Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen
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# Dipeptidyl peptidase IV beyond glucose homeostasis

# A focus on substrates

# Dipeptidyl peptidase IV voorbij de glucose homeostase

# De focus op substraten

Proefschrift voorgelegd tot het behalen van de graad van
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**Lesley Baerts**

**Promotoren:**
**prof. dr. I. De Meester**
**prof. dr. A-M. Lambeir**

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"We choose to go to the Moon in this decade and do the other things,

*not* because they are easy, *but because they are hard*;

because *that goal* will serve to organize and measure the best of our energies and skills,

because *that challeng*e is one that we are willing to accept,

one we are unwilling to postpone, and *one we intend to win* ..."

-John F. Kennedy

# List of Abbreviations

| | |
|---|---|
| ADA | Adenosine deaminase |
| AGE | Advanced glycation end products |
| ANOVA | Analysis of variance |
| ANP | Atrial natriuretic peptide |
| BNP | B-type natriuretic peptide |
| BSA | Bovine serum albumin |
| CCR | Chemokine (CC-motif) receptor |
| CD | Cluster of differentiation |
| cGMP | Cyclic guanosine monophosphate |
| CLCR | Creatinine clearance rate |
| CNS | Central nervous system |
| COX | Cyclo-oxygenase |
| CXCL | Chemokine (CXC-motif) ligand |
| CXCR | Chemokine (CXC-motif) receptor |
| DPP | Dipeptidyl peptidase |
| EDTA | Ethylenediaminetetra-acetic acid |
| EF | Ejection fraction |
| ELISA | Enzyme-linked immunosorbent assay |
| FAP | Fibroblast activation protein α |
| FCS | Fetal calf serum |
| G-CSF | Granulocyte colony-stimulating factor |
| GFR | Glomerular filtration rate |
| GIP | Glucose-dependent insulinotropic peptide |
| GLP-1 | Glucagon-like peptide-1 |

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HF | Heart failure |
| HFpEF | Heart failure with preserved ejection fraction |
| HFrEF | Heart failure with reduced ejection fraction |
| HIV | Human immunodeficiency virus |
| HRP | Horseradish peroxidase |
| ICAM | Intercellular adhesion molecule |
| IH | Ischemic heart disease |
| LPS | Lipopolysaccharide |
| LV | Left-ventricular |
| LVEF | Left-ventricular ejection fraction |
| MALDI | Matrix-assisted laser desorption/ionization |
| MMP | Matrix metalloproteinase |
| MS | Mass spectrometry |
| NHE3 | Sodium-hydrogen exchanger 3 |
| NO | Nitric oxide |
| NPRA | Natriuretic peptide receptor A |
| NPY | Neuropeptide Y |
| NUCB2 | Nucleobindin-2 |
| PACAP | Pituitary adenylate cyclase activating polypeptide |
| PBS | Phosphate-buffered saline |
| PKA | Protein kinase A |
| PKG | Protein kinase G |
| pNA | Para-nitroanilide |
| POP | Prolyl oligopeptidase |
| PYY | Peptide YY |
| RAGE | Receptor of advanced glycation end products |

| | |
|---|---|
| SDF1 | Stromal cell-derived factor 1 |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| VCAM | Vascular cell adhesion molecule |
| VIP | Vasoactive intestinal peptide |

**List of amino acids**

| | | |
|---|---|---|
| A | Ala | Alanine |
| C | Cys | Cysteine |
| D | Asp | Aspartic acid |
| E | Glu | Glutamic acid |
| F | Phe | Phenylalanine |
| G | Gly | Glycine |
| H | His | Histidine |
| I | Ile | Isoleucine |
| K | Lys | Lysine |
| L | Leu | Leucine |
| M | Met | Methionine |
| N | Asn | Asparagine |
| P | Pro | Proline |
| Q | Gln | Glutamine |
| R | Arg | Arginine |
| S | Ser | Serine |
| T | Thr | Threonine |
| V | Val | Valine |
| W | Trp | Tryptophan |
| Y | Tyr | Tyrosine |

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# Chapter 1

# INTRODUCTION

# Chapter 1 - Introduction

This thesis is part of the joined effort of the Laboratories of Medical Biochemistry, Physiopharmacology, Pathophysiology, Toxicology and Medicinal Chemistry of the University of Antwerp to shed a light on the role of dipeptidyl peptidase IV (DPPIV) outside glucose homeostasis. In this collaboration supported by the FWO and BOF (special research fund for research) of the University of Antwerp, the Laboratory of Medical Biochemistry and this doctoral research focused on the biochemical analyses and the underlying mechanisms of the observed effects of DPPIV inhibitors.

Inhibition of the DPPIV enzyme ameliorates glucose control in type 2 diabetes. Since 2006, its inhibitors are a novel class of orally available drugs and are approved by the European and American regulatory authorities for the treatment of type 2 diabetes.

At the beginning of this project, several findings indicated that DPPIV inhibitors had effects outside of glucose homeostasis. First of all, a non-selective DPPIV inhibitor (AB192) prevented ischemia/reperfusion injury in the lung and ameliorated acute graft rejection [1]. Second, DPPIV was shown to be involved in the mobilization of endothelial progenitor cells after ischemia [2]. Lastly, the DPPIV inhibitor vildagliptin resulted in an accumulation of collagen degradation products in rats, revealing that DPPIV was involved in collagen metabolism [3]. In addition, new guidelines had just been published concerning the assessment of cardiovascular safety of new antidiabetic therapies to treat type 2 diabetes mellitus [4]. Type 2 diabetes is accompanied by cardiovascular, renal and bone comorbidities. As DPPIV inhibitors are increasingly used to treat these patients, it is important to evaluate the positive and negative effects on these organ systems.

Currently, it is clear that DPPIV inhibition is a hot topic in cardiovascular, renal and bone pathologies. This can be illustrated by the large, ongoing clinical trials towards the potential role in these diseases (e.g. CAROLINA, CARMELINA and MARLINA).

These provided important incentives to evaluate the physiological role of DPPIV and its substrates in the cardiovascular system, kidney and bone and will help elucidate the complete pharmacological spectrum of DPPIV inhibitors.

## 1.1 Dipeptidyl Peptidases: e pluribus unum

Dipeptidyl peptidases are a group of enzymes capable of splitting off dipeptides from peptides and proteins. According to the Enzyme Commission nomenclature, this group is classified under E.C. 3.4.14 [5]. In humans, the dipeptidyl peptidase family contains membrane-bound and soluble members with varying expression patterns, tissue distributions and compartmentalization. These enzymes have important roles in the regulation of peptide bioactivity, and are drug targets for the treatment of diabetes, cancer and inflammation [6]. All dipeptidyl peptidases, that will be discussed, are serine proteases belonging to the prolyl oligopeptidase family (clan SC, family S9) [7]. This family of enzymes shares a common α/β hydrolase fold and a characteristic catalytic triade comprising of Ser-Asp-His. Furthermore, all these enzymes share a preference towards Xaa-Pro and Xaa-Ala substrates [8, 9]. An overview of the prototypical substrates of the different dipeptidyl peptidases that will be discussed can be found in Figure 1.1. For a more extensive overview of the prolyl oligopeptidase family, we refer to reviews by Polgar *et al.*, Waumans *et al.* and Rea and Fulöp [10–12].

Other members belonging to the dipeptidyl peptidase family fall outside the scope of this thesis as they belong to another type of enzyme (e.g. DPP1 and DPP3, a cysteine and metalloprotease respectively) or are not enzymatically active (e.g. the dipeptidyl peptidase-like enzymes DPP6 and DPP10). Nevertheless, these molecules play crucial roles in inflammation, cancer and neurodegenerative diseases [13–15].

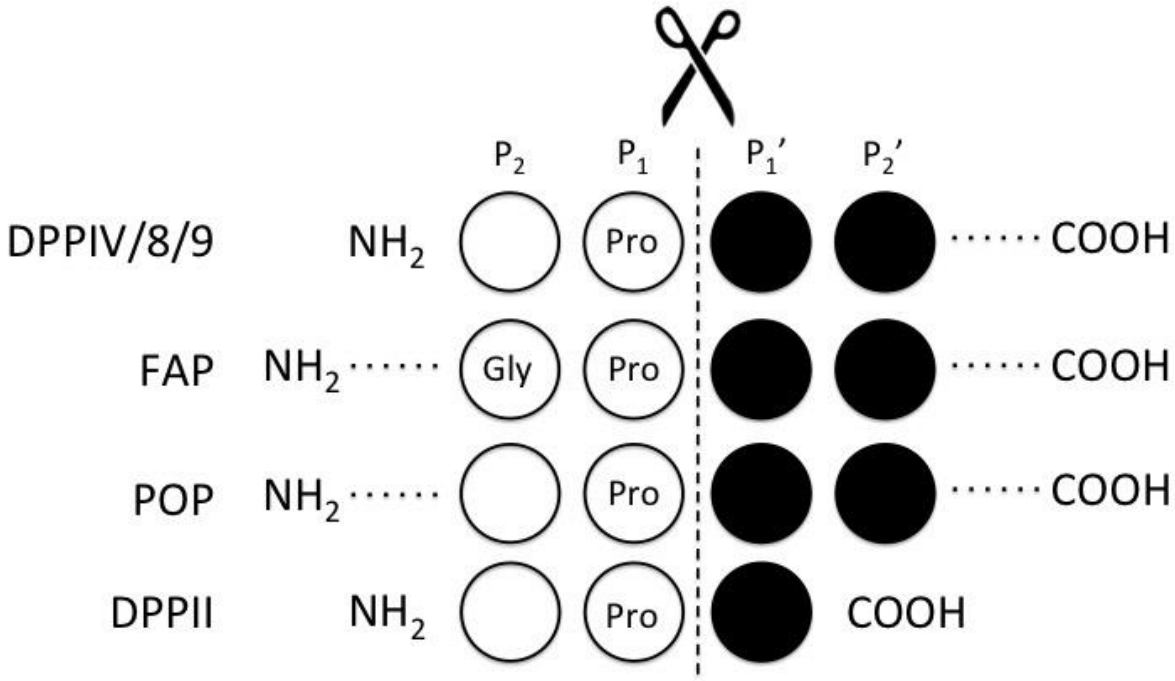



**Figure 1.1: overview of the prototypical substrates of the different dipeptidyl peptidases discussed in this thesis.**

### 1.1.1 Dipeptidyl peptidase IV

DPPIV (E.C. 3.4.14.5) is the best-studied member of all discussed dipeptidyl peptidases and will be the focus of this thesis. DPPIV was originally discovered by Hopsu-Havu after histochemical staining with Gly-Pro-β-naphtylamide of rat liver and kidney [16]. Four years later its presence in human serum was discovered, indicating the existence of a soluble DPPIV molecule besides a membrane-bound form [17–19]. Meanwhile, soluble DPPIV has been described in blood, saliva and seminal fluid [19].

DPPIV is widely expressed in a variety of human tissues, cells and body fluids. Its highest expression in tissues can be found in the kidney, where it is present in the microvilli of the brush border [20]. Other tissues with a high expression are the lung, the liver and the intestines [20, 21]. In all these tissues, DPPIV is attached to the membrane of epithelial and endothelial cells [22–24]. In addition, DPPIV can also be found on the membrane of circulating immune cells, such as T lymphocytes and natural killer cells [11]. It is also known as cluster of differentiation 26 (CD26) [11].

It has been shown that DPPIV is anchored in the cell membrane with its signal peptide [25]. It is hypothesized that the soluble DPPIV is released into the bloodstream after proteolytic cleavage in the stalk region that links it to the transmembrane helix. This is supported by the fact that metalloproteinases (MMP1, MMP2, MMP9 and MMP14) are able to shed DPPIV from human vascular smooth muscle cells and adipocytes [26].

While DPPIV is best known for its peptidase activity, its protein-protein interactions should not be overlooked. Interactions with DPPIV have been described, among others, for collagen, sodium-hydrogen exchanger 3 (NHE3) and adenosine deaminase (ADA) [27]. Due to this last interaction, DPPIV is also known as ADA complexing protein [28]. In addition, its activity as a peptidase depends on dimerization; both active homodimers as well as active heterodimers with fibroblast activation protein α (FAP α) have been described (Figure 1.2) [8]

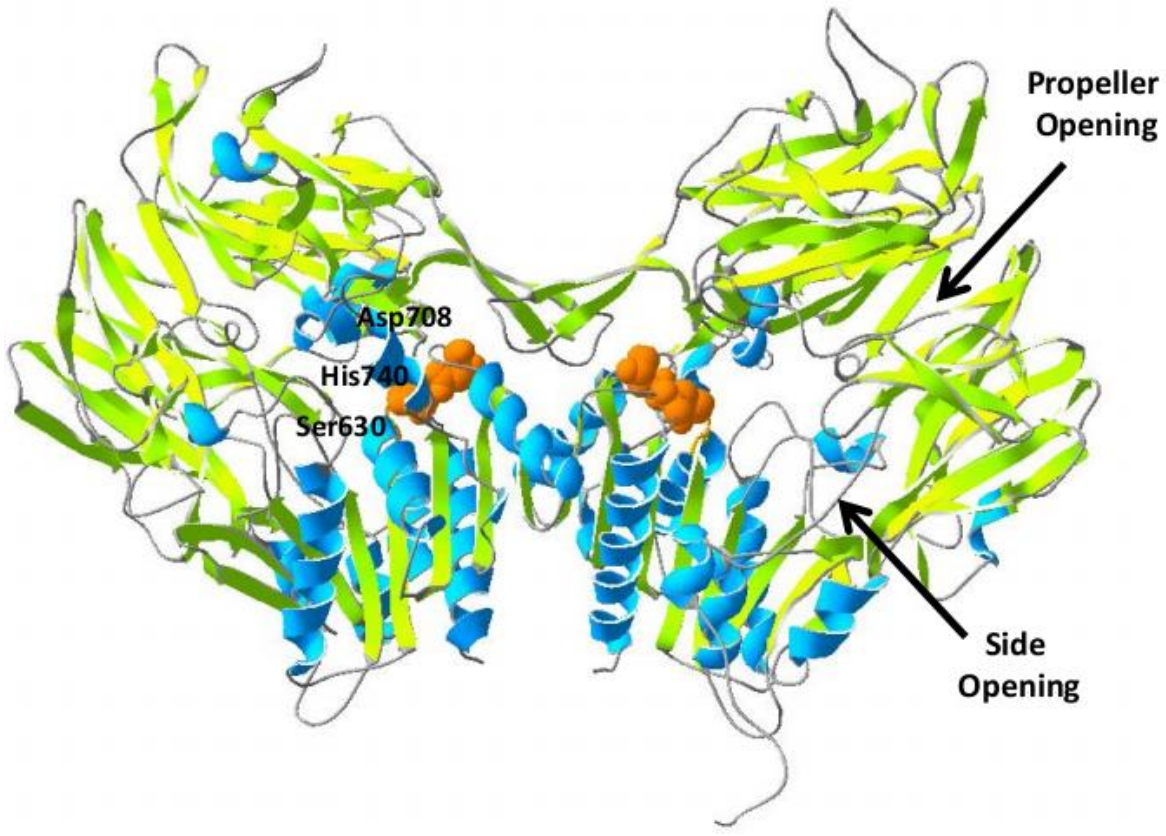



**Figure 1.2: Crystal structure of DPPIV forming a dimer.** DPPIV substrates enter the active site through the side opening. α helices are represented in blue, β sheets in green and the catalytic triade $Ser^{630}$, $Asp^{708}$, $His^{740}$ in orange.

DPPIV is responsible for the cleavage and/or biological inactivation of several neuropeptides, cytokines and hormones (Table 1.1). Substrates can potentially enter the active site of the enzyme through the β-propeller tunnel or through a side opening [8, 29, 30]. However, experimental evidence only exists for the entry of substrates through the side opening. This side opening restricts the access of bulky substrates and requires a free N-terminus of at least six amino acids [30]. As DPPIV substrates are involved in a broad range of diseases, it is not surprising that DPPIV inhibitors can alter several pathological processes. These will be discussed in more detail in the following sections.

### 1.1.2 Fibroblast activation protein α

FAPα (E.C. 3.4.21.B28) was discovered as a cell surface antigen on fibroblasts with the F19 monoclonal antibody [31, 32]. Later it was discovered that the FAPα homodimer is identical to seprase, a gelatinase found in a human melanoma cell line [33]. Similar to DPPIV, dimerization is essential for its enzymatic activity and functional heterodimers with DPPIV have been reported [34]. FAPα mainly functions as an endopeptidase, preferentially cleaving after Gly-Pro moieties [35]. Nonetheless, FAPα also possesses exopeptidase activity and is able to cleave several substrates, in vitro, that are also cleaved by DPPIV [36].

While FAPα expression is low in normal tissues, its expression is strongly upregulated during tissue remodeling, such as wound healing, cancer metastasis or during inflammation [37, 38]. FAPα is upregulated on stromal fibroblasts in most human epithelial tumors [37]. Besides a membranary form there also exists a soluble FAPα. This circulating molecule is also known as antiplasmin cleaving enzyme due to its ability to cleave α2-antiplasmin [39]. Because α2-antiplasmin cleavage results in a rapid cross-linking to fibrin and slows clot lysis, FAPα is thought to be indirectly involved in fibrinolysis [39–41].

**Table 1.1: Overview of *in vivo* DPPIV substrates and the resulting effect of their truncation.**

| Substrate | N-terminus | Function substrate | Effect cleavage | Reference |
|---|---|---|---|---|
| BNP | SP\|KMV... | natriuresis<br>diuresis<br>vasodilation | loss of function | [42] |
| SDF1α | KP\|VSL… | chemotaxis<br>cell proliferation<br>cell survival | antagonism | [43, 44] |
| NPY | YP\|SKP… | vasoconstriction<br>orexigene<br>anti-inflammatory | change in receptor selectivity<br>Y1-R => Y2-R, Y5-R | [45, 46] |
| GLP-1 | HA\|EGT… | anticonvulsive<br>insulin secretion<br>(glucose-stimulated)<br>↓glucagon secretion<br>↓gastric emptying | loss of function | [47–49] |
| GIP | YA\|EGT… | | loss of function | [48, 49] |
| CXCL10 | VP\|LSR… | chemotaxis<br>cell proliferation<br>cell survival | loss of function | [50–52] |
| PYY | YP\|IKP… | vasoconstriction<br>↓gastric emptying | change in receptor selectivity<br>Y1-R => Y2-R | [53] |

ND: not determined

Current FAPα research focuses on its role in cancer, fibrosis and arthritis. As FAPα is only expressed during tissue remodeling, it is considered to be a selective target in these pathologies, leaving healthy tissues unaffected [54].

### 1.1.3 Dipeptidyl peptidase 8 and 9

Between 2000 and 2002, DPP8 and DPP9 (E.C. 3.4.14) were discovered after the characterization of human cDNA clones [55, 56]. Because there is an extensive primary sequence similarity (76% amino acid similarity) and a lack of tools to study these enzymes separately, DPP8 and DPP9 shall be discussed together [55, 57].

Similar to DPPIV, DPP8 and DPP9 preferentially cleave off Xaa-Pro and Xaa-Ala dipeptides. DPP8 and DPP9 share, *in vitro,* several peptide substrates with DPPIV. However, the biological importance of this is not clear as both enzymes are intracellularly localized [58]. It is hypothesized that DPP8 and DPP9 truncate cytosolic peptides or process them prior to secretion [59, 60].

High DPP8 and 9 expressions are found in the brain, the reproductive system and immune system, where they are expressed in the cytoplasm [61–63]. Both enzymes seem to be involved in several processes such as spermatogenesis, apoptosis and inflammation [63–65]. Despite the lack of specific inhibitors, evidence is emerging that DPP8 and DPP9 are involved in distinctive processes of these pathologies [59, 66]. On the one hand, DPP8 seems to be involved in gut inflammation, as was shown in a mice colitis model. On the other hand, DPP9 is involved in the antigen presentation of proline-containing peptides [67].

### 1.1.4 Other related peptidases

#### 1.1.4.1 Dipeptidyl peptidase II

Dipeptidyl peptidase II (DPPII, E.C. 3.4.14.2) was discovered by Mcdonald as an exclusively lysosomal enzyme in the pituitary gland [68]. Other synonyms used are: dipeptidyl aminopeptidase A, DPPV, DPP7 and quiescent cell proline dipeptidase (QPP) [69]. Similar to DPPIV, DPPII is able to release Xaa-Pro or Xaa-Ala dipeptides from peptide molecules. In contrast, its enzyme activity is highest at acidic pH and it has a strong preference towards tripeptide-substrates, while large peptide substrates are not truncated at all [70–72]. Because of this preference, DPPII has also been termed a carboxytripeptidase [69, 73]

DPPII can be found in nearly all organs and tissues, but its highest expression is found in the lysosomes of proximal renal tubule cells, thyroid cells, macrophages and lymphocytes [74, 75]. While its physiological role is still unclear, DPPII seems essential for maintaining lymphocytes in a quiescent

state and its inhibition can result in apoptosis of these cells [75–77]. DPPII might also be involved in neurodegenerative, hematological and inflammatory disorders [69].

#### 1.1.4.2 POP

In 1971, Walter *et al.* discovered POP (EC. 3.4.21.26) as an oxytocin inactivating enzyme in the human uterus [78]. In literature, POP is also known as post-proline cleaving enzyme (PPCE) or prolyl endopeptidase (PEP or PREP) [79]. POP has an endopeptidase activity that is restricted to short peptides (≤ 30 amino acids), preferentially cleaving behind a proline residue [79, 80]. In contrast to FAP, its activity is not restricted to Xaa-Gly-Pro sequences but POP accepts several other amino acids in the P1 and P2 position [80].

POP is ubiquitously expressed in mammals. Its highest expression is found in brain and central nervous system [81, 82]. Although POP is regarded as a soluble cytoplasmic enzyme, it has also been reported in body fluids, such as serum and cerebrospinal fluid [83, 84]. Initially, It was suggested that POP is involved in neurodegenerative diseases through its effect on learning and memory processes [79, 85]. Meanwhile, it has become clear that POP inhibition has a much broader effect than originally was thought. It is now hypothesized that POP inhibition reduces neuroinflammation [86] and reduces α-synuclein aggregation [85, 87]. For a more complete review we refer to reviews by Penttinen *et al.*, Lambeir *et al.* and Van Elzen *et al.* [88–90]

## 1.2 Dipeptidyl Peptidase IV in glucose homeostasis

### 1.2.1 Dipeptidyl peptidase IV in diabetes

DPPIV is probably best known for its role in glucose homeostasis. Its plasma and serum activities are higher in type 2 diabetic patients than in the healthy population [91–94]. As not all reports agree on this [95, 96], Fadini *et al.* performed a meta-analysis showing an increase in DPPIV activity by 33% in type 2 diabetes mellitus [91]. Some authors claim that this increase in DPPIV activity is linked to an altered glucose control [93, 94]. While this is supported by a correlation with HbA1c levels, a marker

for chronic hyperglycemia, a non-pharmacological treatment does not result in lower circulating DPPIV activities [91, 94]. Possibly, DPPIV activities only rise after a certain HbA1c threshold is reached [94]. Another parameter that might influence DPPIV activity is obesity. It is known that obese persons are prone to developing type 2 diabetes mellitus [97]. Intriguingly, DPPIV is expressed in human adipocytes, showing the highest activities in visceral fat tissue [98, 99]. Since adipocytes have been suggested as the main source of circulating DPPIV, its activity might be more dependent on the general metabolic state than on glucose control alone [26, 99].

### 1.2.2 Incretins

DPPIV is also linked to glucose homeostasis, through its effect on the incretin hormones. The incretins are gut hormones secreted from entero-endocrine cells into the circulation several minutes after a meal [100] and stimulate the islets of Langerhans to secrete insulin, leading to glucose uptake [101]. DPPIV is responsible for the proteolytic degradation and inactivation of the incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). DPPIV converts biologically active GIP(1-42), GLP-1(7-36) and GLP-1(7-37) into their inactive counterparts, GIP(3-42), GLP-1(9-36) and GLP-1(9-37) (Figure 1.3) [102, 103].

GIP and GLP-1 exert their biological effect through specific G-protein-coupled receptors, GIP-R and GLP-1R. The GLP-1R is expressed on both α and β islet cells and peripheral organs, while the GIP-R is predominantly found on β cells. The incretin-effect on blood glucose triggered the development of incretin mimetics. Currently, only GLP-1R agonists are used in clinical practice, as GIP-R agonists only show a weak biological effect [104, 105]. Four GLP-1R agonists are commercially available in Belgium. Exenatide (Byetta®), liraglutide (Victoza®) and lixisenatide (Lyxumia®) are registered for once- or twice-daily subcutaneous injection. Albiglutide (Eperzan®) is the only long-acting GLP-1R agonist and has to be injected only once a week [106].

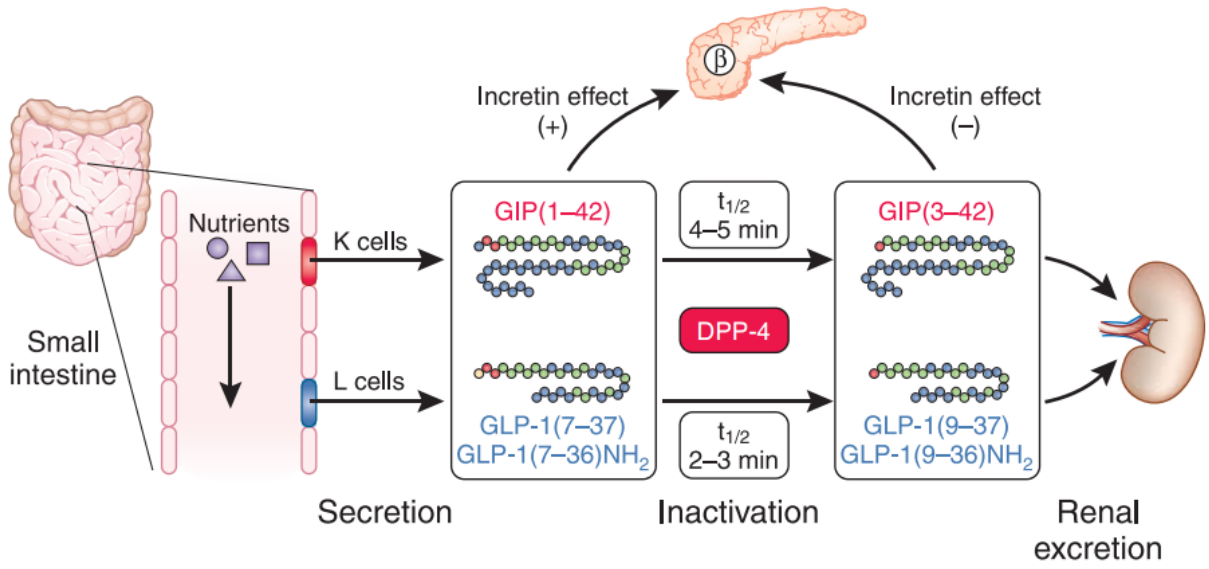



**Figure 1.3: Secretion and metabolism of the incretines (GIP and GLP-1).** K cells secrete GIP, while GLP-1 is secreted from L-cells. Both incretines rapidly undergo proteolytic processing by DPPIV, resulting in inactivation. The circulating incretines are excreted from the kidney [107].

In addition to restoring normoglycemia, the incretin-mimetics show several other advantages. For example, exenatide increases the glucose sensitivity of resistant β cells and stimulates their cell growth and survival. Effects outside of glucose homeostasis have also been described. The GLP-1R agonists promote satiety, inhibit gastric emptying and acid secretion, increase blood pressure and heart rate. For more information on GLP-1 and GIP, we refer to the detailed review by Drucker and Nauck [104].

## 1.3 Dipeptidyl Peptidase IV and their inhibitors

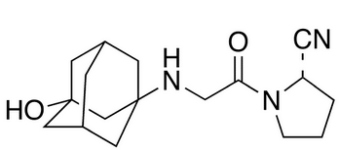
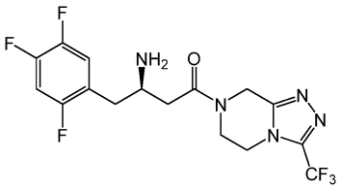
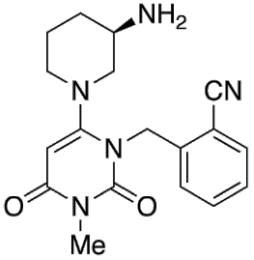
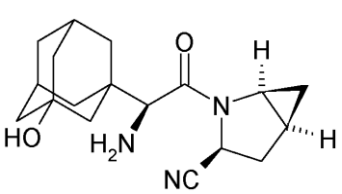
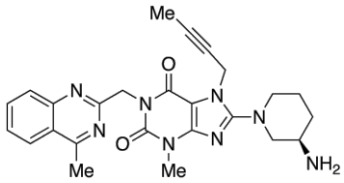
The potential role of DPPIV in glucose homeostasis became apparent after pharmacological inhibition and genetic deletion in mice [108]. Because DPPIV inhibitors prolong the biological activity of the incretins, a similar effect to the incretin mimetics in type 2 diabetes can be expected.

In 2006, sitagliptin was approved for the treatment of diabetes mellitus type 2 by the federal drug and food agency (FDA), becoming the first agent of its class. For the moment, five inhibitors are commercially available in Europe and the United States (Table 1.2). Other inhibitors such as teneligliptin and anagliptin have been approved in Asia [109].

DPPIV inhibitors are regarded as a safe and efficacious treatment of type 2 diabetes mellitus. They have a slightly lower efficacy in lowering HbA1c compared to metformin and GLP-1R agonists, but have a similar efficacy as sulfonylurea, thiazolidinediones and α-glycosidase inhibitors [110, 111]. In addition, they are much better tolerated and most can be orally administered once a day [110, 112]. Contrary to sulfonylurea, DPPIV inhibitors are not associated with an increased risk of hypoglycemia and have been shown to be weight neutral [113]. They show significantly less gastrointestinal side effects as metformin, α-glycosidase inhibitors and GLP-1R agonists (*e.g.* nausea and diarrhea). [110]. In contrast to the thiazolidinediones or glitazones, most DPPIV inhibitors are not associated with an increased cardiovascular risk [114].

After the introduction of the DPPIV inhibitors concern was raised about an increased risk of pancreatitis and infections [115]. However, the analysis of available safety data found no evidence for these associations [115, 116] and the safety of DPPIV inhibitors in monotherapy or as add-on therapy was proven in several studies [117–120]. Currently, DPPIV inhibitors are a second-line therapy, when metformin monotherapy is inadequate in reaching sufficiently low blood glucose levels [121].

**Table 1.2: Overview of the clinically available DPPIV inhibitors in Belgium and their characteristics.**

| | Vildagliptin | Sitagliptin | Alogliptin | Saxagliptin | Linagliptin |
|---|---|---|---|---|---|
| **Drug name** | Galvus® | Januvia® | Vipidia® | Onglyza® | Trajenta® |
| **Manufacturer** | *Novartis Pharma* | *MSD* | *Takeda* | *AstraZeneca* | *Boehringer Ingelheim* |
| **Structure** |  |  |  |  |  |
| **Dosage** | 50 mg; 2xd | 100 mg; 1xd | 25 mg; 1xd | 5 mg; 1xd | 5 mg; 1xd |
| **Metabolism** | glucuronide conjugates | no | no | hydroxylated (to active metabolite) | no |
| **Excretion** | | | | | |
| **unchanged renal (%)** | 23 | 75 | 63 | 24 | 6 |
| **renal clearance (ml/min)** | 243 | 340 | 201 | 76 | 70 |
| **faecal (%)** | 15 | 13 | 11 | 22 | 85 |
| **t1/2 (h)** | 1-4 | 8-24 | 12-21 | 2-4<br>metabolite 3-7 | 10-40 |
| **Dosage adjustment** | | | | | |
| **kidney disease** | impairment=50mg/day | CLCR*30–50=50mg/day | CLCR30–50=12.5mg/day<br>CLCR<30=6.25mg/day | CLCR≤50=2.5mg/day | no |
| **liver disease** | not recommended | CLCR<30=25mg/day | no | no | no |
| **Selectivity** | moderate | high | high | moderate | moderate |

* *CLCR: Creatinine Clearance Rate*

An overview of clinical DPPIV inhibitors available in Belgium and their characteristics is given in Table 1.2. All inhibitors have a similar efficacy after absorption from the gastro-intestinal tract, reaching peak plasma concentrations in 1 to 4 hours [122, 123]. Concomitant food intake does not affect oral absorption [123]. Vildagliptin and saxagliptin are relatively quickly cleared from the circulation. The other inhibitors and an active saxagliptin metabolite, reside much longer in the blood. Consequently, vildagliptin is the only DPPIV inhibitor that has to be dosed twice daily [122]. In the circulation most DPPIV inhibitors show a low and reversible protein binding. Only linagliptin is almost completely protein-bound, predominantly to DPPIV [122, 123]. Moreover, linagliptin is the only inhibitor that is not primarily renally excreted, but is hepatically cleared. An important consequence is that linagliptins dose should not be adjusted during kidney disease [122]. DPPIV inhibitors can also be safely used in patients with mild to moderate hepatic insufficiency. Only vildagliptin is not recommended in these patients due to its association with an increase in hepatic enzymes. Caution is also advised with the use of linagliptin because of its hepatic clearance [122, 123]. Meanwhile, it has become clear that not all inhibitors are selective towards DPPIV. Only sitagliptin and alogliptin can be considered as selective DPPIV inhibitors, while all other gliptins also inhibit related peptidases.

At present, different companies are investigating the use of DPPIV inhibitors outside of type 2 diabetes mellitus. On the one hand, evidence suggests a possible role as add-on therapy to insulin in type 1 diabetes mellitus [124]. On the other hand, experimental and clinical data points to a protective effect against microangiopathy in type 2 diabetic patients, independent from glucose control. Therefore, DPPIV is considered a promising therapeutic target in osteoporosis, cardiovascular disease and nephropathy [125].

## 1.4 Dipeptidyl Peptidase IV beyond glucose homeostasis

While DPPIV and its inhibitors have traditionally been studied in diabetes, there is a growing body of evidence that they might be involved in multiple organ systems. These multiple effects of DPPIV inhibitors are commonly denoted as pleiotropic effects [125, 126]. For the moment, it has been

shown that they are protective in cerebro- and cardiovascular, renal and bone pathologies. However, this list is not exhaustive and multiple other effects have been reported in literature [125, 126] [127].

In the following sections, we will highlight the beneficial effects of DPPIV inhibitors in several organ systems. However, negative effects should not be forgotten as their negative effects tend to be underreported. As the focus of this thesis lies on the effect of DPPIV inhibitors in the cardiovascular system, kidneys and bone, only these will be discussed. For further information on the physiological significance of DPPIV in inflammation, cancer and auto-immune disease, we like to mention the reviews by Havre *et al.*, Thompson *et al.*, Ohnuma *et al.* and Waumans *et al.* [11, 27, 128, 129].

### 1.4.1 Cardiovascular system

While it is longer known that a strict glucose control can lower the cardiovascular risk [130], DPPIV inhibitors seem to have an additional, insulin-independent, mechanism of action [126]. Many potential mechanisms may lie at the base of their positive effects. Prolongation of the biological half-life of the incretins seems an evident possibility, but other substrates should not be overlooked. However, DPPIV could also be involved through a non-enzymatic pathway [131, 132]. It is impossible to suggest a general mechanism valid for all cardiovascular diseases. Instead, we will focus on what is known on DPPIV in the cardiovascular setting.

### 1.4.2 Endothelium

The endothelium is a multifunctional organ involved in regulating vascular tone, inflammation and coagulation. It is considered to be metabolically active, performing autocrine, paracrine and endocrine functions through molecules such as nitric oxide (NO) and prostaglandins. Type 2 diabetes and other pro-inflammatory stimuli are an important cause of endothelial dysfunction. This dysfunction is characterized by a reduced NO bioavailability and constitutes an important risk for the development of cardiovascular diseases [133, 134]. Therefore, much research is focused towards improving endothelial function. DPPIV inhibitors seem to restore the endothelial function in animal

experiments (Figure 1.4) [135–137]. This is not very surprising as DPPIV is anchored in the endothelial membrane and can be found as a soluble form in the bloodstream. This soluble DPPIV is regarded as a pro-inflammatory molecule secreted by adipose tissue. In *in vitro* experiments, addition of soluble DPPIV to human vascular smooth muscle cells results in increased levels of several pro-inflammatory cytokines (Interleukin 6 and monocyte chemotactic protein 1), which is reversed by an experimental DPPIV inhibitor [138].

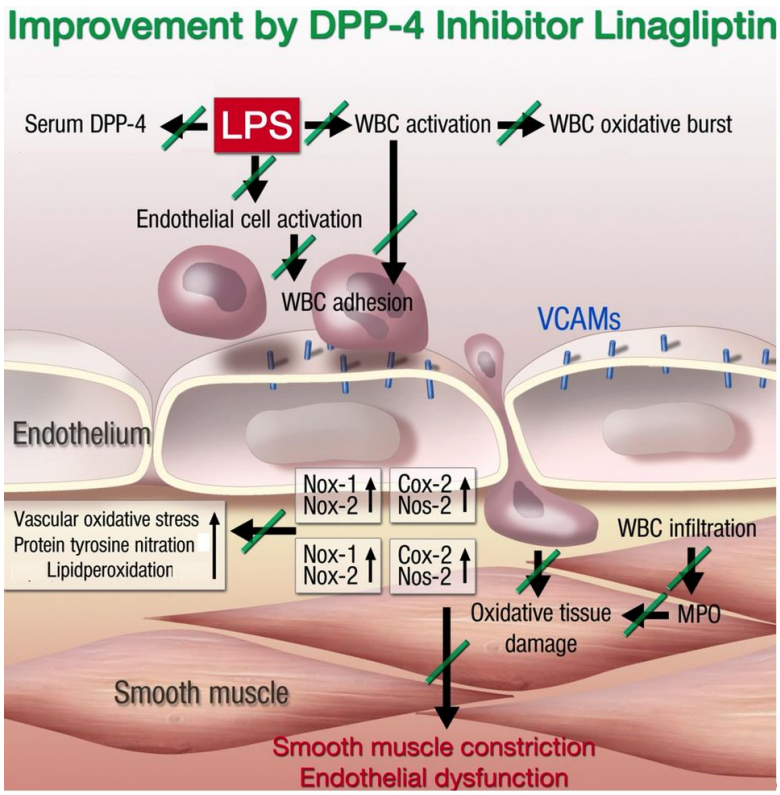



**Figure 1.4: Overview of the protective mechanisms of DPPIV inhibitor linagliptin on the lipopolysaccharide (LPS)-induced dysfunction in endothelium.** LPS treatment activates white blood cells, increases DPPIV serum activity, and activates vascular cells. This leads to the infiltration of WBC to the vascular wall and oxidative damage of the vasculature. Consequently, inducible nitric oxide synthase (Nos-2), NADPH oxidase 1 and 2 (Nox1/2), and myeloperoxidase (MPO) activity increases. Finally, the tissue damage results in smooth muscle constriction and endothelial dysfunction. The green lines represent the inhibitory effects of linagliptin. Adapted from [139].

Besides a direct effect, the protective effect of DPPIV inhibition might arise from the increased half-life of GLP-1. Liu *et al.* showed that sitagliptin improves endothelial function and relaxation in a GLP-1/cAMP mediated pathway [135]. A similar experiment confirmed these findings and showed that the improvement of endothelial function was accompanied by a reduction in oxidative stress [140]. Another group showed that a sitagliptin-analogue improves endothelial function through a GLP-1

mediated increase in NO [136]. Linagliptin effectively reduced oxidative stress and inflammatory markers (VCAM-1 and COX-2) in a non-diabetic rat model of lipopolysaccharide-induced sepsis [137]. Similarly, a GLP-1R agonist lowered ICAM-1 expression and improved endothelial function in Apolipoprotein E negative mice [141]. The same agonist was also shown to increase NO production in streptozotocin-induced diabetic rats [142]. However, these data should be interpreted with caution as it was shown that GLP-1 also can exert its effects independent from the GLP-1R [143].

Another hypothesis is based on a GLP-1 independent NO increase. This increase might be due to a direct effect of the DPPIV inhibitors on the vascular endothelium. In vitro, alogliptin induced vascular relaxation in aortic segments of mice in a cGMP/NO mediated pathway [144]. *In vivo*, sitagliptin and linagliptin increase cGMP in rodent experiments [137, 145]. As cGMP induces NO release and NO is a strong anti-inflammatory molecule it can revert the inflammation underlying the endothelial dysfunction [146].

The last possible hypothesis is based on the recruitment of progenitor cells through an increased half-life of stromal-cell-derived factor 1 α (SDF1α). DPPIV rapidly inactivates SDF1α(1-68), generating SDF1α(3-68) [147]. SDF1α or CXCL12 is a multi-faceted chemokine involved in the chemotaxis of stem cells, cancer cells and normal leukocytes. Together with its receptor, CXCR4, it is implicated in angiogenesis, metastasis and inflammation. The expression of SDF1α is increased under hypoxic conditions. This gradient regulates the mobilization and trafficking of stem cells [148]. A specific subset of these stem cells, called endothelial progenitor cells, is able to restore the endothelial function. Interestingly, the number of endothelial progenitor cells is reduced in diabetes [149, 150]. In a mouse model of carotid injury, sitagliptin improves the functional recovery of the endothelium through an enhanced recruitment of circulating progenitor cells. This effect is abrogated with AMD3100, an antagonist of CXCR4, pointing to the involvement of SDF1α [151].

### 1.4.3 Blood pressure

It is already longer known that diabetes predisposes an individual to arterial hypertension. Likewise, endothelial dysfunction is associated with hypertension [152, 153]. Hypertension occurs twice as frequent in patients with diabetes as in the healthy population [154]. Recently, an increased plasma DPPIV activity was shown to independently correlate with the risk of new-onset hypertension in a healthy Chinese population [155]. DPPIV inhibitors have been shown to increase, decrease or to have no effect at all on blood pressure [156–159]. Nonetheless, a meta-analysis concluded that DPPIV inhibitors have no, or only a small beneficial effect on blood pressure [114].

Although this might seem contradicting, an explanation can be sought in a recent publication in which the effect of DPPIV inhibitors was shown to be context-dependent [160]. While sitagliptin-treatment results in an increase of blood pressure in spontaneously hypertensive rats, blood pressure decreases in rats with the metabolic syndrome (Zucker Diabetic-Sprague Dawley rats). Finally, no effect was observed in normotensive rats [160]. Marney *et al.* showed that the effect of sitagliptin in humans is influenced by the dose of enalapril. In the presence of a low dose, sitagliptin acts synergistically on blood pressure reduction, while in combination with a high dose enalapril this reduction is attenuated [161]. These studies suggest a complicated interplay of several vasoactive DPPIV substrates in which a variety of mechanisms may contribute to the antihypertensive effect of DPPIV inhibitors (Figure 1.5).

The most likely candidates for the rise in blood pressure seem to be neuropeptide Y (NPY) and peptide YY (PYY). Both peptides are able to bind to the Y receptor family. However, DPPIV-mediated truncation from NPY(1-36) and PYY(1-36) to NPY(3-36) and PYY(3-36) alters their Y receptor selectivity [45, 53]. Where the truncated forms bind the Y2 and Y5 receptor and increase nitric oxide formation, the intact peptides bind the Y1 receptor, potentiating angiotensin II mediated vasoconstriction [53, 162].

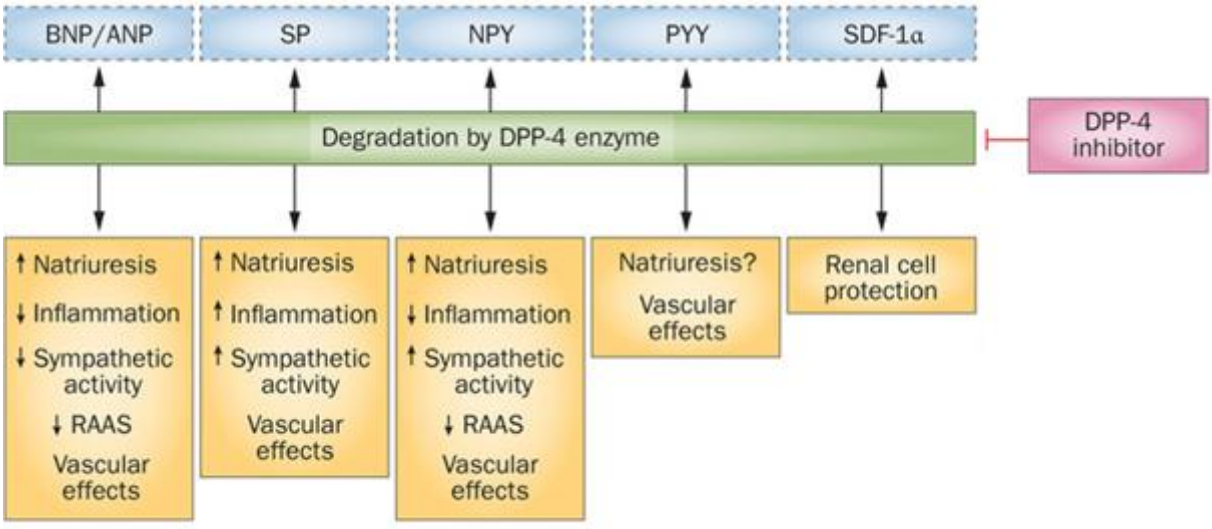



**Figure 1.5: Overview of the important protective renovascular mechanisms of DPPIV inhibitors in hypertension.** Adapted from [163].

Two candidates for the hypotensive effect are GLP-1 and B-type natriuretic peptide (BNP). In a recent meta-analysis, GLP-1R agonists were shown to lower blood pressure more effectively than placebo, insulin or sulfonylurea in patients with T2DM [164]. Another meta-analysis by Robinson *et al.,* confirmed the blood pressure lowering effect compared to placebo [165]. This effect might be mediated through the natriuretic and diuretic actions of GLP-1. Intravenous GLP-1 administration results in a decrease in sodium-hydrogen exchanger 3 (NHE3) activity in the proximal tubules of the kidney [166]. Similarly, DPPIV inhibition decreases NHE3 activity, resulting in an increased sodium excretion and urine output [167]. The natriuretic and diuretic effects of GLP-1 might also be mediated through atrial natriuretic peptide (ANP). GLP1-R activation in the cardiac atria of mice promotes ANP release and results in a reduction of blood pressure [168]. ANP is a cardiac hormone that activates natriuretic peptide receptor A (NPRA), resulting in reduced sodium and water reabsorption [169].

BNP is a DPPIV substrate functionally and structurally related to ANP. In contrast to ANP, BNP is predominantly present in the cardiac ventricles where it is produced and released in the circulation in response to cardiac stress [169]. The cleavage of BNP(1-32) to BNP(3-32) by DPPIV, results in the

loss of its biological activity [170]. Intact BNP(1-32) acts on NPRA, resulting in increased natriuresis and diuresis [169]. After infusion BNP(1-32) lowers the blood pressure in rabbits and heart failure patients [171, 172]. This is supported by the fact that patients with hypertension have reduced circulating BNP(1-32) levels [173].

### 1.4.4 Stroke

Stroke is the acute reduction of blood supply to the brain and can be either hemorrhagic (aneurysm) or ischemic (thrombosis, embolism). About 90% of all strokes are ischemic. WHO has proclaimed that research for efficacious therapeutic options is needed, including agents that are neuroprotective and improve stem cell homing [174].

Interestingly, DPPIV is known to modulate the biological activity of several neuroprotective substrates and neuropeptides (Figure 1.6) [20, 175, 176]. Among them GLP-1 has been most extensively studied. It has been shown in rodents that the GLP-1R is increased in the ischemic region one day after stroke onset [177]. Treatment of diabetic rats with a GLP-1R agonist after stroke attenuates neuronal damage, probably through an anti-inflammatory effect [178, 179]. The same is true for pre-or post-treatment of non-diabetic rodents, as shown by a decrease in stroke size and an improved functional outcome [177, 180, 181].

Intracerebral administration of a non-specific DPPIV inhibitor, IPC1755, or sitagliptin reduces cortical lesions after stroke in rats [182]. Despite their neuroprotective effect, DPPIV inhibitors are not able to cross the blood-brain-barrier [122]. Nevertheless, oral linagliptin pretreatment reduces stroke severity in both diabetic and control mice [183]. The same effect was observed for alogliptin in healthy mice, suggesting an indirect effect through DPPIV substrates [184]. Up to date, the neuroprotective effect was only shown for DPPIV inhibitor pretreatment [185]. A recent study of sitagliptins efficacy in the Taiwanese population did not shown any effect on cerebrovascular outcome [186].

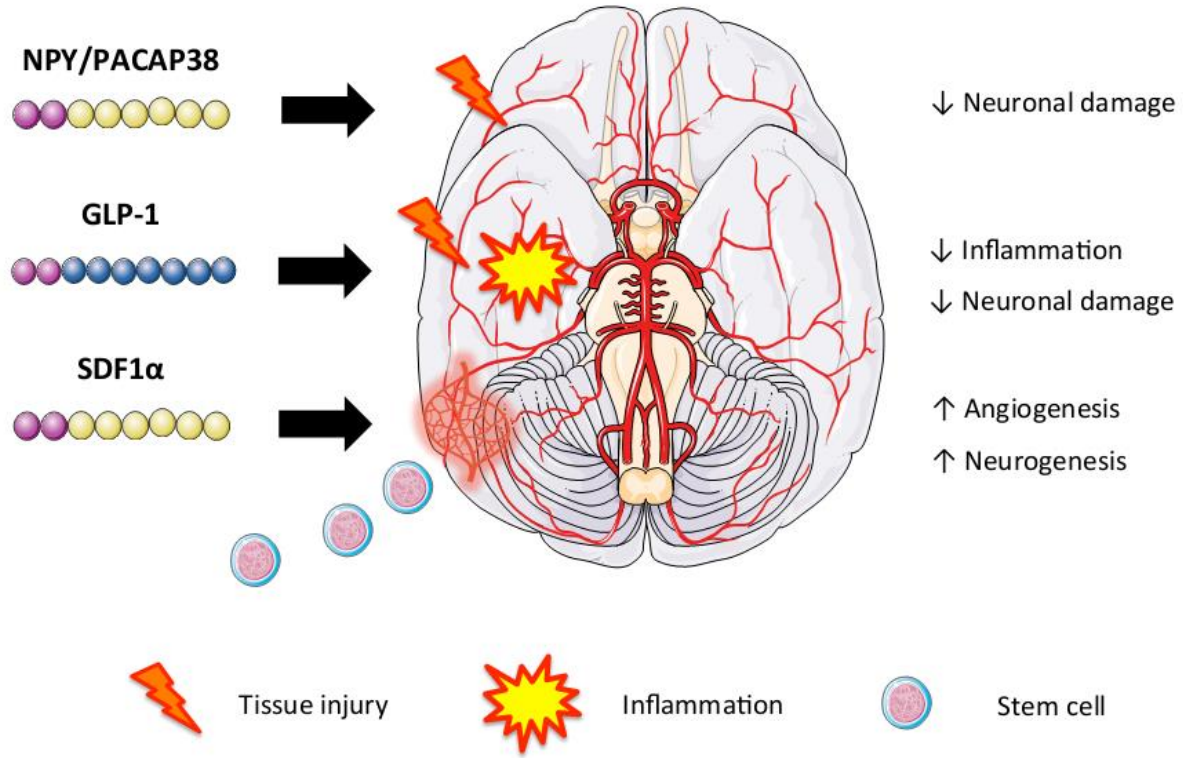



**Figure 1.6: Overview of the important protective mechanisms of DPPIV inhibitors in stroke.**

Even though stroke data are scarce about the effect of sitagliptin on substrates beside GLP-1, their effect cannot be excluded. For example, it has been shown that NPY polymorphisms are associated with an increased stroke risk [187]. In women, plasma NPY concentrations seem to be decreased after ischemic stroke [188]. The intravenous administration of a related peptide, PACAP38, before or within 4 hours of stroke onset lowers the brain damage by half [189]. The same neuroprotective effect, compared to PACAP38, was not observed for the related molecule, vasoactive intestinal peptide (VIP) [176].

Finally, a DPPIV substrate that might be involved in stroke pathology is SDF1α. It has been shown to improve functional response in the long term in an adenoviral delivery experiment 1 week after stroke [175]. In the clinic, targeted delivery is a more feasible alternative. Using this technique, Kim *et al.* demonstrated that SDF1α increased neurogenesis and angiogenesis, but did not have an anti-

inflammatory effect nor did it increase cell survival [190]. The same might also hold true for diabetic animals, but this has not been tested. However, it was shown that the SDF1α expression is reduced in the plasma and brain of obese diabetic rats (db/db) [191].

### 1.4.5 Myocardial infarction

Myocardial infarction is characterized by myocardial cell death due to prolonged ischemia [192]. Studies in diabetic and non-diabetic animals have already shown the protective effect of DPPIV inhibitor treatment, before and after myocardial infarction (Figure 1.7) [193–195]. It is known that hypoxia upregulates DPPIV expression and increases its shedding. Consequently, the inactivation of several DPPIV substrates may be increased [26, 196, 197].

Huisamen *et al.* showed that a 4-week pretreatment with an experimental DPPIV inhibitor of obese pre-diabetic mice, resulted in smaller infarct sizes accompanied by an increase in GLP-1 [198]. Similarly, vildagliptin and sitagliptin reduce infarct size through a GLP-1/PKA mediated pathway [193]. Conversely, Yin et al. reported no effect on infarct size in non-diabetic rats with immediate or late vildagliptin inhibition, despite an increase in GLP-1 levels [199]. In patients with acute myocardial infarction and reduced left-ventricular ejection fraction (LVEF), GLP-1 infusion after reperfusion effectively improves LVEF and functional recovery of the heart [200]. In pigs with myocardial infarction, GLP-1R agonist post-treatment reduced the myocardial infarct size [201]. In contrast with these results is the finding that the pretreatment of pigs with a GLP-1R agonist does not reduce infarct size 2 hours post infarct [202]. The same was observed with GLP-1 [203]. These results indicate the importance of the time of administration.

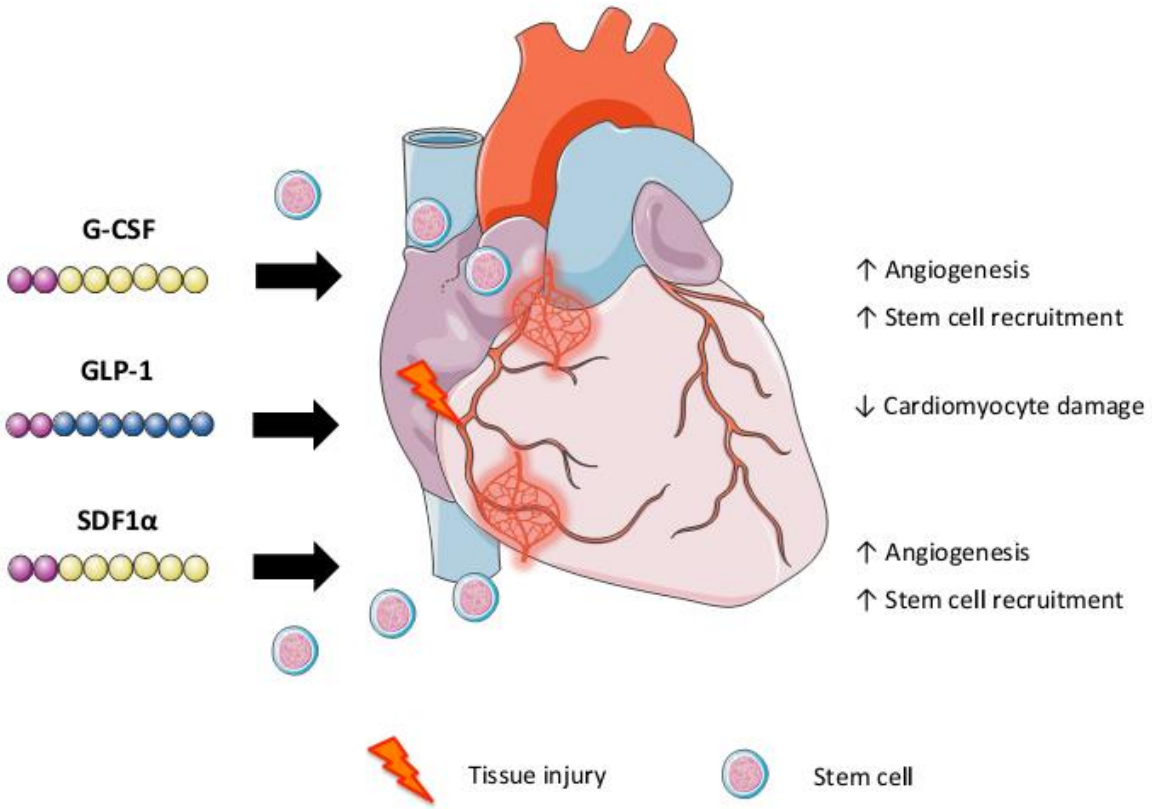



**Figure 1.7: Overview of the important protective mechanisms of DPPIV inhibitors in myocardial infarction.**

Clinical data suggest that the modulation of the SDF1α/CXCR4 axis also results in an improved myocardial repair. Therefore, prolongation of SDF1α bioactivity is one of the most plausible explanations for the beneficial effect of DPPIV inhibitors. Similar to DPPIV, SDF1α is upregulated during hypoxia [204]. The local increase in SDF1α is a powerful chemoattractant for circulating progenitor cells and promotes their survival, thus increasing myocardial repair [205]. DPPIV inhibitors prevent the inactivation of SDF1α and result in an increased neovascularization and survival which can be abolished by the use of the CXCR4 blocker, AMD3100 [206]. In addition, the local injection of a protease-resistant SDF1α after ischemia/perfusion increases angiogenesis and improves outcome, without affecting infarct size [207].

In recent years, stem cell therapy and mobilization has emerged as a potential treatment of myocardial infarction. Granulocyte colony stimulating factor (G-CSF) is able to induce stem cell mobilization from the bone marrow and is considered to be a potential treatment of acute

myocardial infarction [208]. Its administration in mice mobilizes stem cells and improves cardiac function after infarction [209]. However, sitagliptin monotherapy is more effective compared to G-CSF in the induction of stem cell mobilization. The combination of both molecules is even more effective and was shown to be a safe and promising therapy after acute myocardial infarction in the SITAGRAMI trial [210]. [211].

The microRNA 29 family might also be involved in the beneficial effect of DPPIV inhibitors. This microRNA is downregulated after myocardial infarction in the region of the infarct region and its downregulation is thought to be associated with a worse outcome [212]. Interestingly, linagliptin is able to increase the expression of the microRNA 29 family in the endothelium of diabetic mice [213].

### 1.4.6 Heart failure

Heart failure is a clinical syndrome characterized by dyspnea and edema. It often arises as one of the complications of acute myocardial infarction or chronic hypertension [214]. While DPPIV inhibitors are regarded as cardiovascular safe drugs, caution has been advised with regard to the development of heart failure. This effect has only been observed with saxagliptin and it is not yet clear if this is a class effect or that it is restricted to saxagliptin itself [114, 215].

Nevertheless, DPPIV inhibitors have been shown to be beneficial in several studies. Plasma DPPIV activity increases upon induction of heart failure in rats and is further increased in the presence of diabetes [216]. This increase in DPPIV activity was also reported by another group [217]. In addition, this group observed an inverse correlation between DPPIV activities and LVEF [217]. In both studies, the DPPIV inhibitor-treated group showed less cardiac dysfunction and remodeling [216, 217]. In diabetic mice, sitagliptin improves left-ventricular function independently from glucose control [145]. Furthermore, sitagliptin was shown to improve the glomerular filtration rate and stroke volume in pigs with heart failure. Interestingly, this treatment potentiated the effect of exogenous BNP [218]. In humans, patients with chronic heart failure have higher DPPIV activity as controls [217]. High DPPIV serum levels in patients with acute heart failure are also associated with a three-fold higher

mortality risk within 6 months due to heart failure [219]. In a small cohort of T2DM patients with coronary artery disease who underwent (dobutamine) stress echocardiography, addition of sitagliptin to the treatment regimen resulted in an increased myocardial performance and improvement of ischemia [220].

The restoration of cardiac dysfunction during DPPIV inhibitor-treatment might be related to BNP. While high BNP immunoreactivities are used as a marker for heart failure, it is known that biologically active BNP(1-32) is decreased or absent in heart failure [221, 222]. Clinical BNP levels correlate well with circulating BNP(3-32), indicating the importance of DPPIV [222]. In an ongoing clinical trial (NCT02312427), the influence of several DPPIV inhibitors on BNP levels in T2DM patients is investigated [223].

DPPIV inhibitors might also affect the natriuretic peptide receptors. It was recently shown that these are deregulated in obesity and type 2 diabetes mellitus. While natriuretic peptide receptor A, the physiological BNP receptor, is downregulated; its clearance receptor, natriuretic peptide receptor C seems to be upregulated by obesity and type 2 diabetes [224]. Improved glycemic control during DPPIV inhibitor-treatment might contribute to a normalization of natriuretic peptide receptor expression.

GLP-1 has also been attributed cardioprotective effects in heart failure. GLP-1 treatment of heart failure prone rats resulted in preservation of LVEF [225, 226]. A one-time use of GLP-1R agonists was shown to effectively reduce the pulmonary wedge pressure, a measure of left ventricular output, in T2DM patients with chronic heart failure [227]. These effects could not be replicated in a non-diabetic population with heart failure [228]. However, in human (dobutamine) stress echocardiography, a GLP-1R agonist protects the heart from ischemic left-ventricular dysfunction [229].

The prolonged half-life of biologically active SDF1α might also contribute to an improvement in cardiac function. This has been shown for SDF1α gene therapy in rats and in a phase I study [230]

[231]. However, a follow-up study (STOP-HF trial, NCT01643590) could only demonstrate the clinical safety of this approach [232]. In contrast with these results, high circulating SDF1α levels have been linked to the development of heart failure and its severity in hospitalized patients [233–235].

DPPIV inhibitors might also have a direct effect on the failing myocardium. In salt-sensitive hypertensive rats with heart failure, linagliptin treatment protects against fibrosis independently from blood glucose and blood pressure [236]. As vildagliptin and sitagliptin have a similar effect as linagliptin, it might be concluded that DPPIV inhibition in general has anti-fibrotic effects [145, 237]. This fibrosis is a consequence of ventricular remodeling and leads to loss of ventricular function. DPPIV inhibition also increases titin phosphorylation. Titin is a cardiac protein that determines myocardial stiffness and is regulated by its phosphorylation. DPPIV inhibition results in an improvement of left ventricular function and passive stiffness [145]. As it is becoming increasingly clear that endothelial dysfunction and heart failure can be considered as different parts of the same disease, the positive effects of DPPIV inhibition on the endothelium might also contribute to a protective effect in heart failure [238].

### 1.4.7 Clinical trials

Currently, the results of several large trials on the cardiovascular endpoints of DPPIV inhibitors have been published. Meanwhile, several others are still ongoing. An overview of these trials can be found in Table 1.3.

The **SAVOR TIMI trial** examined the effect of saxagliptin treatment in a mature (>40y) type 2 diabetic population with cardiovascular disease or at high risk. Compared to placebo, saxagliptin did not reduce or increase cardiovascular mortality, myocardial infarction or ischemic stroke when added to the standard of care in these patients. Strikingly, saxagliptin did increase the hospitalization rate for heart failure, in particular during the first year of therapy. A combination of biomarkers and clinical factors was able to identify a high risk population. In general, patients with a high overall risk for

heart failure (history of heart failure, high NT-proBNP levels, …) had the highest hospitalization rate. Nevertheless, no sign of volume overload or increase in NT-proBNP levels was observed [215, 239].

The **EXAMINE trial** explored the effect of alogliptin in adult T2DM patients with a high cardiovascular risk compared to placebo. No increase in cardiovascular mortality or morbidity was observed for alogliptin after 18 months. However, no decrease was observed either. An analysis of a subgroup with heart failure did not reveal more hospitalizations due to heart failure or worse outcomes. The same was true for patients without a history of heart failure, although a small increase (1%) was observed compared to the placebo group. Nevertheless, use of diuretics and NT-proBNP levels were similar in both treatment groups. In addition, overall cardiovascular mortalities were lower after alogliptin treatment [240, 241].

The TECOS trial enrolled T2DM patients with an existing cardiovascular disease, eligible for secondary prevention. As the patients had a modest hyperglycemia, the addition of sitagliptin to their therapy could be expected. As the study just ended, no results have been made public concerning the effect of sitagliptin on cardiovascular effects [242]. Other completed studies are SAXATH and VIVIDD, but their results are not yet published.

**Table 1.3: Overview of clinical trials with a cardiovascular endpoint.** Completed studies are indicated in bold.

| Name | Code | Treatment | Inclusion criteria | Primary outcome | Number of patients |
|---|---|---|---|---|---|
| **EXAMINE** | **NCT00968708** | **Alogliptin (25mg; 1xd)<br>Placebo** | **T2DM (HbA1C 6,5-11%)<br>>18y<br>ACS** | **Primary Major Adverse Cardiac Events ( %)** | **5400** |
| CAROLINA | NCT01243424 | Linagliptin (5mg; 1xd)<br>Glimepiride (1-4mg; 1xd) | T2DM (HbA1C 6,5-8,5%)<br>45 - 80y<br>Cardiovascular disease | Time to cardiovascular event | 6000 |
| CARMELINA | NCT01897532 | Linagliptin (5mg; 1xd)<br>Placebo | ≥40y<br>T2DM (HbA1C 6,5-10%)<br>Cardiovascular risk | Time to cardiovascular event | 8300 |
| n.a. | NCT02350478 | Linagliptin (5mg; 1xd)<br>Placebo | T2DM (diet or metformin)<br>40 - 80y<br>Coronary atherosclerosis | endothelial function (flow) | 50 |
| **SAVOR-TIMI** | **NCT01107886** | **Saxagliptin (5mg; 1xd)<br>Placebo** | **≥40y<br>T2DM (HbA1C ≥ 6,5%)<br>Cardiovascular risk** | **Participants with CV death, non-fatal MI or non-fatal ischemic stroke** | **18206** |
| **SAXATH** | **NCT01552018** | **Saxagliptin (5mg; 1xd)<br>Placebo** | **T2DM (HbA1C > 6,5%)<br>18 - 80y<br>metformin or glimepiride treatment** | **Inflammatory atherosclerosis biomarkers** | **12** |
| n.a. | NCT02377388 | Saxagliptin (5mg; 1xd)<br>Placebo | T2DM (HbA1C 6,5-12%)<br>>18y<br>AMI<br>Aspirin + P2Y12 receptor blocker | Changes on platelet aggregability | 80 |
| SCARF | NCT02481479 | Interventional<br>Saxagliptin start-up | T2DM (HbA1C 7,5-9,5%)<br>>18y<br>metformin | Change in LVEF | 40 |

| Name | Code | Treatment | Inclusion criteria | Primary outcome | Number of patients |
|---|---|---|---|---|---|
| **n.a.** | **NCT00657280** | **Interventional**<br>**Sitagliptin (100mg; 1xd)** | **Heart failure (NYHA I-III)**<br>**stable treatment regimen (>3m)**<br>**>18y** | **Myocardial Glucose Uptake** | **16** |
| **TECOS** | **NCT00790205** | **Sitagliptin (100mg; 1xd)**<br>**Placebo** | **T2DM (HbA1C 6,5-8,0%)**<br>**Cardiovascular disease** | **Time to cardiovascular event** | **14724** |
| **n.a.** | **NCT01863147** | **Sitagliptin (100mg; 1xd)**<br>**Acarbose (150mg; 1xd)** | **T2DM (HbA1C > 7,5%)**<br>**55 - 70y**<br>**CAD or MI** | **Left ventricular mass**<br>**Left ventricular volume** | **66** |
| SITAGRAMI | NCT00650143 | Sitagliptin (100mg; 1xd; 28d)<br>+ G-CSF (10µg/kg/d; 5d)<br>Placebo | Acute STEMI<br>>18y | Change of global myocardial function | 174 |
| **VIVIDD** | **NCT00894868** | **Vildagliptin (50mg; 2xd)**<br>**Placebo** | **18-80y**<br>**T2DM (> 3m)**<br>**Heart failure (NYHA I-III)**<br>**LVEF < 40%** | **Change in left ventricular ejection fraction** | **798** |
| VAAST | NCT01604213 | Vildagliptin (50mg; 1/2xd)<br>+ metformin<br>Metformin | T2DM (HbA1C ≥ 6,5%)<br>>21y<br>Stable ischemic heart disease | Reduction in serum levels of Interleukin 6 | 60 |
| **n.a.** | **NCT02456428** | **Incretine-based drug**<br>**other oral antidiabetic drug**<br>**Insulin**<br>**Placebo** | **New T2DM patient**<br>**>18y** | **Heart failure hospitalization** | **1499650** |
| n.a. | NCT02312427 | Interventional<br>1month without DPPIV inhibitor | T2DM (HbA1C < 9%)<br>>20y<br>BNP (100 - 2000 pg/ml)<br>DPPIV inhibitor treatment | Serum BNP level | 50 |

### 1.4.8 Other pathologies

#### 1.4.8.1 Osteoporosis

Osteoporosis is characterized by a low bone mass, increased bone fragility and decreased bone quality. Patients with type 2 diabetes have an increased risk of developing osteoporosis and have an increased risk of fractures. This might be caused by a change in bone quality due to microvascular complications [243].

DPPIV inhibitors have been shown to protect against bone fractures and osteoporosis in several rodent models. Sitagliptin improves bone mass and strength in diabetic rats, independent from blood glucose [244]. A similar treatment of non-diabetic mice resulted in an increase in vertebral bone mineral density [245]. A large meta-analysis of human clinical trials supports these data and shows a reduction in fractures in patients treated with DPPIV inhibitors [246]. Interestingly, the osteoprotective effect seems to be linked to the duration of treatment as the risk of fractures is lowest in patients treated for over one year [246, 247].

Several authors claim that the protective effect of the DPPIV inhibitors is due to the increased half-life of the incretins GLP-1 and GIP [246–248]. This is indeed supported by the fact that GLP-1 and a GLP-1R agonist increase bone mineral density in diabetic and non-diabetic animals [249, 250]. In addition, GLP-1R knock-out mice suffer from low bone mass and bone fragility due to increased osteoclast-mediated bone resorption [251]. Similarly, GIP-R knock-out mice show a decreased bone mass, while the reverse is true for GIP overexpressing mice [252, 253]. In vitro, GIP itself has an anabolic effect and stimulates collagen type I mRNA expression [254]. These changes in collagen might be important in type 2 diabetes as these patients do not show a change in bone mass but rather a poor bone quality due to changes in collagen [255]. Intriguingly, vildagliptin causes an accumulation of collagen-derived peptides, possibly due to an alteration in the collagen metabolism [3].

A growing body of evidence points to the involvement of advanced glycation end products (AGEs) and their receptors (RAGEs) in diabetic-induced osteoporosis [256]. Vildagliptin treatment reduced both AGEs and RAGEs vascular expression [257]. This might provide another mechanism for the osteoprotective effect of DPPIV inhibitors, as AGEs increase bone fragility.

#### 1.4.8.2 Kidney disease

The most common cause of kidney disease is diabetic nephropathy. It occurs in 40% of all diabetic patients and is diagnosed by the presence of albuminuria (>300 mg/d) or proteinuria (>500 mg/d). As most DPPIV inhibitors undergo a renal clearance, some concerns have been raised about their safety in patients with renal impairment. All inhibitors can be safely used in kidney disease after dose adjustment, only for linagliptin no dose adjustment is in order [122, 258].

Several animal models have demonstrated the renoprotective effect of DPPIV inhibitors in diabetic, as well as non-diabetic, nephropathy. After induction of glomerulonephritis in non-diabetic animals, alogliptin-treatment tended to result in less kidney injury and proteinuria and was accompanied by a reduction of inflammation [259]. Sitagliptin-treatment of diabetic rats effectively prevented diabetic nephropathy through a reduction in inflammation and apoptosis [260]. Sitagliptin and vildagliptin in rats also protect the kidneys against ischemia reperfusion injury. This was shown by a reduction in apoptosis of glomerular and tubular cells [261, 262]. Sitagliptin is able to prevent lipid nephrotoxicity in Apolipoprotein E negative mice independent from an effect on blood glucose or lipid levels [263].A similar effect was observed in mice on a high fructose and high fat diet [264]. In human type 2 diabetic patients sitagliptin also has a beneficial effect on nephropathy, as determined by albuminuria [265, 266].

Several mechanisms can be thought to be responsible for this renoprotective effect. First of all, it is known that chronic hyperglycemia leads to the development of diabetic nephropathy. Consequently, antidiabetics are able to prevent this evolution. Secondly, it is increasingly acknowledged that inflammation is a hallmark of the development and progression of diabetic nephropathy [267].

Interestingly, DPPIV inhibitors have an anti-inflammatory activity, both in and outside of nephropathy [268–270].

Thirdly, DPPIV inhibitors can influence the natriuretic and diuretic activity. As mentioned earlier, DPPIV inhibitors are thought to have an antihypertensive effect. High blood pressure causes shear-stress and results in glomerular and arterial damage [271]. DPPIV inhibitors also interact with a sodium-hydrogen exchanger, NHE3, in the proximal tubules of the rat kidney. Inhibition decreases the NHE3 activity and potentially influences volume homeostasis and thus blood pressure [167]. In addition, DPPIV might also influence natriuresis and diuresis through the prolonged biological half-life of its substrates. DPPIV substrates that have been shown to influence these parameters are, among others, BNP, GLP-1 and NPY [272–274]. The prolongation of the biological half-life of SDF1α may also contribute to the renoprotective effect in renal ischemia reperfusion injury [275] [276]. In addition, it was shown that SDF1α might be involved in the prevention of deterioration in chronic kidney disease in rats through its effect on renal microvasculature [277, 278].

Since the advent of DPPIV inhibitors for treatment of type 2 diabetes, a substantial body of evidence has shown that they exert effects beyond glucose homeostasis. The research reported in this doctoral thesis must be seen in this light.

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# Chapter 2

AIMS

# Chapter 2 - Aims

At the beginning of this doctoral thesis, DPPIV inhibitors were just suggested to have positive effects on the micro- and macrovascular complications of type 2 diabetes. At the same time, the cardiovascular safety of the new antidiabetics was questioned. Our research investigated the physiological level of two DPPIV substrates in heart failure, the effect of DPPIV inhibitors on dipeptides in osteoporosis and the DPPIV enzyme activities following stroke onset. This allowed us to better define the pharmacological profile of DPPIV inhibitors outside glucose homeostasis. These four aims are shown schematically in Figure 2.1.

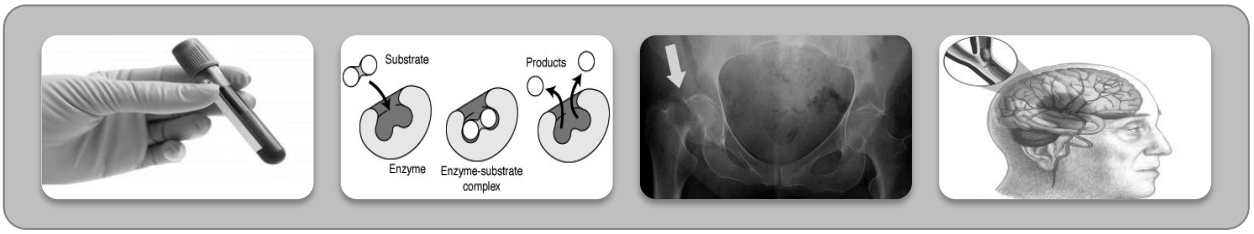


**Figure 2.1: Overview of the aims of this doctoral thesis.**

Our first aim (**aim 1**) was to optimize the blood sample collection protocol for DPPIV substrates. As SDF1α is one of the substrates with the shortest *in vivo* half-life [1], we optimized our protocol based on this chemokine. SDF1α is a multifaceted molecule that is investigated in different pathologies such as cardiovascular disease, HIV infection and cancer. In these domains SDF1α is often measured using commercial immunoassays. Even though, proteolytic degradation can result in an altered immunoreactivity, these specifications are generally not provided by the manufacturers. As SDF1α is proposed to prevent the development of heart failure [2], we decided to investigate its levels in this pathology with the optimized sampling protocol.

Over 40 *in vivo* and *in vitro* DPPIV substrates have been reported in literature. These substrates span a broad range of physiological activities ranging from chemotaxis to natriuresis and diuresis [3]. Several other DPPIV substrates are thought to exist. During this thesis, we came across another possible DPPIV substrate, namely nesfatin-1. This molecule shares similarities with B-type natriuretic peptide (BNP). Nesfatin-1 interacts with the BNP receptor and is able to activate it, resulting in the modulation of myocardial function[4]. Because nesfatin-1 might be responsible for the cardiovascular protection observed with DPPIV inhibitors, the second aim (**aim 2**) was to investigate if nesfatin-1 is a DPPIV substrate.

During DPPIV-mediated cleavage, both a cleaved substrate and a dipeptide arise. While most interest goes out to the cleaved substrate, the resulting dipeptides should not be forgotten. These Xaa-Pro dipeptides have been shown to possess biological activity, increasing bone growth and promoting osteoblast differentiation *in vitro* [5, 6]. Therefore, our third aim (**aim 3**) was to develop a method to determine circulating dipeptides and amino acids in biological fluids. Because amino acids are readily measured in plasma using mass spectrometry [7], a similar approach was taken for the analysis of dipeptides, in particular the collagen-derived Xaa-Pro dipeptides. Our interest is based on the finding that vildagliptin-treatment of rats results in an accumulation of collagen-derived peptide fragments. Moreover, DPPIV has a collagen type I binding site and possesses a substrate specificity for denatured collagen [8, 9]. As collagen is the most abundant protein in the human body, derived circulating dipeptides can be expected to be present in high levels. This facilitates the detection of the Xaa-Pro dipeptides. In the second part of this aim, we investigated the effect of sitagliptin on collagen breakdown in diabetic osteoporosis using a mass spectrometric method.

DPPIV is negatively involved in myocardial and renal ischemia/reperfusion injury. However, its involvement in cerebral ischemia/reperfusion or stroke is understudied. Increased glucose levels at admission are associated with worse outcomes and antidiabetics reduce stroke severity [10]. DPPIV inhibitor pretreatment is neuroprotective, reducing neuronal loss and improving functional outcome

in rodents [11]. As this neuroprotection has only been shown in animal models and the expression of DPPIV after acute stroke is unknown, our last aim (**aim 4**) was to characterize the circulating DPPIV activity in stroke.

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**Chapter 3**

# MATERIALS & METHODS

# Chapter 3 - Materials and methods

## 3.1 Inhibitors

Diisopropyl fluorophosphate (DFP) was purchased from Acros. Sitagliptin (SG) was extracted from Januvia tablets (Merck) by a three-step procedure as described in [1]. Vildagliptin (VG) was custom-synthesized by GLSynthesis, Inc. (purity >98%, consistent with $^{1}$H NMR). Complete protease inhibitor cocktail tablets were purchased from Roche and used according to manufacturer's instructions. This cocktail inhibits a broad spectrum of serine, cysteine and metalloproteases.

## 3.2 Enzymes

### 3.2.1 Dipeptidyl peptidase IV

Soluble human DPPIV was purified from seminal fluid as described previously [2]. One unit (U) of activity is described as the amount of enzyme required to catalyze the conversion of 1 µmol of substrate per minute (0.5 mM Gly-Pro-*p*-nitroanilide in 50-mM Tris buffer; pH 8.3) at 37 °C.

### 3.2.2 Fibroblast activation protein α

Recombinant murine fibroblast activation protein (FAP) was purified from human HEK293 kidney cells.

## 3.3 Enzyme activities

### 3.3.1 Dipeptidyl peptidase IV

DPPIV activities were measured and *in vivo* inhibition was estimated as reported by Matheeussen et al. [1]. In short, 10 µL serum was mixed with 100 µL of a 50-mM Tris buffer (pH 8.3) containing 0.5 mM Gly-Pro-4-methyl-β-Naphtylamide. The release of 4-methyl-β-Naphtylamide was measured for 10 min at 37 °C ($\lambda_{ex}$ = 340 nm; $\lambda_{em}$ = 430 nm). Fluorescence intensity was related to a 4-methyl-β-

naphtylamide standard curve (0.75 – 20 µM) diluted in the same buffer. From these activities the *in vivo* inhibition could be estimated [1].

### 3.3.2 Fibroblast activation protein α

FAP activity was measured using Z-Gly-Pro-AMC. Standards and samples were diluted in a 0.1 M Tris buffer (pH 8.0) containing 300 mM NaF, 1 mM $NaN_3$, 1 mM EDTA and 50 mM salicylic acid. Substrate (final concentration 184 µM) was added and incubated for 2 h at 37 °C. Reactions were stopped with 1.5 M acetic acid and fluorescence was measured ($\lambda_{ex}$ = 370 nm, $\lambda_{em}$ = 440 nm). In these assay conditions all measured activity is attributable to FAP when measured in serum or plasma.

### 3.3.3 Gelatinases

MMP2 and 9 gelatinolytic activities were measured through gelatin zymography based on the work of Kleiner [3]. Gels were prepared by incorporating 0.1% gelatin type A in a 7.5% polyacrylamide mixture (BioRad Mini-PROTEAN; 8.3 x 7.3 cm; 0.75 mm). Serum samples were diluted to 4 g protein/L in zymography sample buffer (100 mM Tris, 1.25% SDS, 5% Glycerol and 0.01% Bromophenol blue at pH 6.8). A sample volume of 10 µL was loaded onto the gel and run for 2 h at 80 V. Afterwards, SDS was washed out by incubating the gels for 1h on a horizontal rocker in washing buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$ and 2.5% Triton X-100 at pH 7.5). The gels were then transferred to enzyme buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij 35 at pH 7.5) and incubated overnight at 37 °C followed by staining of the gels with Coomassie. Results were analyzed using Totallab software and normalised using a control serum sample.

### 3.3.4 Prolidase

Prolidase activities were determined by measuring the production of proline from Glycylproline (Gly-Pro) [4]. Serum was incubated for 2h at 37 °C after which 20 µL was taken and added to 20 µL of the substrate solution (94 mM Gly-Pro, 500 mM Tris and 1mM $MnCl_2$, pH 8.0). The reaction was stopped after 30 min at 37 °C by adding 200 µL trichloroacetic acid (0.45 M). In the blanc vials the substrate

solution was added after stopping the reaction. The samples were centrifuged and 200 µL of both acetic acid and Chinard's reagent (2.5% Ninhydrin, 30 mL glacial acetic acid and 20 mL 6M phosphoric acid) was added to 100 µL of the supernatant. This mixture was heated to 90 °C for 15 min. 200 µL was added to a 96-well plate and absorbance was measured at 515 nm.

## 3.4 Immunoassays and antibodies

An overview of all used immunoassays can be found in Table 3.1. All immunoassays were performed as instructed by the manufacturer. Only for the Nesfatin-1/NUCB2 duoset the reagent diluent was replaced by 25% of Fetal Calf Serum in PBS for the preparation and dilution of the standards and samples.

**Table 3.1: Overview of the commercial immunoassays used in this doctoral thesis.**

| Manufacturer | Immunoassay | Component | Species | Component specifics |
|---|---|---|---|---|
| RnD systems | CXCL12/SDF-1 Duoset | Capture Ab | Mouse | anti-human/mouse SDF1 |
| | | Detection Ab | Goat | anti-human/mouse SDF1 (biotinylated) |
| | | Enzyme conjugate | | Streptavidin-HRP |
| | Nesfatin-1/NUCB2 duoset | Capture Ab | Sheep | anti-human nesfatin-1 |
| | | Detection Ab | Sheep | anti-human nesfatin-1 (biotinylated) |
| | | Enzyme conjugate | | Streptavidin-HRP |
| Peprotech | SDF1α mini EDK | Capture Ab | Rabbit | anti-human SDF1α |
| | | Detection Ab | Rabbit | anti-human SDF1α (biotinylated) |
| | | Enzyme conjugate | | Avidin-HRP |
| Raybiotech | SDF1α ELISA Kit | Capture Ab | Mouse | anti-human SDF1α |
| | | Detection Ab | Goat | anti-human SDF1α (biotinylated) |
| | | Enzyme conjugate | | Streptavidin-HRP |

*Ab* *Antibody*
*EDK* *ELISA Development Kit*
*ELISA* *enzyme-linked immunosorbent assay*

For the immunoaffinity coupled to gel filtration chromatography, a nesfatin-1 /NUCB2 polyclonal sheep antibody (catalog no. AF5949, RnD systems) was covalently linked to magnetic S-Tosyl beads.

## 3.5 Western blotting

Western blotting was done with a 10% SDS-PAGE gel. After transfer, the membrane was blocked in 2% tween 20 in PBS and incubated for 2 h at room temperature with the biotinylated primary antibody and 20 min with a streptavidin-HRP solution (catalog no. DY5949 part 843232 and part 890803, RnD systems). The membrane was developed with the supersignal west femto chemiluminescent substrate (Thermo Fischer).

## 3.6 *In vitro* peptide cleavage

### 3.6.1 Stromal-cell-derived factor 1 α

To study SDF1α truncation *in vitro*, the recombinant peptide provided with the CXCL12/SDF-1 RnD duoset was used for spiking (500 pg/ml). SDF1α was completely truncated through an incubation of one hour at 37 °C in the presence of DPPIV [5]. As a control, DPPIV was first inhibited by a 10-min pre-incubation at 4 °C with 1 mM DFP. To study *ex vivo* cleavage, serum (Bio-Rad; level 2 liquid assayed multiqual chemistry control serum) was spiked with SDF1α and incubated at room temperature (25 °C) or 37 °C for one hour. As a control, the serum was pre-incubated with protease inhibitors (100 µM sitagliptin and 1x complete protease inhibitor cocktail).

### 3.6.2 Nesfatine-1

Recombinant human nesfatin-1, produced in E. *coli* was obtained from peprotech (Catalog Number: 300-67). To study the decay curves of nesfatin-1, 2 µl of enzyme solution was incubated with 1 µl of nesfatin-1 at 37 °C (final concentration DPPIV: 25 U/l and FAP 11 U/l) in 7 µl of 10 mM ammonium bicarbonate solution (pH 7.4). At regular time intervals 1.5 µl samples were withdrawn and quenched with a 1.5 µl mixture of acetonitrile and 0.2% formic acid containing 4mg/ml α-Cyano-4-

hydroxycinnamic acid (CHCA, Sigma-Aldrich). 0.4 µl of this mixture was spotted on a stainless steel MALDI target plate (Applied Biosystems) and allowed to air-evaporate.

Mass spectrometric analysis was performed on a 4800 MALDI TOF/TOF analyzer (Applied Biosystems/MDS SCIEX) equipped with a Nd:YAG 200-Hz laser. The samples were analyzed in linear mode. Absolute concentrations were calculated based on the amount of nesfatin-1 present in the sample and the nesfatin-1(1-82) to nesfatin-1(3-82) ratio.

Steady-state kinetic parameters were determined by the incubation of different nesfatin-1 concentrations (0.5 – 20 µM) with fixed enzyme concentrations. The ratio of the intact and cleaved molecule was used to calculate the amount of substrate converted as described by Lambeir *et al.* [5].

## 3.7 Dipeptide and amino acid analysis

A Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS) method was developed based on the EZ-FAAST kit from Phenomenex [6]. The method was optimized for the analysis of two amino acids (proline, Pro and hydroxyproline, Hyp) and four Pro-containing dipeptides (Pro-Hyp, Gly-Pro, Pro-Pro and Ala-Pro). In short, the procedure involves solid phase extraction, followed by derivatization of the extracted dipeptides and amino acids. Internal standard (methionine-d3; 100 µL; 0.8 µM for dipeptides and 40 µM for amino acids) was added to 100 µL serum. Water (150 µL) was added to decrease the viscosity of serum. The extraction and derivatization was performed with the sorbent tips and reagents provided by the manufacturer of the EZ FAAST kit. An Agilent LC1290 Infinity system was used with a Kinetex Phenomenex C18 100 x 2.1 mm, 2.6 µm column. The mobile phase consisted of 10 mM ammonium formate in water (solvent A) and 10 mM ammonium formate in methanol (solvent B), in an elution gradient consisting of 30% B, then to 90% B in 4 min, stay at 90% B for 2 min, then to 30% B in 0.1 min, stay at 30% B for re-equilibration for 4 min. The injection volume was 10 µL and the flow was 0.3 mL/min. An Agilent 6410 triple quadrupole mass spectrometer was used in electrospray ionization in positive mode, with the following source

parameters: gas temperature 350°C, gas flow 8 L/min, nebulizer 20 psi, capillary voltage 4000 V. The mass spectrometry parameters can be found in Table 3.2, intra-day and inter-day precision are given in Table 3.3.

**Table 3.2: MRM transitions, dwell times (ms), fragmentor and collision energy voltages (V) for the targeted amino acids and dipeptides.**

| Compound name | Precursor Ion | Product Ion | Dwell time (ms) | Fragmentor (V) | Collision Energy (V) |
|---|---|---|---|---|---|
| **Hyp** | 260.2 | 200 | 10 | 70 | 0 |
| | 260.2 | 172.1 | 10 | 70 | 10 |
| | 260.2 | 157 | 10 | 70 | 5 |
| **Pro** | 244.3 | 184 | 10 | 70 | 0 |
| | 244.3 | 156.2 | 10 | 70 | 5 |
| | 244.3 | 114 | 10 | 70 | 5 |
| **Pro-Hyp** | 357.2 | 297 | 10 | 90 | 10 |
| | 357.2 | 184 | 10 | 90 | 10 |
| | 357.2 | 156.3 | 10 | 90 | 10 |
| **Pro-Pro** | 341 | 281.2 | 10 | 80 | 10 |
| | 341 | 184.2 | 10 | 80 | 10 |
| | 341 | 156 | 10 | 80 | 15 |
| **Ala-Pro** | 315 | 158.2 | 10 | 80 | 10 |
| | 315 | 130.1 | 10 | 80 | 15 |
| | 315 | 70.2 | 10 | 80 | 15 |
| **Gly-Pro** | 301.3 | 241 | 10 | 100 | 5 |
| | 301.3 | 158.2 | 10 | 100 | 10 |
| **IS-d3MET** | 281.1 | 221 | 10 | 80 | 10 |
| | 281.1 | 193.2 | 10 | 80 | 10 |
| | 281.1 | 142 | 10 | 80 | 10 |

## 3.8 Immunoaffinity coupled to gel filtration chromatography

A nesfatin-1 /NUCB2 polyclonal sheep antibody (catalog no. AF5949, RnD systems) was covalently linked to magnetic S-Tosyl beads (catalog no. PR-MAG00069, Mobitec). This was done in a ratio of 20 µg antibody per mg beads, according to the protocol of the M-280 Tosylactivated Dynabeads® (Rev.010).

150 µl of the final bead solution was incubated with 750 µl serum on a tube rotator overnight at 4 °C. Afterwards, beads were washed 3 times with 1 ml ammonium bicarbonate buffer (50 mM, pH 7.6). Elution was done twice with 80 µl glycine (100 mM, pH 2.5) and pooled in a tube containing 10µl tris buffer (1.5 M, pH 8.8).

150 µl of the eluate was applied on a gel filtration column (sephacryl S100 HR, volume 42 ml, diameter 10 mm) and run at 1 ml/min with ammonium bicarbonate buffer (50 mM, pH 7.6). Fractions of 1 ml were collected and concentrated in a rotary evaporator (Savant Speedvac) before western blot analysis.

**Table 3.3: Intra-day and inter-day precision (expressed as RSD%) for the analysis of amino acids and dipeptides.**

| | | | Intra-day precision | | | |
|---|---|---|---|---|---|---|
| **RSD (%)** | **PRO** | **HYP** | **PRO-HYP** | **GLY-PRO** | **ALA-PRO** | **PRO-PRO** |
| DAY 1 (n=5) | 17 | 10 | 10 | 11 | 7 | 11 |
| DAY 2 (n=5) | 12 | 8 | 9 | 13 | 6 | 10 |
| DAY 3 (n=5) | 12 | 9 | 8 | 15 | 12 | 10 |

| | | | Inter-day precision | | | |
|---|---|---|---|---|---|---|
| **RSD (%)** | **PRO** | **HYP** | **PRO-HYP** | **GLY-PRO** | **ALA-PRO** | **PRO-PRO** |
| DAY 1-3 (n=15) | 13 | 11 | 9 | 13 | 16 | 11 |

## 3.9 Statistical analysis

All statistical analyses were performed by the *Statistical Package for the Social Sciences* (SPSS) version 20. All measurements are reported as mean ± standard error of the mean (SEM), unless stated otherwise.

In chapter 4, each measurement was performed 5 times to determine the specificity of the different immunoassays. All groups were compared using a Kruskal-Wallis-test, followed by a Mann-Whitney U-test when significant to compare groups head-to-head. *Post-hoc* Bonferroni correction was applied for the number of comparisons. Cardiovascular parameters, SDF1α, nesfatin-1/NUCB2 concentrations and DPPIV activities in the patient samples were analyzed using a one-way ANOVA followed by Bonferroni's post-hoc tests if necessary. In chapter 7, a one-way ANOVA or t-test for independent samples was used to compare enzyme activities between groups, while a t-test for paired samples was used for time-points within a group. Bonferroni post hoc analysis or correction was applied where necessary. Correlations between stroke severity, outcome or evolution and changes in enzyme activity over 7 days after stroke onset ($\Delta DPPIV_{day7}$ and $\Delta FAP_{day7}$) were assessed with bivariate analysis. For continuous data such as infarct volume a Pearson correlation was used, while ordinal data, for example stroke progression, was assessed with a Spearman rank correlation coefficient. Statistical differences were determined to be significant when the p-value was below 0.05.

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# Chapter 4

# SDF1α sample collection and immunoassays

Adapted from: **Baerts L**, Waumans Y, Brandt I, Jungraithmayr W, Van der Veken P, Vanderheyden M, De Meester I.; Circulating Stromal Cell-Derived Factor 1α Levels in Heart Failure: A Matter of Proper Sampling; PLoS One. 2015 Nov 6; 10(11):e0141408.

# Chapter 4 - SDF1α sample collection and immunoassays

## 4.1 Abstract

The chemokine Stromal cell-derived factor 1α (SDF1α, CXCL12) is currently under investigation as a biomarker for various cardiac diseases. The correct interpretation of SDF1α levels is complicated by the occurrence of truncated forms that possess an altered biological activity. We studied the immunoreactivities of SDF1α forms and evaluated the effect of adding a DPPIV inhibitor in sampling tubes on measured SDF1α levels. Using optimized sampling, we measured DPPIV activity and SDF1α levels in patients with varying degrees of heart failure.

The immunoreactivities of SDF1α and its degradation products were determined with three immunoassays. A one hour incubation of SDF1α with DPPIV at 37 °C resulted in 2/3 loss of immunoreactivity in each of the assays. Incubation with serum gave a similar result. The extent of *ex vivo* cleavage of endogenous SDF1α under similar conditions was determined to be around 75%. Using appropriate sampling, SDF1α levels were found to be significantly higher in those heart failure patients with a severe loss of left ventricular function. DPPIV activity in serum was not altered in the heart failure population. However, the activities were found to be significantly decreased in patients with high SDF1α levels.

We propose that all samples for SDF1α analysis should be collected in the presence of at least a DPPIV inhibitor. In doing so, we found higher SDF1α levels in subgroups of patients with heart failure. Our work supports the need for further research on the clinical relevance of SDF1α levels in cardiac disease.

## 4.2 Introduction

In recent years, the chemokine Stromal cell-Derived Factor 1α (SDF1α or CXCL12) has been shown to play a role in cardiovascular diseases [1] and to be a promising biomarker [2, 3]. Together with its receptor, CXCR4, SDF1α is involved in the homing of progenitor/stem cells thereby favoring the repair of injured myocardium through angiogenesis [1, 4–6]. In addition, there is a growing interest in SDF1α as a cardiovascular biomarker. Elevated levels are associated with a risk of heart failure [2], the extent of coronary artery disease [3], and right ventricular dysfunction in patients with idiopathic pulmonary hypertension [7].

Similar to other chemokines, an intact N-terminus is essential for its biological activity [8]. Work by Crump et al. showed that loss of the N-terminal lysine, generating SDF1$\alpha_{2\text{-}68}$, results in a complete loss of bioactivity [9]. *In vivo*, N-terminal trimming is often initiated by dipeptidyl peptidase IV (DPPIV). Trimming by DPPIV results in the formation of SDF1$\alpha_{3\text{-}68}$ which lacks chemotactic properties [9, 10]. In this regard, Christopherson et al. showed that DPPIV inhibition significantly increases homing and engraftment of hematopoietic stem cells [11]. Other enzymes that might play a role in N-terminal cleavage are leukocyte elastase, matrix metalloproteases 1, 2, 3, 9, 13 and 14, and cathepsin G generating SDF1$\alpha_{4\text{-}68}$, SDF1$\alpha_{5\text{-}68}$ and SDF1$\alpha_{6\text{-}68}$ respectively. As mentioned, these cleavage products lack biological activity [12–14].

All these findings clearly demonstrate a crucial role for DPPIV and other proteases in modulating the biological activity of SDF1α. Moreover, DPPIV inhibitors or protease-resistant SDF1α analogs might become novel therapies in pathologies such as ischemic heart disease and heart failure [1, 15–18]. In this case, the distinction between intact and cleaved SDF1α will become increasingly important to assess the biologically active SDF1α levels. Unfortunately, at present no commercially available immunoassay claims to discriminate between the intact and cleaved, and thus inactive, forms of SDF1α.

In this study, we first report on the difference in immunoreactivity between intact SDF1α and its cleavage products in commercial immunoassays. The addition of a DPPIV inhibitor to plasma tubes, as a means to prevent *ex vivo* proteolysis, profoundly affected the measurements. Based on these observations, a universal formula is proposed to calculate the percentage of *in vivo* truncated SDF1α. Secondly, the use of SDF1α and DPPIV as biomarkers were analyzed in patients with varying degrees of heart failure [19, 20].

## 4.3 Heart failure population

Consecutive patients (age 65 ± 11 years) with no heart failure (n = 34), a diagnosis of HFpEF (heart failure with preserved ejection fraction, > 40% and evidence of a diastolic left-ventricular dysfunction; n = 28) or HFrEF (heart failure with reduced ejection fraction, ≤ 40%; n = 30) [21] and a recent episode of decompensated heart failure, necessitating IV diuretic therapy, referred for diagnostic left and right heart catheterization were included in the study. Patients with HFrEF were further divided in those with compensated (characterized by a normal preload reserve) or decompensated heart failure (characterized by an impaired preload reserve) [22]. In addition, patients were categorized according to the ejection fraction (EF) in those with 'normal' ≥ 60%; 'slight loss' 59-51%; 'loss' 50-35%; 'severe loss' <35%. Patients with renal insufficiency defined by an estimated GFR (according to the modification of diet in renal disease study equation) below 60 ml/min/1.73 $m^2$ or patients that received DPPIV inhibitors at the time of the study were excluded. All patients gave oral informed consent, a procedure which, at 2006, was approved by the local medical ethical committee of the OLV hospital, Aalst, Belgium. The study complied with the declaration of Helsinki. Patients were informed that the blood could be stored for the subsequent analysis of biomarkers. The oral informed consent was documented in the electronic or paper patient file and the study was approved by the local ethical committee.

Before diagnostic catheterization when the patient was in a stable hemodynamic condition five milliliter of whole blood was drawn from the femoral vein for subsequent measurements. Blood was

collected in 7.5-ml EDTA tubes (S-monovette®; Sarstedt) with or without DPPIV inhibitor to prevent *ex vivo* cleavage (VG, 120 µM final concentration in the tube). The samples were centrifuged for 15 min (2000 x g) and were subsequently frozen at –80° C until further analysis without undergoing additional freeze-thaw cycles. Blood collected from patients with heart failure symptoms and without HFpEF or HFrEF collected were chosen as control samples.

Catheterization of the left and right sides of the heart was performed unblinded from the right femoral artery and vein. Pulmonary capillary wedge pressure was measured by use of a Swan-Ganz catheter whereas LV pressure was recorded with a catheter, positioned in the left ventricular cavity. LV angiograms were obtained in left and right anterior oblique position. Left ventricular volumes and EF were derived from the single plane angiogram using the area-length method. An impaired preload reserve was defined by the presence of LVEDP ≥ 16 mm Hg [22].

## 4.4 Results

### 4.4.1 Specificity of SDF1α ELISAs

#### 4.4.1.1 SDF1α and DPPIV

The immunoreactivity of intact vs cleaved SDF1α was tested by incubating SDF1α with DPPIV (materials and methods 3.6.1). After one hour at 37 °C, a significantly lower immunoreactivity was detected for the DPPIV-generated $SDF1\alpha_{3\text{-}68}$. This effect was observed with different lot numbers (RnDsystems) and different commercial ELISA kits (Raybiotech, Peprotech) (Figure 4.1A). As expected, the incubation of $SDF1\alpha_{1\text{-}68}$ with inactivated DPPIV did not result in a difference in immunoreactivity.

#### 4.4.1.2 SDF1α in serum

To study the cleavage of SDF1α by proteases other than DPPIV, serum was spiked with SDF1α and incubated at 25 °C and 37 °C for one hour. As control, serum was inhibited beforehand with a

combination of sitagliptin and Roche protease inhibitor cocktail. Compared to control a significantly lower SDF1α immunoreactivity was found at 25 °C and 37 °C for all kits (Figure 4.1B). No differences in SDF1α immunoreactivity could be detected between the different kits and 25 °C or 37 °C.

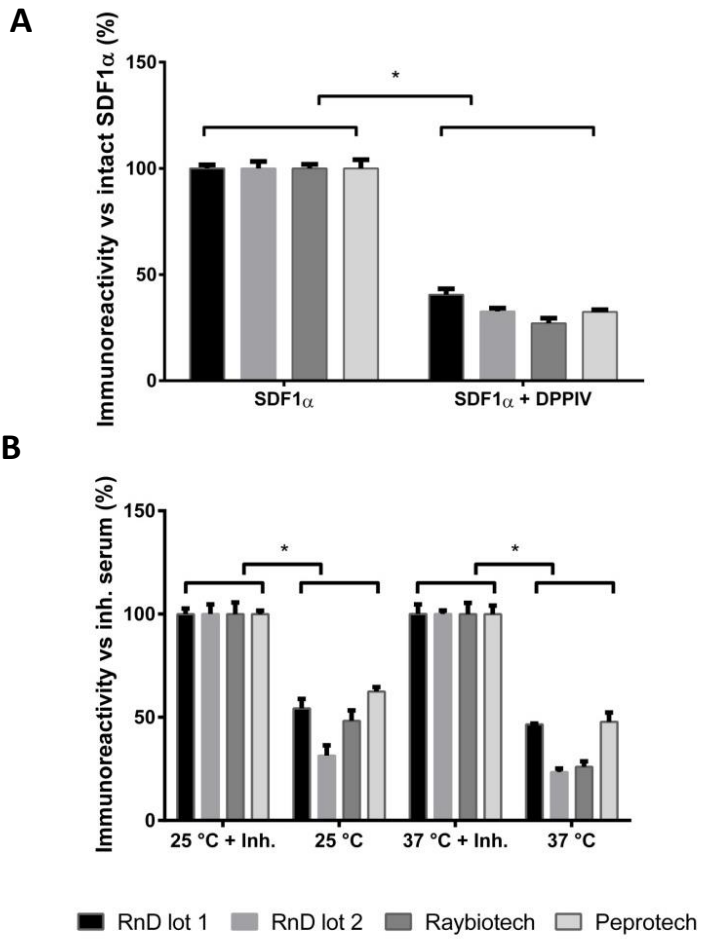


**Figure 4.1: The SDF1α immunoreactivity measured by commercially available kits.** (A) SDF1α (500pg/ml) incubated in PBS for 1 h at 37 °C was set at 100% immunoreactivity. Incubation in the presence of DPPIV (25 U/l) resulted in a significantly lower immunoreactivity compared to intact SDF1α (RnD lot 1: 40.5 ± 2.8%; RnD lot 2: 32.7 ± 1.5%; Raybiotech: 27.1 ± 2.4%; Peprotech 32.5 ± 1.2%; *p < 0.05; results ± SEM; n = 5). (B) The immunoreactivity of pure SDF1α (500pg/ml) spiked into serum and incubated for 1 h at 25 °C or 37 °C was measured with different commercial kits. As the 100% reference, SDF1α spiked into inhibited serum (DPPIV inhibitor and Roche protease inhibitor cocktail) was chosen. *Ex vivo* degradation in serum significantly decreased the immunoreactivity of SDF1α (25 °C: RnD lot 1: 54.3 ± 4.1%; RnD lot 2: 31.4 ± 6.4%; Raybiotech: 48.3 ± 5.1%; Peprotech: 62.4 ± 2.3% and 37 °C: RnD lot 1: 46.6 ± 0.4%; RnD lot 2: 23.5 ± 1.7%; Raybiotech: 25.9 ± 2.7%; Peprotech 47.8 ± 1.9%; *p < 0.05; results ± SEM; n = 5).

### 4.4.2 SDF1α in blood samples

#### 4.4.2.1 Ex vivo cleavage of endogenous SDF1α

To examine the effect of *ex vivo* cleavage of endogenous SDF1α in plasma of healthy volunteers (n = 13). Blood was collected in EDTA tubes with or without DPPIV inhibitor, immediately processed and frozen at -80 °C. The samples were thawed at 4 °C and analyzed immediately. A significantly lower

immunoreactivity was found in tubes that did not contain DPPIV inhibitor as compared to DPPIV inhibited samples (100%) (Figure 4.2).

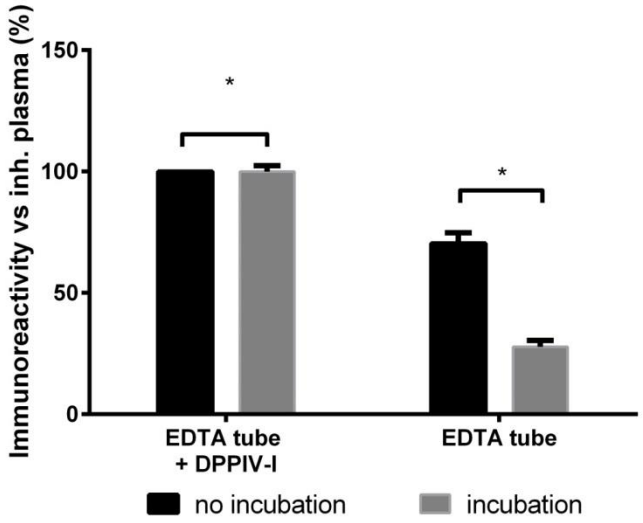


**Figure 4.2: The average SDF1α immunoreactivity of healthy plasma with the RnD SDF1α duoset** when immediately analysed (no incubation, n = 13, range [749-1776 pg/ml]) or after an incubation of 1 h at 37 °C (n = 7, range [832-1776 pg/ml]). Blood was collected in tubes with or without DPPIV inhibitor (DPPIV-I). A significantly lower immunoreactivity was found in regular tubes versus the DPPIV inhibitor containing tubes (no incubation: 67.6 ± 3.5%; incubation: 27.8 ± 2.8%; *$p < 0.05$; results ± SEM).

Since samples are often not immediately analyzed, we also determined the immunoreactivity of endogenous SDF1α after an incubation of one hour at 37 °C (n = 7). As expected, the immunoreactivity of the samples without DPPIV inhibitor was significantly lower compared to the DPPIV-inhibited samples (Figure 4.2).

Finally, the immunoreactivity observed in DPPIV inhibited samples did not rise further after addition of Roche Protease Inhibitor cocktail on top of the DPPIV inhibitor.

#### 4.4.2.2 Circulating intact SDF1α

From these data and the immunoreactivity of SDF1α spiked in serum, we propose a universal formula to estimate the percentage of circulating truncated SDF1α. As an example, we describe the calculation of the *in vivo* truncated SDF1α with RnD lot 2. In Figure 4.1B we observed that, after one hour at 37 °C, the immunoreactivity drops to 23%.

$$IR_{\%} = (SDF1\alpha_{in\_vivo\_truncated\%}) + (SDF1\alpha_{ex\_vivo\_truncated\%})$$

$$IR_{\%} = (SDF1\alpha_{in\_vivo\_truncated\%}) + 23\% * (100\% - SDF1\alpha_{in\_vivo\_truncated\%})$$

$$IR_{\%} - (23 * (100\% - SDF1\alpha_{in\_vivo\_truncated\%})) = (SDF1\alpha_{in\_vivo\_truncated\%})$$

$$IR_{\%} - (23\%) + (23\% * SDF1\alpha_{in\_vivo\_truncated\%}) = (SDF1\alpha_{in\_vivo\_truncated\%})$$

$$IR_{\%} - 23\% = (SDF1\alpha_{in\_vivo\_truncated\%}) - (23\% * SDF1\alpha_{in\_vivo\_truncated\%})$$

$$IR_{\%} - 23\% = SDF1\alpha_{in\_vivo\_truncated\%} * (100\% - 23\%)$$

$$\frac{IR_{\%} - 23\%}{(100\% - 23\%)} = SDF1\alpha_{in\_vivo\_truncated\%}$$

*$IR_{\%}$: immunoreactivity after incubating tube without inhibitors for 1 h at 37 °C*

*$SDF1\alpha_{in\_vivo\_truncated\%}$: immunoreactivity of the in vivo truncated molecule*

Solving this equation, we find that 7% of the immunoreactivity of the remaining immunoreactivity (28%, Figure 4.2) in these samples is due to *in vivo* truncated SDF1α. Consequently, the amount of *in vivo* truncated SDF1α is approximately 25%. In other words, three quarters of all circulating SDF1α is N-terminally intact.

#### 4.4.2.3 SDF1α levels, DPPIV activity and hemodynamic parameters

Characteristics of the study population are summarized in Table 4.1. In the entire study population, SDF1α levels ranged from 491 to 2550 pg/ml, median 1033 [915-1143] pg/ml, as measured from samples collected on a mixture of DPPIV and protease inhibitors.

Patients were divided into tertiles of SDF1α. A significantly lower DPPIV activity was found in patients with low compared to those with high SDF1α levels. Other cardiovascular parameters did not differ between groups (Figure 4.3).

Although no difference in SDF1α levels was observed between controls, HFpEF and HFrEF patients (Figure 4.4A). Patients with severe LV dysfunction had significantly higher SDF1α concentrations (Figure 4.4B) DPPIV activity was similar in all of the investigated heart failure subgroups.

**Table 4.1: Patients characteristics according to tertiles of SDF1α levels.**

| | | SDF1α Tertiles | | | |
|---|---|---|---|---|---|
| | | Tertile 1 (n = 32) | Tertile 2 (n = 32) | Tertile 3 (n = 31) | p-value |
| **Mean SDF1α** | pg/ml | 836 | 1044 | 1369 | |
| | *range* | *491 - 955* | *959 - 1119* | *1120 - 2550* | |
| **Age** | years | 64 | 66 | 66 | 0.750 |
| | *SD* | *8* | *12* | *10* | |
| **Men** | n | 24 | 18 | 18 | 0.129 |
| | *%* | *75* | *56* | *58* | |
| **Heart Rate** | bpm | 73 | 67 | 72 | 0.386 |
| | *SD* | *15* | *11* | *16* | |
| **Ejection Fraction** | %max | 71 | 65 | 63 | 0.224 |
| | *SD* | *18* | *17* | *20* | |
| **EDP** | mmHg | 19 | 14 | 19 | 0.078 |
| | *SD* | *8* | *4* | *16* | |
| **EDVI** | $ml/m^2$ | 71 | 76 | 72 | 0.709 |
| | *SD* | *26* | *27* | *19* | |
| **ESVI** | $ml/m^2$ | 24 | 29 | 27 | 0.632 |
| | *SD* | *21* | *21* | *21* | |
| **DPPIV activity** | U/l | 24 | 23 | 20 | **0.042** |
| | *SD* | *5* | *7* | *7* | |
| **10-year survival** | n | 22 | 24 | 20 | 0.399 |
| | *%* | *69* | *75* | *65* | |

DPPIV: Dipeptidyl Peptidase IV;

SDF1α: Stromal cell-Derived Factor 1 alpha

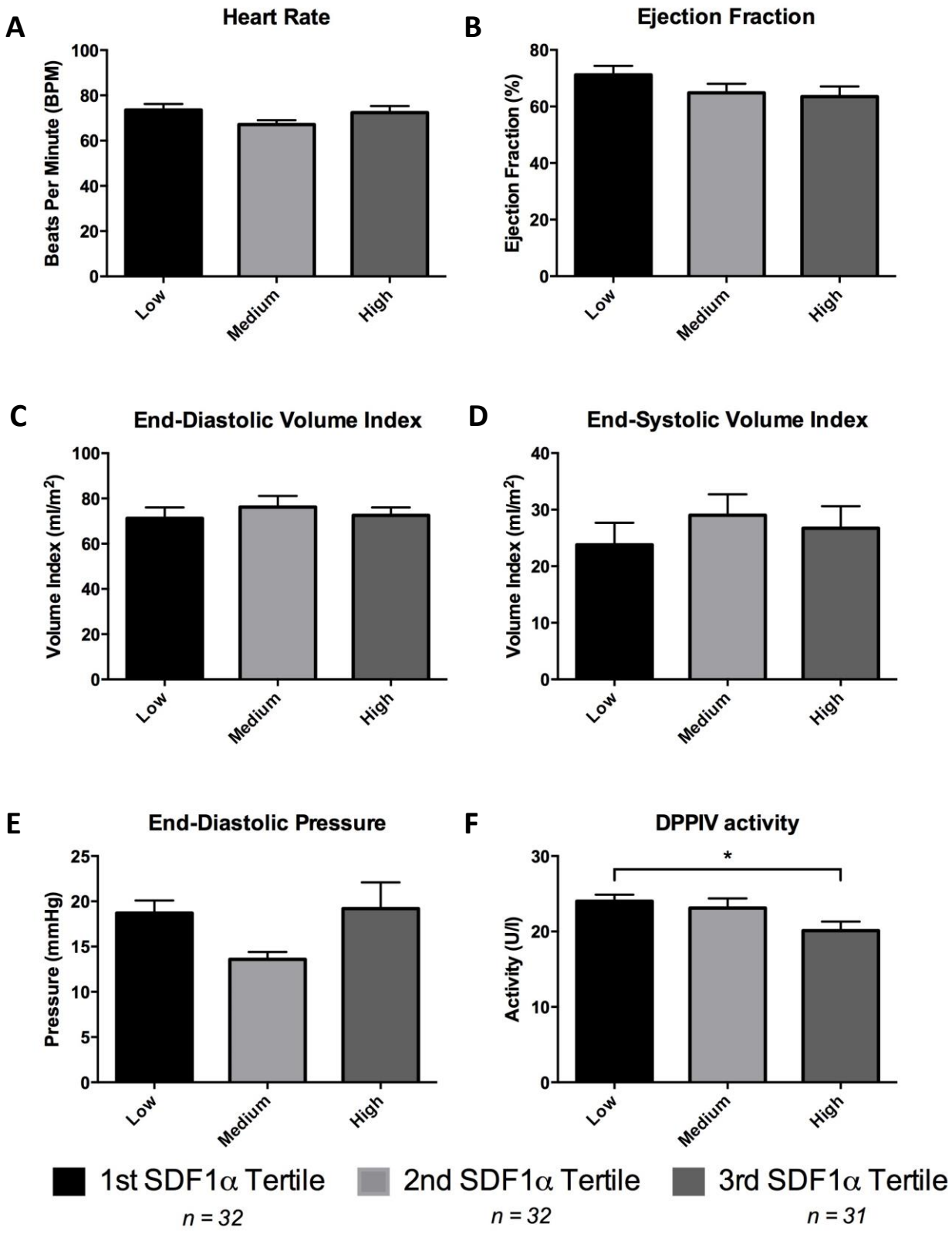


**Figure 4.3: Comparison of cardiovascular parameters between SDF1α tertiles** (Low = First Tertile, Medium = Second Tertile and High = Third Tertile). No significant differences were observed in the Heart Rate (A), Ejection Fraction (B), End-Diastolic Volume Index (C), End-Systolic Volume Index (D) and End-Diastolic Pressure (E). The first and third tertile had significantly different DPPIV activities (F). All samples were collected on a mixture of DPPIV and protease inhibitors.

## 4.5 Discussion

### 4.5.1 Quantification of the in vivo circulating SDF1α

SDF1α currently receives a lot of interest within cardiovascular research [2, 5, 18]. Although current clinical immunoassays have a high sensitivity and reproducibility, it remains unknown which kind of fragments these antibodies exactly recognize. As this leads to misinterpretation, a method to quantitate the *in vivo* circulating intact SDF1α was developed using commercially available immunoassays.

Our study revealed that frequently used commercially available immunoassays react differently towards intact SDF1α as compared to DPPIV-truncated SDF1α. This might have clinical consequences as DPPIV circulates freely in the blood and is anchored in the endothelial cell membrane. Consequently, DPPIV is able to lower the immunoreactivity both *in vivo* as well as *ex vivo* [23], resulting in an underestimation of physiological concentrations. In addition, other proteases might also contribute to SDF1α degradation [12–14]. However, the remaining immunoreactivity after incubation with DPPIV and serum was similar, which suggests that the observed *ex vivo* loss in signal is due to N-terminal truncation. The relative contribution of DPPIV was confirmed *ex vivo* with healthy plasma samples and is in line with previous findings of Kanki *et al.* [1]. This loss in immunoreactivity most likely reflects the immunogenic properties of the highly basic N-terminus. Even though we found this to be true for all tested immunoassays, these characteristics are often poorly specified by the manufacturer.

Based on our findings, we decided to propose a method to calculate the percentage of circulating intact SDF1α. We found that about 75% of all circulating molecules are intact in the plasma of healthy volunteers. This is the first study to estimate the *in vivo* N-terminal truncation. Furthermore, this method can be used to elucidate the *in vivo* effect of DPPIV inhibitor.

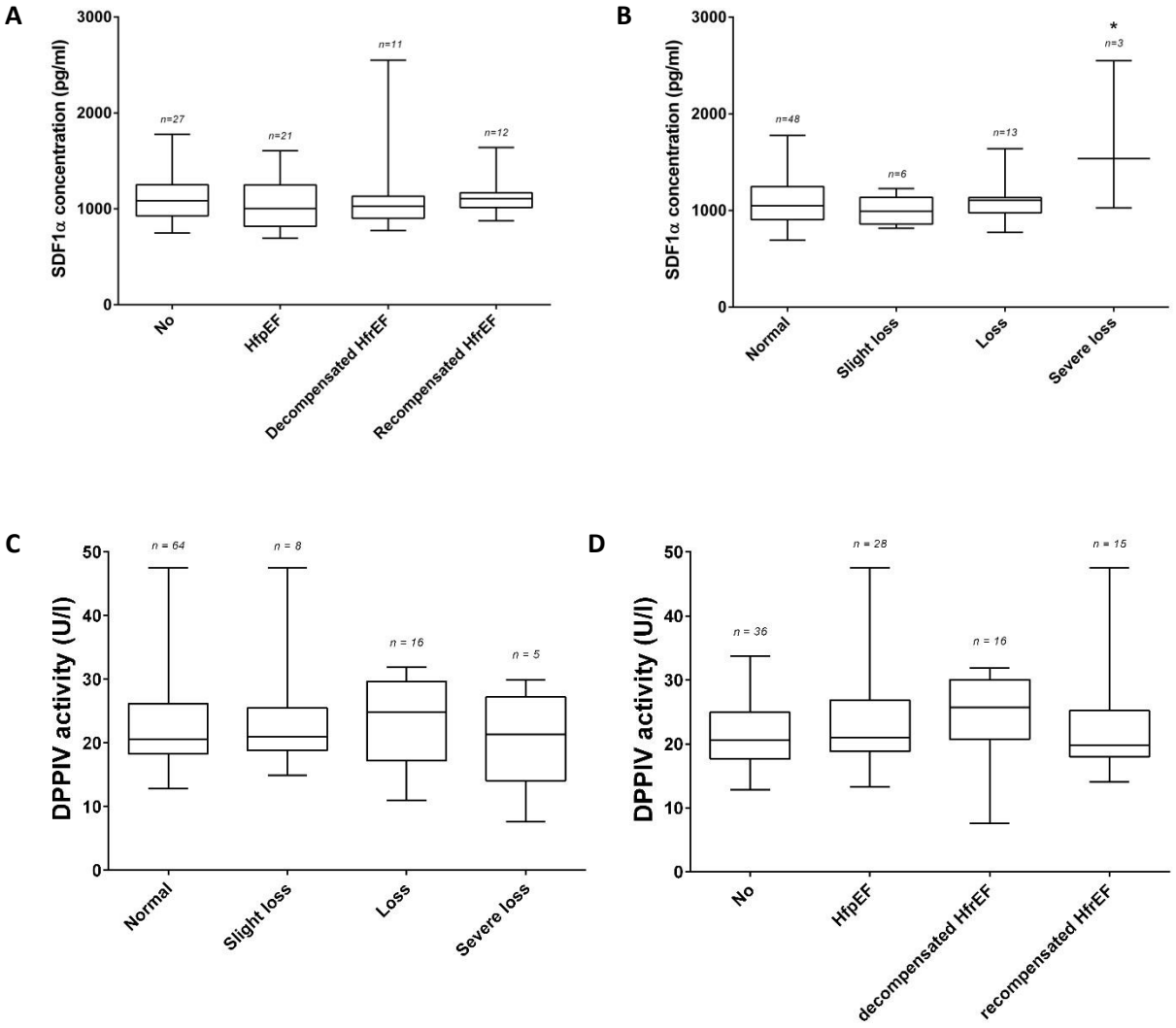


**Figure 4.4: SDF1α concentrations and DPPIV activities in patient samples collected in tubes with DPPIV inhibitor.** (A) No difference was found between patients with a different type of LV dysfunction (none 1096 ± 47 pg/ml; HFpEF 1043 ± 55 pg/ml; decompensated HFrEF 1201 ± 145 pg/ml; recompensated HFrEF 1109 ± 51 pg/ml). (B) For the different severities of LV dysfunction a significant difference was found in patients with a severe loss of LV function (normal 1076 ± 36 pg/ml; slight loss 1002 ± 62 pg/ml; loss 1090 ± 55 pg/ml; severe loss 1705 ± 447 pg/ml;). (C) No difference was found between patients with a different type of LV dysfunction (none 21.7 ± 0.9 U/l; HFpEF 23.0 ± 1.3 U/l; decompensated HFrEF 24.0 ± 1.8 U/l; recompensated HFrEF 22.3 ± 2.0 U/l). (D) or for the different severities of LV dysfunction (normal 22.2 ± 0.8 U/l; slight loss 24.2 ± 3.6 U/l; loss 23.6 ± 1.6 U/l; severe loss 20.8 ± 3.7 U/l; *p < 0.05).

### 4.5.2 Clinical Implication

The *ex vivo* truncation, which occurs during sample handling, storage or even incubation of the ELISA plate, results in an underestimation of the physiological SDF1α concentrations. Of note, even under optimal pre-analytical conditions, there is a significant (more than 30%) loss of SDF1α-immunoreactivity in samples that did not contain a DPPIV inhibitor.

Our results show that *ex vivo* cleavage of SDF1α by DPPIV can lead to misinterpretations of laboratory results. This is especially relevant when dealing with patients that are treated with DPPIV inhibitors or that have aberrant DPPIV activities, for example due to hyperglycemia or hypoxia [24, 25]. To overcome this problem, we highly recommend collecting all samples in tubes containing at least a DPPIV inhibitor to block any additional *ex vivo* cleaving. The clinical importance of this procedure is illustrated by the fact that an intact N-terminus is linked to SDF1α's cardioprotective effects [1].

Our data is in line with other *in vivo* studies utilizing DPPIV inhibitors and measuring SDF1α levels. Most studies use the RnD immunoassay and all groups reported significantly higher SDF1α levels upon treatment with DPPIV inhibitor [15, 26, 27]. Taking our results into account, these findings are suggestive of higher levels of intact and thus active circulating SDF1α.

The stabilization of *in vivo* intact SDF1α by DPPIV inhibition can be a valuable therapeutic strategy after myocardial infarction. Zaruba et al. showed that genetic deletion or pharmacological inhibition of DPPIV in combination with Granulocyte-Colony-Stimulating Factor led to improved heart function and survival [16]. Another strategy is to locally inject a protease-resistant SDF1α. This improves cardiac function after cardial ischemia and might provide an additional therapy for heart failure [1, 28].

### 4.5.3 DPPIV activity and SDF1α as biomarkers

In recent years, DPPIV activity and SDF1α have been suggested as possible biomarkers for heart failure [2, 19, 20]. Therefore, we evaluated both in patients referred for elective diagnostic cardiac catheterization.

We found DPPIV activities to be lower in patients with high SDF1α levels. This difference in activity could point to the *in vivo* post-translational regulation of SDF1α [29]. In addition, patients with a severe loss of LV function showed a marked increase in SDF1α. Several mechanisms might be

responsible for this observation. First, the higher wall stress with concomitant subendocardial ischemia may induce SDF1α production thereby mobilizing stem cells to the injured myocardium. Secondly, apart from beneficial effects, SDF1α might also have detrimental effects and depress LV function. It was recently shown that SDF1α has a negative inotropic effect through binding with its receptor CXCR4 [30]. Our results, in combination with a recent finding [2], point to the possible role of SDF1α as a biomarker in heart failure and warrant further investigation.

Surprisingly, no difference in DPPIV activities could be found between any of the investigated groups. This is in contrast with recent data demonstrating increased DPPIV activity levels in patients with heart failure [19, 20]. One study focused on diastolic heart failure and only found a weak correlation with DPPIV activity in peripheral venous plasma [19], while a second study only included patients with a Left Ventricular Ejection Fraction lower than 45% [20]. As we included a more heterogeneous group of heart failure patients, this could partially account for the discrepancy.

## 4.6 Conclusion

We demonstrated that the N-terminal truncation of SDF1α profoundly affects the immunoreactivity measured by ELISA irrespective of the commercially available kit used. Additionally, we found that in the absence of protease inhibitors *ex vivo* cleavage cannot be prevented and that more than a third of the immunoreactivity is lost. We therefore recommend collecting all samples in tubes with protease inhibitors, at least including a DPPIV inhibitor. The possible value of DPPIV activities and SDF1α levels as biomarkers for heart failure was also evaluated. DPPIV activities were found to be lower in patients with high SDF1α levels. DPPIV activities were similar in the investigated heart failure subgroups. In contrast, SDF1α plasma levels were significantly elevated in patients with a severe loss of LV function. The observation of lower DPPIV activity in patients with high SDF1α as well as the presence of elevated SDF1α in patients with severe LV dysfunction warrants further investigation. Therefore, additional research is needed into the use of SDF1α as a biomarker and the role of DPPIV as a target in patients with heart failure.

## 4.7 Limitations

The main limitation is the small number of severe heart failure patients included in our study. The data concerning this population should be interpreted with caution and confirmed with a larger population. A second limitation is the missing information regarding the epitopes recognized by the antibodies. Unfortunately, these could not be obtained from the suppliers as they considered it to be proprietary information. In addition, our proposed formula is based on a single time point and only gives a rough estimation of the circulating intact SDF1α. Another limitation is that the influence of C-terminal truncation on SDF1α immunoreactivity was not investigated in a direct manner. The last limitation is that while patient samples were collected on a mixture of DPPIV and protease inhibitors, the method did not allow to determine the amount of *in vivo* intact versus *in vivo* truncated SDF1α.

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# Chapter 5

## Nesfatin-1/NUCB2 and DPPIV

In preparation

# Chapter 5 - Nesfatin-1/NUCB2 and DPPIV

## 5.1 Abstract

Nesfatin-1 was originally discovered as a satiety molecule, generated from nucleobindin-2 (NUCB2). It was recently discovered that nesfatin-1 is implicated in the cardiovascular system. Nevertheless, much remains unknown about its physiology. Meanwhile, it is clear that nesfatin-1 shares similarities with B-type natriuretic peptide (BNP), a validated biomarker in heart failure. Both peptides can activate natriuretic peptide receptor A and have a proline N-terminally in the penultimate position, a hallmark of dipeptidyl peptidase IV (DPPIV) substrates.

The substrate properties of nesfatin-1 for soluble dipeptidyl peptidases, present in the circulation, were investigated by an *in vitro* incubation. The circulating nesfatin-1 molecular forms were characterized using immunoaffinity coupled to gel filtration chromatography. The circulating levels were determined with an immunoassay in patients suspected of heart failure.

Nesfatin-1 was shown to be a substrate of dipeptidyl peptidase IV, but was more rapidly cleaved by fibroblast activation protein. Only the precursor molecule, NUCB2, was detected in the serum of both healthy controls and heart failure patients. Additional western blot experiments demonstrated the presence of a high molecular weight NUCB2. Determining NUCB2 concentrations revealed a trend towards lower levels in patients with decompensated heart failure with reduced ejection fraction.

Our data suggest that further research of nesfatin-1 in the circulation should focus on NUCB2. In addition, the use of NUCB2 as a biomarker in heart failure deserves further investigation.

## 5.2 Introduction

Nesfatin-1 was originally discovered as a 82 amino acid peptide, generated from Nucleobindin 2 (NUCB2, ± 50 kDa) [1]. Immunohistological staining revealed the presence of nesfatin-1/NUCB2 in the appetite regulating regions of the hypothalamus [1]. Interestingly, intracerebrovascular and peripheral injection decreased food intake in a dose-dependent manner[1, 2].

Subsequent research mainly focused on the anorexigenic properties of this molecule. Co-localization experiments in the brain showed proximity of nesfatin-1/NUCB2 with pro-opiomelanocortin (POMC), vasopressin, oxytocin, somatostatin, corticotropin-releasing-hormone and growth-hormone-releasing-hormone [3, 4].

However, recently the focus has been shifted from its appetite regulating properties to a cardiovascular role [5]. This interest stems from the finding that nesfatin-1 induces a tachycardic and hypertensive effect after intracerebral injection [6, 7]. Subsequently, Angelone *et al.* showed that nesfatin-1 also has a direct effect on the heart. In addition, nesfatin-1 immunoreactivity was shown in the heart and plasma [8]. Its precursor, NUCB2, has also been implicated in heart failure. Atrial mRNA levels were shown to be reduced in women with coronary injury. In addition, the authors suggested that this might also be the case for nesfatin-1 and NUCB2 protein levels [9]. Later it was shown that patients with an acute myocardial infarction have 20% lower nesfatin-1 plasma levels than controls or patients with stable angina pectoris [10]. Similarly, serum nesfatin-1 was shown to be decreased in diabetic patients with peripheral artery disease [11].

It is important to note that the pathways induced by nesfatin-1 and its physiology are not yet fully understood. For example, a specific receptor is not yet known. However, due to a similarity with the B-type natriuretic peptide (BNP) it is able to stimulate natriuretic peptide receptor A (NPRA) [8]. Moreover, like BNP, it possesses an N-terminal X-Pro motive, characteristic of a dipeptidyl peptidase

IV (DPPIV) substrate. This sequence, consisting of Val-Pro-Ile, is similar to the apparent DPPIV inhibitors diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) [12].

Many other signaling molecules and receptors, besides NPRA, have been shown to be implicated in its physiological effects [13, 14]. Furthermore, it is still unclear which molecular forms circulate in the bloodstream [5]. For the moment, nesfatin-1 has only been unequivocally observed in rat cardiac tissue and cerebrospinal fluid [1, 8].

In this work we had three aims. First of all, we sought to investigate if nesfatin-1 is a substrate of DPPIV and fibroblast activation protein (FAP), two soluble dipeptidyl peptidases present in the circulation [15, 16]. Second, we wanted to determine the circulating NUCB2 molecular forms and fragments as we wanted to study its serum levels in heart failure.

## 5.3 Results

### 5.3.1 Nesfatin-1 substrate properties

Nesfatin-1 decay curves were determined with DPPIV (25 U/l) and FAP (11 U/l), a related extracellular protease (materials and methods 3.6.2). Both proteases were able to degrade nesfatin-1(1-82) to nesfatin-1(3-82). However, degradation by FAP was much faster in the experimental conditions used. The $K_m$ was determined to be higher than 20 µM for both (Figure 5.1). The specificity constant ($k_{cat}/K_m$) was determined to be $(0.006 \pm 0.002)*10^{-6}\ M^{-1}s^{-1}$.

### 5.3.2 Nesfatin-1 inhibitory properties

As nesfatin-1 was found to be a slow substrate for DPPIV, just as the apparent DPPIV inhibitors diprotin A and B, the ability of nesfatin-1 to inhibit 25 U/l DPPIV was tested with Gly-Pro-*p*-nitroanilide (0.5 mM). No inhibition could be observed in a concentration range from 0.1 µM to 10 µM nesfatin-1. As small molecule substrates might be able to “bypass” inhibitors in the catalytic center, resulting in cleavage of Gly-Pro-*p*-nitroanilide, we repeated the experiments with SDF1α, a

well-known peptide substrate. At the highest concentration (10 µM) nesfatin-1 did not prevent SDF1α cleavage (data not shown).

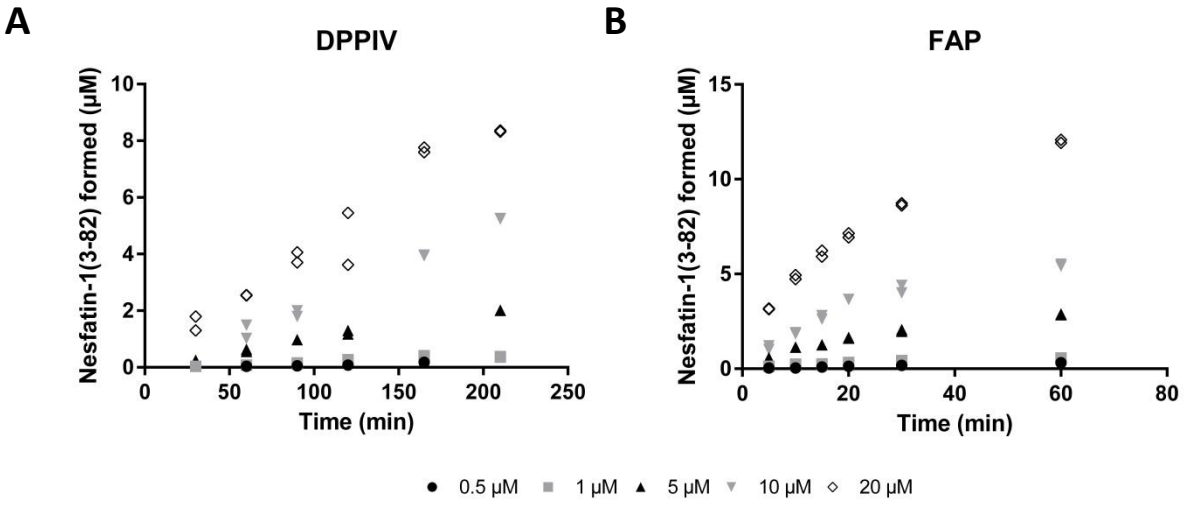


**Figure 5.1: Generation of nesfatin-1(3-82) from different concentrations of intact nesfatin-1(1-82) by DPPIV (25 U/l) and FAP (11 U/l).** (A) The $K_m$ was determined to be higher than 20 µM for DPPIV. (B) The $K_m$ was also higher than 20 µM for FAP. The starting concentrations of intact nesfatin-1 are indicated below the graphs.

### 5.3.3 Circulating molecular forms of nesfatin-1/NUCB2

Nesfatin-1 /NUCB2 molecules were enriched from serum on antibody-coated magnetic beads. Gel filtration chromatography was then used to determine whether the circulating molecules were nesfatin-1, NUCB2 or a mixture of both (materials and methods 3.8). As nesfatin-1 is suspected to have similar properties as BNP, this experiment was done for both healthy controls and patients with heart failure (n = 3 for both groups, selected from the heart failure population mentioned in 4.3 heart failure population). After gel filtration, a protein peak was only detected in the high molecular weight fraction, corresponding with NUCB2. In contrast, serum spiked with recombinant nesfatin-1, showed an additional peak in the low molecular weight fraction. This finding was confirmed using western blot analyses. Besides a band around 50 kDa, corresponding with NUCB2, a second band was detected which might be a glycosylated form (Figure 5.2). No difference in UV elution profile was found between samples from healthy controls and patients with heart failure.

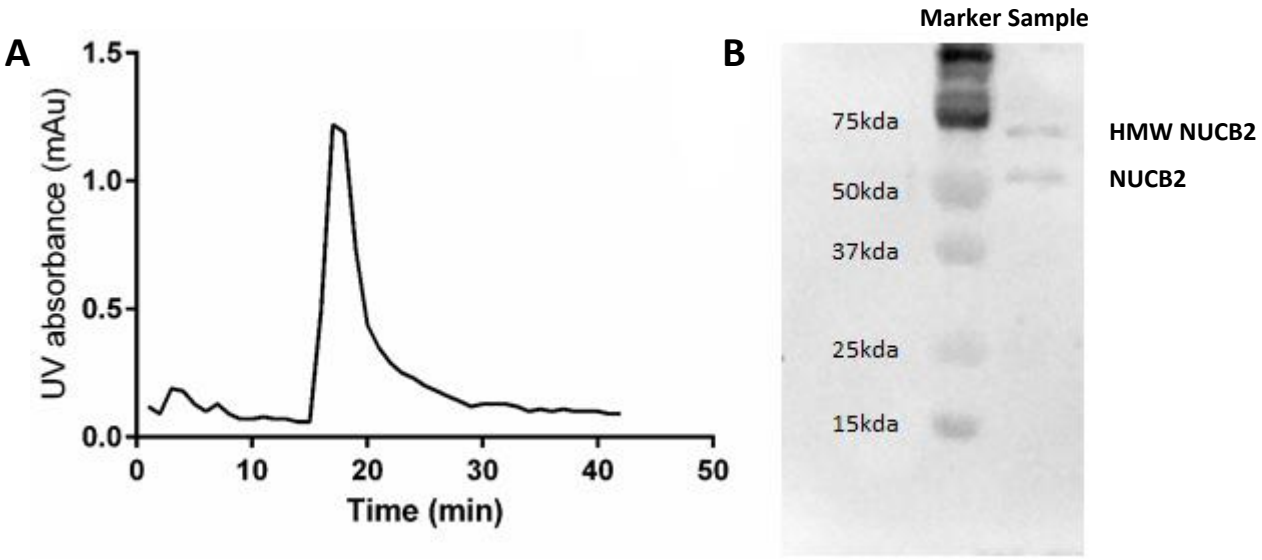


**Figure 5.2: Analysis of circulating nesfatin-1/NUCB2 molecular forms in healthy controls and heart failure patients.** (A) UV-elution profile of heart failure serum after immuno-affinity enrichment followed by gel filtration. A peak corresponding to NUCB2 was eluted after 17 min. (B) Western blot analysis of the same sample revealed two bands. One corresponding to NUCB2 and another at a higher molecular weight (HMW NUCB2), possibly corresponding to glycosylated NUCB2.

### 5.3.4 NUCB2 serum levels in humans

As there is a certain similarity between nesfatin-1 and BNP resulting in NPRA activation, NUCB2 levels were determined in the same population suspected of having heart failure (n = 62), mentioned in chapter 4 (4.3 heart failure population). Patient characteristics are shown in Table 5.1. NUCB2 concentrations ranged from < 30 to 1422 pg/ml, median 451 [interquartile range: 269-571] pg/ml.

Patients were divided in tertiles based on their NUCB2 concentrations and their cardiovascular parameters were compared. No significant difference was found in heart rate, ejection fraction, end-diastolic pressure, end-diastolic volume (EDV) or end-systolic volume (ESV). However, a trend was observed in both the EDV and ESV towards higher values in patients with low and medium NUCB2 concentrations. This corresponds with the lower nesfatin-1 levels found in patients with decompensated heart failure with reduced ejection fraction (HFrEF; Figure 5.3).

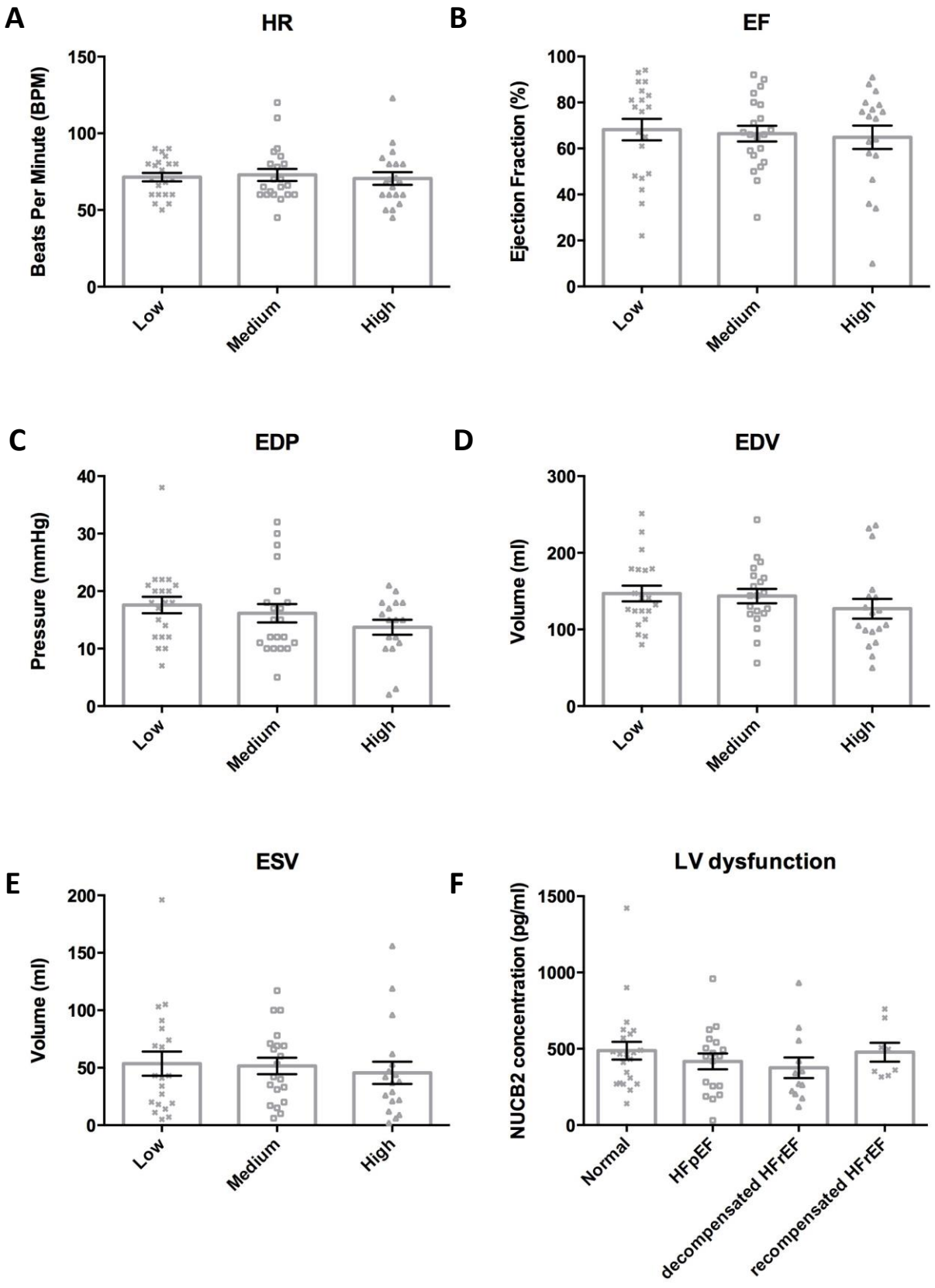


**Figure 5.3: Comparison of cardiovascular parameters between NUCB2 tertiles** (Low = First Tertile, Medium = Second Tertile and High = Third Tertile). No significant differences were observed in the Heart Rate (A), Ejection Fraction (B), End-Diastolic Pressure (C), End-Diastolic Volume (D) and End-Systolic Pressure (E). **NUCB2 concentrations in patients with different left-ventricular dysfunctions** (F). No significant difference was observed between any of the groups (HFpEF: heart failure with preserved ejection fraction; HFrEF: heart failure with reduced ejection fraction).

## 5.4 Discussion

In past years, it has become clear that nesfatin-1/NUCB2 has a role outside of appetite regulation [14]. While increasing amounts of evidence implicate nesfatin-1/NUCB2 in the cardiovascular system, much remains unknown about its physiology [5]. Recently, Angelone *et al.* showed that nesfatin-1 is able to produce cGMP through NPRA due to a structural similarity with BNP, a well-known biomarker in heart failure [8, 17]. In addition, nesfatin-1/NUCB2 contains a penultimate proline at its N-terminus, another characteristic shared with BNP. As this N-terminal proline is the hallmark of a DPPIV substrate and this residue is strongly conserved between mammals in the NUCB2 orthologues (Figure 5.4), we speculated that this might be of biological significance. Therefore, we studied the possible role as a substrate of DPPIV, the circulating nesfatin-1/NUCB2 molecules and its levels in patients suspected of heart failure.

### 5.4.1 Nesfatin-1 is a DPPIV and FAP substrate

Nesfatin-1 was shown to be a novel substrate of both DPPIV and FAP in vitro. While the $K_m$ was above 20 µM, a relatively fast cleavage to nesfatin-1 (3-82) was observed for FAP. This is in accordance with Keane *et al.* who showed that FAP is capable of cleaving neuropeptides [18]. Even though we do not yet know the physiological significance of this cleavage, it can be speculated that this may lead to a reduction of cGMP release, as is the case with BNP(3-32) [19]. Because nesfatin-1 is generated from the N-terminus of NUCB2 we speculate that NUCB2 might also be cleaved by the same proteases. Interestingly, Zhang *et al.* identified NUCB1 as a DPPIV, DPP8 and DPP9 substrate [20].

While we mainly focused on the cardiovascular importance of nesfatin-1/NUCB2, the cleavage could also be important for appetite regulation by increasing the half-life of central nesfatin-1. This might, in part, explain the weight neutrality of DPPIV inhibitors in type 2 diabetic patients [21].


**Figure 5.4: Sequence alignment of mammalian nesfatin-1 orthologues.** Proprotein convertase 1/2 (PC1/2) cleavage position is indicated and cleavage result in the generation of nesfatin-1 (in black) and a C-terminal part (in grey). The fibroblast activation protein and dipeptidyl peptidase IV (DPPIV) cleavage site and glycosylation sites (as predicted with NetOGlyc 4.0 server) within nesfatin-1 are also indicated.

**Table 5.1: Patient characteristics according to tertiles of nesfatin-1 levels.**

| | | | **NUCB2 Tertiles** | |
|---|---|---|---|---|
| | | **Low** | **Normal** | **High** |
| | | **(n = 21)** | **(n = 21)** | **(n = 20)** |
| **Mean NUCB2** | pg/ml | 255 | 451 | 642 |
| | range | 30-215 | 324-504 | 508-1422 |
| **Age** | years | 67 | 65 | 67 |
| | SD | 8 | 10 | 13 |
| **Men** | n | 11 | 16 | 14 |
| | % | 52 | 76 | 70 |
| **Heart Rate** | bpm | 71 | 73 | 71 |
| | SD | 13 | 18 | 18 |
| **Ejection Fraction** | %max | 68 | 66 | 65 |
| | SD | 21 | 16 | 21 |
| **EDP** | mmHg | 18 | 16 | 18 |
| | SD | 7 | 7 | 20 |
| **EDV** | ml | 147 | 144 | 127 |
| | SD | 46 | 42 | 54 |
| **ESV** | ml | 54 | 52 | 46 |
| | SD | 47 | 32 | 41 |
| **DPPIV activity** | U/l | 23 | 24 | 20 |
| | SD | 6 | 4 | 5 |

*NUCB2 - Nucleobindin-2*

In our experiments, we showed that nesfatin-1 does not function as an apparent endogenous inhibitor for small synthetic substrates as well as physiological peptide-substrates. This is surprising as it has an N-terminal Val-Pro-Ile motive, and thus some similarity to diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) [12]. However, other DPPIV substrates are known to have a similar motive (for example CXCL10 (Val-Pro-Leu)). Intriguingly, the specificity constant ($k_{cat}/K_m$) was determined to be $0.006*10^{-6}$ $M^{-1}s^{-1}$ and is approximately a 1000-fold and 100-fold lower than SDF1α and GLP-1, respectively. In comparison to CXCL10, $k_{cat}/K_m$ is about ten times slower, possibly owing to the unfavorable acidic residue in the P2' position [22]

### 5.4.2 NUCB2 is the circulating molecule

We are the first to report that NUCB2, and not nesfatin-1, is the main circulation molecular form in serum of both healthy controls and heart failure patients. From gel filtration and western blotting experiments it was clear that only NUCB2 circulated. Therefore, we should rethink peripheral experiments and focus on NUCB2.

While it was unclear which molecular form circulates in the human body, it is not surprising that NUCB2 is the circulating molecule. Barnikol-Watanabe *et al.* showed that besides its expression in the cytosol and on the plasma membrane, it can be secreted into the medium [23].

It is important to note that there might be a spatial heterogeneity in the molecular forms found. For example, it was shown that nesfatin-1 is present in the brain where it is generated locally from NUCB2 by proprotein convertases 1 and 2 [1]. However, these convertases are restricted to the secretory granules of neuroendocrine cells [24]. Therefore, this pathway might be biologically irrelevant in the circulation, even though other proprotein convertases might be involved in the proteolytic processing.

Besides NUCB2, we also observed a higher molecular weight band on western blot. This band might correspond to a glycosylated NUCB2. Using a bioinformatics tool on NUCB2 with its signal peptide

(NUCB2(1-420), NetOGlyc 4.0 server)[25], we found five possible mucin type GalNAc O-glycosylation sites ($Thr_{32}$, $Ser_{42}$, $Thr_{163}$, $Thr_{322}$ and $Ser_{408}$), two of which lay within nesfatin-1. The presence of a glycosylated form in the circulation would be in contrast with its gastro-intestinal expression where NUCB2 was found not to be glycosylated [26]. Nevertheless, this might also be explained by spatial heterogeneity.

### 5.4.3 NUCB2 in heart failure

In previous publications it was shown that nesfatin-1/NUCB2 plays a role in cardiovascular diseases, such as ischemic cardiomyopathy and acute myocardial infarction [8–10]. However, we are the first to assess the study of NUCB2 as a biomarker in patients suspected of heart failure. While none of the measured cardiovascular parameters were significantly different between the NUCB2 tertiles, we could observe that patients with decompensated HFrEF tended towards lower NUCB2 levels.

While we mainly focused on the similarity with BNP, and thus the activation of NPRA, it should be noted that effects mediated through other receptors should not be overlooked. As Yosten and Samson showed, nesfatin-1 also exerts cardiovascular effects through the melanocortin pathway [6, 27].

## 5.5 Conclusion

In this study we have shed a new light on the nesfatin-1/NUCB2 physiology in the circulation. First of all, nesfatin-1 was shown to be a novel substrate of DPPIV and FAP. Second, in the circulation only NUCB2 was detected. Therefore, we propose that future research in the circulation should focus on NUCB2. Finally, as our results show a trend towards lower levels in decompensated HFrEF, we suggest studying the role of NUCB2 in a larger population of heart failure patients.

## 5.6 Limitations

These are preliminary results and should be interpreted as such. We can only speculate about the glycosylation and possible *in vivo* proteolytic cleavage as we did not perform any experiments to investigate these. In addition, only nesfatin-1 was shown to be a DPPIV and FAP substrate but no kinetic constants were determined.

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.

# Chapter 6

# Effect of sitagliptin on collagen metabolism

Adapted from: **Baerts L**, Glorie L, Maho W, Eelen A, Verhulst A, D'Haese P, Covaci A, De Meester I.; Potential impact of sitagliptin on collagen-derived dipeptides in diabetic osteoporosis; Pharmacol Res. 2015 Oct;100:336-40.

# Chapter 6 - Effect of sitagliptin on collagen metabolism

## 6.1 Abstract

It is known that diabetes poses an increased risk of osteoporosis. While a disturbed collagen metabolism is proposed as a possible cause, much remains unknown about the enzymes involved and the changes in collagen-derived dipeptides and amino acids. Therefore, we sought to study this intricate pathway and the effect of dipeptidyl peptidase IV (DPPIV) inhibitors.

Control (CON) and streptozotocin-nicotinamide-induced diabetic rats (DM) were treated for 12 weeks with vehicle (VH) or sitagliptin (SG), a DPPIV inhibitor (Con/VH, Con/SG, DM/VH and DM/SG). The activities of four key enzymes involved in collagen breakdown were determined in serum (DPPIV, matrix metalloproteinase 2 and 9 and prolidase). Dipeptide (Ala-Pro, Gly-Pro, Pro-Pro and Pro-Hyp) and amino acid (Pro and Hyp) concentrations were measured by liquid chromatography coupled to mass spectrometry.

We found three-fold higher MMP9 activities in DM/VH than in controls, while in DM/SG this rise was attenuated. MMP2 and prolidase did not differ in the investigated groups. Furthermore, we are the first to report on two-fold higher Ala-Pro and Pro-Pro levels in diabetic rats compared to controls. In contrast, Pro-Hyp concentrations were lower in diabetes (DM/VH and DM/SG).

DPPIV inhibition does not seem to have a direct influence on the collagen metabolism in streptozotocin-nicotinamide-induced diabetic rats. Instead, it probably acts through its effect on osteoprotective substrates. In diabetes, increased MMP9 activities seem to favour the production of Ala-Pro and Pro-Pro containing collagen fragments. The high Pro-Hyp levels in untreated controls

might have a bone-stimulating effect. Nevertheless, the biological significance of these dipeptides is not yet clear and should be further investigated.

## 6.2 Introduction

It has become clear that diabetes is associated with an increased risk of osteoporosis [1, 2]. While the exact mechanism behind this phenomenon is not clear, there is growing evidence of an altered collagen metabolism [1, 3].

Collagen is one of the most abundant proteins in the human body and is an essential component of the connective tissue [1]. Its unique triple helical structure is formed by the interaction of its main amino acid building blocks: glycine, proline and hydroxyproline [4].

Type I collagen is the most common type and can be found in virtually any tissue, including bone, where it is responsible for its tensile strength [1, 4]. Therefore, it is not surprising that the proteolytic degradation products of collagen can be used as markers for bone diseases, such as osteodystrophy and osteoporosis [5–7].

The proteolytic degradation of collagen and the generation of dipeptides can be seen as an intricate network of enzymes with gelatinases (MMP2 and MMP9), dipeptidyl peptidase IV (DPPIV) and prolidase as key enzymes [8–11].

The breakdown is initiated by collagenases, such as matrix metalloproteinase 1 (MMP1) [4]. The resulting fragments rapidly denature at 37 °C and are further degraded by gelatinases [12]. This is supported by the correlation of circulating MMP2 levels with bone turnover in postmenopausal women [13]. By combining these reports with the work of Nyman *et al,* it becomes clear that the gelatinases play an important role in bone integrity [14].

The produced fragments are then further degraded by DPPIV, generating proline-containing dipeptides [15]. Recent peptidomics data stress the role of DPPIV in the degradation of collagen. In

these studies, treatment of rats with the irreversible DPPIV inhibitor AB192, as well as the clinically used vildagliptin, resulted in elevated levels of collagen fragments [9, 10]. In addition, a meta-analysis suggested that treatment with DPPIV inhibitors lowers the risk of bone fractures in type 2 diabetic patients [16]. We recently confirmed these observations in diabetic rats treated with the DPPIV inhibitor, sitagliptin [17].

The iminodipeptides formed during the breakdown of collagen can be split further into amino acids by prolidase. This enzyme regulates the degradation of iminodipeptides and its activity correlates with the amount of collagen breakdown [11, 18]. The authors also suggest that serum prolidase activity could be used as a marker for osteoporosis in diabetes [18]. Finally, the amino acids and remaining dipeptides are cleared by the kidney [11, 19].

In this study, we investigated the collagen-derived dipeptide metabolism in healthy and diabetic rats. We also examined the effect of sitagliptin, a DPPIV inhibitor, on the enzymatic activities and amino acid and dipeptide levels of these animals. Since DPPIV inhibitors are increasingly used in type II diabetes and have been shown to increase bone strength [17], it is interesting to investigate their effect on the underlying collagen metabolism.

## 6.3 Osteoporotic rats

Rat serum was used from the study reported by Glorie *et al*. [17]. All procedures were carried out in accordance with the "*guide for the care and use of laboratory animals"* of the United States National Institute of Health. In short, 64 Wistar rats of 10-weeks old were divided in four groups (N=16 each): a vehicle-treated (Con/VH) and a sitagliptin-treated control group (Con/SG) on the one hand and a vehicle-treated (DM/VH) and a sitagliptin-treated diabetic group (DM/SG) on the other hand. Diabetes was induced by intravenously injecting streptozotocin (65 mg/kg) 15 min after an intraperitoneal nicotinamide injection (230 mg/kg), both diluted in physiological solution. Sitagliptin (2 g/L) was added to the drinking water [17]. This dose was shown to be efficient in achieving a

biologically significant *in vivo* DPPIV inhibition of >85%. After 6 and 12 weeks, eight animals of each group were sacrificed by exsanguination through the abdominal aorta. To prevent *ex vivo* proteolytic cleavage, all blood samples were collected in tubes containing sitagliptin (final conc. 100 µM) and Roche Protease Inhibitor Cocktail. These samples were used for the dipeptide and amino acid analysis. Enzyme activities were performed on a separate blood sample taken form the tail vein. Renal function as assessed by creatinine clearance did not differ between groups [17].

## 6.4 Results

### 6.4.1 Biochemical characterization

DPPIV activities were measured in serum and the *in vivo* inhibition estimated (materials and methods 3.3). All treated rats had a physiological relevant inhibition. In addition, DPPIV activities did not differ between untreated diabetic (DM/VH) and control rats (Con/VH) [17].

These activities can be found, together with glucose, calcium, phosphate and creatinine levels, in Table 6.1.

### 6.4.2 Effect on matrix metalloproteinase activity

After 6 weeks, the relative MMP2 activity in the sitagliptin-treated diabetic rats (DM/SG) differed significantly from the other groups. After 12 weeks, there was no significant difference in MMP2 activity left between any of the groups. Nonetheless, the diabetic rats (DM/VH and DM/SG) tended to have lower activities compared to the controls (Figure 6.1A).

Immediately after induction of diabetes, the MMP9 activities of the untreated diabetic group (DM/VH) were found to be significantly higher than those of the control groups (Con/VH and Con/SG). 6 weeks later, these differences were evened out. At the end of the study (12 weeks), the untreated diabetic rats (DM/VH) had three-fold higher MMP9 activities compared to controls

(Con/VH). Sitagliptin treatment of diabetic rats (DM/SG) resulted in significantly lower activities (Figure 6.1B).

**Table 6.1: Overview of biochemical parameters in the serum of the different groups.**

| | | Con/VH | Con/SG | DM/VH | DM/SG |
|---|---|---|---|---|---|
| **Glucose** | | | | | |
| *mg/dl ± SD* | Initial glucose | 122 ± 13 | 127 ± 10 | 120 ± 9 | 119 ± 13 |
| | Glucose (6w) | 122 ± 30 | 121 ± 12 | 493 ± 152 | 383 ± 176 |
| | Final glucose (12w) | 136 ± 15 | 120 ± 6 | 393 ± 137 | 390 ± 170 |
| **Calcium** | | | | | |
| *mg/dl ± SD* | Initial calcium | 9.89 ±0.46 | 10.31 ± 0.46 | 10.66 ± 0.31 | 11.29 ± 0.43 |
| | Calcium (6w) | 8.40 ± 0.89 | 9.72 ± 0.69 | 9.6 ± 0.39 | 10.58 ± 0.47 |
| | Final calcium (12w) | 9.43 ± 0.57 | 9.22 ± 0.32 | 8.79 ± 0.56 | 10.07 ± 0.27 |
| **Phosphate** | | | | | |
| *mg/dl ± SD* | Initial phosphate | 9.21 ± 1.56 | 9.28 ± 1.35 | 9.80 ± 1.15 | 10.42 ± 1.17 |
| | Phosphate (6w) | 8.26 ± 1.44 | 9.24 ± 1.77 | 7.78 ± 1.02 | 9.58 ± 1.46 |
| | Final phosphate | 6.36 ± 0.79 | 8.94 ± 1.43 | 8.31 ± 6.42 | 7.59 ± 1.74 |
| **Creatinine** | | | | | |
| *mg/dl ± SD* | Initial creatinine | 0.70 ± 0.06 | 0.72 ± 0.07 | 0.64 ± 0.06 | 0.63 ± 0.07 |
| | Creatinine (6w) | 0.83 ± 0.07 | 0.76 ± 0.07 | 0.86 ± 0.13 | 0.93 ± 0.04 |
| | Final creatinine (12w) | 0.70 ± 0.06 | 0.72 ± 0.06 | 0.69 ± 0.06 | 0.87 ± 0.06 |
| **DPPIV activity** | | | | | |
| *U/l ± SD* | Initial DPPIV activity | 16.0 ± 3.0 | 16.0 ± 1.1 | 15.4 ± 2.6 | 16.0 ± 1.7 |
| | DPPIV activity (6w) | 16.4 ± 3.3 | n.a. | 21.2 ± 4.1 | n.a. |
| | Final DPPIV activity (12w) | 16.0 ± 2.9 | n.a. | 17.1 ± 3.1 | n.a. |
| **%in vivo DPPIV inhibition** | | | | | |
| *% ± SD* | Inhibition (6w) | | 85 ± 6 | | 83 ± 9 |
| | Inhibition (12w) | | 82 ± 6 | | 87 ± 6 |

### 6.4.3 Effect on prolidase activity

The only trend in changes of prolidase activities was found after 6 weeks in the sitagliptin-treated control group (Con/SG). No significant differences between the groups could be found at the end of the study, even though the diabetic rats (DM/VH and DM/SG) tended to have higher serum prolidase activities (Figure 6.2).

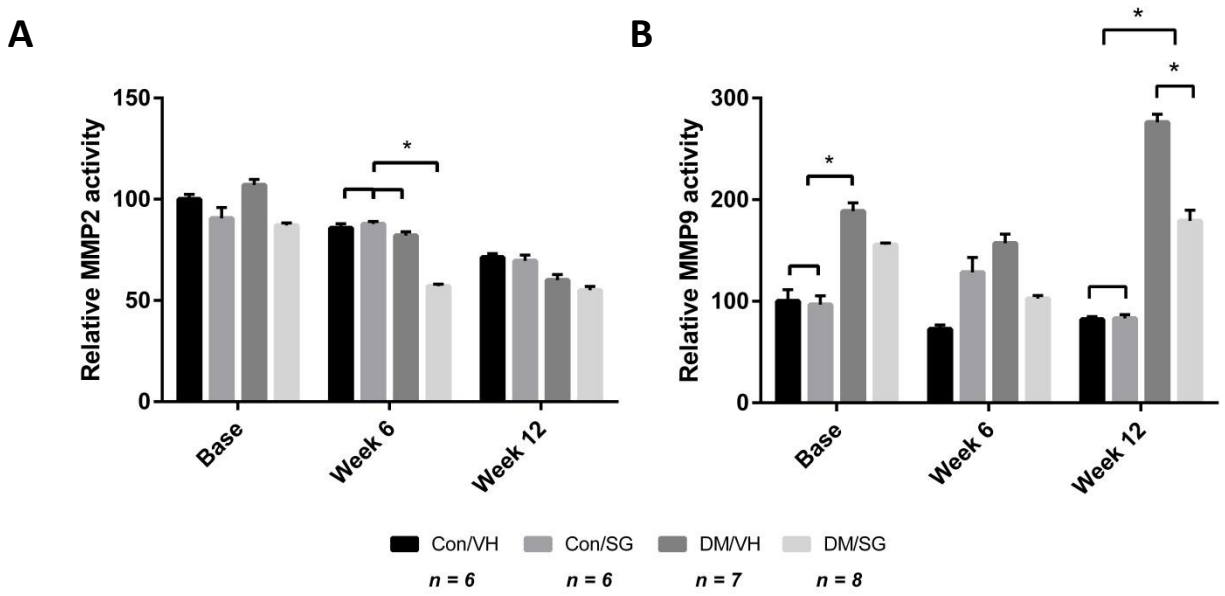


**Figure 6.1: Relative Matrix MetalloProteinase (MMP) activities in rat serum compared to vehicle-treated controls at base.** (A) At week 6, the diabetic sitagliptin-treated group (DM/SG) had a significantly lower MMP2 activity compared to the other groups. At week 12, no significant difference was found in MMP2 activity between the different groups. (B) At base a significant difference in MMP9 activity was found in the diabetic vehicle-treated group (DM/VH) immediately after induction of diabetes compared to the controls (Con/VH and Con/SG). After 12 weeks, diabetic animals had significantly higher MMP9 activities compared to controls. Treatment of the diabetic group with sitagliptin resulted in lower MMP9 activities. (Groups: CON = Control, DM = Diabetes Mellitus, VH = Vehicle-treated, SG = Sitagliptin-treated; * p<0.05).

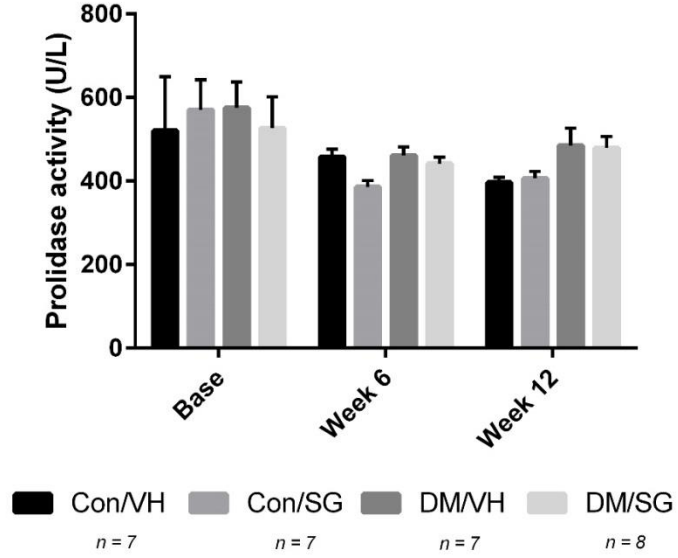


**Figure 6.2: Prolidase activities measured in rat serum.** No significant difference was found between the different groups at base, week 6 or week 12. (Groups: CON = Control, DM = Diabetes Mellitus, VH = Vehicle-treated, SG = Sitagliptin-treated; * p<0.05).

### 6.4.4 Dipeptide and amino acid levels

After 12 weeks of treatment, Ala-Pro and Pro-Pro concentrations significantly differed between untreated controls (Con/VH) and sitagliptin-treated diabetic animals (DM/SG; materials and methods 3.7). In addition, Pro-Pro levels in sitagliptin-treated controls (Con/SG) were significantly lower than in their diabetic counterparts, while Gly-Pro remained constant between the different groups (Figure 6.3A). The Pro-Hyp levels were on average ten times higher than the other dipeptides. These were significantly higher in untreated controls (Con/VH) compared to the diabetic animals (DM/VH and DM/SG). Sitagliptin-treated controls (Con/SG) showed a clear trend towards lower levels, but significance was not reached (Figure 6.3B).

At the end of the experiment, Hyp levels remained constant between the different groups. In contrast, Pro levels tended to be higher in diabetic rats compared to the controls (Figure 6.3C). Similar trends in the dipeptide and amino acid levels were found at week 6

## 6.5 Discussion

It is well known that patients with diabetes have an increased risk of bone fractures [2]. While this risk seems to be related with an altered collagen metabolism [1, 3], there is still an incomplete understanding of this process. Therefore, our first goal was to study the enzymes involved in collagen breakdown as well as the resulting dipeptides and amino acids after onset of diabetes. In addition, new antidiabetic agents, such as DPPIV inhibitors, seem to have an anti-osteoporotic effect independent from glycemic control [16, 17]. Nonetheless, the mechanism behind this remains unclear. Therefore, our second goal was to investigate the effect of sitagliptin, a DPPIV inhibitor, on the collagen-derived dipeptides and amino acids. For all these reasons, a unique and well-characterized set of diabetic and control animals was chosen [17].

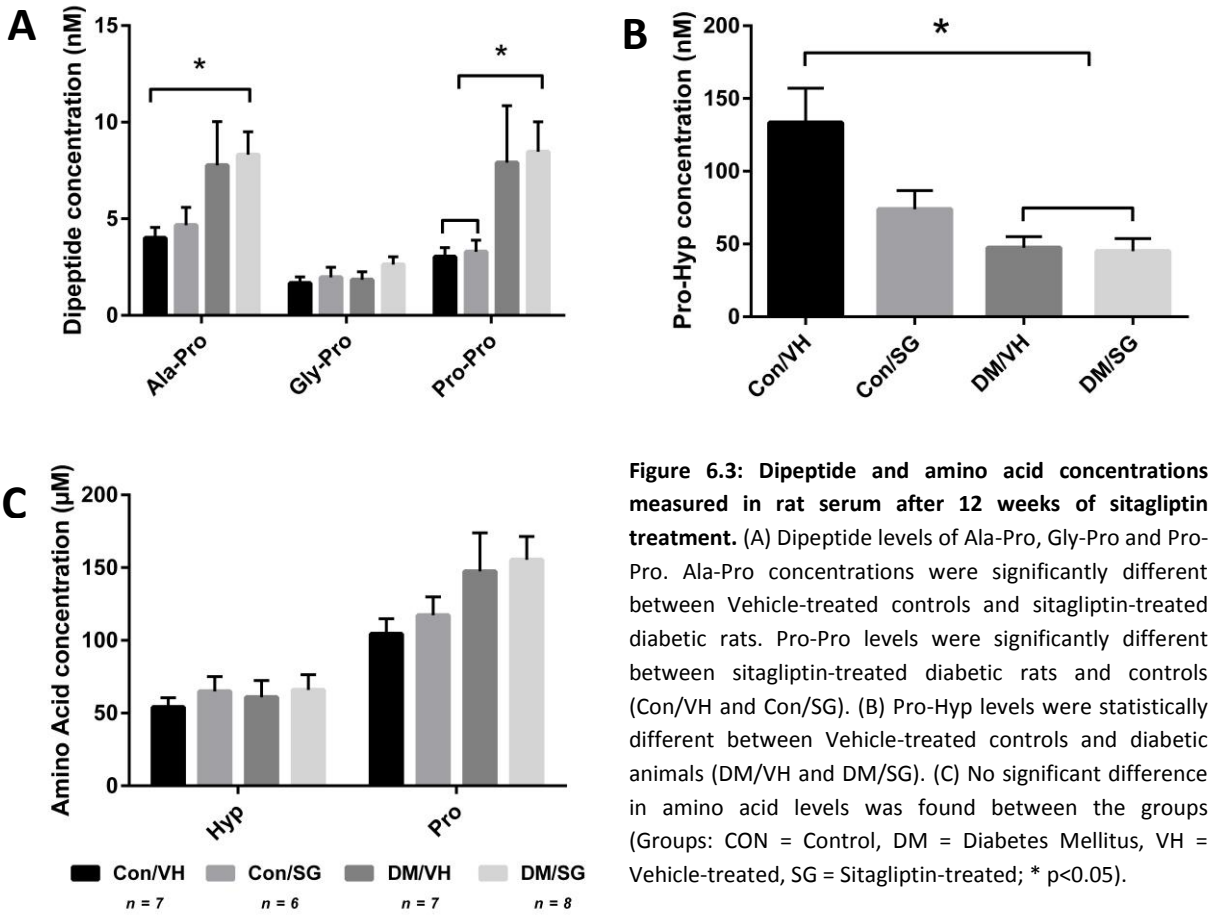


**Figure 6.3: Dipeptide and amino acid concentrations measured in rat serum after 12 weeks of sitagliptin treatment.** (A) Dipeptide levels of Ala-Pro, Gly-Pro and Pro-Pro. Ala-Pro concentrations were significantly different between Vehicle-treated controls and sitagliptin-treated diabetic rats. Pro-Pro levels were significantly different between sitagliptin-treated diabetic rats and controls (Con/VH and Con/SG). (B) Pro-Hyp levels were statistically different between Vehicle-treated controls and diabetic animals (DM/VH and DM/SG). (C) No significant difference in amino acid levels was found between the groups (Groups: CON = Control, DM = Diabetes Mellitus, VH = Vehicle-treated, SG = Sitagliptin-treated; * $p<0.05$).

### 6.5.1 Sitagliptin attenuates rise in MMP9 activity in diabetes

After 12 weeks we found that MMP9 activities were three times higher in diabetic rats compared to controls. Moreover, these activities lowered significantly after treatment with sitagliptin. In contrast, only a trend towards lower MMP2 activities was found in diabetic animals. While it is known that MMP9 activities rise in diabetes, the effect on MMP2 is less conclusive in the literature and seems to depend on the type of diabetes [20, 21]. The higher MMP9 activities can be responsible for the recruitment of osteoclasts towards the bone [22]. Based on the observation that sitagliptin does not influence MMP9 activity in controls, we concluded that this DPPIV inhibitor most probably has an indirect effect on MMP9 activity. This is most likely caused by the effect on the inflammatory response in these animals.

### 6.5.2 Sitagliptin does not influence prolidase activity

While sitagliptin treatment did not influence the prolidase activities, diabetic animals tended to have higher activities than controls. These higher activities in diabetics are in line with findings of Eren *et al.* and Uzar *et al.* , but contrasts with the results of Erbagci *et al.* [18, 23, 24]. Nonetheless, these higher prolidase activities might reflect the increased collagen breakdown generally found in diabetes [3, 17].

### 6.5.3 Sitagliptin prevents Pro-Hyp generation in controls

To our knowledge, we are the first to report dipeptide and amino acid concentrations in rat serum. Compared to published data of human serum, dipeptides and amino acid concentrations were on average five times lower in this study using rats [25]. This could reflect a higher renal clearance due to a difference in renal peptide transport as there exist important interspecies differences in kidney blood flow, glomerular filtration rate and renal organic anion and cation transporters [26, 27].

We are the first to show that a streptozotocin-induced diabetic state possibly induces increased levels of Pro-Pro and Ala-Pro. Combining these results with those from the enzymatic activities supports the hypothesis that MMP9 is involved in the generation of Ala-Pro or Pro-Pro containing collagen fragments. Nevertheless, the contribution of other proteases cannot be fully excluded from this process.

The lower Pro-Hyp levels in the diabetic group may in part be explained by the higher prolidase activities. As Pro-Hyp is by far the preferred substrate of bacterial prolidase and probably mammalian prolidase, it will preferentially be cleaved [28]. In addition, Pro-Hyp concentrations are 10 times higher than the other dipeptides, which promotes the degradation of Pro-Hyp.

The lower Pro-Hyp levels in diabetic animals are surprising in view of earlier findings suggesting increased urinary levels of Pro-Hyp to be a marker for osteoporosis [19]. However, the data found in literature is scarce, especially with regard to serum levels. Moreover, the high Pro-Hyp levels in

controls can also be regarded as beneficial. It was recently shown that this dipeptide promotes osteoblast differentiation by regulation of osteoblast-associated genes [29]. In addition, Pro-Hyp also stimulates the growth of fibroblasts on collagen [30]. The latter finding might also explain the decreased wound healing in diabetics. The lower Pro-Hyp levels in sitagliptin-treated control animals are not surprising as this may reflect the inhibition of DPPIV [31]. However, it does not seem to play a similar role in diabetic rats.

No other effects of sitagliptin on the generation of dipeptides were found. This is probably due to the low dipeptide levels, making significant differences hard to measure. In addition, we previously showed that changes in collagen metabolism only start after 12 weeks of treatment, as measured by a C-terminal peptide of collagen type I [17]. It could be that maintenance of this therapy is necessary to induce significant differences in dipeptide levels.

We suggest that the protective effect of sitagliptin against the development of osteoporosis is not linked to a direct effect on the collagen metabolism in this animal model. The most probable explanation is that the treatment results in an increased half-life of biologically active DPPIV substrates. Important candidates for this could be: glucagon-like peptide 1 and gastric intestinal peptide, but others, such as neuropeptide Y and substance P, should also be considered [32–36].

## 6.6 Conclusion

Of all the investigated collagen-derived dipeptides and amino acids, sitagliptin only seems to affect the Pro-Hyp levels. Therefore, its osteoprotective effect in streptozotocin-nicotinamide-induced diabetic rats does not seem to be directly linked with collagen-derived dipeptides. It most likely prolongs the biological activity of DPPIV substrates involved in bone metabolism. There is a three-fold upregulation of MMP9 activity and a doubling of Ala-Pro and Pro-Pro levels in this diabetes model. Although the biological role of these dipeptides is not yet clear, our data may provide evidence for an active regulation of their levels. While high Pro-Hyp levels are generally regarded as a

marker for osteoporosis, we hypothesize that this circulating dipeptide functions as a bone stimulating factor. However, more research is needed towards the physiological significance of these circulating proline-containing dipeptides.

## 6.7 Limitations

Only a limited set of dipeptides, amino acids and enzymes were measured. A selection was made based on the abundance and likelihood of involvement. However, the effect of other enzymes on the generation of these dipeptides cannot be excluded. Similarly, the levels of other dipeptides and amino acids might be influenced by diabetes or sitagliptin.

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**Chapter 7**

# Circulating DPPs in stroke

Submitted

# Chapter 7 - Circulating DPPs in stroke

## 7.1 Abstract

Dipeptidyl peptidase IV (DPPIV) inhibition may be a promising therapeutic strategy for acute stroke treatment, given its potential to prolong the biological half-life of neuroprotective substrates. A related protease, fibroblast activation protein (FAP), was recently shown to inactivate the same substrates. Therefore, it should also be investigated as a potential target in stroke. We aimed to investigate whether stroke severity and outcome correlates with DPPIV and FAP activities and their time course of expression (kinetics) shortly after acute ischemic stroke.

DPPIV and FAP activities were analyzed in the serum of 50 hyperacute stroke patients at admission, 1 day, 3 days and 7 days after stroke onset and in 50 age-matched healthy controls. This was done as part of the Middelheim's Interdisciplinary Stroke Study.

DPPIV activity tended to increase shortly after stroke compared to the control population. DPPIV and FAP activities steadily decreased in the first week after stroke onset. Higher infarct volumes (≥ 5 ml) and a more severe stroke (NIHSS > 7) at admission were correlated with a stronger decrease in the activities of both enzymes. Moreover, these patients more often developed a progressive stroke, were more often institutionalized and had a higher degree of disability after three months (modified Rankin scale ≥ 4).

Patients with a higher DPPIV activity at admission and stronger decrease in the activity of both DPPIV and FAP during the first week after stroke onset had a more severe stroke and worse short-term and long-term outcomes.

## 7.2 Introduction

In recent years, evidence which points to a role of dipeptidyl peptidase IV (DPPIV) in stroke has been growing. Darsalia *et al.* showed that the DPPIV inhibitor linagliptin counteracts stroke in the normal and diabetic mouse brain [1]. This is supported by a meta-analysis that found a trend towards a lower stroke incidence in patients with type 2 diabetes using DPPIV inhibitors [2]. The protective effect is not only attributable to an improved glycemic control alone but also to the prolonged half-life of neuroprotective DPPIV substrates [1, 3, 4].

Several DPPIV substrates have been shown to be implicated in stroke. For example, stromal-cell-derived factor 1, neuropeptide Y and B-type natriuretic peptide are correlated with stroke outcome and are proposed as biomarkers [5, 6]. Interestingly, fibroblast activation protein (FAP) a DPPIV-related serine protease and member of the same prolyl oligopeptidase family, is able to inactivate several of these substrates *in vitro* [7]. This is in agreement with Röhnert *et al.,* who suggested that DPPIV and proteases with similar substrate specificity are potential therapeutic targets in cerebral ischemia [8]. For more information on DPPIV and its substrates in ischemia we refer to the review by Matheeussen et al [9].

In this study we aimed to investigate if stroke severity, progression and outcome were correlated with serum DPPIV and FAP activities and their kinetics shortly after stroke onset.

## 7.3 Stroke population

Stroke samples were collected as part of the Middelheim's Interdisciplinary Stroke Study (ZNA Middelheim Hospital, Antwerp; 2005-2008). Patients with ischemic stroke underwent neuroimaging and were clinically, biochemically, neuropsychologically and electrophysiologically evaluated. The results of other biochemical analyses have been reported elsewhere [10–14]. Serum samples from 50 patients, who did not receive thrombolytic treatment, were selected for analysis of DPPIV and FAP activities. Leftover samples from healthy subjects were used to create an age-matched control group

of 50 samples (Table 7.1). This study was conducted according to the Declaration of Helsinki and was approved by the Ethics Committees of ZNA Antwerp and the University of Antwerp.

**Table 7.1: Characteristics of the entire stroke study population.**

| | | Patients | Controls |
|---|---|---|---|
| Age | (years) | 75 ± 7 | 73 ± 5 |
| Gender | (male) | 26 (52%) | 31 (62%) |
| Caucasian | (%) | 100% | 100% |
| Time to blood sampling after onset stroke symptoms | (h) | 3 ± 2 | |

**Table 7.2: Stroke characteristics of the patients.**

| Stroke characteristic | | Value |
|---|---|---|
| Stroke severity | NIHSS at admission | 9 ± 9 |
| | Infarct volume | 4.3 ml [IQR; 0.8-40.] |
| Stroke etiology (TOAST criteria) | Atherothrombotic | 16% |
| | Cardioembolic | 53% |
| | Lacunar | 12% |
| | Specific | 2% |
| | Undetermined | 16% |
| Subacute stroke evolution (after 7 days) | Normalized gain in NIHSS | 46% ± 60% |
| | Progressive stroke (EPSS criteria) | 16% |
| Short-term stroke outcome | Institutionalization | 43% |
| | mRS score after 7 days | 3 ± 2 |
| | Mortality after 7 days | 2% |
| Long-term stroke outcome | mRS score after 3 months | 3 ± 2 |
| | Mortality after 3 months | 12% |

*EPSS European Progression Stroke Study Criteria*

*mRS modified Rankin Scale*

*NIHSS National Institutes of Health Stroke Scale*

Trained stroke physicians determined neurological impairment according to the National Institutes of Health Stroke Scale (NIHSS) at admission, day 1, day 3 and day 7. Progressing stroke was evaluated as described by the European Progressing Stroke Study criteria. A brain MRI scan was performed to confirm the diagnosis of acute ischemic stroke. The infarct volume was assessed by two independent

neurologists, as previously described [10, 14]. After three months, stroke outcome was assessed with the modified Rankin Scale, severe disability was defined as an mRS score ≥ 4 [14] (Table 7.2).

Venous blood samples were collected at admission, 1 day, 3 days and 7 days after stroke onset, in serum tubes. After a centrifugation at 2000 ×*g* for 15 min at 4°C, serum was frozen in liquid nitrogen and stored at −80 °C.

## 7.4 Results

### 7.4.1 DPPIV and FAP activity in serum

DPPIV and FAP activities from the patient samples are shown in Figure 7.1. DPPIV showed a trend towards an increase immediately after stroke onset and decreased afterwards. FAP activities decreased significantly in the first week after admission. The lowest activities in patients were found at day 7 (materials and methods 3.3).

At admission, DPPIV and FAP activities were significantly correlated. This correlation was also seen for the kinetics of both enzymes over 7 days. On average, activities decreased by 11% for DPPIV and 19% for FAP. Enzyme activities of both enzymes at admission progressed independently from age, gender or interval between stroke onset and time of blood sampling (Table 7.1).

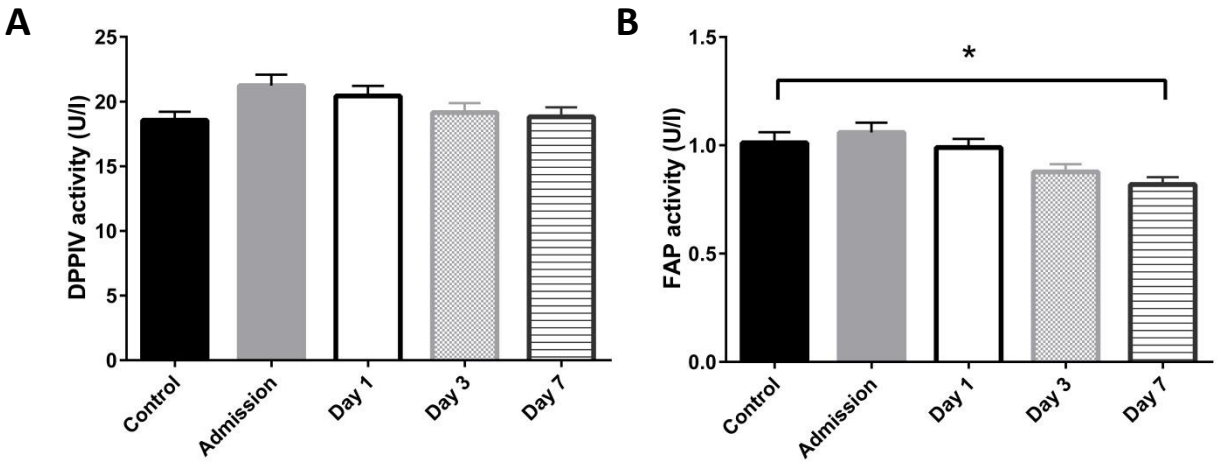


**Figure 7.1: Enzyme activities of the total study population and controls.** (A) DPPIV activity at admission showed a trend towards higher levels compared to controls and tended to decrease afterwards. (B) FAP activity decreased steadily after stroke and reached statistical significance 7 days after stroke (* $p < 0.05$).

### 7.4.2 Relation between patient characteristics and enzyme activity

No relation was observed between the enzyme activities or their kinetics and risk factors associated with stroke (arterial hypertension, atrial fibrillation, heart failure, familial stroke, previous stroke, previous myocardial infarction, diabetes mellitus, dyslipidemia, smoking or alcohol abuse), with the exception of DPPIV kinetics and dyslipidemia. Peripheral artery disease was correlated with DPPIV activities at admission (Table 7.1).

Both systolic and diastolic blood pressures, at presentation, were correlated with DPPIV and FAP activities at the same time and with their kinetics after 7 days. The same was true for HDL levels at admission. Glycemia at admission and after 7 days was correlated with the change in DPPIV activity over the same period. Inflammatory parameters were also correlated with the enzyme activities. FAP admission levels inversely related with CRP levels at admission and its kinetics with CRP levels after 7 days. All the data is summarized in Table 7.1.

### 7.4.3 DPPIV and FAP activity in relation to stroke severity

DPPIV activities at admission were significantly higher in patients with higher infarct volumes (≥ 5 ml) and showed a trend towards a more pronounced decrease of DPPIV and FAP during the first week after stroke onset (Figure 7.2A and 7.2C).

Patients were divided in two groups based on their NIHSS score. Mild stroke was defined as NIHSS ≤ 7 and more severe stroke as NIHSS > 7. Patients with a more severe stroke at admission showed a more pronounced decrease in DPPIV activity during the first week after stroke (Figure 7.2B, 7.2D and Table 7.3).

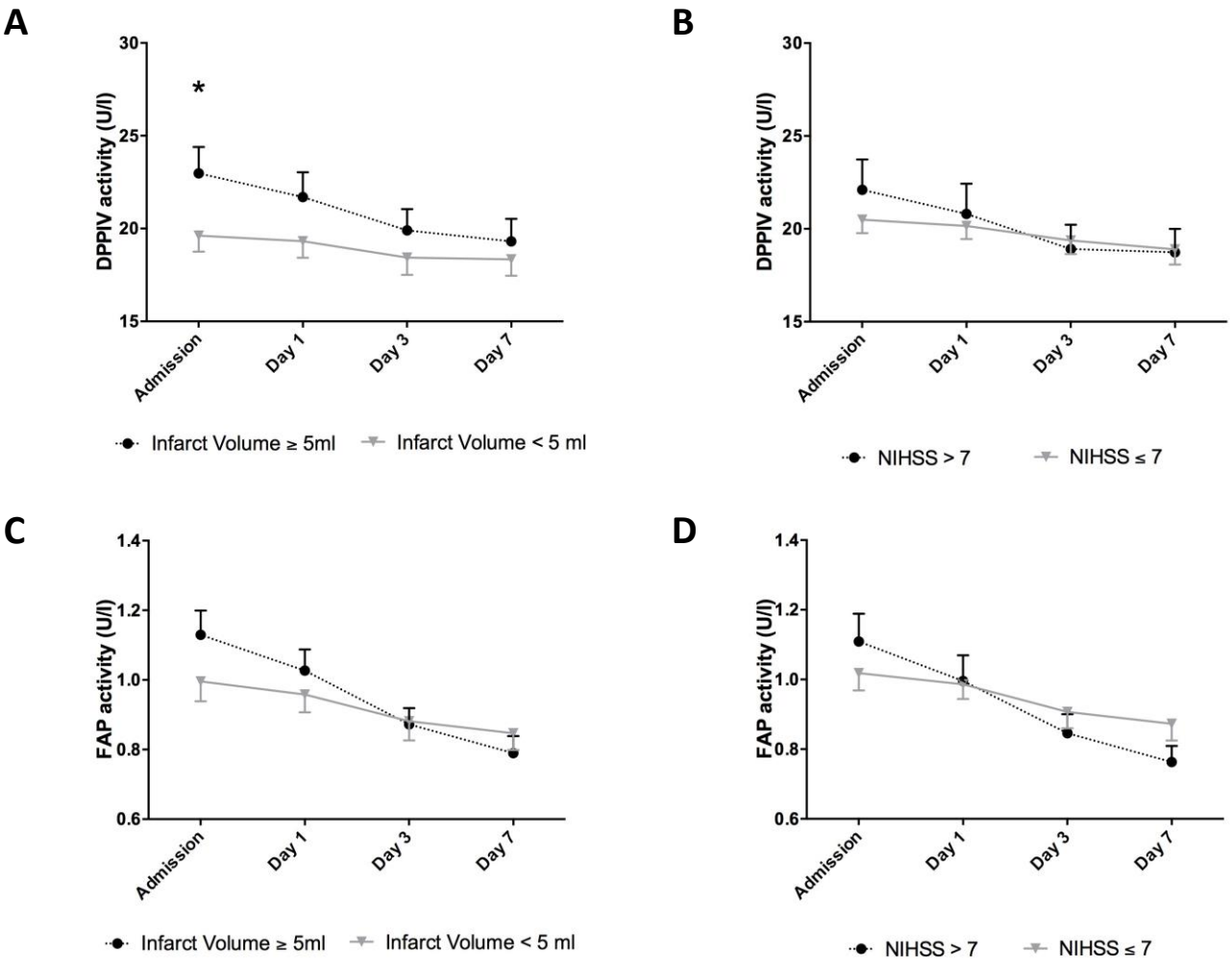


**Figure 7.2: Enzyme activity kinetics and stroke severity.** (A) Patients with large infarct volumes (≥ 5 ml) had significantly higher DPPIV activities at admission. (B) Its activity did not differ at any time-point between patients with high or low NIHSS scores (≤ 7). (C) FAP activity did not differ significantly between patients with large or small infarct volumes (D) or high or low NIHSS scores (* $p < 0.05$).

### 7.4.4 DPPIV and FAP activity in relation to short-term stroke outcome and evolution

Patients who developed progressive stroke, according to the EPSS criteria, had a more pronounced decrease in DPPIV and FAP activity than those with a more favorable outcome. In addition, changes in NIHSS during the first week were related with DPPIV levels at admission and the kinetics of both enzymes over 7 days (Figure 7.3A and 7.3C). Patients who were institutionalized after discharge had higher DPPIV and FAP activities at admission and a more pronounced decrease towards day 7 (Figure 7.3B and 7.3D).

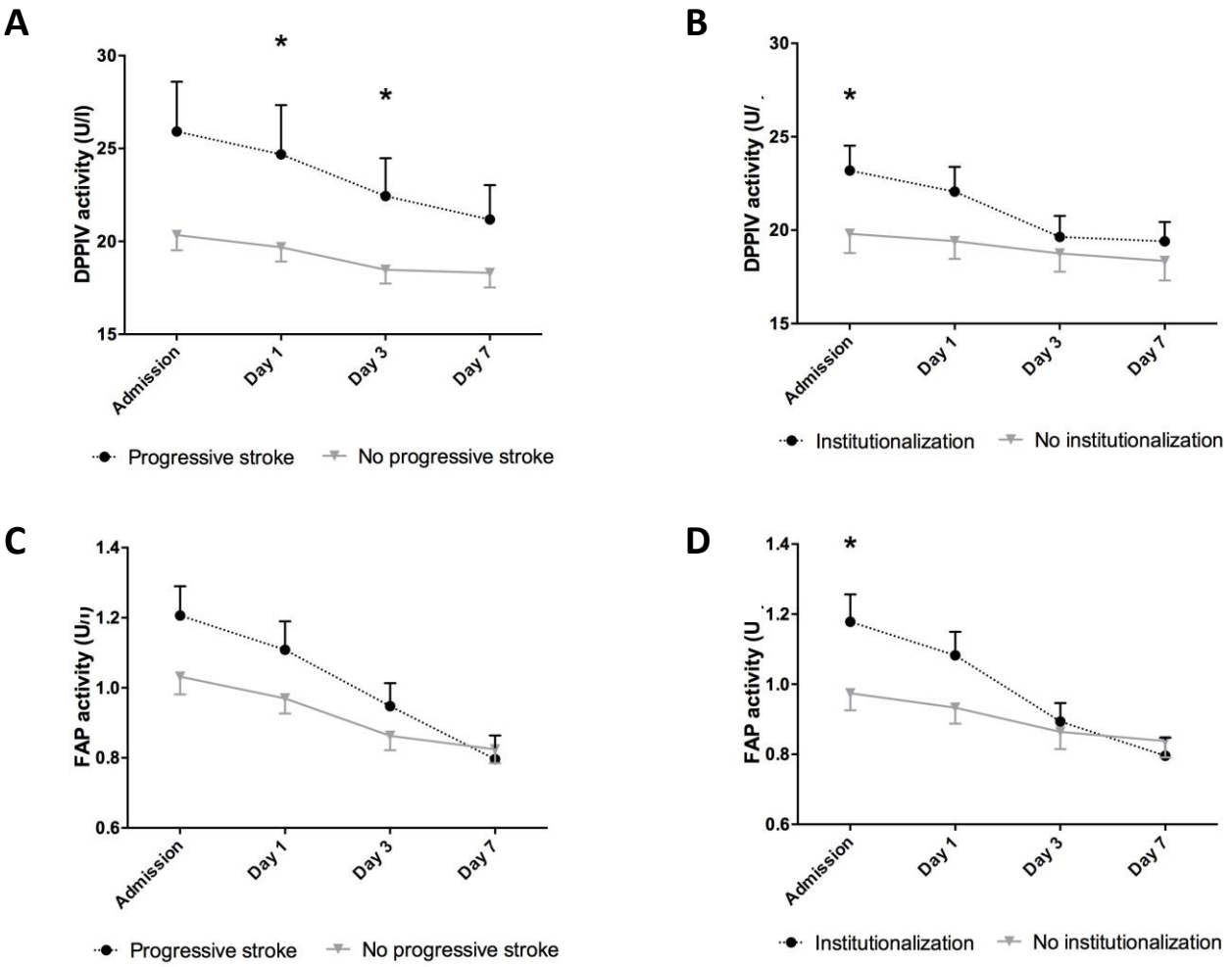


**Figure 7.3: Enzyme activity kinetics and short-term outcome.** (A) DPPIV activities were higher in patients with progressive stroke after 1 and 3 days. (B) Patients who were institutionalized after discharge had significantly higher DPPIV activities at admission. (C) FAP activities tended to be higher at admission, 1 day and 3 days after stroke in patients who developed a progressive stroke. (D) At admission, FAP activities were significantly higher in patients who had to be institutionalized after discharge (* $p < 0.05$).

### 7.4.5 DPPIV and FAP activity in relation to long-term stroke outcome

Long-term outcome was assessed with the mRS score after 3 months. Patients with a severe disability (mRS ≥ 4) had a more pronounced decrease in DPPIV and FAP activities during the first week after stroke onset (Figure 7.4).

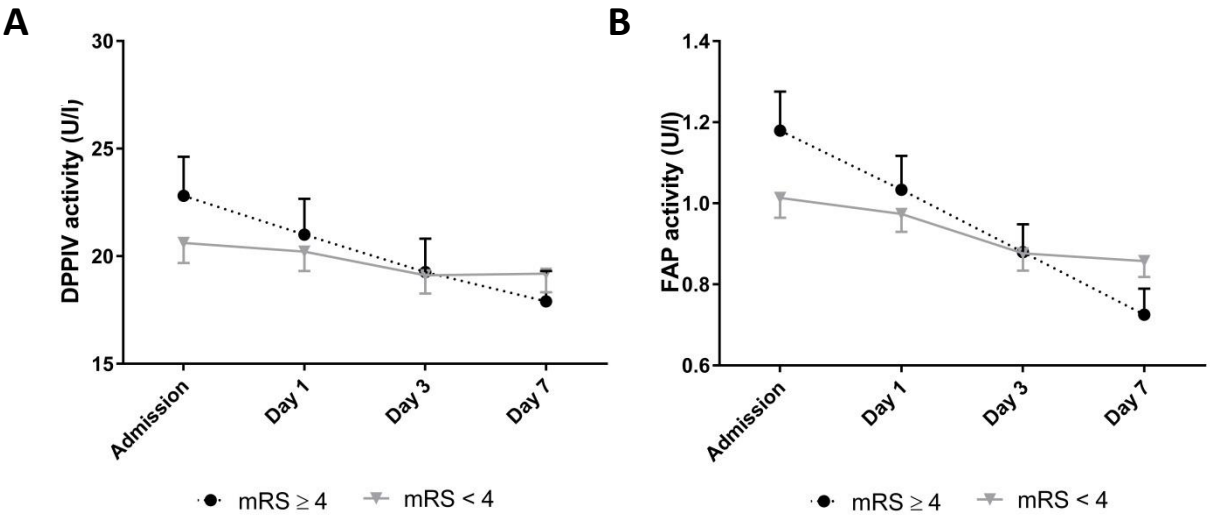


**Figure 7.4: Enzyme activity kinetics and long-term outcome.** No difference could be found in DPPIV (A) or FAP activity (B) between patients with a severe disability and no or mild disability at any of the time-points (* $p < 0.05$).

### 7.4.6 Result validation

In order to validate the results, hematocrit was chosen as an internal standard. As erythrocytes have a long half-life, this can be considered as a stable parameter. No significant difference was observed in change in hematocrit after 7 days between most of the investigated subgroups. In the groups with a change in hematocrit, the DPPIV or FAP activities showed the reverse trend (i.e. increased where hematocrit declined), excluding that differences between groups were influenced by factors such as changes in plasma volume.

**Table 7.3: The correlation between the parameters analyzed and DPPIV and FAP activities at admission and their kinetics.**

| | | DPPIV activity at admission | | | $\Delta DPPIV_{day7}$ | | | FAP activity at admission | | | $\Delta FAP_{day7}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Pearson correlation | T-value | P-value | Pearson correlation | T-value | P-value | Pearson correlation | T-value | P-value | Pearson correlation | T-value | P-value |
| | Age | -0.022 | | 0.88 | 0.102 | | 0.506 | 0.065 | | 0.654 | 0.164 | | 0.28 |
| | Gender | | 1.791 | 0.08 | | 1.545 | 0.13 | | 0.567 | 0.574 | | 0.962 | 0.342 |
| | Interval stroke onset and blood sampling | 0.17 | | 0.239 | n.a. | | n.a. | 0.088 | | 0.542 | n.a. | | n.a. |
| Cardiovascular | | | | | | | | | | | | | |
| | Arterial hypertension | | 0.344 | 0.733 | | -0.062 | 0.951 | | 0.108 | 0.915 | | -0.35 | 0.728 |
| | Atrial fibrillation | | -0.227 | 0.822 | | -0.842 | 0.404 | | -0.098 | 0.922 | | -0.14 | 0.89 |
| | Heart failure | | 0.592 | 0.557 | | -0.111 | 0.913 | | 1.456 | 0.152 | | 1.328 | 0.191 |
| | Peripheral artery disease | | 2.447 | **0.018*** | | 1.213 | 0.232 | | 2.29 | **0.026*** | | 1.939 | 0.059 |
| | Familial stroke | | 0.02 | 0.984 | | 0.397 | 0.693 | | -0.758 | 0.452 | | 0.05 | 0.96 |
| | Previous stroke | | -0.031 | 0.975 | | 0.157 | 0.876 | | -1.72 | 0.092 | | -1.08 | 0.286 |
| | Previous myocardial infarction | | -0.17 | 0.866 | | -1.221 | 0.229 | | 0.86 | 0.394 | | 0.2 | 0.843 |
| | Diabetes mellitus | | 0.222 | 0.825 | | -1.025 | 0.311 | | 0.598 | 0.552 | | -0.376 | 0.709 |
| | Dyslipidemia | | -0.859 | 0.395 | | -2.571 | **0.014*** | | -0.659 | 0.513 | | -1.543 | 0.13 |
| | Previous smoking | | 0.73 | 0.469 | | 0.401 | 0.691 | | 0.364 | 0.718 | | 0.487 | 0.629 |
| | Current smoking | | 1.268 | 0.211 | | 1.574 | 0.123 | | 1.416 | 0.163 | | 0.992 | 0.393 |
| | Alcohol abuse | | 0.47 | 0.64 | | 0.303 | 0.764 | | -0.383 | 0.703 | | -0.027 | 0.978 |
| Biochemistry | | | | | | | | | | | | | |
| | $Glycemia_{admission}$ | 0.211 | | 0.142 | 0.322 | | **0.031*** | 0.003 | | 0.983 | 0.162 | | 0.287 |
| | $Glycemia_{day7}$ | 0.126 | | 0.409 | 0.346 | | **0.020*** | -0.121 | | 0.427 | 0.081 | | 0.595 |
| | Total triglycerides | 0.031 | | 0.835 | 0.035 | | 0.828 | -0.109 | | 0.465 | -0.116 | | 0.464 |
| | Total cholesterol | 0.154 | | 0.3 | -0.101 | | 0.526 | 0.264 | | 0.072 | 0.137 | | 0.386 |
| | High-density lipoprotein | 0.459 | | **0.001*** | 0.348 | | **0.026*** | 0.413 | | **0.004*** | 0.468 | | 0.088 |
| | Low-density lipoprotein | -0.003 | | 0.986 | -0.273 | | 0.084 | 0.12 | | 0.427 | -0.36 | | 0.821 |
| | $CRP_{admission}$ | -0.207 | | 0.149 | -0.035 | | 0.819 | -0.342 | | **0.015*** | -0.132 | | 0.387 |
| | $CRP_{day7}$ | 0.007 | | 0.963 | 0.331 | | 0.026 | 0.043 | | 0.78 | 0.466 | | **0.001*** |
| | $Sedimentation\ rate_{admission}$ | -0.158 | | 0.31 | 0.006 | | 0.972 | -0.258 | | 0.095 | -0.117 | | 0.483 |
| | $Sedimentation\ rate_{day7}$ | 0.007 | | 0.965 | 0.293 | | 0.056 | -0.272 | | 0.077 | 0.042 | | 0.79 |
| Stroke | | | | | | | | | | | | | |
| | Infarct volume | 0.323 | | **0.022*** | 0.382 | | **0.010*** | 0.162 | | 0.262 | 0.299 | | **0.046*** |
| | $NIHSS\ score_{admission}$ | -0.073 | | 0.617 | 0.307 | | **0.040*** | -0.09 | | 0.534 | 0.249 | | 0.099 |
| | $\Delta NIHSS\ score_{admission\text{-}day7}$ | -0.285 | | **0.045*** | -0.089 | | 0.561 | -0.353 | | **0.012*** | -0.248 | | 0.101 |
| | Progressive stroke | | -1.959 | 0.081 | | -2.339 | **0.024*** | | -1.425 | 0.161 | | -2.316 | **0.025*** |
| | Institutionalization | | -2.038 | **0.047*** | | -3.444 | **0.001*** | | -2.311 | **0.025*** | | -4.19 | **0.000*** |
| | $mRS\ score_{day7}$ | 0.166 | | 0.249 | 0.55 | | **0.000*** | 0.091 | | 0.529 | 0.528 | | **0.000*** |
| | $mRS\ score_{month3}$ | 0.03 | | 0.839 | 0.495 | | **0.001*** | 0.028 | | 0.849 | 0.474 | | **0.001*** |

## 7.5 Discussion

In recent years, there has been a growing interest in dipeptidyl peptidases in stroke. As most research focuses on the effects of inhibition or on the role of physiological substrates of DPPIV [1, 8, 15], there exists a knowledge-gap about DPPIV activity levels in stroke. While the involvement of DPPIV activity and related proteases has been reported in cerebral ischemia induced inflammation in rats [8], we are the first to report on the kinetics of DPPIV and FAP activity in patient serum in hyperacute stroke and the 7 days following stroke onset. While DPPIV activities only tended to increase shortly after stroke and decrease thereafter, FAP activities decreased significantly during the first week after stroke onset. Both enzymes reached their lowest levels at day 7.

The increase in soluble DPPIV shortly after stroke onset is in agreement with Röhrborn *et al.* who showed *in vitro* that DPPIV is shed during hypoxia [16]. After admission this hypoxic state is relieved and the activity lowers to baseline. Since we did not observe a significant increase in FAP activity, we have no indication for a similar mechanism for FAP.

This hypothesis might help explain the beneficial effect of a pretreatment with DPPIV inhibitors in an animal model [1]. This is most likely a class effect as both linagliptin and alogliptin show a beneficial effect [1, 15]. As inhibitors prevent the sudden rise in DPPIV activity, the biological half-life of neuroprotective substrates such as glucagon-like peptide 1 (GLP-1) is extended [3]. However, the effect of GLP-1 on hyperglycemia should not be neglected, as hyperglycemia is associated with worse outcomes in stroke [17].

The fact that we did not find a correlation between cardiovascular risk factors and enzyme activities, especially of DPPIV, was not expected. A recent meta-analysis found a reduction in all-cause mortality and major cardiovascular events during DPPIV inhibitor treatment [2]. This might be explained by the fact that the meta-analysis only included patients with diabetes mellitus type 2.

We showed that both the systolic and the diastolic blood pressure increased with higher DPPIV and FAP activities. The role of DPPIV in blood pressure is controversial. While the majority of authors state that DPPIV inhibitors can lower blood pressure, others claim a rise [9, 18]. However, the truth probably lies in the middle as Jackson *et al.* showed that the effect of DPPIV inhibitors is context dependent [18]. We are the first to show a correlation of FAP levels with blood pressure. Nonetheless, the mechanism behind this remains unclear as FAP expression is tightly regulated [19]. An explanation might be sought in the overlap with DPPIV concerning its cardiovascular-active substrates.

The correlation between glycemia and DPPIV kinetics could be anticipated, as its best known substrate, GLP-1, is involved in glucose homeostasis [9]. The correlation of inflammation with FAP activity is equally unsurprising as it was shown earlier that its expression is induced during inflammation through TNFα [20].

## 7.6 Conclusion

This study describes the decrease in DPPIV and FAP activities during the first week after stroke onset and its correlation with stroke severity, progression and outcome. These novel data help us to better understand the biological significance of both enzymes in stroke and warrant further investigation of the potential benefit of DPPIV inhibitors in stroke in humans.

## 7.7 Limitations

Our results are only based on a limited number of patients. This is due to the fact that the collection of samples from hyperacute stroke patients is difficult. Therefore, the results should be interpreted with caution and stimulate further research. While there seems to exist a correlation between DPPIV/FAP activity and stroke outcome it is not clear whether the change in enzyme activity is the cause or the result of a worse stroke outcome.

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**Chapter 8**

# Conclusion and perspectives

# Chapter 8 – Conclusion and perspectives

At the beginning of this doctoral thesis, DPPIV inhibitors were just suggested to have positive effects on the micro- and macrovascular complications of type 2 diabetes. At the same time, the cardiovascular safety of the new antidiabetics was questioned. As the **pharmacological profile of DPPIV inhibitors** was not completely understood, we aimed to better define its cardiovascular and osteoprotective profile.

The common thread in this doctoral research on **DPPIV** outside glucose homeostasis is the assumption that it **exerts its effect through its substrates**. This is based on the original discovery that DPPIV inhibitors improve glucose homeostasis by increasing the half-life of the incretines like GLP-1. As with GLP-1, this requires the identification of a potential substrate, proof that its N-terminus is key to its biological activity and demonstration that DPPIV is responsible for its modification. All three of these points are equally challenging. An important advancement in the development of DPPIV inhibitors for type 2 diabetes was the production of an antibody that could discriminate between the intact and the truncated form of GLP-1. **By optimizing sample handling and the immunoassay for intact SDF1α (in chapter 4), our work provides a useful tool that could open up new avenues in SDF1α related research well beyond cardiovascular pathologies.** The advantages of an immunoassay are its high sensitivity and specificity combined with a high-throughput, without the need for specific equipment. Regardless of these benefits, **methods such as liquid chromatography coupled to mass spectrometry seem crucial** for the simultaneous analysis of all proteolytically modified peptides. Several attempts were undertaken for the development of a mass spectrometric method, but, to our disappointment, all of these fell short of our analytical requirements.

Besides proteolytic degradation, many other forms of **post-translational modifications** are known to occur and **influence the biological activity of peptides and proteins**. One of these modifications is

the citrullination of arginine moieties by peptidylarginine deiminase. As SDF1α contains N-terminally several arginine residues ($Arg_8$, $Arg_{12}$, and $Arg_{20}$), which are potentially citrillunated, this can result in a reduction of its chemotactic activity [1]. It would be interesting to investigate the effect of citrullination on DPPIV-mediated cleavage and on SDF1α immunoreactivity. What's more, similar to SDF1α and DPPIV, peptidylarginine deiminases are deregulated in inflammation. Therefore, the extent of citrullination should be determined in inflammatory diseases to investigate a possible link between DPPIV and peptidylarginine deiminase activity. As DPPIV inhibitors have anti-inflammatory properties, this provides an alternative mechanism in which DPPIV inhibitors retain SDF1α's biological activity.

As several publications, including this thesis, **focus on the positive effects of DPPIV inhibitors, its negative effects tend to be overlooked**. The increased half-life of DPPIV substrates should be regarded as a double-edged sword. For example, it is known that tissues that express high SDF1α levels are among the most common sites of metastasis. Conversely, CXCR4, the SDF1α receptor, is overexpressed in many tumors including ovarian, breast and prostate cancer. In a mouse breast cancer model CXCR4 antagonism inhibits tumor growth and metastasis. Abrogation of the SDF1α/CXCR4 axis decreases metastasis rate and tumor growth in both breast and prostate cancer [2, 3]. As mentioned, DPPIV inhibitors increase the biological half-life of SDF1α, thereby enhancing the risk of cancer. Indeed, DPPIV inhibitors have been associated with an increase in pancreatic cancer and are linked to the progression and metastasis of pancreatic cancer [4]. From the SAVOR-TIMI and EXAMINE trial we now know that alogliptin and saxagliptin do not increase the risk for pancreatic cancer. Nevertheless, the effect of DPPIV inhibitors on SDF1α levels and the safety of their long-term use in a population at risk for cancer should be further investigated.

The other side of the coin is that SDF1α is able to prevent human immunodeficiency virus-1 (HIV-1) infection of peripheral blood mononuclear cells through CXCR4. CXCR4 functions as a co-receptor for HIV-1. Together with CCR5, this co-receptor is essential for viral entry of HIV-1. CCR5 antagonists (*e.g.*

maraviroc) are routinely used in the clinic to treat HIV-infection and blocking this co-receptor reduces viral load [5]. Truncation of SDF1α results in a decrease of its antiviral activity, suggesting that DPPIV inhibition in HIV patients might be beneficial [6]. In addition, HIV-infection is slowly transforming into a chronic condition, due to improved treatment strategies. The prevalence of common western diseases, such as diabetes, has increased to about 40% in these patients [7]. Consequently, DPPIV inhibitors might provide additional benefit in diabetic HIV-positive patients. The first results of a small pilot study with sitagliptin, revealed no increase in the number of adverse effects [7].

Chapter 5 contains the **discovery of a new DPPIV substrate**, nesfatin-1, with an only recently recognized role in the cardiovascular system. In vitro, **nesfatin-1 is a moderate substrate** in terms of $k_{cat}/K_m$. There is a longstanding discussion in the field of peptidases whether in vitro biochemical data are somehow predictive of physiological significance. It also is a reminder that GLP-1, which is cleaved after an alanine residue, is a much better substrate than expected. **There still is a lack of understanding of the DPPIV-substrate interactions on a molecular level.**

It is known that the glycosylation status affects protein-protein interactions and the biological activity. Indeed, glycosylation of proBNP prevents the generation of the active hormone BNP. Similarly, glycosylation can prevent the liberation of nesfatin-1 from NUCB2. As NPRA activation has only been shown for intact nesfatin-1(1- 82), the cGMP production of NUCB2 should be assessed in cardiomyocytes.

One of the large obstacles in the treatment of heart failure is the **lack of therapeutic options in heart failure with preserved ejection fraction (HFpEF)**, previously known as diastolic heart failure. HFpEF is thought to **arise as a consequence of a continuous pro-inflammatory state leading to endothelial dysfunction**. This dysfunction reduces nitric oxide and cGMP bioavailability and lowers PKG activity in cardiomyocytes, resulting in titin hypophosphorylation, cardiomyocyte stiffness and fibrosis [8]. Interestingly, **DPPIV inhibitors are able to restore all of these dysfunctions (chapter 1) and their protective effect in HFpEF should be explored *in vivo***.

During this doctoral thesis we explored a somewhat **alternative hypothesis**, namely that DPPIV does not only convert peptide substrates but also **produces bioactive products, namely proline-containing dipeptides**. The optimization of analytical methods for detecting dipeptides in serum turned out to be something out of a nightmare, both experimentally and what concerns the interpretation. Nevertheless, we were able to show (in chapter 6) that **DPPIV inhibition alters collagen metabolism**. These results warrant further investigation of DPPIV and its inhibition in **diseases associated with altered collagen metabolism** such as fibrosis of the heart and atherosclerosis.

Myocardial fibrosis is caused by an increase in collagen type I synthesis and deposition [9]. This is supported by the fact that circulating collagen fragments are increased by fibrosis and might be related with the type of left-ventricular dysfunction [10]. As shown in chapter 6, DPPIV inhibition alters collagen metabolism and matrix metalloproteinase activity in rats. Given the changes in collagen metabolism and the increase in MMP activity in heart failure, **a parallel can be drawn between heart failure and the osteoporotic rats (from chapter 6)** [11]. Characterizing collagen metabolism in heart failure and determining the involvement of four key proteases (MMP2, MMP9, DPPIV and prolidase) will increase our understanding of fibrosis in heart failure.

It is known that collagen is a critical component of atherosclerotic plaques. Collagen constitutes up to 60% of total protein in the plaque. Its presence in the fibrous cap lends structural integrity and mechanical strength against plaque rupture. Even though, DPPIV is restricted to the endothelial cells of neovessels in human plaques, circulating DPPIV can still contribute to plaque instability and plaque growth. This, in part, explains the reduction in plaque size after DPPIV inhibitor treatment observed in Apolipoprotein E negative mice [12–14]. Other pathologies associated with an aberrant collagen metabolism that should be investigated are: pulmonary fibrosis, cancer metastasis and scleroderma. It should also be noted that several chemokine substrates of DPPIV appear to mediate fibrosis [15].

In chapter 7, we provide evidence for the **involvement of DPPIV in acute ischemic stroke**. This provides a rationale for the use of DPPIV inhibitors in the prevention of stroke. The **use of DPPIV inhibitors in type 2 diabetic patients with an increased risk of stroke seems logical**. It has been shown that several DPPIV substrates are related to the severity and outcome of stroke [16, 17]. Besides the incretins, the levels of DPPIV substrates and their biologic inactivation have not been extensively studied in patients. Among these substrates SDF1α has been shown to be one of the most promising molecules and is involved in neuroprotective pathways [18, 19]. As shown in myocardial infarction, local injection of protease resistant SDF1α might prove effective in the reduction of infarct size and short- and long-term outcomes.

For the moment, several companies are developing once-weekly DPPIV inhibitors, culminating in the development of trelagliptin and omarigliptin. Although these inhibitors seem to be as efficient as the current generation of inhibitors, their effects outside diabetes have not yet been studied. Trelagliptin and omarigliptin result in a similar inhibition of DPPIV as the once-daily molecules and are expected to provide the same micro- and macrovascular protection. Nonetheless, their effectiveness in reducing these complications and their cardiovascular safety still has to be tested in large clinical trials.[20].

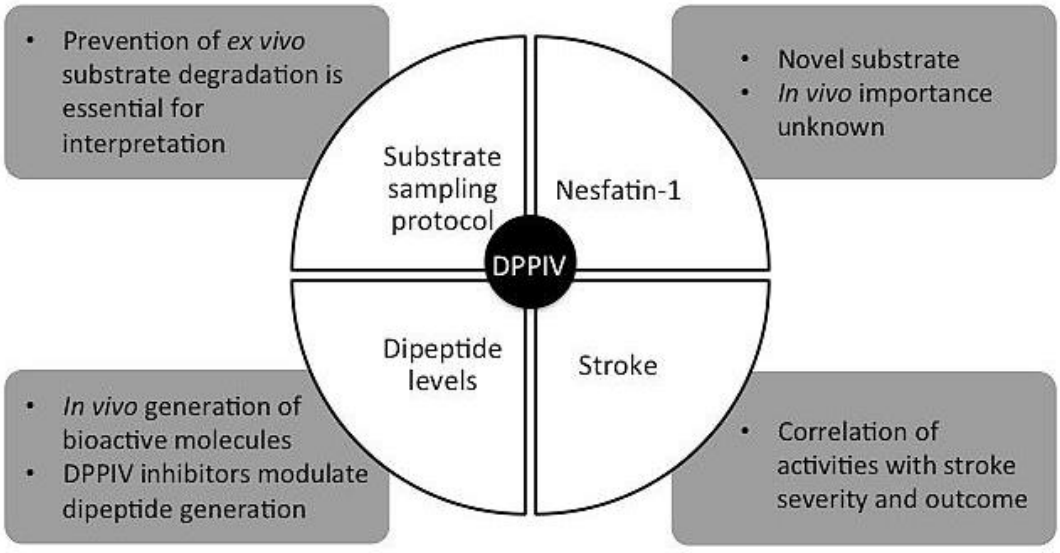


**Figure 8.1: Overview of the parts of the pharmacological profile of DPPIV inhibitors investigated in this thesis.**

While most of our findings are not groundbreaking, such as the fact that nesfatin-1 levels were not linked with cardiovascular parameters, we also **contributed significantly to DPPIV research** in two ways. We have shown that **DPPIV** not only alters receptor selectivity, but also is **responsible for the generation of biologically active molecules *in vivo***. However, our biggest achievement is the fact that we proved that the **analysis of DPPIV substrates and the interpretation of its physiological levels are critically dependent on sample handling**.

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# Chapter 9

Summary

# Chapter 9 - Summary

DPPIV is an exopeptidase able to split off Xaa-Pro or Xaa-Ala dipeptides from the N-terminus of peptide substrates. This process modulates the biological activity of cytokines and (neuro)hormones, resulting in an altered interaction with their receptor. DPPIV inhibitors are routinely used as a second-line therapy, after metformin, in the treatment of type 2 diabetes. The knowledge about these inhibitors has progressed immensely since the development of the first generation of inhibitors (vildagliptin and sitagliptin). DPPIV inhibitors might provide new possibilities in the treatment and prevention of the micro- and macrovascular complications in diabetes, such as stroke, myocardial infarction and nephropathy (**chapter 1**). This doctoral thesis aimed to shed a light on the mechanisms underlying these glucose-independent mechanisms (**chapter 2**). **Chapter 3** described the materials and methods used.

In **chapter 4,** a sampling protocol was optimized for the collection of blood when DPPIV substrates are to be analyzed. We found that enzymatic conversion of SDF1α by DPPIV lowers its immunoreactivity *in vitro* and *ex vivo*. Even under optimal pre-analytical conditions, *ex vivo* truncation results in the underestimation of SDF1α concentrations. The collection of blood in tubes containing DPPIV inhibitors, and a general cocktail of protease inhibitors, effectively blocks this cleavage. In the absence of a specific DPPIV inhibitor a loss in immunoreactivity could not be prevented. Therefore, it is highly recommended to collect all samples in tubes containing at least a DPPIV inhibitor to block *ex vivo* degradation.

In addition, a method to quantitate the *in vivo* circulating, and N-terminally intact, SDF1α was developed. As commercial SDF1α immunoassays react differently towards intact than DPPIV-truncated SDF1α, we used this characteristic to calculate the percentage of *in* vivo circulating intact SDF1α. We found that about 75% of all circulating molecules are intact in the plasma of healthy

volunteers. This method can be used to elucidate the *in vivo* effect DPPIV inhibitor-treatment in humans and in animal experiments.

The optimal sample handling protocol was used for the analysis of SDF1α levels in patients referred for elective diagnostic cardiac catheterization because of suspected heart failure. In patients with high SDF1α levels DPPIV activities were lower and these patients showed a severe loss of LV function. Our results combined with other recent findings indicate the potential of SDF1α as a biomarker in heart failure [1–3]. Interestingly, no difference in DPPIV activities could be found between any of the investigated heart failure groups.

**Chapter 5** investigates nesfatin-1 as a possible DPPIV substrate. Originally nesfatin-1 and its precursor NUCB2 were discovered as appetite regulating molecules. Meanwhile, it is clear that they are also involved in the cardiovascular system, through its similarity to BNP. Nesfatin-1 and its mammalian orthologues contain an N-terminal proline in the penultimate position, marking it as a potential DPPIV substrate. Therefore, we studied its potential as a DPPIV substrate.

Nesfatin-1 was shown to be a novel substrate of both DPPIV and FAP. Despite a $K_m$ above 20 μM, nesfatin-1 cleavage by FAP was relatively fast. Because nesfatin-1 is generated from the N-terminus of NUCB2, we speculate that NUCB2 might also be a DPPIV and FAP substrate. Analysis of the main circulating forms revealed that NUCB2, and not nesfatin-1, is the predominant protein in healthy controls and heart failure patients. Further investigation revealed the presence of another, higher molecular weight form. We speculate that this molecule corresponds to glycosylated NUCB2. The possibility of glycosylation was investigated using a bioinformatics tool (NetOGlyc 4.0). NUCB2 was found to have five possible glycosylation sites of which two lay within nesfatin-1.

Similar to SDF1α, NUCB2 levels were evaluated in patients suspected of heart failure. No cardiovascular parameter was significantly related to NUCB2 concentrations. However, patients with decompensated HFrEF tended to have lower NUCB2 levels.

Diabetic patients are known to have an increased risk of bone fractures. This risk seems related to alterations in their collagen metabolism. As DPPIV is related to collagen metabolism and is able to interact with collagen type I, the effect of sitagliptin on collagen-derived dipeptides was investigated in diabetic and non-diabetic rats using a streptozotocin-nicotinamide model (in **chapter 6**).

Sitagliptin-treatment for 12 weeks did not result in changes in MMP2 or prolidase activity. MMP9 activities were altered by diabetes and untreated diabetic rats had three-fold higher MMP9 activities compared to control animals. Treatment of the diabetic rats dampened the increase in MMP9. Since it is known that MMP9 activities increase in response to inflammatory stimuli, the effect of sitagliptin might be caused by its anti-inflammatory properties.

Compared to humans, dipeptides and amino acid concentrations were on average five times lower in rats, which reflect their higher renal clearance. We are the first to show that a diabetic state possibly increases Pro-Pro and Ala-Pro levels, while leaving Gly-Pro constant. Pro-Hyp levels were ten times higher than these of other dipeptides. Compared to controls, diabetes reduced Pro-Hyp levels significantly, while sitagliptin-treated controls only tended to have lower levels. The decreased Pro-Hyp levels in diabetes might be detrimental, as Pro-Hyp promotes osteoblast differentiation and stimulates the growth of fibroblasts on collagen.

Hyp and Pro amino acid levels were unchanged by sitagliptin treatment or diabetes. This suggests that the degradation of collagen-derived dipeptides is not altered.

Taken together, the results of dipeptide levels and protease activities indicate an involvement of MMP9 in the generation of Ala-Pro or Pro-Pro containing collagen fragments. Nevertheless, the contribution of other proteases cannot be excluded.

As DPPIV inhibition did not result in a change in glucose levels, we can conclude that our findings are independent from an effect on glycemia. Furthermore, our data indicates that the osteoprotective effect of sitagliptin is not linked to a direct effect on the collagen metabolism in streptozotocin-

nicotinamide-induced diabetic rats. It most likely reflects the increased half-life of biologically active DPPIV substrates such as GLP-1 and GIP.

In **chapter 7** we investigated DPPIV and FAP activities in acute ischemic stroke. This was the first study reporting on the kinetics of DPPIV and FAP activity in patient serum in hyperacute stroke. DPPIV activities tended to increase shortly after stroke and returned to baseline after seven days. Hypoxia probably increases circulating DPPIV shortly after onset. As hypoxia is relieved after admission, DPPIV activity returns to normal. In contrast, FAP activities decreased constantly after onset and reached their lowest levels after one week.

No correlation was observed between cardiovascular risk factors and DPPIV or FAP activity. However, both higher systolic and higher diastolic blood pressure were associated with increased enzyme activities. We are the first to show that FAP levels are correlated with blood pressure. Similar to DPPIV, FAP might regulate blood pressure through the modulation of cardiovascular-active DPPIV substrates. We also confirmed the correlation between glycemia and DPPIV activity and inflammation with FAP activity.

In conclusion we can state that DPPIV inhibitors are promising molecules for the treatment of micro- and macrovascular complications of diabetes. They offer additional benefits in type 2 diabetes besides their effect on blood glucose, suggesting them as a logical choice when add-on therapy is needed.

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**Chapter 10**

# Samenvatting

# Chapter 10 - Samenvatting

DPPIV is een exopeptidase dat Xaa-Pro en Xaa-Ala dipeptides afsplitst van peptiden. Dit proces wijzigt de biologische activiteit van ondermeer cytokines en (neuro)hormonen. Inhibitoren van DPPIV verlengen het half-leven van de incretine hormonen en herstellen hierbij de bloedsuikerspiegel. Momenteel worden de DPPIV-inhibitoren in de kliniek gebruikt als tweede-lijnstherapie in de behandeling van type 2 diabetes, indien metformine in monotherapie onvoldoende blijkt. Onze kennis van deze inhibitoren en hun effecten is exponentieel toegenomen sinds de ontwikkeling van de eerste generatie. DPPIV-inhibitoren bieden mogelijk bescherming tegen de micro- en macrovasculaire complicaties van diabetes, zoals beroerte, hartinfarct of nieraandoeningen. Dit doctoraat had als doel een licht te werpen op de mechanismes achter deze bloedsuikerspiegel-onafhankelijke effecten.

In **hoofdstuk 1** introduceerden we de peptidase familie en gaven we een overzicht van de verkrijgbare DPPIV-inhibitoren en de data omtrent hun cardioprotectief, osteoprotectief en nefroprotectief effect. **Hoofdstuk 2** besprak de doelstelling van deze thesis en **hoofdstuk 3** de methoden en reagentia die hiervoor gebruikt werden.

In **hoofdstuk 4,** hebben we een staalname-protocol geoptimaliseerd voor (bloed)stalen die DPPIV-substraten bevatten. We ontdekten dat DPPIV de SDF1α immunoreactiviteit verlaagde in zowel *in vitro* als *ex vivo* experimenten. Zelfs onder optimale pre-analytische omstandigheden resulteerde dit in een onderschatting van de SDF1α spiegels. Het verzamelen van bloedstalen in tubes met DPPIV-inhibitoren, naast een brede mix van inhibitoren, verhinderde deze knipping. In de afwezigheid van een specifieke DPPIV-inhibitor kon een verlies in immunoreactiviteit niet worden afgeremd. Daarom is het aanbevolen is om alle stalen af te nemen op tubes die minstens een DPPIV-inhibitor bevatten, om de *ex vivo* knipping te voorkomen.

Aangezien commerciële SDF1α immunoassays verschillend reageren op intact en DPPIV-geknipt SDF1α, besloten we deze eigenschap te gebruiken om de hoeveelheid *in* vivo circulerend, N-terminaal intact SDF1α te bepalen. In het plasma van gezonde vrijwilligers bleek 75% van alle circulerende moleculen intact te zijn. De voorgestelde methode kan gebruikt worden voor onderzoek naar het *in vivo* effect van DPPIV-inhibitoren in zowel patiënten als proefdier-experimenten.
Het geoptimaliseerde staalname-protocol werd eveneens gebruikt voor de analyse van SDF1α spiegels in patiënten met een vermoeden van hartfalen. Patiënten met hoge SDF1α spiegels hadden lagere DPPIV activiteiten, bovendien hadden deze patiënten een sterk gedaalde linker-ventriculaire functie. Onze resultaten wijzen op het potentieel van SDF1α als biomerker in hartfalen. Er werd geen verschil in DPPIV activiteit teruggevonden tussen de onderzochte patiëntengroepen.

**Hoofdstuk 5** onderzocht nesfatin-1 als een potentieel DPPIV-substraat. Hoewel nesfatin-1 en zijn precursor NUCB2 origineel beschreven werden als eetlustregulerende hormonen, is het ondertussen duidelijk dat ze ook betrokken zijn in het cardiovasculair systeem. Nesfatin-1 en zijn zoogdier orthologen bevatten N-terminaal een Xaa-proline sequentie, het typische kenmerk van een DPPIV substraat.

Nesfatin-1 bleek een substraat te zijn van zowel DPPIV als FAP. Ondanks een $K_m$ groter dan 20 µM, werd nesfatin-1 vrij snel gesplitst door FAP. Aangezien nesfatin-1 vrijgesteld wordt uit de N-terminus van NUCB2, speculeren we dat ook NUCB2 een DPPIV- en FAP-substraat is. Analyse van de circulerende moleculen toonde aan dat NUCB2, en niet nesfatin-1, het voornaamste hormoon is in gezonde vrijwilligers en in patiënten met hartfalen. Daarnaast wees verder onderzoek op de aanwezigheid van een andere vorm met een hoger moleculair gewicht. We vermoeden dat deze molecule overeenkomt met een geglycosyleerde vorm van NUCB2. De mogelijkheid van glycosylatie werd onderzocht aan de hand van bioinformatica. NUCB2 bleek vijf potentiële glycosylatieplaatsen te bezitten, waarvan er twee binnen nesfatine-1 lagen.

Net als bij SDF1α, onderzochten we NUCB2 spiegels in patiënten met een vermoeden van hartfalen. De patiënten werden opgedeeld in tertielen op basis van hun NUCB2 concentratie, maar geen enkele cardiovasculaire parameter was significant gewijzigd tussen deze groepen. De patiënten met gedecompenseerd HFrEF hadden over het algemeen lagere NUCB2 spiegels.

Bij patiënten met diabetes ziet men een toename in het aantal botfracturen. Dit risico is te wijten aan een gewijzigd collageen metabolisme. Aangezien DPPIV nauw verbonden is met het collageen metabolisme, werd het effect van sitagliptine op van collageen afkomstige dipeptides onderzocht in diabetische en niet-diabetische ratten met behulp van een streptozotocine-nicotinamide proefdier-model (in **hoofdstuk 6**).

Een behandeling van 12 weken met sitagliptine gaf geen verschil in MMP2- of prolidase-activiteit. MMP9-activiteiten waren gewijzigd in diabetes en onbehandelde diabetische ratten hadden driemaal hogere MMP9-activiteiten in vergelijking met controledieren. Sitagliptine-behandeling van de diabetische ratten verminderde de toename in MMP9. Een mogelijke verklaring hiervoor is te vinden in het anti-inflammatoire effect van sitagliptine dat zo de stijging in MMP9 verhindert.

In vergelijking met mensen, lagen de dipeptide en aminozuur spiegels in ratten ongeveer vijf maal hoger. Dit wordt mogelijk verklaard door de hogere renale klaring in ratten. Het ontwikkelen van diabetes verhoogt schijnbaar de Pro-Pro en Ala-Pro spiegels, terwijl diabetes geen effect heeft op Gly-Pro. De Pro-Hyp concentraties lagen tien maal hoger dan deze van de andere dipeptiden. In vergelijking met controledieren, namen de Pro-Hyp spiegels in diabetes significant af. De lagere Pro-Hyp spiegels in diabetes zijn mogelijk schadelijk, aangezien Pro-Hyp de osteoblast differentiatie en fibroblast groei stimuleert. De concentraties van de aminozuren Hyp en Pro werden niet beïnvloed door sitagliptine of diabetes. Dit wijst erop dat de afbraak van collageen afkomstige dipeptides niet gewijzigd is in de proefdieren.

De resultaten van de dipeptiden samen met deze van de protease-activiteiten wijzen op de betrokkenheid van MMP9 in het ontstaan van Ala-Pro of Pro-Pro bevattende collageen fragmenten. Desalniettemin, kan de bijdrage van andere enzymen niet worden uitgesloten.

Aangezien DPPIV-inhibitie niet leidde tot wijzigingen in bloedsuiker spiegel, kunnen we concluderen dat onze bevindingen onafhankelijk zijn van een mogelijk effect op de glycemie. Daarenboven, wijzen onze resultaten op het feit dat het osteoprotectieve effect van sitagliptine niet het gevolg is van een direct effect op het collageen metabolisme in dit proefdiermodel. Het weerspiegelt eerder een toename in het half-leven van osteoprotectieve DPPIV-substraten zoals GLP-1 en GIP.

In **hoofdstuk 7** onderzochten we de DPPIV- en FAP-activiteiten in patiënten met een acute ischemische beroerte. Dit is de eerste studie die de wijzigingen in DPPIV- en FAP-activiteit in serum van patiënten met een beroerte in de hyperacute fase beschrijft. De DPPIV-activiteiten namen schijnbaar toe kort na de beroerte en herstelden zich na zeven dagen. Daarentegen bleven de FAP-activiteiten stijgen tot de zevende dag na opname.

Er werd geen correlatie gevonden tussen cardiovasculaire risico factoren en DPPIV- of FAP-activiteiten. Een verhoging in sytolische of diastolische bloeddruk was geassocieerd met een verhoging in de activiteit van beide enzymes. FAP en DPPIV oefenen dit effect mogelijks uit door modulatie van cardiovasculair actieve substraten. De reeds eerder gerapporteerde relatie tussen, enerzijds glycemie en DPPIV-activiteit en anderzijds inflammatie en FAP-activiteit werd eveneens bevestigd.

Tot slot kunnen we stellen dat DPPIV-inhibitoren veelbelovende geneesmiddelen zijn voor de behandeling van de micro- en macrovasculaire complicaties van diabetes. Daarenboven bieden ze voordelen aan diabetische patiënten onafhankelijk van een verbetering in bloedsuikerspiegel en werd hun veiligheid in verschillende klinische studies bewezen. Dit maakt van DPPIV-inhibitoren een logische eerste keuze indien add-on therapie nodig is.

Scientific curriculum vitae

# Scientific curriculum vitae

**Personalia**

| | |
|---|---|
| Name | Lesley Baerts |
| Day of birth | July 6, 1986 |
| Place of birth | Antwerp, Belgium |
| Nationality | Belgian |

**Education**

| | |
|---|---|
| 1998 - 2004 | Humaniora, option Latin-Sciences<br>Sint-Norbertuscollege, Antwerp, Belgium |
| 2004 - 2009 | Master in Pharmaceutical Sciences, magna cum laude<br>University of Antwerp, Belgium<br><br>Thesis: Ontwikkeling en validatie van een methode voor de bepaling van het B-type natriuretisch peptide (BNP)<br>Supervisor: Prof. Dr. I. De Meester<br>Prof. Dr. A-M. Lambeir |
| 2009 - | PhD-student in Pharmaceutical Sciences<br>Laboratory of Medical Biochemistry<br>University of Antwerp, Belgium<br><br>Thesis: Dipeptidyl peptidase IV beyond glucose homeostasis:<br><br>A focus on substrates<br><br>Supervisor: Prof. Dr. I. De Meester<br>Prof. Dr. A-M. Lambeir |

**Scientific publications**

Peer reviewed papers:

2011 Jungraithmayr W, De Meester I, Matheeussen V, **Baerts L**, Arni S, Weder W; CD26/DPP-4 inhibition recruits regenerative stem cells via stromal cell-derived factor-1 and beneficially influences ischaemia-reperfusion injury in mouse lung transplantation; Eur J Cardiothorac Surg. 2011 Dec 21.

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2013 S. De Schutter, E. Leuridan, K. Maertens , **L. Baerts**, P. Van Damme, I. De Meester; Quantification of Bordetella Pertussis toxin antibodies in breast milk: optimization of sample preparation and evaluation of commercial ELISA's; 10th International Symposium on Bordetella, 8/9/13 – 11/9/13, Dublin, Ireland

2014 **L. Baerts**, Y. Waumans, I. Brandt, M. Vanderheyden and I. De Meester; Cleavage of SDF1α by Dipeptidyl peptidase IV and other proteases affect Immunoreactivity in Commercial Immunoassays; Belgian Peptide Group Meeting 2014, 10/2/14 – 11/2/14, Ghent, Belgium. **Poster presentation.**

2015 L. Baerts, L. Glorie, W. Maho, A. Eelen, A. Verhulst, P. D'Haese, A. Covaci, I. De Meester; Impact of Sitagliptin on collagen-derived dipeptides in diabetes; Research day of the Faculty of Pharmaceutical Sciences, 20/5/15. **Oral presentation.**

**L. Baerts**, Y. Waumans, I. Brandt, W. Jungraithmayr, P. Van Der Veken, M. Vanderheyden, I. De Meester; Circulating SDF1α levels in heart failure: a matter of proper sampling; Heart Failure 2015, European Society of Cardiology (ESC), 23/5/2015-26/5/2015, Seville. **Poster presentation.**

L. Baerts, L. Glorie, W. Maho, A. Eelen, A. Verhulst, P. D'Haese, A. Covaci, I. De Meester; Impact of Sitagliptin on collagen-derived dipeptides in diabetes; Forum of pharmaceutical sciences, Blankenberge, Belgian Society of Pharmaceutical Sciences (BSPS), 28/5/15-29/5/15. **Oral presentation.**

**L. Baerts**, Y. Waumans, I. Brandt, W. Jungraithmayr, P. Van Der Veken, M. Vanderheyden, I. De Meester; Characterization of immunoassays: lessons learned from DPP4 substrates; Research day of the Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, 30/10/15. **Oral presentation.**

# Dankwoord

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