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Understanding the biological role of the carnosine-carnosinase system in diabetes & beyond

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**UNDERSTANDING THE BIOLOGICAL ROLE OF THE
CARNOSINE-CARNOSINASE SYSTEM
IN DIABETES & BEYOND**

STELLINGEN / PROPOSITIONS

Belonging to this PhD Thesis

Understanding the biological role of the carnosine-carnosinase system in diabetes & beyond

by María Angélica Rodríguez-Niño

1. Urinary Carnosinase-1 concentrations in healthy individuals reflect local renal Carnosinase-1 synthesis. Under circumstances of an impaired glomerular filtration barrier, Carnosinase-1 might be filtered reaching the urinary compartment. (*This thesis*).
2. Reduced serum Carnosinase-1 levels are a common denominator in advanced stages of diseases, in which catabolism prevails *e.g.*, chronic kidney disease. (*This thesis*).
3. High urinary Carnosinase-1 is independently associated with risk of late graft failure in kidney transplant recipients. (*This thesis*)
4. Urinary carnosine and anserine are associated with positive outcomes after kidney transplantation. (*This thesis*).
5. Glucose-lowering effects account as one of the primary mechanisms by which carnosine affects the resistance of the afferent glomerular arteriole. (*This thesis*).
6. The scavenging activity of carnosine towards Methylglyoxal is limited. (*This thesis*).
7. Current pharmacological strategies interfering with Carnosinase-1 activity represent a new therapeutic target for counteracting the negative effects of Carnosinase-1 on histidine-containing dipeptides (*This thesis*).
8. „Der Kopf ist rund, damit das Denken die Richtung ändern kann” (*Francis Picabia*).
9. “And what can life be worth if the first rehearsal for life is life itself?” (*Milan Kundera, The Unbearable Lightness of Being*).
10. “That’s the thing about human life – there is no control group, no way to ever know how any of us would have turned out if any variables had been changed.” (*Daniel Keyes, Flowers For Algernon*).

PhD Dissertation, University of Groningen, Groningen, The Netherlands.

This dissertation includes summaries in Dutch, German, as well as in Spanish.

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Understanding the biological role of the carnosine – carnosinase system in diabetes & beyond

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

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Para mi familia...

TABLE OF CONTENTS

		INTRODUCTION & SCOPE	12
PART	A	RELEVANCE OF CARNOSINASE-1 IN RENAL FUNCTION	
	1	Detection of carnosinase-1 in urine of healthy individuals and patients with type 2 diabetes: correlation with albuminuria and renal function <i>Amino acids 2019, 51: 17-25</i>	31
	2	Serum carnosinase-1 and albuminuria rather than CNDP1 genotype correlate with urinary carnosinase-1 in diabetic and non-diabetic patients with chronic kidney disease <i>J Diabetes Res 2019; 6850628</i>	47
PART	B	URINARY HISTIDINE- CONTAINING DIPEPTIDES IN LONG-TERM OUTCOMES AFTER KIDNEY TRANSPLANTATION	
	3	Urinary Carnosinase-1 excretion is associated with urinary Carnosine depletion and risk of graft failure in kidney transplant recipients: results of the TransplantLines Cohort Study <i>Antioxidants 2021; in press</i>	71
	4	Meat intake and risk of mortality and graft failure in kidney transplant recipients <i>Am J Clin Nutr 2021; in press</i>	95

PART	C	A GLANCE INTO CARNOSINASE´S POTENTIAL PROTECTIVE MECHANISMS	
	5	Modulatory effects of carnosine and carnosinase-1 on diabetes-induced afferent arteriole vasodilation: implications for glomerular hyperfiltration <i>Submitted for publication</i>	123
	6	Methylglyoxal induces p53 activation and inhibits mTORC1 in human umbilical vein endothelial cells <i>Sci Rep 2021; 11: 8004</i>	145
		DISCUSSION, LIMITATIONS & FUTURE PERSPECTIVES	169
		SUMMARY Nederlandse samenvatting Deutsche Zusammenfassung Resumen en español	181
		APPENDIX Acknowledgments List of publications List of contributing authors	201

INTRODUCTION & SCOPE

DIABETES AND THE NATURAL COURSE OF DIABETIC NEPHROPATHY

Diabetes Mellitus is a metabolic disorder characterized by chronic hyperglycemia affecting more than 425 million worldwide¹. It imparts high morbidity and mortality and, as such, it is a major health problem that urges to take immediate action to reduce the burden on health care systems². Aside from age and lifestyle-related factors, genetic factors contribute to the development of type 2 diabetes (T2D) with currently more than 60 genetic traits reported to be associated with the risk to develop diabetes³.

Approximately one third of patients with type 1 or type 2 diabetes develop diabetic nephropathy (DN), which is considered one of the most devastating microvascular complications of diabetes⁴. It is by far the most common cause of chronic kidney disease (CKD) worldwide, frequently leading to end-stage renal disease (ESRD) and the need for renal replacement therapy, i.e. kidney transplantation or dialysis⁵. Kidney transplantation is considered the preferred therapy as it improves life quality and overall survival at lower healthcare costs in comparison to dialysis⁶.

DN typically develops through sequential phases, commonly starting with a supraphysiologic increase in glomerular filtration rate (GFR) known as hyperfiltration⁷. Hyperfiltration occurs in response to functional nephron mass reduction, as well as to hemodynamic and metabolic changes prevailing in diabetes and obesity⁷. It is postulated that hyperfiltration is associated with the initiation and progression of DN^{8,9}, as it predisposes to nephron damage by increasing glomerular capillary hydrostatic pressure. The latter is a result of alterations in pre and post-glomerular arteriole resistances¹⁰. In the classical course of DN, hyperfiltration is followed by intermittent episodes of microalbuminuria which progress to persistent microalbuminuria in one third of the patients, and reflects loss of permselectivity of the glomerular basement membrane. The stage of microalbuminuria is followed by macroalbuminuria and progressive decline in GFR^{11,12}. All stages are accompanied by structural pathological changes such as podocyte injury, mesangial expansion and glomerulosclerosis¹³.

Current treatment modalities for DN focus on four main targets: cardiovascular risk reduction, glycemic control, blood pressure control and inhibition of the renin-angiotensin system (RAS)¹⁴. Observational data from large clinical intervention trials in patients with T2D and proteinuria, i.e. the Irbesartan Diabetic Nephropathy Trial (IDNT) and the Reduction of Endpoints in Non-Insulin Dependent Diabetes Mellitus With the Angiotensin II Antagonist Losartan (RENAAL) trial have demonstrated that RAS blockade with angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) retards disease onset and progression of albuminuria, independently of blood pressure control^{15–17}. Additional novel therapies such as the use of sodium-glucose cotransporter-2 (SGLT2) inhibitors have been recently approved for the treatment of diabetes and have shown positive effects on renal and metabolic outcomes¹⁴. Complementary to pharmacological interventions, nutritional assessment and dietary interventions play key roles in halting further kidney deterioration in DN. The KDIGO (Kidney Disease Improving Global Outcome) panel of 2020 suggests in their guidelines to maintain dietary intake of 0.8 gr protein/kg weight/day¹⁸ in patients with dia-

betes and CKD, which is the recommended amount from the World Health Organization for the general population¹⁹. Despite the controversy, and according to the KDIGO guidelines, it is emphasized that there is not enough evidence demonstrating the beneficial effect of dietary protein intake restriction on renal or other clinical outcomes¹⁸.

CARBONYL AND OXIDATIVE STRESS IN DIABETIC NEPHROPATHY: THE ROLE OF METHYLGLYOXAL

The value of appropriate glycemic control in diabetic patients is indisputable, yet some patients with T2D and adequate glycated hemoglobin A1C levels still develop microvascular complications²⁰. This raises the question as to whether additional mechanisms might be involved in onset and disease progression of such complications²¹.

Compelling evidence has suggested a pivotal role for methylglyoxal (MGO), a reactive carbonyl species (RCS), in diabetic complications. In line with this, elevated levels of MGO concentrations in serum and tissues of patients with diabetes and ESRD have been reported^{22,23}. MGO is a reactive metabolite and a byproduct of carbohydrate, lipid, and amino acid metabolisms. MGO reacts with proteins and nucleic acids causing protein malfunction and formation of advanced glycation end-products (AGEs)²⁴. Exogenous administration of MGO in animals or in in vitro systems results in similar pathological changes as seen in diabetic conditions, e.g. microvascular damage and insulin resistance^{25–27}. In agreement with this, elevating levels of endogenous MGO by knockout of one of MGO's detoxifying enzymes, i.e. glyoxalase-1, is sufficient to recapitulate the phenotype of T2D, namely obesity, insulin resistance and hyperglycemia²¹. Similarly, knockdown of glyoxalase-1 in a non-diabetic mice model, results in renal lesions similar to the ones found in diabetic mice models²⁸. MGO might also elicit an inflammatory response through the formation of reactive oxygen species (ROS). Pathological concentrations of MGO provoke a rapid production of ROS in human aortic endothelial cells²⁹ and they also elicit a strong regulation of cell cycle arrest-associated genes, compatible with a possible activation of the p53 pathway³⁰.

The tumor suppressor p53 determines cell fate in response to DNA damage³¹ and plays a critical role in the regulation of other essential signaling pathways such as the mammalian target of rapamycin complex 1 (mTORC1) pathway³². The mTORC1 is a nutrient sensing pathway, and as such coordinates anabolic (protein, nucleotide and lipid synthesis, and cell growth) and catabolic processes e.g., autophagy³², which is a stress-response mechanism implicated in the degradation of damaged organelles and protein aggregates³³. Dysregulation of autophagy has been implicated in DN³⁴. Autophagy appears to play a predominant role in maintaining the filtration barrier function of podocytes³⁵. In line with this, impaired podocyte autophagy has been reported in rodent models of DN and in patients with diabetes and massive proteinuria³⁶.

In view of the compelling evidence suggesting an important role for glycoxidative stress in DN, compounds counteracting this are certainly relevant herein. In the following section, I will discuss the pathophysiological role of carnosine, a naturally occurring dipeptide with promising protective properties, in DN.

PATHOPHYSIOLOGICAL ROLE OF CARNOSINE: A VERSATILE NATURALLY OCCURRING DIPEPTIDE

In 1900, the Russian biochemist V. Gulewitsch was the first to discover a non-protein nitrogen-containing compound in meat extract, which was named carnosine from the Latin *caro*, *carnis* – meat^{37,38}. Carnosine is a natural occurring dipeptide, composed of β -alanine and L-histidine, and synthesized by the carnosine synthase (CARNS) enzyme³. The rate-limiting precursor for carnosine synthesis appears to be β -alanine⁴⁰. In humans, CARNS is present in brain, liver, heart, skeletal muscle, and more recently it was found to be expressed in kidney⁴¹.

In mammalian tissues, carnosine is widely distributed but predominantly found in the olfactory bulb of the brain and in skeletal muscle. In the latter, carnosine can be found in relatively high concentrations (10-40 mmol/kg of dry tissue)⁴⁰. Interestingly, the species with the highest muscle carnosine concentrations, are the ones which muscles are exposed to frequent anaerobic periods, e.g. whales and hunting animals^{42,43}. Especially birds, but also all mammals, with the exception of *Homo sapiens*, and some fish also harbour the methylated form of carnosine (β -alanine-1-methyl-histidine), also known as anserine, which comes from the Latin term *anser*, standing for goose⁴⁴ (**Figure 1**).

Whereas anserine is abundantly present in poultry and some fish, carnosine is predominantly present in red meat and seafood^{44,45}. Because of the abundance of carnosine and anserine in animal skeletal muscle, measurements of these dipeptides and their metabolites in urine⁴⁶ and serum are used as biomarkers for the dietary assessment of meat intake in nutrition science^{47,48}.

Figure 1. Chemical structure of the histidine-containing dipeptides anserine and carnosine. Adapted from Boldyrev et. al ⁴⁴.

Based on their chemical structure, carnosine, anserine and other related dipeptides such as homocarnosine (GABA-histidine) are collectively called histidine-containing dipeptides (HCDs). In the following I will mainly focus on carnosine, its role in muscle physiology and its potential therapeutic use in conditions of glycoxidative stress.

Endowed with a plethora of biological properties, carnosine has been subject of research in a wide range of scientific fields, particularly in muscle physiology, in metabolic disorders, in oxidative stress conditions and in sport medicine. Carnosine acts as a physiological buffer and this property is mostly attributed to its characteristic imidazole ring and its pKa (6.72), which is close to the physiological pH⁴⁴. In cardiac myocytes, Swietach et al. demonstrated that HCDs behave as diffusible cytoplasmatic buffers by exchanging H⁺ and Ca²⁺ ions, allowing for Ca²⁺ transport against a proton gradient^{49,50}. The imidazole group of L-histidine, together with the small size of HCD (~250 Da) contribute to these fluxing properties. In line with the above, carnosine plays an important role in the muscle, specifically in the contractile function of skeletal fibers and calcium handling⁴⁴. Furthermore, human studies have shown that the degree of acidosis during high-intensity exercise is alleviated when muscle carnosine content is increased⁵¹. Thus, it is not surprising that high carnosine concentrations in muscle appear to improve high-intensity exercise performance⁵¹. Consequently, increasing intramuscular carnosine content through beta-alanine supplementation^{52,53} is widely popular among elite athletes as an ergogenic aid⁵⁴.

CARNOSINE IN DIABETES

In search for potential candidate genes for susceptibility to develop DN, the first evidence that brought to light the potential protective effect of carnosine in DN was reported by Janssen et al. Accordingly, a tri-nucleotide repeat polymorphism (CTG)_n in the *carnosine dipeptidase 1* (*CNDP1*) gene was associated with the risk of developing DN⁵⁵.

Although the beneficial effect of carnosine in metabolic diseases, e.g., diabetes and hyperlipidemia is widely recognized, relatively few human trials have been reported. In streptozotocin (STZ)-induced diabetic rats, carnosine supplementation reduced hyperglycemia and hyperlipidemia⁵⁶, and in Zucker obese rats, it restrained the development of dyslipidemia⁵⁷. Moreover, in animal models of T2D, e.g., db/db and ob/ob diabetic mice, glucose-lowering effects were reported.

Even though the mechanisms underlying carnosine's effect on glucose homeostasis are not fully unrevealed, evidence supports the role of pancreatic β -cell mass preservation and an insulinotropic effect. As such, carnosine treatment in diabetic mice resulted in pancreatic islet hyperplasia⁵⁸ and in significantly higher insulin levels^{58,59}. Consistent with these data, in *in vitro* studies, carnosine treatment was shown to stimulate insulin secretion in isolated mouse pancreatic islets and in INS-1 pancreatic β -cells. Of note, carnosine treatment appeared to enhance glucose uptake by skeletal muscle cells⁶⁰, which raises the question as to what extent carnosine might positively affect insulin sensitivity. In a small randomized trial with overweight and obese pre-diabetic subjects, glucose tolerance and insulin sensitivity appeared to improve upon carnosine supplementation for 12 weeks⁶¹. Currently supplementation with carnosine to improve cardiometabolic health and cognitive function in patients with pre-diabetes and T2D is being studied (Trial registration: NCT02917928).

Numerous mechanisms may underlie the beneficial effect of carnosine to improve metabolic status. Discussed are carnosine's antioxidant properties, its ability to reverse protein

glycation, and its ability to reduce AGE and advanced lipoxidation end-product (ALE) formation. Carnosine's antioxidant activity involves scavenging of reactive oxygen species (ROS) such as hydroxyl- and superoxide radicals, but also the upregulation of antioxidant enzymes⁴⁴. Accordingly, carnosine treatment was found to reduce markers of lipid oxidation, and to restore depleted levels of glutathione (GSH) as well as to improve basal activities of SOD and glutathione peroxidase (GPX) in liver and heart of aged rats⁶².

Carnosine covalently binds to RCS resulting in the formation of unreactive covalent adducts^{63–65}. As such, carnosine strongly reacts with 4-hydroxy-trans-2,3-nonenal (4-HNE), the most abundant class of ALE precursors, and forms stable carnosine-HNE reaction adducts *in vivo*^{59,65,66}. As a result, carnosine supplementation is associated with increased urinary excretion of adducts and a decrease of urinary markers of AGEs and ALEs⁶⁷. Indeed, recent data from a clinical trial confirmed carnosine's "trapping effect" on RCS in patients with obesity⁶⁸, supporting the role of carnosine in detoxification of RCS. The anti-glycating properties of carnosine have been repeatedly reported *in vitro* and *in vivo* studies. Compelling evidence suggests that carnosine acts at different stages of AGEs formation, i.e. from reversing the formation of Schiff bases (first product of protein glycation) to reversal of preexisting glycated proteins^{69–71}.

Since the production and accumulation of ALEs and AGEs are important mediators in diabetic complications, the use of carnosine has gained scientific interest, and numerous *in vivo* and *in vitro* diabetes relevant studies have reported positive results to diminish or prevent diabetes associated complications. Whereas evidence for the beneficial effect of carnosine in conditions of glycoxidative stress has increased over the past decades, similar evidence for anserine is scarce. This is surprising, considering that anserine is more resistant to enzymatic degradation than carnosine and consequently also has a superior serum stability compared to carnosine⁷². The few available studies have shown that anserine improves proteinuria and vascular permeability in diabetic mice⁷³ and reduces protein carbonylation *in vitro*⁷³. The latter findings have been corroborated in a recent randomized trial in healthy individuals subjected to exercise protocols⁷⁴. Anserine administration might also exert hypoglycemic effects⁷⁵. Based on the current evidence, the role of carnosine as a therapeutic agent appears promising. Yet, the translation of experimental *in vitro* and *in vivo* findings to the human are somehow limited, in view of the fact that in humans, but not in rodents, HCDs (particularly carnosine) are rapidly degraded in serum by the enzyme carnosinase-1⁷⁶.

In the following section, I will discuss this enzyme as the limiting factor for the bioavailability of circulating carnosine.

CARNOSINASE-1: THE LIMITING FACTOR OF CARNOSINE'S PROTECTIVE EFFECTS

Two forms of carnosinase have been identified: carnosinase-1 or serum carnosinase (CN1), and the more ubiquitously expressed tissue carnosinase or cytosolic nonspecific dipeptidase (CN2)^{77,78}. Both enzymes exist as homodimers and have a high degree of homology. However, in comparison to CN2, CN1 appears to have more restricted substrate specificity and a higher catalytic rate⁴⁴. Importantly, CN1 is present in serum of humans and absent in most mammals. Therefore, extrapolation of findings on supplementation studies in rodents to humans should be done with caution.

CN1 is encoded by the *CNDP1* gene located on chromosome 18 and was first described by Teufel et. al as a dipeptidase belonging to the metalloproteases family. CN1 is the rate-limiting enzyme for the hydrolysis of carnosine into β -alanine and L-histidine, and also degrades anserine into β -alanine and 3-methylhistidine and homocarnosine into γ -aminobutyryl and L-histidine⁷⁷. One of the first reports suggesting a link between DN and CN1 came from the finding that a tri-nucleotide ((CTG)_n) repeat polymorphism in the *CNDP1* gene was associated with the susceptibility to develop DN in patients with T2D. Patients with T2D and homozygous for the short allelic variant (CTG)₅, also referred as the *Mannheim allele*, have a lower risk of developing DN⁵⁵. Thereafter, multiple studies have consistently confirmed the association between this polymorphism and susceptibility to develop DN in T2D of different ethnicities^{79,80}. Yet, studies in patients of african-american and mexican-american ethnicity have failed to confirm this^{81,82}. The association between the *CNDP1* (CTG)_n polymorphism and DN has been suggested to be sex-specific⁸³.

Subsequently, studies in diabetic animal models have been carried out to gain mechanistic cues into the effect of carnosine on CKD. Ob/ob mice crossed into a BTBR genetic background develop a more severe DN phenotype⁸⁴. In these BTBR^{ob/ob} mice carnosine treatment attenuated albuminuria, reduced mesangial matrix expansion and improved other glomerular anomalies of DN⁵⁹. A similar protection against the development of albuminuria was observed in Zucker and in diabetic rats upon carnosine feeding^{57,85}. Furthermore, inhibition of pro-apoptotic signaling and podocyte loss were prevented by carnosine feeding in STZ-diabetic induced rats⁸⁶. Moreover, carnosine treatment appeared to accelerate wound healing and increased expression of growth factors and cytokine genes involved in reparative processes in db/db mice⁸⁷.

In contrast to patients with T2D, an association between the *CNDP1* (CTG)_n polymorphism and susceptibility to develop DN in patients with type 1 diabetes is more controversial. While in a genome-wide association study (GWAS) in Caucasian patients with type 1 diabetes, an association between end-stage renal disease (ESRD) and *CNDP1* was observed⁸⁸, this was not found in another case-control study of Caucasian patients with type 1 diabetes⁸⁹. Likewise, in another study with white European patients with type 1 diabetes, no protective association was found between the homozygous *CNDP1* (CTG)₅ genotype and DN, but rather an increased risk for progression to ESRD late after baseline⁹⁰. The association of the *CNDP1* gene and kidney disease has also been investigated in non-diabetic nephropathies. Accordingly,

studies have shown that the (CTG)₅ is associated with a slower progression to CKD and to slightly correlate with renal survival in patients with glomerulopathies but not in patients with tubulointerstitial disease^{91,92}.

The (CTG)_n polymorphism is located within the signal sequence of the *CNDP1* gene and of functional relevance as it influences the efficacy of CN1 secretion⁹³. Accordingly, the shortest allelic form i.e. (CTG)₅ has been associated with poor CN1 secretion resulting in low carnosinase activity in serum⁹³. The current hypothesis explaining the protective effect of *CNDP1* gene puts forward that low serum CN1 activity, generally found in *CNDP1* (CTG)₅ homozygous individuals, promotes higher circulating and tissue carnosine concentrations, thereby affording protection against hyperglycemia-mediated tissue damage^{55,93,94} (**Figure 2**). Apart from the (CTG)_n polymorphism, age^{95,96}, gender⁹⁷ and the substrate type (homocarnosine and anserine versus carnosine)^{95,98} appear to influence CN1 enzymatic activity. Furthermore, it has been shown that hyperglycemia and RCS enhance CN1 enzymatic activity^{99,100}. Of note, a recent study suggested that renal function might also influence CN1 concentrations in serum of patients with T2D¹⁰¹.

Figure 2. Proposed mechanism explaining the protective effect of the *CNDP1* (CTG)₅ homozygous genotype and predisposition to develop diabetic nephropathy. Adapted from Boldyrev et. al and Everaert et al.^{44,94}

In humans, CN1 expression was initially found in liver and to a lesser extent in brain, and some years later it was reported to be also expressed in kidney^{41,55,77,80}. Janssen et.al showed an elevated expression in glomeruli of patients with DN and corroborative studies have also shown marked differences in renal CN1 expression between healthy subjects and patients with DN^{41,55}. Peters et. al reported that CN1 is primarily localized in the distal tubules and glomeruli of healthy individuals, while in T2D patients with DN the expression of CN1 is elevated and much more localized to proximal tubuli⁴¹.

Although some epidemiological studies on one hand did not show an association between the CNDP1 gene and DN, animal studies on the other hand provide substantiated evidence supporting the negative impact of CN1 on glucose and lipid metabolism and DN. As such, overexpression of human CN1 in db/db mice resulted in worsening of glucose metabolism, reflected by increased fasting plasma glucose, HbA1c levels and glycosuria⁵⁸. In the BTBR^{ob/ob}, CN1 overexpression accelerated hyperlipidemia, and aggravated albuminuria, mesangial expansion and glomerular hypertrophy¹⁰². In another study, using the same animal model, CN1 overexpression was associated with a more severe renal histology, and increased albuminuria accompanied with a higher mortality rate¹⁰³. Collectively, these studies have shown that overexpression of human CN1 in diabetic mice resulted in reduced amount of carnosine in serum and in kidney. Based on carnosine's protective properties, carnosine depletion might account for the disease-aggravating effect of CN1 overexpression.

SCOPE OF THE THESIS

In human, both carnosine degrading and synthesizing enzymes, *i.e.*, CN1 and Carnosine synthase (CARNS) are expressed in the kidney⁴¹. While polymorphisms that are associated with susceptibility to develop DN have been described for CN1, no such polymorphisms for CARNS have been reported. Most research attention in this field has focused on dissecting the impact of serum CN1 on diabetic kidney disease, whereas the appearance and role of CN1 in the urine has been neglected.

The studies performed in the **Part A** of this doctoral thesis particularly focus on the role of urinary CN1 in the progression of kidney disease (**Figure 3**). As CN1 is expressed in the kidney^{41,55,80} and targeted to the secretory pathway⁷⁷, we therefore tested in **Chapter 1** the hypothesis that CN1 would be detected in urine as a consequence of renal secretion. To this end, we studied urinary CN1 excretion in a large cohort of healthy individuals (n=243) and patients with T2D (n=361) enrolled in the DIABetes and LiFestyle Cohort Twente-1 (DIALECT) cohort, and investigated the association of urinary CN1 with renal function and albuminuria in a cross-sectional manner. Since in rodents CN1 does not have a signal peptide, is not secreted⁷⁷. By making use of BTBR^{wt/wt} mice overexpressing human CN1, we validated the specificity of the methods used for the detection of CN1 in urine.

In **Chapter 2** we intended to gain more insight into the origin of urinary CN1 under healthy and kidney disease conditions *i.e.*, to what extent the glomerular filtration barrier integrity contributes to the appearance of CN1 in urine. For this purpose, urinary CN1 together with serum CN1 concentrations were studied in a cohort of patients with diabetic and non-diabetic

tic kidney disease (n= 111) and in group of healthy subjects (n= 24). As the (CTG)_n polymorphism within the CNDP1 gene determines the secretion efficacy of CN1 into the circulation⁹³, we assessed if this polymorphism also influences the extent of urinary CN1 concentrations.

Figure 3. Studies on the relevance of carnosinase-1 and renal function (Chapters 1 & 2). Image created with BioRender.com. CN1: Carnosinase-1. T2D: type 2 diabetes, CKD: chronic kidney disease.

In **Part B** we studied the protective role of the histidine containing-dipeptides: anserine and carnosine, in the context of long-term outcomes in a well-defined large cohort of kidney transplant recipients (KTR) (**Figure 4**). In these patients, both immunological and non-immunological factors play a role in decline of kidney function, with the non-immunological factors showing great resemblance to the factors involved in decline in kidney function in diabetes¹⁰⁴. KTR therefore serve as an interesting clinical model to be investigated in the context of carnosine. In **Chapter 3** we report on urinary measurements of carnosine and CN1 in this KTR population and in a cohort of healthy kidney donors. Here, we explore the association of CN1 with carnosine in both populations. Because high CN1 concentrations may lead to depletion of renal carnosine stores, we proposed that high urinary CN1 might indirectly render the kidney more susceptible to damage in circumstances of high and continuous inflammation or oxidative stress, which might be the case in KTR. We studied this by first assessing the association of urinary CN1 with urinary inflammatory markers. Subsequently we studied the prospective association between urinary CN1 and the risk of late graft failure in KTR.

Urinary 1-methylhistidine, which is a product of anserine metabolism⁴⁴, and urinary 3-methylhistidine have been proposed as putative biomarkers of white and red meat intake, respectively^{46,47,105,106}. Therefore, in **Chapter 4** we first evaluated the association of these urinary metabolites with meat consumption as estimated by a validated food-frequency questionnaire. Although the impact of protein intake on kidney health has been extensively studied, controversy and gaps in knowledge persist. As such, some studies suggest that the protein source i.e., animal versus plant may have different effects on renal outcomes¹⁰⁷. In this chapter we further investigated the prospective associations of these urinary biomarkers i.e., 1-methylhistidine and 3-methylhistidine with graft failure and mortality in KTR.

Figure 4. Studies on urinary histidine-containing dipeptides in long-term outcomes after kidney transplantation (Chapters 3 & 4). Image created with BioRender.com. CN1: Carnosinase-1.

In **Part C** we carried out two further studies using in vitro and in vivo models to gain further understanding of the effect of 1) alterations in glomerular hemodynamics and 2) elevated concentrations of MGO, both of these being proposed as contributing factors of diabetes initiation and/or disease progression^{7,108}. We also studied the effect of carnosine herein (**Figure 5**). Diabetes-induced glomerular hyperfiltration has been postulated as a pathophysiologic and prognostic factor for diabetic kidney disease^{8,9}. Compelling evidence suggest that oral carnosine supplementation and overexpression of human CN1 in diabetic mice might have antagonistic effects on kidney disease progression^{59,103}. Yet, the effect of the CN1-carnosine system on glomerular hyperfiltration remains unexplored. In **Chapter 5** we aimed to investi-

gate the effect of these two interventions on the vasotone of the afferent and efferent arteriole. To this end, we used a three-dimensional approach together with the conventional two-dimensional histology to assess the lumen area of the afferent arteriole from BTBR^{ob/ob} diabetic mice.

Glomerular hypertrophy and the expression of nephrin expression, a podocyte injury marker, were also assessed. In **Chapter 6** we aimed to provide formal proof of p53 activation upon MGO exposure in human umbilical endothelial cells, and its consequences on two p53 downstream targets: mTORc1 and autophagy pathways. In view of carnosine antioxidant and carbonyl-sequestering properties, we also investigated whether MGO-driven cellular effects are counteracted by the co-treatment with carnosine, and whether, and to what extent, carnosine influences the redox milieu.

Figure 5. Studies on: glance into carnosine's potential mechanism (Chapters 6 & 7). Image created with BioRender.com. hCN1: human carnosinase-1, MGO: Methylglyoxal, ROS: reactive oxygen species.

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PART A

RELEVANCE OF CARNOSINASE-1
IN RENAL FUNCTION