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## Diabetic nephropathy : pathology, genetics and carnosine metabolism

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# 1

## GENERAL INTRODUCTION



Diabetic nephropathy, a progressive kidney disease due to longstanding diabetes, is the leading cause of end stage renal disease in the Western world (1). However, not all patients with diabetes-mediated hyperglycemia will develop this disease (2;3;4). It appears that both environmental and genetic factors play a role in the development of diabetic nephropathy, making diabetic nephropathy a complex disease.

One of the aims of this thesis was to create a histopathological classification of diabetic nephropathy. Another goal was to create a systematic overview of genetic associations in diabetic nephropathy. Furthermore, we focused more on one of these genetic variants, the number of trinucleotide repeats coding for leucine in the *CNDP1* gene. Concerning this genetic variant, we examined the genotypic distribution in 3 ethnic groups. Additionally, we investigated whether the association between this genetic variant and diabetic nephropathy was sex-specific. Furthermore, the association between the *CNDP1* genotype and other progressive glomerular diseases was studied. Finally, as the *CNDP1* gene encodes for the enzyme carnosinase and carnosinase breaks down carnosine, which is known to have many protective capacities, we searched for determinants of carnosine levels.

The outline of this introduction is as follows. First, an introduction on diabetes, the kidney and diabetic nephropathy (part I) will be given, followed by an introduction on genetics in diabetic nephropathy (part II). Continuing with the *CNDP1* gene, the enzyme it codes for, carnosinase-1 and its substrate L-carnosine (part III).

## **PART I – DIABETES, THE KIDNEY AND DIABETIC NEPHROPATHY**

### ***Diabetes***

Diabetes mellitus is a metabolic disorder of multiple causes which is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism. The word diabetes originates from two Greek words; 'dia' (=διά) means 'through' and 'bainein' (=βαίνειν) means 'to pass'. The word mellitus comes from Latin, meaning 'honey sweet'. Therefore, diabetes mellitus could be translated as 'honey sweet passage'.

The two most common types of diabetes mellitus are type 1 and type 2 diabetes. Type 1 diabetes, accounting for 5-10% of cases of diabetes in populations of European origin, is associated with primary beta cell failure, mostly a result of autoimmune

destruction. Type 2 diabetes, the most common type in all populations, is characterized by high glucose in the context of insulin resistance and relative insulin deficiency. The number of type 2 diabetes patients has rapidly increased in the past few decades and is still rising further. In 2002, the number of diabetes patients worldwide was estimated at 173 million and has been predicted to increase to 350 million in 2030 (1).

Approximately 5% of the diabetes patients have maturity onset diabetes of the young (MODY). MODY is caused by a genetic defect leading to pancreatic beta cell dysfunction. One percent of the diabetes patients have mitochondrial diabetes, due to defects in the mitochondria.

Long-term hyperglycemia can lead to severe complications later in life. These complications of diabetes are divided in macrovascular and microvascular complications. Macrovascular disease is mainly confined to cardiovascular disease. Microvascular complications occur mainly in the eyes, kidneys, peripheral lower limbs and nerves, resulting in diabetic retinopathy, diabetic nephropathy, diabetic foot and diabetic neuropathy respectively. In this thesis we will further focus on diabetic nephropathy due to type 1 and type 2 diabetes.

### ***The kidney***

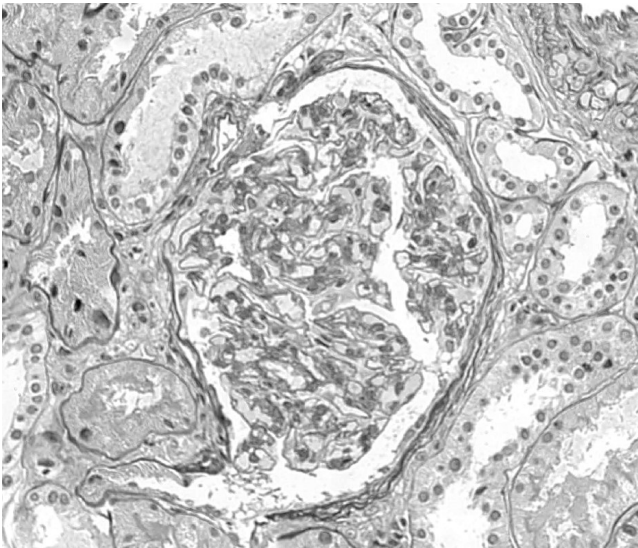
To understand the pathology of diabetic nephropathy we first briefly describe the anatomy and function of the human kidney. The kidneys are the main excretory organs of the human body. They regulate the extracellular volume, water and salt balance, and acid-base homeostasis. Furthermore, kidneys are endocrine organs, producing hormones with a role in erythropoiesis, calcium metabolism and blood pressure regulation.

Each kidney weighs approximately 150 grams in adults, and is located in the retroperitoneum. A kidney has three major components: the cortex, the medulla and the collecting system. The cortex, the outer layer of the kidney, consists mainly of glomeruli and convoluted tubuli. Situated more to the centre is the medulla consisting of pyramidal structures of parallel arranged tubular structures with apical papillae. The bases of the pyramids are at the corticomedullary junction and the apices extend into the collecting system. In the collecting system the pre-urine goes from the minor calyces, which receive pre-urine from a medullary papilla, to the major calyces, pelvis and finally it enters the ureter.

The blood supply of each kidney is by a single renal artery originating from the abdominal aorta. The main renal artery branches form anterior and posterior divisions

at the hilus and divides further into the interlobar arteries which run between lobes. Interlobar arteries extend to the corticomedullary junction and give rise to arcuate arteries, which arch between cortex and medulla. Afferent arterioles branch off from the arcuate arteries, each directing to a single glomerulus. A glomerulus represents a spherical bag of capillary loops arranged in several lobules. The capillaries come together to exit the glomerulus through the efferent arteriole. In most nephrons, the efferent arterioles branch off to form another vascular bed, the peritubular capillaries, which surround the tubules.

A nephron is a functional unit of the kidney and consists of a glomerulus with attached tubuli. Each kidney has approximately one million nephrons. The glomerulus consists of 4 types of cells: the mesangial cell, endothelial cell, visceral epithelial cell (podocyte), and parietal epithelial cell. The mesangial cells are responsible for the production of the mesangial extracellular matrix. Mesangial cells have numerous functions including contraction, production of extracellular matrix, secretion of inflammatory and other active mediators and phagocytosis. Mesangial cells and their mesangial matrix together form the mesangium.



**Figure 1.** Histology of a normal glomerulus

The blood from the capillary network of the glomerulus is filtered into the tubuli. The filtration barrier of the glomerulus consists of endothelial cells, glomerular basement membrane and podocytes. Endothelial cells of the glomerulus are thin and have multiple fenestrae with a glycocalyx, extracellular polymeric material at the apical site of the endothelial cell. The glomerular basement membrane is a 300-350 nm thick network that surrounds the glomerular capillaries. The outer aspects of the glomerular capillaries are covered with podocytes. Each podocyte has a large body with small finger-like processes that interdigitate with similar structures from adjacent cells. These structures cover the glomerular capillaries forming slit pores. Blood is filtered through these slits, known as a slit diaphragm. After passing through the glomerular filtration barrier and leaving large and negatively charged molecules in the capillaries, the filtrated blood enters the Bowman's space which is aligned by parietal epithelial cells. The Bowman's space is continuous with the tubular system.

The remaining portion of the nephron consists of tubuli. First, the filtrated blood enters the proximal tubule, then enters the loop of Henle and finally the distal tubules. These structures concentrate the pre-urine and reabsorb essential molecules, but also secretion from blood to tubuli occurs. The tubuli are supported by the interstitium, which consists of thin connective tissue in peritubular and periarterial spaces.

### ***Diabetic nephropathy***

The UK prospective diabetes study (UKPDS) and diabetes control and complications trial (DCCT) have established an initiating role for hyperglycemia in developing microvascular complications such as diabetic nephropathy (2;5).

There are four main hypotheses about how hyperglycemia causes diabetic nephropathy:

- 1) increased polyol pathway flux,
- 2) increased advanced glycation end product formation,
- 3) activation of the protein kinase C isoforms,
- 4) increased hexosamine pathway flux.

Each of these hypotheses will be described in detail below:

1) Aldose reductase is the first enzyme in the polyol pathway and an oxidoreductase that catalyses the NADPH-dependent reduction of a wide variety of carbonyl compounds, such as glucose. Aldose reductase has a low affinity for glucose and in the normal situation the metabolism of glucose by this pathway is minimal. In a hyperglycemic environment, increased intracellular glucose results in its increased enzymatic conversion

to sorbitol, with concomitant decrease in NADPH. In the polyol pathway, sorbitol is oxidized to fructose by the enzyme sorbitol dehydrogenase with NAD<sup>+</sup> being reduced to NADH. The most accepted theory how the polyol pathway is involved in causing diabetic nephropathy is as follows. Reduction of glucose to sorbitol consumes NADPH. As NADPH is required for generating glutathione, and glutathione protects cells from oxidative stress, this could induce intracellular oxidative stress (6;7).

2) Advanced glycation end products (AGE) are irreversibly damaged proteins or lipids resulting from a chain of chemical reactions after an initial glycation reaction (8). AGE formation is increased in diabetes due to increased intracellular glucose. Intracellular and extracellular AGEs and its precursors damage cells by three mechanisms. First, intracellular proteins modified by AGEs have an altered function. Second, extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with the receptors for matrix proteins, such as integrins on cells. Third, plasma proteins modified by AGE precursors bind to AGE receptors on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production of reactive oxygen species.

3) Intracellular increase of glucose augments the synthesis of a molecule called diacylglycerol (DAG), which is a critical activating cofactor for the classic isoforms of protein kinase C (PKC),  $\beta$ ,  $\delta$  and  $\alpha$ . PKC has an effect on expression of a variety of genes. Its overactivation leads to blood flow abnormalities, vascular permeability, capillary and vascular occlusion, pro-inflammatory gene expression and oxidative stress (9-11).

4) In the hexosamine pathway, fructose-6-phosphate is diverted from glycolysis leading to an increase in uridine diphosphate-*N*-acetylglucosamine. Overmodification by this glucosamine of serine and threonine residues leads to pathological changes in gene expression and protein function. Although a role for this relatively newly identified pathway in diabetic nephropathy is evident (12;13), the exact pathogenic mechanisms still need to be established.

Brownlee (14) hypothesized that these four pathways can be linked together by a common initiating factor: superoxide formation by the mitochondria. Increased hyperglycemia-derived electron donors from the tricarboxylic acid cycle (also known as the citric acid cycle), NADH and FADH<sub>2</sub>, generate a high mitochondrial membrane potential, by pumping protons across the mitochondrial inner membrane. This inhibits electron transport, increasing the half-life of free radical intermediates of co-enzyme O, which reduce O<sub>2</sub> to superoxide. Hyperglycemic induced superoxide formation



by the mitochondria decreases GAPDH which converts glyceraldehyde-3-P into 1,3-diphosphoglycerate in the tricarboxylic acid cycle. As a result glyceraldehyde-3-P, which is a precursor for AGE and PKC, increases. This initiates AGE formation and PKC activation. Due to the reduced conversion by GAPDH, the molecules upstream the tricarboxylic acid cycle will also increase. These are fructose-6-P, activating the hexosamine pathway, and glucose itself, increasing the polyol pathway activity.

Early in the course of diabetes, abnormalities in blood flow and increased vascular permeability occur. In this stage, there is decreased activity of vasodilators, such as nitric oxide and increased activity of vasoconstrictors, such as angiotensin II and endothelin-1 and elaboration of permeability factors such as vascular endothelial growth factor (VEGF). Later, abnormalities in the extracellular matrix contribute to an irreversible increase in vascular permeability. With time, microvascular cell loss occurs, in part as result of apoptosis, and there is progressive capillary occlusion due to both extracellular matrix overproduction induced by growth factors such as transforming growth factor  $\beta$  (TGF- $\beta$ ) and deposition of hyaline material. Together, these changes lead to oedema, high blood pressure in the glomerulus and ischemia, finally leading to glomerulosclerosis.

The abovementioned mechanisms are not confined to diabetic nephropathy. They also play a role in macrovascular complications and other microvascular complications.

### *Clinical features*

Clinically, the natural history of diabetic nephropathy is described to consist of five stages (15). The first stage is characterized by hyperfiltration and hypertrophy of the glomerulus, leading to higher glomerular filtration rate and renal enlargement. This stage is still reversible but is associated with an increased risk of developing more advanced diabetic nephropathy (16). Stage 2 develops silently over many years and is characterized by morphologic lesions without signs of clinical disease. Then diabetic nephropathy progresses from microalbuminuria (incipient diabetic nephropathy, stage 3) to macroalbuminuria (overt nephropathy, stage 4) and finally, end stage renal disease (advanced diabetic nephropathy). Microalbuminuria can still regress in contrast to the later stages. When high blood pressure in the stage 4 diabetic nephropathy (macroalbuminuria) is left untreated, renal function (GFR) declines, the mean fall rate being around 1 ml/min/month. Long-term antihypertensive treatment reduces the fall rate by about 60% and thus postpones end stage renal disease considerably (15). End stage renal disease refers a stage in which patients require dialysis or kidney transplantation, because the kidneys do not function anymore.

There is still debate whether diabetic nephropathy due to type 1 and type 2 diabetes can be considered the same disease. Two early studies showed a similar course in clinical diabetic nephropathy in type 1 and type 2 diabetes (17;18). However, several differences have been described between diabetic nephropathy in type 1 and type 2 diabetes. Diabetic nephropathy due to type 1 diabetes almost always coincides with diabetic retinopathy (19) in contrast to type 2 diabetes in which this parallel is less clear. However, the relationship between diabetic nephropathy and retinopathy in type 2 diabetes varies depending on the diabetes regimen (20). In patients treated with insulin, the relationship between nephropathy and the severity of retinopathy is similar to that in type 1 diabetes (21). Furthermore, some claim that diabetic nephropathy develops more often in type 1 diabetes than in type 2 diabetes. The difficulty in type 2 diabetes is that many die due to cardiovascular disease before reaching the clinical stage of diabetic nephropathy. If we look at Pima Indians who have type 2 diabetes relatively early in life and are known for their relatively low cardiovascular mortality risk, 65% of the diabetes type 2 patients will develop ESRD (22), suggesting that cardiovascular disease in Caucasians might influence the incidence rate of diabetic nephropathy due to type 2 diabetes.

For pathologists, diabetic nephropathy due to type 1 and type 2 diabetes appears to be undistinguishable (23;24). However, on the genetic level, there seem to be differences in genetic susceptibility between diabetic nephropathy due to type 1 and type 2 diabetes (25-29). This is suggestive for different mechanisms in type 1 and type 2 diabetes leading to similar histopathologic appearance of diabetic nephropathy.

### *Histopathology*

Diabetic nephropathy causes pathological abnormalities in the glomerulus and tubulus extraglomerular vessels and interstitium. The first pathological sign of diabetic nephropathy is enlargement of the glomerulus which corresponds to the clinical stage of glomerular hyperfiltration (stage 1) (15). Additionally, glomerular basement thickening occurs in an early stage and is seen to be present after 2 years of diabetes duration (30). When the disease progresses the mesangium starts to expand, corresponding to the clinical stage of both micro- and macroalbuminuria.

Two forms of diabetic nephropathy are described; diffuse versus nodular glomerulosclerosis (31). This designation is primarily of descriptive value, because the distinctions do not have clear-cut clinical significance, although nodular glomerulosclerosis is more often described in severe cases (31;32). Diffuse diabetic glomerulo-

sclerosis is less specific for diabetic glomerulosclerosis than nodular glomerulosclerosis. The nodular lesions of diabetic glomerulosclerosis are also known as Kimmelstiel-Wilson lesions.

Glomerular hyalinosis is common in diabetic glomerulosclerosis. These hyaline lesions may result from insudation or exudation of plasma proteins from vessels followed by entrapment in the matrix. The hyalinosis can occur anywhere in the glomerular tufts, but there are two characteristic patterns: capsular drops and hyaline caps (also known as fibrin caps). Capsular drops are spherical accumulations of hyaline material adjacent to or within the Bowman's capsule. The hyaline caps occupy the capillary lumen instead of being attached to Bowman's capsule.

Arteriolosclerosis and arteriosclerosis often accompany diabetic glomerulosclerosis. Arteriolar hyalinosis at the glomerular hilum is present in diabetic glomerulosclerosis and typically affects both the afferent and efferent arterioles. Hypertensive hyaline arteriolar sclerosis affects the afferent but not the efferent arteriole (33).

The earliest tubular change is thickening of tubular basement membrane that is analogous to the glomerular basement membrane thickening. When the disease progresses, tubules become atrophic and, fibrosis and chronic inflammation are present in the interstitium. Mononuclear cells can also be found in the interstitium in diabetic nephropathy. Inflammatory infiltrates of the interstitium, predominantly by T lymphocytes and macrophages, have been described (34). These chronic tubulointerstitial changes are not specific to diabetic nephropathy.

#### *Pathological classification of diabetic nephropathy*

Although for many kidney diseases pathological classification schemes exist (35-37), for diabetic nephropathy no uniform internationally accepted classification scheme has been developed yet. Classification schemes improve communication between and among renal pathologists and clinical nephrologists, provide logistical structure for prognostic and interventional studies and assist clinical management and efficiency (38).

In the past, several attempts have been made in classifying diabetic nephropathy. In 1959, Gellman *et al.* (39) proposed a systematic evaluation examining glomeruli, tubules, arterioles and the interstitium of kidney biopsies with diabetic nephropathy. The unique feature of this paper was that it presented for the first time associations between histopathological findings in renal biopsies with diabetic nephropathy and

severity of clinical parameters (39). Due to its extensive and elaborate nature, this evaluation system turned out to be unsuitable for practical usage.

More recently, in 1993, Gambará *et al.* (40) suggested to distinguish three classes of type 2 diabetes related nephropathy: class 1, typical diabetic glomerulopathy; class 2, aspecific glomerular and tubulointerstitial lesions, and class 3, different glomerular diseases superimposed on diabetic lesions. This system does not distinguish in severity of the lesions; it also contains a rather broad 'aspecific' category.

In 1996, Fioretto *et al.* (41) proposed another categorization of lesions for diabetic nephropathy in type 2 diabetes, also distinguishing between three classes: Class I, normal or near normal renal structure; Class II, typical diabetic nephropathy; Class III, atypical diabetic nephropathy. Although this system proved useful in evaluating renal biopsies for research purposes, it is not practical for clinical use. There are four drawbacks to this system:

- it does not discriminate between damage from other causes (e.g. vascular damage) and diabetic nephropathy,
- it does not distinguish in severity of the lesions,
- it is only suitable for 'proven' diabetic nephropathy at the electron microscopy level,
- the category 'atypical' is too broad to make a reliable evaluation of the biopsy.

In 2002, Mazzucci *et al.* (42) published an evaluation system for type 2 diabetic nephropathy that was based upon Gambará's scheme from 1993 (40). Mazzucci *et al.* (42) revised the Gambará scheme by re-naming class 2 into a category mainly characterized by changes related to vascular damage. In addition to this revision, they split class 3 into two subtypes: 3a) glomerular diseases superimposed on diabetic glomerulosclerosis and 3b) glomerular disease as the only renal change. They concluded that a renal biopsy should play a central role in management of type 2 diabetic patients with proteinuria.

None of these classification schemes were used in clinical practice. A standardized classification could encourage international uniformity in classifying diabetic nephropathy, facilitate experiments, be applied in multi-center clinical trials, and ultimately lead to improvement in the care of diabetic nephropathy. A proposal for such a classification scheme, based on severity of diabetic nephropathy, is made in chapter 2 by a group of experts.

## PART II – GENETICS IN DIABETIC NEPHROPATHY

Francis Harry Compton Crick, James Dewey Watson and Maurice Hugh Frederick Wilkins received the Nobel Prize in Medicine for their discoveries in the molecular structure of DNA in 1962. Since then, many techniques have been developed to investigate variations in this DNA molecule in relation to disease. Several distinctions in genetic variation between individuals can be made based on variation in the DNA sequence and allele frequency. At the DNA level, there are insertion/deletions, copy number variations, microsatellites, and single nucleotide polymorphisms (SNP). Insertion/deletion are insertions or deletion in a certain part of the DNA sequence. Copy number variation is the same but larger, also referred to as duplications and insertions. Microsatellites are repeated sequences of which the CA repeat is the most common one. A single nucleotide polymorphism is a variant in a single nucleotide. Furthermore, based on allele frequency, the distinction is made between a polymorphism (minor allele frequency greater or similar to 1%) and a mutation, which is more rare (minor allele frequency <1%).

1 In the beginning, genetic mapping was used to search for causal mutations of diseases that run in families and are inherited by the principles of Mendel. These Mendelian diseases, also called monogenetic disorders, are caused by a mutation in a single gene. Duchenne muscular dystrophy is an example of a Mendelian disease. The disorder is caused by a mutation in the gene *DMD*, which encodes for the protein dystrophin, an important structural component within muscle tissue. Due to a single gene mutation the protein is not produced adequately, leading to the severely disabling phenotype of Duchenne. In contrast to Mendelian diseases, complex diseases are disorders in which the cause is considered to be a combination of several genetic effects and environmental influences. For example, type 1 and 2 diabetes, diabetic nephropathy and cardiovascular disease are such complex diseases.

### **Genetic mapping**

Genetic mapping in the last century has resulted in a rapid increase in the understanding of disease pathology in many Mendelian diseases. These were found by a family-based approach based on the principle of linkage. Linkage is the tendency of certain loci or alleles to be inherited together. In family based linkage studies, it has been investigated which DNA markers (microsatellites) are more often inherited in affected family members compared to unaffected family members. When a genetic marker is

tightly linked (i.e. often inherited) with the disease, additional DNA markers near this marker are sought and studied. This process is often referred to as positional cloning and finally leads to the gene involved in the disease. This is a most useful approach in Mendelian disease, but in complex disease one gene does not ultimately lead to the disease. Therefore some relatives might be affected without the risk allele of the genetic variant and unaffected relatives might have this risk allele. Especially when a risk allele and disease are common ( $> 5\%$  allele frequency), the inheritance pattern of the risk allele might not be so informative. An impractically large number of families would be needed to study a complex disease to find a small enough region to ultimately lead to one gene.

A candidate gene is a gene which is suspected to be involved in diabetic nephropathy based on the literature. To investigate a candidate gene association studies are used. Association studies investigate whether a risk allele occurs more frequently in subjects with the disease than in individuals without the disease. In contrast to familial studies, in association studies a problem called population stratification can occur. Population stratification refers to the problem that results in genetic association studies are due to ancestral differences in cases versus controls instead of related to the disease. Population stratification can only occur when either allele frequency or the disease risk differs between ancestral backgrounds. Another problem is that a clear biological explanation beforehand is needed to choose such a candidate gene and from Mendelian diseases we have learned that most genes found, were completely unexpected.

A new approach, developed to investigate complex disease, is the genome wide association scan (GWAS). This is an association study of the genome with common SNPs used as genetic markers. Since many SNPs are present in the human genome, this gives a good coverage of the human genome. A great advantage of the GWAS is that it does not need a biological explanation beforehand. It maps the genome with common SNPs either directly involved in disease pathology or indirectly through tagSNPs, which are potentially linked with risk alleles. Another advantage is that population stratification can be corrected for in this approach, as with the many SNPs the ancestry of the individual can be determined.

### ***Models in complex genetics***

Three models have been proposed to explain the genetic component in complex disease; the common disease-common variant model (CD-CV model), the rare alleles of major

effect (RAME) model and the infinitesimal model.

The CD-CV model assumes that common variants contribute to risk, each explaining a small proportion of disease liability. Due to their high frequencies, these variants may explain a large part of the population risk. The genome-wide association scan (GWAS), based on the CD-CV model, has proven to be powerful (43). An example of a common disease variant consistently replicated is *APOE* in Alzheimer's disease and heart disease (44). In contrast, the RAME model postulates that common diseases are in fact genetically heterogeneous and caused by *de novo* mutations. A rare variant in each individual (or family) with large effect may contribute to risk. Support for this model comes from studies of rare genetic variants in the high density lipoprotein C (HDL-C) gene (45;46). Rare variants were more often present in patients with low HDL-C (<fifth percentile) than with high HDL-C (>95<sup>th</sup> percentile). These findings were replicated in an independent study population (45). That the GWAS provides solid evidence for novel gene associations does not ultimately mean that the CD-CV model explains the genetic risk in complex diseases. Common SNPs identified by GWAS could be associated with rare mutations. Therefore, some common SNPs may be a proxy for a rare variant with large effect. Rare variants with large effect may provide clear information on disease etiology.

The infinitesimal model assumes that liability for disease follows an asymptotic distribution, as shown for the continuous trait height. Hundreds of genes would be involved, covering a wide range of frequencies (47). This model has gained support in GWAS, where odds ratios of 1.2 may each account for a small fraction of the liability.

### ***Genetic susceptibility of diabetic nephropathy***

In type 1 diabetes it has clearly been shown that some patients develop diabetic nephropathy within the first fifteen years and after that the incidence decreases (4). This suggests that some patients seem to be more susceptible to develop diabetic nephropathy. This is less clearly shown in type 2 diabetes as probably many die of cardiovascular disease before reaching the age to develop diabetic nephropathy. Several studies indicate that a separate genetic risk factor exists for diabetic nephropathy in type 1 or type 2 diabetes. Evidence for a genetic predisposition is that diabetic nephropathy seems to aggregate in certain families (48;49). Furthermore the prevalence of diabetic nephropathy varies significantly among ethnic groups (48).

### **Genetics in diabetic nephropathy**

The first studies performed in the search for genes in diabetic nephropathy were linkage analyses in family studies. Several genome-wide linkage scans have been published in diabetic nephropathy in type 1 and type 2 diabetes. Although most of these analyses evaluated small numbers of families from different ethnic groups, several consistent regions of linkage have been detected (50-56). These regions can be helpful in choosing candidate genes, but these linkage studies were underpowered for directing to one specific gene.

In genetic association studies several candidate genes for diabetic nephropathy have been investigated. The frequently investigated genes were the genes involved in the renin-angiotensin-system. The most studied variant, the insertion/deletion polymorphism in intron 16 of the gene coding for angiotensin-converting enzyme (ACE), has shown to have a small effect in a meta-analysis of 53 studies (57). Furthermore, genetic variants in genes coding for aldose reductase (58), endothelial nitric oxide (59-61), manganese superoxide dismutase (62), vascular endothelial growth factor (63), TGF- $\beta$  (64), apolipoprotein E (65-67), inflammatory cytokines (68) among several other candidates were studied in relation to diabetic nephropathy. A broad definition of diabetic nephropathy was used in these studies, from hyperfiltration and microalbuminuria to biopsy proven diabetic nephropathy.

Combining the two approaches, also referred to as large scale mapping, has led to some success. A good example is the *CNDP1* gene. A region on chromosome 18q22-22.3 was found to be associated with diabetic nephropathy in large Turkish families who were not treated for this disease, making this an ideal population to study for this purpose (50). Genes of this relatively small region were searched for up- or downregulation in diabetic nephropathy. A few genes were differentially expressed in diabetic nephropathy and one of these genes was the *CNDP1* gene. Genetic variations were sought in this gene and tested in a genetic association study. A polymorphism in exon 2 was found to be associated with diabetic nephropathy (26). This is an example of a successful finding, however, most found regions in diabetic nephropathy are larger. Furthermore, microarray analyses are known for the high false-positivity rate and finding the right variant is also a challenge considering multiple testing. Therefore, only few successful examples of this approach are known.

A few GWAS have been performed in diabetic nephropathy (69;70), leading to



several variants in or near genes which were not hypothesized before. Examples are genetic variants in the engulfment and cell motility 1 gene (*ELMO1*) (71) and cysteinyl-tRNA synthetase gene (*CARS*) (70). Most of the found variants will not prove to be causal and the relevance of these findings still needs to be assessed.

### **PART III - *CNDP1*, CARNOSINASE AND CARNOSINE IN DIABETIC NEPHROPATHY**

#### ***Carnosine***

In 1900 carnosine ( $\beta$ -alanine-L-histidine), as the name implies, was first isolated from meat by Gulewitsch and Amiradzibi (72). Related compounds to carnosine are anserine, homocarnosine, ophidine, carcinine and N-acetyl carnosine. In the human body, carnosine and related compounds are found in high concentrations in muscle and nervous tissue, but are also present in several organs such as the kidney, liver and small intestine. In the muscle, carnosine makes up a significant fraction of the water soluble nitrogen-containing compounds. The most convincing role of carnosine in the muscle was considered to be the control of intracellular hydrogen ion concentration. It was presumed that this property explains its predominant association with white, fast glycolytic type IIb muscles which possess relatively few mitochondria. Therefore these fibers will more often need anaerobic activity, which generates lactic acid. The importance of carnosine as a physicochemical buffer within human muscle was examined by calculating its buffering ability over the physiological pH range. From the range of carnosine concentrations observed (7.2-30.7 mmol/kg dry muscle mass), it was estimated that the dipeptide could buffer between 2.4 and 10.1 mmol H<sup>+</sup>/kg dry mass over the physiological pH range 7.1-6.5, contributing on average, approximately 7% to the total muscle buffering. This suggests that in humans, in contrast to many other species, carnosine is of only limited importance in preventing the reduction in pH observed during exercise (73). Further evidence that carnosine is more than a physiological buffer, comes from Severin and co-workers (74;75). They showed that after addition of carnosine in medium surrounding a fatigued skeletal muscle, the muscle working was restored and because of special precautions in their study, it was shown that it did not depend on the pH-buffering capacity of carnosine.

The protective effect of carnosine can also be explained by its antioxidant properties.

Carnosine is capable of preventing the accumulation of oxidized products derived from the lipid components of biological membranes (73;76;77). This is rather surprising considering that carnosine is water soluble and remote from the site at which the peroxidation of membrane lipids takes place. However, both carnosine and anserine potentiate the effect of lipid soluble antioxidants such as vitamin E (78). Detailed studies of the time course of lipid peroxidation in the sarcoplasmic reticulum suggest that the ability of carnosine to inhibit the accumulation of thiobarbituric acid-reactive products is mediated by the result of direct interaction of the dipeptide not only with free radicals generated within the system, but also with primary molecular products of the lipid peroxidation (76). The antioxidant effect of carnosine has been demonstrated at both cell and tissue level as well as in suspensions of lipids derived from cell membranes. Carnosine is able to suppress peroxidation induced both enzymatically and non-enzymatically and eliminates the products of free radical reactions. These effects are not confined to the muscle and are accomplished by membrane stabilizing action as shown by preservation and recovery of intact cell membrane structures.

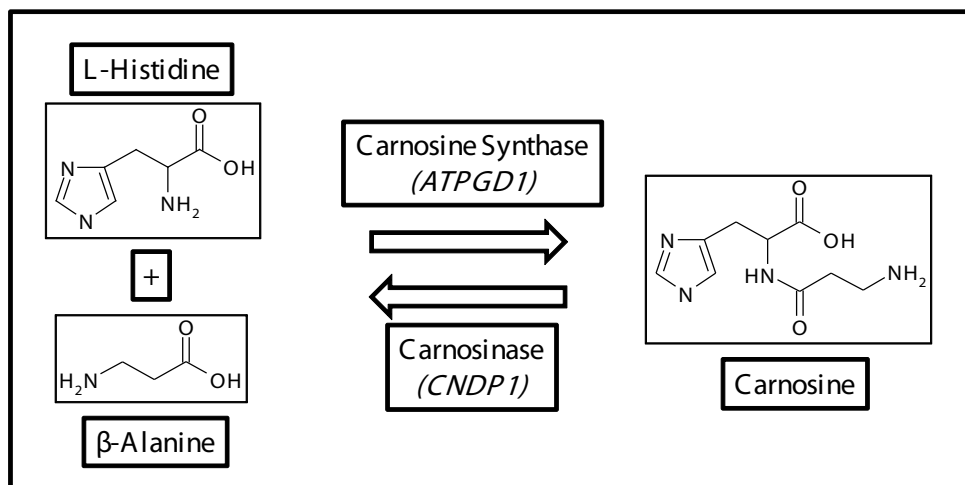
Kohen *et al.* (79) have demonstrated that these compounds can act as antioxidants as a result of their ability to scavenge single oxygen, peroxy radicals and hydroxyl radicals. Carnosine and its analogues have been shown to be efficient chelating agents for copper and other transition metals. Since human skeletal muscle contains one-third of the total copper in the body (20-47 mmol/kg) and the concentration of carnosine is relatively high, the complex of carnosine:copper was thought to be of biological importance. Kohen *et al.* results indicated the complex of copper:carnosine can dismutate superoxide radicals released by neutrophils (79). Furthermore, carnosine has shown to attenuate oxidative damage to DNA molecules (80) in the presence of iron and copper ions.

Carnosine has also been shown to have AGE inhibitory actions; protein crosslinks induced by methylglyoxal (a precursor of AGE) were found to be eliminated in the presence of carnosine (81;82). Although the mechanism by which carnosine inhibits the formation of AGE is unknown, it is likely that the free amino group derived from  $\beta$ -alanine competes with the amino groups of proteins in their reaction with precursors of AGEs (75). Apart from the abovementioned protective capacities, carnosine has also shown to inhibit formation of foam cells *in vitro* (83) and is a natural ACE inhibitor (84).

While carnosine is mainly found in skeletal muscle, homocarnosine concentration

is higher in the brain. It has been postulated that carnosine is a neurotransmitter. However, no receptors for carnosine have been found in nervous tissues (85). The role of carnosine in the brain is still under debate (86).

Investigating this dipeptide gained popularity for its anti-aging potential; it suppresses cultured human fibroblasts senescence and delays aging in senescence-accelerated mice (87). The exact reason why carnosine has this effect is not known, but it is probably due to the combination of abovementioned protective effects. In humans, so far no relation with carnosine and longevity have been found (88). Carnosine has also been investigated in age-related diseases such as Alzheimer (89) and Parkinson disease (90), but also in a wide variety of disorders and diseases, such as autism (91), cataract (92) and, cancer (93). The exact role of carnosine in these diseases still needs to be established.



**Figure 2.** Carnosine metabolism

### **Carnosine metabolism**

Carnosine and related compounds are produced by carnosine synthase of which the genetic sequence has recently been identified (94) as ATP-grasp domain containing protein 1, *ATPGD1*. Carnosine is synthesized by muscle cells, glial cells and oligodendrocytes (85). The kidney brush border also possesses a carnosine transport system (95;96). The majority of circulating carnosine is internalized by proximal tubular epithelial cells of the kidney via oligopeptide transporters Pept1 and Pept2 (97;98). Dibutyryl cyclic AMP and other agents that can, directly or indirectly, activate cyclic AMP-dependent protein

kinases strongly lower the rate of carnosine synthesis (99).

Carnosinase degrades carnosine into L-histidine and  $\beta$ -alanine (figure 2). Classical carnosinase was first described by Hanson and Smith (100), who found hog kidney carnosinase in 1949. Later, a distinction was made in two isoforms of carnosinase; serum carnosinase (also known as carnosinase-1) and tissue carnosinase (also known as carnosinase 2) (101). Teufel *et al.* showed that in fact there were two different genes coding for each of the enzymes (102). Tissue carnosinase is not capable of degrading carnosine under physiological condition in humans and has been shown to have rather a broad specificity (102). Therefore, this enzyme should be renamed into 'non-specific dipeptidase' and serum carnosinase into the genuine carnosinase (94;102). The following part will only describe data on genuine carnosinase. The physiological function of this carnosinase seems to be the hydrolysis of homocarnosine in the brain and the splitting of carnosine and anserine in the blood stream. Carnosinase is present in plasma and the brain and is specific to carnosine and related compounds.

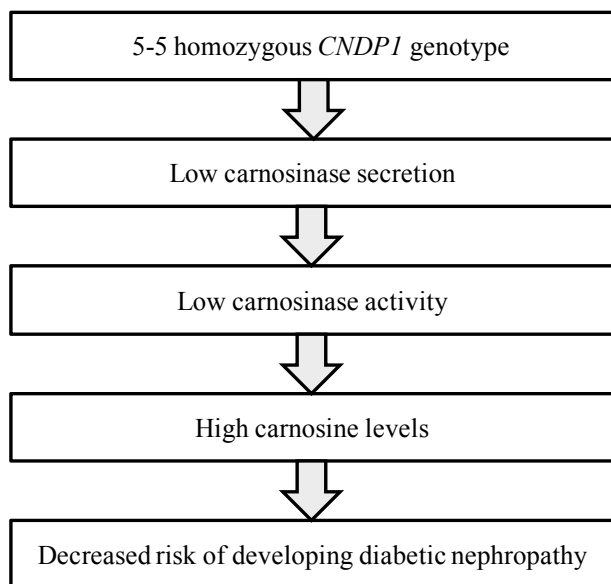
Carnosinase activity is nearly absent in neonates (103), low in children (although protein level are similar in children and adults) and seems to increase with age (104). Women have slightly higher carnosinase levels than men (105). In the literature, some cases have been described with serum carnosinase deficiency, leading to severe neurological symptoms such as epilepsy, myoclonic seizures, microcephaly, blindness and mental retardation (103;106-108). On the other hand, subjects with carnosinase deficiency without any symptoms (103) have been described and the degree of serum carnosinase deficiency does not seem to correlate with the severity of the disease (106). However, it seems evident that even without a clear dose-response relationship, carnosinase deficiency is often associated with mental retardation (106).

### ***Carnosinase-carnosine metabolism in diabetes and diabetic nephropathy***

The most important clue that carnosine metabolism plays a role in diabetic nephropathy comes from genetic studies (26;50). A region on chromosome 18q22-22.3 was found to be associated with diabetic nephropathy in large Turkish families (50). A polymorphism in the *CNDP1* gene, which encodes serum carnosinase and is located in the chromosomal region 18q22-22.3, was found to be associated with diabetic nephropathy (26). Freedman *et al.* (25) confirmed this finding in European Americans with end stage diabetic nephropathy due to type 2 diabetes, but the association between diabetic nephropathy and the *CNDP1* gene could not be confirmed in patients with diabetic

nephropathy due to type 1 diabetes (27;28). The polymorphism is a microsatellite of CTG repeats (coding for the amino acid leucine) in exon 2 of the *CNDP1* gene, which varies between 0, 4, 5, 6, 7 and 8 repeats. Alleles with null, four and eight repeats are rare (25;26;88). Patients with homozygosity for 5 leucine repeats demonstrated a reduced susceptibility (2.0-fold reduced risk) for developing diabetic nephropathy due to type 2 diabetes compared with individuals with 6–8 repeats (25;26). Increasing numbers of leucine repeats in the leader peptide, have shown to increase the secretion of serum carnosinase (109) and to lead to higher serum carnosinase activity (26). Carnosines and related dipeptides are generally known for their protective effects. In the pathogenesis of diabetic nephropathy oxidative stress plays a central role. Therefore, of particular interest in relation to diabetic nephropathy is the scavenging of reactive oxygen species effect of carnosine (110). Formation of AGE is increased in a diabetic environment and has a major role in the development of diabetic nephropathy (111). Carnosine is able to scavenge precursors of AGE, such as methylglyoxal, and facilitates the degradation of AGEs (81). Furthermore, carnosine has shown to inhibit angiotensin converting enzyme (84), potentially lowering blood pressure. Elevated blood pressure could accelerate the development of diabetic nephropathy. Finally, TGF- $\beta$  induced synthesis of extracellular matrix components (26) is thought to induce fibrosis and can be reduced by carnosine. This might explain why lower carnosinase levels (because of the 5-5 *CNDP1* homozygous genotype) leading to high circulating carnosine concentrations, are protective in diabetic nephropathy (figure 3).

Carnosine is mainly present in meat and fish and therefore it is interesting to see that vegetarians have higher levels of a precursor of AGE, which carnosine can scavenge, than carnivores. Furthermore, carnosine content increases with exercise (112;113), which has been shown to be body beneficial in diabetes, apart from the fact that it decreases mass content (114).



**Figure 3.** Hypothesis of *CNDP1* genotype in relation to diabetic nephropathy

### ***Carnosine in animal models***

With exception of the Goldhamster, carnosinase is not present in serum of rodents. However, rodents have a functional carnosinase coded by *CNDP1*, but it is not excreted from the cell into the serum. Several studies have been performed with carnosine treatment in rodents with diabetic nephropathy (115-117). Ob/ob mice, a leptin-deficient mouse used as a model for type 2 diabetes and diabetic nephropathy, were made transgenic for human carnosinase under a liver-specific promoter (117). Human carnosinase was present in serum of these transgenic mice and in this unique model, carnosinase could be overexpressed. When the animals were treated with carnosine, the hyperglycemia was reduced. The histological features of the kidney, serum creatinine levels and albumin creatinine levels in the carnosine-treated mice versus the untreated mice did not differ. These data suggest that the protective effect of the 5-5 homozygous genotype in diabetic nephropathy is primarily regulated by improved glycemic control (117). The obese Zucker rat, used as a model for metabolic syndrome (which includes type 2 diabetes) associated with kidney damage, was also treated with carnosine. Carnosine led to a reduced albumin excretion and improved kidney function in these rats. Furthermore, carnosine reduced the plasma triglycerides and insulin resistance but not plasma glucose (115). The characteristic pattern of lipid abnormalities in

patients with diabetes consists of moderate elevation in triglycerides, low high-density cholesterol values, and an increase in small dense low-density lipoprotein particles. Possibly in this rat model the improvement of the kidney is primarily due to improvement in the lipid abnormalities. In male streptozocin-treated Sprague-Dawley rats, used as a model for type 1 diabetes with diabetic nephropathy, the treatment with carnosine was also investigated. In these rats carnosine treatment reduced the amount of albumin excretion, but not plasma glucose (116). This is in contrast to streptozocin-treated Balb/cA mice, which did show improved glycemic control, lower triglyceride concentrations after carnosine treatment. In the latter study, the effect of carnosine on the kidney was not investigated (118). Clearly, more studies have to be done in mice to establish the role of carnosine in diabetic nephropathy.

### ***Outline of this thesis***

This thesis, with as a central theme diabetic nephropathy, comprises 7 chapters. In **chapter 2** a histological classification of diabetic nephropathy is proposed, classifying diabetic nephropathy according to severity of the disease. The idea is that this will induce clinical and pathological uniformity, leading to better patient care and scientific communication. As diabetic nephropathy has a strong genetic component, **chapter 3** covers the genetic variants reproducibly associated with diabetic nephropathy in a meta-analysis study. Chapters 4, 5 and 6 focus on one genetic variant known to be involved in diabetic nephropathy, the leucine repeat in exon 2 of the *CNDP1* gene. In **chapter 4**, a population of South Asians Surinamese immigrants, who have an increased risk of developing diabetic nephropathy, was investigated for the frequency of the protective 5-5 homozygous genotype of the *CNDP1* gene. **Chapter 5** describes the relation between the *CNDP1* genetic variant and gender in diabetic nephropathy in three independent diabetic nephropathy groups. In **chapter 6** the relation between the *CNDP1* genotype with glomerular kidney diseases other than diabetic nephropathy was investigated. **Chapter 7** relates more to the physiological aspect of carnosine, investigating determinants such as gender, age, diet, *CNDP1* genotype, carnosinase and testosterone in relation to carnosine content in muscle.

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