consequently, systemic pressure is transferred to the glomerular capillary loops, resulting in hyperfiltration [23, 24]. This may explain the benefit of anti-hypertensive treatment in the treatment of DN [25, 26], although also additional renoprotective effects beyond blood pressure control have
been discussed [27, 28]. Along with the progression of DN, the GFR declines in a linear manner [23] and is accompanied by macroalbuminuria. Depending on the type of diabetes, overt nephropathy (i.e. persistent macroalbuminuria at levels of ≥300 mg/24 hours) may develop after many years. Conversion to macroalbuminuria likely will progress to ESRD [29].

DN is characterized by mesangial matrix expansion, thickening of the GBM and nodular glomerulosclerosis (Kimmelstiel–Wilson nodules). The histo-pathological classification of DN is given in table.1 [30].

<table>
<thead>
<tr>
<th>Class</th>
<th>Description</th>
<th>Inclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mild or nonspecific light microscopic changes and EM-proven GBM thickening</td>
<td>Biopsy does not meet any of the criteria mentioned below for class II, III, or IV. GBM &gt; 395 nm in female and &gt;430 nm in male individuals who are 9 years of age and older.</td>
</tr>
<tr>
<td>IIa</td>
<td>Mild mesangial expansion</td>
<td>Biopsy does not meet criteria for class III or IV. Mild mesangial expansion in &gt;25% of the observed mesangium.</td>
</tr>
<tr>
<td>IIb</td>
<td>Severe mesangial expansion</td>
<td>Biopsy does not meet criteria for class III or IV. Severe mesangial expansion in &gt;25% of the observed mesangium.</td>
</tr>
<tr>
<td>III</td>
<td>Nodular sclerosis (Kimmelstiel–Wilson lesion)</td>
<td>Biopsy does not meet criteria for class IV. At least one convincing Kimmelstiel–Wilson lesion.</td>
</tr>
<tr>
<td>IV</td>
<td>Advanced diabetic glomerulosclerosis</td>
<td>Global glomerular sclerosis in &gt;50% of glomeruli. Lesions from classes I through III.</td>
</tr>
</tbody>
</table>

1.1.3 Diabetic nephropathy and risk factors

The incidence of DN is approximately 40% in type 1 and type 2 diabetic patients [31] and is the leading cause of ESRD [32]. Major risk factors for development and progression of DN include hyperglycemia, duration of diabetes, obesity, blood
pressure, dyslipidemia, life style, age and gender [33]. Even though glycemic control and hypertension are the major therapeutic targets for the treatment of diabetic patients, it should be emphasized that DN can still develop in patients with well-controlled blood glucose concentrations and treated with anti-hypertensive medications. However, progression of renal function deterioration is significantly retarded by latter treatment modality [4, 26]. Based on numerous epidemiologic studies there is no compelling evidence that the susceptibility to develop DN is genetically determined [34, 35]. In addition, several genome wide linkage studies have revealed an association between DN and susceptibility loci on different chromosome [36-38]. Since the serum carnosinase gene (CNDP1) is amongst the susceptibility loci reported in literature and topic of this thesis, in the following I will first discuss the physiology of histidine containing dipeptides (HCD) with emphasis on carnosine.

1.1.4 Physiology of histidine containing peptide

The most commonly found HCD in human are carnosine, anserine and homocarnosine. Carnosine can be converted into β-analyl-3-methyl- or 5-methyl-histidine, also known as anserine and ophidine (= balenine) respectively, by methylation of the histidine moiety. These methylated analogues of carnosine are formed by methyltransferase activity, in which the methyl group of S-adenosyl-methionine (SAM) acts as methyl donor and catalysis is provided by carnosine N-methyltransferase [39]. Under physiological condition, synthesis of carnosine is catalyzed by an ATP dependent carnosine synthase enzyme, which is present in skeletal- and heart muscles, brain [40] and liver [41]. The rate-limiting precursor for the synthesis of carnosine is β-alanine [42], and therefore, the increase of β-alanine concentrations in
these tissue can result in increased carnosine levels. Homocarnosine ($\gamma$-aminobutyryl-L-histidine) is mostly present in cerebrospinal fluid and believed to be a depot for the neurotransmitter $\gamma$-Aminobutyric acid (GABA) [43] (Fig.1).

![Diagram of various dipeptides]

**Fig.1 Naturally occurring histidine-containing dipeptides (1–5) and some relevant synthetic derivatives (6–9) [44].**

Current knowledge on the physiological relevance of individual HCD is fragmentary, yet there are a number of studies that outline the role of carnosine and homocarnosine in muscle and brain.

Because of its pH buffering capacity carnosine is widely used in the field of sports nutrition [45]. The majority of research relating to the ergogenic effects of elevated muscle carnosine content has been performed by food supplementation either via chicken breast extracts, high in HCD content, or via $\beta$-alanine [42, 46, 47]. The acid dissociation constant (pKa) of carnosine [48, 49] suggests that carnosine may attenuate the reduction in blood pH during strenuous exercise, and thus may suppress a loss of force in muscle [50]. At the same time, muscle carnosine content
positively correlates with high intensity exercise performance [51] and fast-twitch muscle fibers [52].

It is reported that carnosine can readily pass through the blood brain barrier (BBB), acts as transmitter precursor of the histaminergic neuron system and effectively regulate brain histamine levels [53-55]. Synthesized from histidine by a unique enzymatic reaction mediated by L-histidine decarboxylase, histamine mediates multiple biological activities through four types of receptors (histamine receptors (HRs)): H1R, H2R, H3R, and H4R [56]. Since histamine cannot cross the BBB, brain histamine levels are strictly dependent on the conversion of histidine by L-histidine decarboxylase. As carnosine can freely cross the BBB, carnosine could serve as a reservoir for histamine. Indeed, recent studies by Zhu et al. demonstrated that carnosine can activate histamine neurons in a histidine decarboxylase dependent fashion [57]. Similarly Li et al [58] showed that orally administered carnosine significantly elevated brain histamine levels in restraint-stressed mice. Utilizing a model of permanent middle cerebral artery occlusion in mice, Shen et al [59] found that carnosine significantly improved neurological function and decreased infarct size in both L-histidine decarboxylase knockout and the corresponding wild-type mice to the same extent. These findings suggest that the neurological effects of carnosine are not solely due to its degradation by carnosinase (CNDP1) and subsequent conversion of L-histidine to histamine. Carnosine also decreases glutamate levels and preserves the expression of glutamate transporter-1 (GLT-1) in astrocytes exposed to ischemia in vivo and in vitro [59].

Like carnosine, degradation of homocarnosine results in the release of L-histidine. However, degradation of homocarnosine also gives rise to GABA. GABA is the most abundant inhibitory neurotransmitter in the human brain and is of major interest to the clinical neuroscience community [60]. Abnormal GABA levels are postulated to play
an important role in various neurological disorders, in particular epilepsy [61] and in several psychiatric disorders [62-64].

1.1.5 Carnosine and diabetic nephropathy

L-carnosine is a major HCD found in skeletal muscle [65], brain [66] and less abundantly in other tissues, e.g. kidney and spleen. The suggestion that L-carnosine may affect diabetic complications emerged from the finding that a polymorphism in the precursor protein of the carnosine degrading enzyme, CNDP1, is a susceptibility locus for developing DN in type 2 diabetic patients [67]. The association between DN and CNDP1 has been confirmed in other studies [68-71], and seems to be stronger in female [70] than male patients probably due to the fact that CNDP1 activity / concentration is lower in male subjects. The most compelling evidence to support the beneficial effect of carnosine in diabetes comes from animal studies where in a model of type 2 diabetes carnosine feeding retards the on-set of diabetes and its complications, while over-expression of CNDP1 significantly increased the progression to diabetes [72]. In type 2 diabetic patients and elderly people muscle carnosine concentrations are reduced [73]. In concordance to this, CNDP1 activity / concentration is increasing with age [74, 75] and is significantly higher in diabetic patients compared to age and sex matched healthy controls [76]. Preliminary data suggest that CNDP1 activity decreases as a consequence of exercise training (unpublished data). However, whether this type of life style change is instrumental to the beneficial effect of exercise training on glycemic control in type 2 diabetic patients [77] is still elusive.

Oxidative stress may cause protein modifications, either directly via reactive oxygen species (ROS), or indirectly by reactive carbonyl products formed by auto-oxidation of carbohydrates, lipids or amino acids. While auto-oxidation of carbohydrates yields
precursors of advanced glycation end-products (AGE), e.g. glyoxal, methylglyoxal and glycoaldehydes, lipid peroxidation also generates precursors of advanced lipoxidation end-product (ALE), e.g. malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [78, 79]. Hyperglycemia is associated with both glycative and oxidative stress as a consequence of an increased availability of carbohydrates in the circulation and an increased production of mitochondrial derived ROS [80]. It has been proposed that accelerated chemical modification of proteins during hyperglycemia contributes to the pathogenesis of diabetic complications. This is substantiated by the finding that ALE and AGE modified proteins accumulate in renal lesions in patients with DN [81-83]. AGE and ALE can evoke a variety of biological responses, e.g. stimulation of extracellular matrix production, induction of inflammatory responses and inhibition of proliferation, which perpetuates the progression of diabetic lesions [84-86]. Although several compounds have been developed as AGE inhibitors and are being tested in animal models of diabetes and in clinical trials, the mechanism of action of these inhibitors is poorly understood. In general, they are thought to function as nucleophilic traps for reactive carbonyl intermediates in the formation of AGE/ALE or alternatively via metal chelation and prevention of metal-catalyzed auto-oxidation. In vitro and in vivo models have suggested that HCD may have the capacity to quench reactive carbonyl products [87-90] and to prevent auto-oxidation via metal chelation [91]. In addition, it has been suggested by Hipkiss et al [92] that carbonylated proteins may be converted to protein-carbonyl-carnosine adducts (“carnosylated protein”) at the carbonyl groups, which protect them from degradation and/or cross-linking. These properties indeed may explain the efficacy of oral carnosine treatment in rodent diabetic models [72, 93], yet, there is a huge discrepancy between in vivo and vitro carnosine concentrations required for their anti-oxidative and anti-glycative properties.
Moreover, from a translational point of view, animal models may significantly differ with respect to the enzymes involved in carnosine metabolism, e.g. *CNDP1* which is not present in serum of rodents. Also the properties of carnosine to act as antioxidant in the presence of transition metal ions is controversially discussed [94-97]. Apart from the in vivo findings that carnosine treatment in diabetic models is associated with reduced HbA1c and AGE levels, it has also been reported that carnosine diminishes apoptosis of glomerular cells in STZ rats [93]. In vitro findings also suggest that carnosine may function as an ACE inhibitor [98, 99]. Since the IC$_{50}$ value for ACE inhibition by carnosine is estimated to be 5.2 mM [98], the physiological relevance of this finding is questionable.

1.1.6 Serum carnosinase

Metallopeptidases of the M20/28 family play diverse functions throughout all kingdoms of life, ranging from a general role in the hydrolysis of late products of protein degradation to specific biochemical functions in protein maturation, tissue repair, and cell-cycle control [100]. For example, Lactobacillus sp. aminopeptidase V (PepV) and Salmonella typhimurium peptidase T (PepT) function in amino acid utilization, whereas Escherichia coli allantoate amidohydrolase and yeast β-alanine synthase are enzymes of the catabolic pathway of nucleotides, respectively.

Carnosinases, also belonging to the M20 family, are dipeptidases which hydrolyze Xaa-His dipeptides including carnosine. In human two carnosinase enzymes are found, i.e. serum carnosinase (CN-1; monomeric molecular weight 70KDa; isoelectric point: 4.4) [101] and tissue carnosinase (CN-2, monomeric molecular weight 53KDa), encoded by the *CNDP1* and *CNDP2* genes respectively. CN-1 is mainly synthesized by hepatocytes from where it is secreted into the circulation as a specific carnosine-hydrolase. Also in the cerebellar cortex CN-1 is expressed and localized in adjacent
neuronal projections of homocarnosine expressing Purkinje cells [102]. The hydrolyzing activity of CN-1 is not only restricted to carnosine, but also anserine, homocarnosine and probably ophidine are genuine substrates for CN-1 [103]. CN-1 deficiency has been described in several sib-ships in conjunction with tremor, myoclonic seizures, hypotonia, and profound psychomotor retardation [104-108]. CN-2 is a cytosolic nonspecific dipeptidase with broad substrate specificity and hydrolyzes carnosine only under non-physiological conditions (pH optimum: pH=9.5) in the presence of Mn$^{2+}$ [109]. CN-2 mostly distributes in kidney, liver, spleen and cerebral cortex [110].

Like other members of the M20 family, CN-1 is composed of two structural domains of which one adopts an $\alpha/\beta/\alpha$ sandwich fold that features a dinuclear zinc-binding site [111]. The other smaller domain is inserted into the middle of the metal-binding domain and, as in most M20 family enzymes, mediates homodimerization of CN-1 (Fig. 2).

![Domain A](image1.png) ![Domain B](image2.png)

Fig.2 **Crystal structure of CN-1 monomer** (adapted based on data in Pubmed, available in http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi). CN-1 contains two structural domains, i.e. the catalytic domain (domain A) and dimerization domain (domain B).
Two active sites per dimer are located at the interface between one metal-binding domain and the two associated dimerization domains. Catalysis can occur independently in the two active sites. CN-1 may exist in two conformations, i.e. an open state and closed state conformation. Closure movement is required for catalysis, which occurs as a result of approximately $30^\circ$ rigid body rotation of the catalytic domain relative to the dimerization domain. The closed state conformation is stabilized by metal-ion binding. In CN-1 (MEROPS accession number MER015142), H478 and E200 chelate zinc 1, and H132 and D228 chelate zinc 2. As a bridging ligand, D165 completes the penta-dentate coordination sphere of both zinc ions. Any mutation of H132, D165, or E200 would lead to the loss of CN-1 activity, indicating the relevance of these residues for binding transition metals and hence for enzyme activity [109].

The concentration and activity of CN-1 in serum are affected by many factors (Fig. 3).

![Factors influencing CN-1 activity](adapted from [112]).

Serum CN-1 concentration and activity are genetically determined by the $(CTG)_n$ polymorphism [67, 113]. This tri-nucleotide repeat encodes different numbers of leucine and is located in the hydrophobic part of the CN-1 signal peptide. It is believed that the hydrophobic part in signal peptides not only functions as a type of
anchor in the endoplasmic reticulum membrane during protein synthesis but also directs the synthesized protein to the secretory pathway. Therefore, the shorter (CTG)$_5$ allelic variant might be less efficiently secreted. Support for this assumptions comes from the findings of Riedl et al that COS-7 cells secrete significantly less CN-1 when transfected with a $CNDP1$ cDNA plasmid that contains the shorter allelic variant [113]. Likewise it also explains why $CNDP1$ (CTG)$_5$ homozygous individuals have lower serum CN-1 concentrations and activities [67]. Apart from genotype, serum CN-1 concentrations and activities are influenced by N-glycosylation of CN-1 [76] and gender [73, 114]. The CN-1 hydrolyzing activity can be modulated by divalent metal ions, such as Cd$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Mg$^{2+}$ [115, 116], and by competing substrates, such as anserine and homocarnosine [114, 117]. Because elite athletes performing high intensive exercise training have lower serum CN-1 activity compared to untrained individuals [118], it has been postulated that serum CN-1 concentration might be modulated by the type of training activities, yet formal proof for this assumption is lacking.

So far, the existing methods for measuring CN-1 were mostly based on time consuming activity measurement. More recently two ELISA systems for CN-1 concentration measurement have been described by Adelmann et al [119]. One is using a commercially available polyclonal anti-CN-1 IgG (ATLAS) and the other is using an in-house-made monoclonal anti-CN-1 IgG (RYSK173). While a good correlation between CN-1 concentration and enzyme activity was observed in the ATLAS-based ELISA, the RYSK173-based ELISA only recognized a proportion of total CN-1. Because the proportion of RYSK173 could be increased by addition of EDTA or protein denaturation it is believed that RYSK173 recognizes a quality rather than a quantity of CN-1[119]. Interestingly there is an inverse correlation between the
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proportion of RYSK173 recognized CN-1 and CN-1 activity, thus suggesting that this antibody recognizes a CN-1 form with low activity.

1.1.7 CNDP1 and diabetic nephropathy

Type 2 diabetes (T2DM) patients homozygous for the CNDP1 (CTG)₅ repeat have a reduced risk to develop DN as compared to all other genotypes [67]. It seems that the association of the CNDP1 polymorphism with DN is depending on the type of diabetes [120, 121], ethnicity [122] and gender [70]. Although the histo-pathology of DN is quite similar for type 1 and type 2 diabetes, there is no single study thus far, in which an association of the CNDP1 polymorphism and DN in type 1 diabetic patients has been observed [120, 121]. The explanation for this discrepancy between DN in type 1 and type 2 diabetes is so far still unclear. Also the prevalence of the (CTG)₅ allele strongly varies with different ethnicities. While homozygosity for the (CTG)₅ allele is more frequent in the European population (38.6% in healthy controls and 29.3% in DN-ESRD patients) [68], this genotype seems to be much more rare in the Chinese population. A study in peritoneal dialysis patients revealed that the majority of patients (80.3%) were homozygous for the (CTG)₆ allele whereas the percentage of (CTG)₅ homozygous patients was less than 1% [123]. In South Asian Surinamese, the frequency of (CTG)₅ homozygosity is also lower as compared to White Dutch (23% vs. 41.3%) [124]. The former ethnicity seems to be more susceptible to develop DN as compared to Dutch Europeans [125].
1.2 Aims of the study

Even though a number of studies have reported on the association of the *CNDP1* gene and diabetic nephropathy (DN) in Caucasian type 2 diabetes mellitus (T2DM) patients, there are a number of issues that need to be addressed in terms of the enzyme itself, the sex specificity of the association, and particularly on why serum carnosinase 1 (CN-1) may be involved in the susceptibility for - or progression to - DN in T2DM patients. The overall aim of this thesis is to obtain a better understanding of the carnosine-carnosinase system and its relation to DN.

We have previously demonstrated that monoclonal antibody RYSK173 only recognizes a fraction of total serum CN-1, which ranges in healthy individuals from 0.5 to 2% [119]. We also have observed that a high proportion of RYSK173 recognized CN-1 is associated with low CN-1 activity. In Chapter 2 we therefore further elucidated why this antibody is reacting in this manner and tried to give a biological plausibility for its behavior. To this end we studied how it reacts with recombinant CN-1 expressed in endothelial cells, once it is secreted in the supernatant and when it is still present in the cell. Experiments were performed to elucidate the influence of metal ions on recognition of CN-1 by RYSK173 and finally we performed epitope mapping to delineate the RYSK173 epitope on CN-1.

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 other ethnicities, e.g. Afro-Americans. In Chapter 3 we therefore re-evaluated this association in an independent cohort of T2DM patients (n=272). We assessed whether the association was still found when only biopsy proven DN was considered and, if so, whether it was gender specific. Since it has also been suggested that female *CNDP1* (CTG)\(5\) homozygous T2DM patients have an increased risk for cardiovascular mortality, we also assessed in a cross-sectional
design whether the frequency of this genotype changes over time on dialysis in T2DM patients, since T2DM patients on dialysis are a population at particularly high risk for cardiovascular mortality. It would be expected that particularly in female T2DM patients this frequency will be low, so female T2DM patients will reside a long time on dialysis more pronouncedly.

Even though homozygosity for the CNDP1 (CTG)$_5$ allele may afford protection against DN, a significant number of T2DM patients carrying this genotype still develop DN. In Chapter 4 we therefore tested the hypothesis that T2DM patient homozygous for the CNDP1 (CTG)$_5$ allele have a significant higher serum CN-1 concentration and activity as compared to those without nephropathy. The presence of CN-1 in urine, CN-1 expression in proximal tubules and the relation between low serum CN-1 and carnosinasuria or protein-energy wasting was also studied.

Tissue iron accumulation has been reported to be an alternative/additional mechanism by which organs/tissues are damaged in hyperglycemic patients. In Chapter 5 we tested whether hyperglycemia makes endothelial or renal epithelial cells more susceptible to iron mediated damage and whether this can be prevented by carnosine treatment.

Oral carnosine treatment has been shown to be effective in a variety of diabetic models. The pitfall of these models is however that no severe renal pathology is observed. Hence, current studies do not allow assessing whether oral carnosine treatment has a salutary effect on late renal pathologic changes in hyperglycemic animals. In Chapter 6 we therefore tested the efficacy of oral carnosine treatment in the BTBR ob/ob model, in which profound mesangiolysis and glomerulosclerosis has been described.
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