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DPP4 beyond glucose homeostasis

Attenuation of acute kidney injury and diabetic bone loss through DPP4 inhibition

Dipeptidyl peptidase 4 - meer dan glucose homeostase

Vermindering van acute nierschade en diabetes-gerelateerd botverlies door DPP4 inhibitie

Proefschrift voorgelegd tot het behalen van de graad van doctor in de Biomedische Wetenschappen
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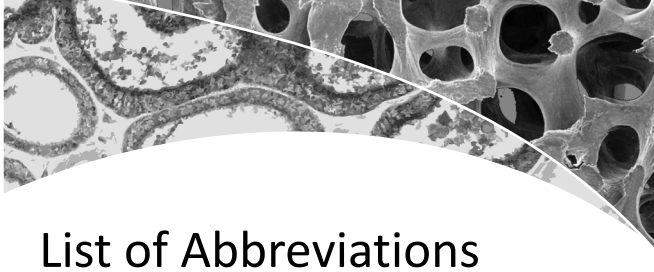
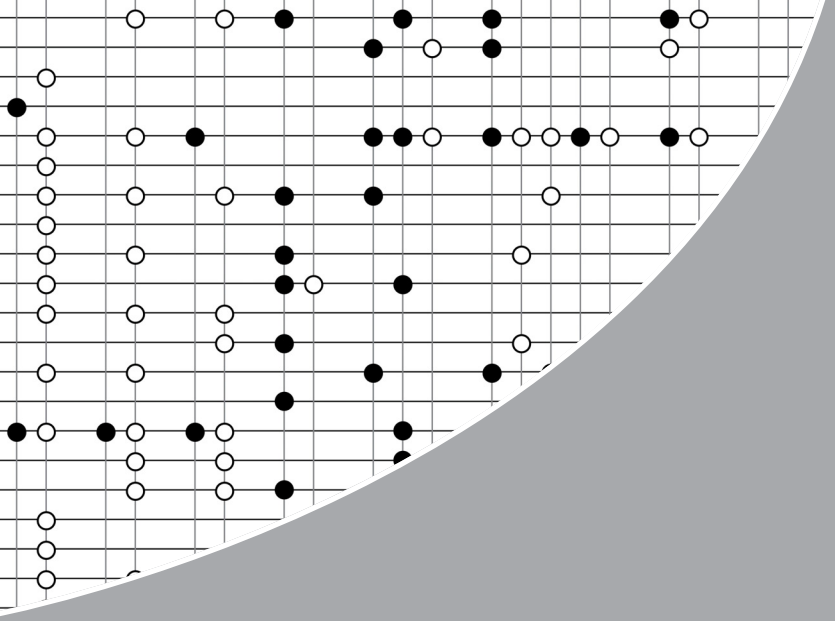


List of Abbreviations

ACE	Angiotensin converting enzyme
ADA	Adenosine deaminase
α-MSH	α-melanocyte stimulating hormone
AGEs	Advanced glycation end-products
AjAR	Adjusted mineral apposition rate
AKI	Acute Kidney Injury
Akt (= PKB)	Protein kinase B
ALEs	Advanced lipoxidation end-products
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APN(R)	Adiponectin (receptor)
AST	Aspartate aminotransferase
ATN	Acute tubular necrosis
ATP	Adenosine triphosphate
BAr	Bone area
Bax	B-cell CLL/lymphoma-2-associated X protein
Bcl-2	B-cell CLL/lymphoma-2
BFR	Bone formation rate
BMD	Bone mineral density
BMI	Body mass index
BMP(2)	Bone morphogenetic protein (2)
BMU	Basic multicellular unit
BNP	B-type/brain natriuretic peptide
BS	Bone surface
BV	Bone volume
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium sensing receptor
CatK	Cathepsin K
CD26	Cluster of differentiation 26
CKD	Chronic kidney disease
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
Coll1	Collagen type 1
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CSF	Colony stimulating factor
CTX-1	C-terminal telopeptide / C-terminal collagen 1 crosslinks
CXCR4	C-X-C chemokine receptor type 4
DAP12	DNAX-activating protein 12
DEXA	Dual-energy X-ray absorptiometry
dlPm	Double-labelled perimeter
DM	Diabetes mellitus
DPP4/8/9	Dipeptidyl peptidase 4/8/9
EMA	European Medicines Agency
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
EPm	Eroded perimeter
EPO	Erythropoietin
FADD	Fas-associated death domain protein
FAPα	Fibroblast activation protein α
FDA	US Food and Drug Administration
FGF(23)	Fibroblast growth factor 23
G-CSF	Granulocyte-colony stimulating factor
GFR	Glomerular filtration rate
GIP(R)	Glucose-dependent insulinogenic peptide (receptor)
GLP-1/2(R)	Glucagon-like peptide 1/2 (receptor)
GLUT2	Glucose transporter 2
GOA	Concerted Research Action (Geconcerteerde Onderzoeksactie)
GRP	Gastric releasing peptide
HFD	High fat diet fed
HDL	High density lipoprotein
HIF-1	Hypoxia inducible factor 1
HO-1	Heme oxygenase 1
HRT	Hormone replacement therapy
HSC	Hematopoietic stem cell
Hsf1	Heat shock factor protein 1
Hsp(32)	Heat shock protein (32)
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Interferon γ
IGF-1(R)	Insulin growth factor 1 receptor

IL(-1/6/7/8/10/12/16/18)	Interleukin 1/6/7/8/10/12/16/18
IP10	Interferon γ-induced protein 10
IRF-1	Interferon regulatory factor 1
IRI	Ischemia reperfusion injury
ISOM	Inner stripe of outer medulla
I-TAC	Interferon-inducible T-cell α chemoattractant
KIM-1	Kidney injury molecule 1
L-FABP	Liver-type fatty acid-binding protein
LDL	Low density lipoprotein
LP(R)	Leptin (receptor)
M-CSF	Macrophage colony stimulating factor
MAPK	Mitogen activated protein kinase
MAR	Mineral apposition rate
MCP-1	Monocyte chemoattractant protein 1
MDA	Malondialdehyde
MdAr	Mineralized area
MIG	Monokine induced by interferon γ
MIP-1α	Macrophage inflammatory protein 1 α
MLT	Mineralization lag time
MMP(2/9)	Matrix metalloproteinase 2/9
MPO	Myeloperoxidase activity
MSC	Mesenchymal stem cell
MTAL	Medullary thick ascending limb
NAD	Nicotinamide adenine dinucleotide
NGAL	Neutrophil gelatinase-associated lipocalin
NHE3	Sodium hydrogen antiporter (exchanger) 3
nk-1R	Neurokinin 1 receptor
NO(S)	Nitric oxide (synthase)
NPY	Neuropeptide Y
NSAIDs	Non-steroidal anti-inflammatory drugs
OAr	Osteoid area
OB	Osteoblast
ObPm	Osteoblast perimeter
OC	Osteoclast
OcPm	Osteoclast perimeter
OCN	Osteocalcin
OGTT	Oral glucose tolerance test
OMT	Osteoid maturation time
OPC	Osteoprogenitor cell
OPG	Osteoprotegerin
OPm	Osteoid perimeter
OPN	Osteopontin
OSOM	Outer stripe of outer medulla
OST-PTP	Osteotesticular protein tyrosine phosphatase
OVX	Ovariectomy
PACAP	Pituitary adenylate cyclase-activating peptide
PAC1	PACAP receptor 1
PAS-PCNA	Periodic acid Schiff/proliferating cell nuclear antigen
PCB	Polychlorinated biphenyl
PCNA	Proliferating cell nuclear antigen
PDC	Plasmacytoid dendritic cells
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PPAR(γ)	Peroxisome proliferator-activated receptor (γ)
PTC	Proximal tubular cell
PTH	Parathyroid hormone
PYY	Pancreatic peptide / Peptide tyrosine tyrosine
RAGE	Receptor for AGEs
RANK(L)	Receptor activator of nuclear factor κB (ligand)
RBF	Renal blood flow
ROI	Region of interest
ROS	Reactive oxygen species
RPF	Renal plasma flow
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Runx2	Runt-related transcription factor 2 (=Cbfa1)
SDF-1α	Stromal-cell derived factor 1 α
SERMs	Selective estrogen receptor modulators

SG	Sitagliptin
SHBG	Sex hormone binding globulin
SMI	Structure model index
SOD	Superoxide dismutase
SP	Substance P
STAT1α	Signal transducer and activator of transcription α
STZ	Streptozotocin
SU	Sulfonylurea
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAL	Thick ascending limb
TBA	Thiobarbituric acid
tBMD	Trabecular bone mineral density
TbN	Trabecular number
TbSp	Trabecular spacing
TbTh	Trabecular thickness
TGF-β	Transforming growth factor β
TLR2/4	Toll-like receptor 2/4
TNF-α	Tumor necrosis factor α
TRADD	TNF receptor-associated death domain
TRAP	Tartrate resistant acid phosphatase
TS	Tissue surface
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TV	Tissue volume
UcOCN	Uncarboxylated osteocalcin
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VG(1/10)	Vildagliptin (1 or 10 mg/kg dose)
VIP	Vasoactive intestinal peptide
VOI	Volume of interest
VPAC1/2	Receptor for VIP and PACAP 1/2
WHO	World Health Organization
Y1-5	NPY receptor 1-5
ZDF	Zucker diabetic fatty (rat)



Chapter 1
Introduction

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Chapter 1: Contents

1.1. Introduction

In this *first* chapter, a general summary will be given about dipeptidyl peptidase 4 (DPP4), its expression, function and its various interactions. DPP4 is an exopeptidase which plays an important role in the regulation of function of various protein substrates throughout the body, thus participating on different levels in metabolic, neurological and immunological mechanisms which will be discussed further. At the end of this section it will be explained how the regulation of incretins by DPP4 made it a potential target for drug development in case of disturbances of the glucose metabolism in diabetic patients, and how this increased interest for DPP4 inhibition has resulted in various studies investigating its potentially beneficial or harmful effects throughout the human body. Although DPP4 inhibitors have frequently been used worldwide to treat diabetes type 2 patients, the complete pharmacological profile and the long term effects of DPP4 inhibitors have not yet been revealed.

In the *second* chapter, an introduction is given about acute kidney failure and the underlying mechanisms as well as the current knowledge about the role of DPP4 and its substrates in kidney function and failure. Results of our study regarding the effects of DPP4 inhibition by vildagliptin in an unilateral model of ischemia reperfusion injury will be presented. In the *third* chapter, the pathophysiology of osteoporosis and diabetic bone pathology is described, with emphasis on the function of DPP4 and its substrates in the bone. Also, the results of studies regarding the effect of DPP4 inhibition by sitagliptin in a model of postmenopausal osteoporosis as well as diabetic bone pathology will be presented.

In a general discussion, the fourth chapter, these results and those of other studies will be summarized to open perspectives for the application of DPP4 inhibitors in future scientific research, as well as their potential for further therapeutic development.

The studies that are summarized in this thesis are part of a Concerted Research Action (GOA) conducted by the University of Antwerp, which is aimed at better understanding the effects of chronic dipeptidyl peptidase (DPP) inhibition on pre-defined aspects of cardiovascular, renal and bone pathophysiology, including the development of selective inhibitors and study of expression of DPP4 as well as its substrates. The results presented in this thesis have been published as two articles[1,2] meant to contribute to the search for unknown effects of DPP4 inhibition in the kidney and the bone. The acquisition of further insight in the pluripotent effects of DPP4 inhibition through its many substrates is particularly interesting for the diabetic population that is currently being treated with DPP4 inhibitors. The fact that this population is at

high risk for kidney, bone and cardiovascular pathology due to glucose intolerance adds to the relevance of the presented research. It is our hope that these findings may contribute to the development of therapeutic methods involving DPP4 inhibition to prevent and aid patients suffering from renal injury, and osteoporotic and diabetic bone pathology.

1.2. Dipeptidyl Peptidase 4

1.2.1. Function of dipeptidyl peptidases

Dipeptidyl peptidases (DPP) are proteases that remove two peptides from the amino terminus of target polypeptides. As of this moment, 11 different dipeptidyl peptidases have been identified in living organisms, which have a regulatory role in cellular processes through the deactivation or modulation of the activity of other bioactive peptides. Dipeptidyl peptidase 4 (DPP4) is ubiquitously expressed in the human body and has been examined most intensively among the DPP-family[3]. Other important members are dipeptidyl peptidase 1 or Cathepsin C, dipeptidyl peptidase 2[4], dipeptidyl peptidase 8 (DPP8) and dipeptidyl peptidase 9 (DPP9)[5], which are also expressed throughout the human body and of which the latter two are very similar to DPP4 and share some of its substrates. DPP4, DPP8 and DPP9 are highly conserved proteins with similar expression levels in rat and human, which are upregulated in specific pathophysiological conditions[6]. The highest DPP8 mRNA expression levels are found in the testes and in the placenta, and the resulting protein is upregulated in activated T-cells and expressed in all currently examined lines of B- and T-cells. DPP9 mRNA levels are high in skeletal muscle, heart, liver and peripheral blood leukocytes. High expressions of DPP8/9 have also been reported in the rat and human brain[7,8].

1.2.2. Expression and activity of DPP4

DPP4, also known as cluster of differentiation 26 (CD26) or adenosine deaminase complexing protein 2, is a conserved 220 kDa dimeric N-glycosylated endogenous type II plasma membrane glycoprotein exopeptidase, belonging to the prolyl oligopeptidase family, and is ubiquitously expressed, mostly on endothelial and epithelial cells in a variety of tissues. Highest expression levels in humans have been reported to occur in the gastrointestinal epithelial cells[9], hepatocytes[10], the bone marrow[11] and the kidney[12-14]. DPP4 expression was also observed in the bone[15,16], brain[17], pancreas[18], lungs[6] and endothelial and smooth muscle cell surfaces[19]. Furthermore, DPP4 was found to be expressed on the surface of immune cells[20,21]. DPP4 exists in a membrane-

bound form, but also in a soluble form as a result of the cleavage of the extracellular domain from the membrane-anchored hydrophobic part, without losing enzymatic activity[3]. The origin of the soluble form of DPP4 is not completely understood but part is known to originate from bone marrow derived cells[11]. The normal concentration of DPP4 in the human plasma is about 25 units/mL. In rat serum, activity of DPP4 at basal levels varies around 16 units/mL. The dimeric transmembrane domain of DPP4 is very stable, contributes to the quaternary structure of the enzyme, and also enhances its enzymatic activity[22]. DPP4 is a homodimer, but was also found to form heterodimers with fibroblast activation protein α (FAPα), a related protein, on the membranes of fibroblasts and melanocytes. DPP4 peptidase specifically cleaves off X-Pro and X-Ala dipeptides from polypeptide substrates (three or more amino acids) or from dipeptides linked to C-terminal fluorogenic or chromogenic compounds. Substrates for cleavage by DPP4 are often N-terminally prepared by cleavage by another aminopeptidase[23].

1.2.3. Physiological role of DPP4

The catalytic activity of this oligopeptidase has been shown to exert major influence on the glucose metabolism, signal transduction, cell migration, cell differentiation and the immune system by deactivating or modulating the activity of its substrates. These substrates will be extensively discussed in section 1.3. Apart from its catalytic activity, it interacts with several other proteins like adenosine deaminase (ADA), the HIV gp120 protein, fibronectin, collagen, the chemokine receptor CXCR4, and the tyrosine phosphatase CD45[24].

DPP4 was found to regulate the activity of colony-stimulating factors (CSF) and other cytokines involved in hematopoiesis in the bone marrow[25]. DPP4 truncation of certain chemokines, colony stimulating factors, and interleukins is thereby responsible for an altered regulation of hematopoietic progenitor cells towards more mature blood cells and to other cell types[26].

On the surface of immune cells, DPP4 is most commonly referred to as CD26. CD26 participates in the immune response in the plasma from the surface of specific populations of $CD4^+$ and $CD8^+$ T-cells, B-cells and natural killer cells. High levels of CD26 have been reported as a late marker of activated T-lymphocytes[20,27,28] and are more specifically expressed by $CD4^+CD45RO^+$ memory T-cells[21]. After activation, a $CD4^+CD26^{high}$ T-cell responds maximally to certain recall antigens[29] and exerts a potent co-stimulatory T-cell activation signal which was found independent from its proteolytic activity[20,30] (figure 1.1). High surface expression of CD26 is associated with the production of Th1 cytokines[31-33]. CD26 is also highly expressed on a subset of $CD8^+$ T-cells. $CD26^{high}CD8^+$ T-cells belong to the early effector memory cell subset, and CD26-

mediated co-stimulation of $CD8^+$ T-cells has a cytotoxic effect through TNF-α, interferon gamma (IFN-γ) and Fas ligand[33]. Targeting CD26 in $CD8^+$ T cells was earlier proposed to have therapeutic potential[33]. Although the T-cell co-stimulatory signal of CD26 is independent of its proteolytic activity, the inhibition of DPP4 was also shown to suppress mitogen-mediated cell proliferation in T-lymphocytes[34].

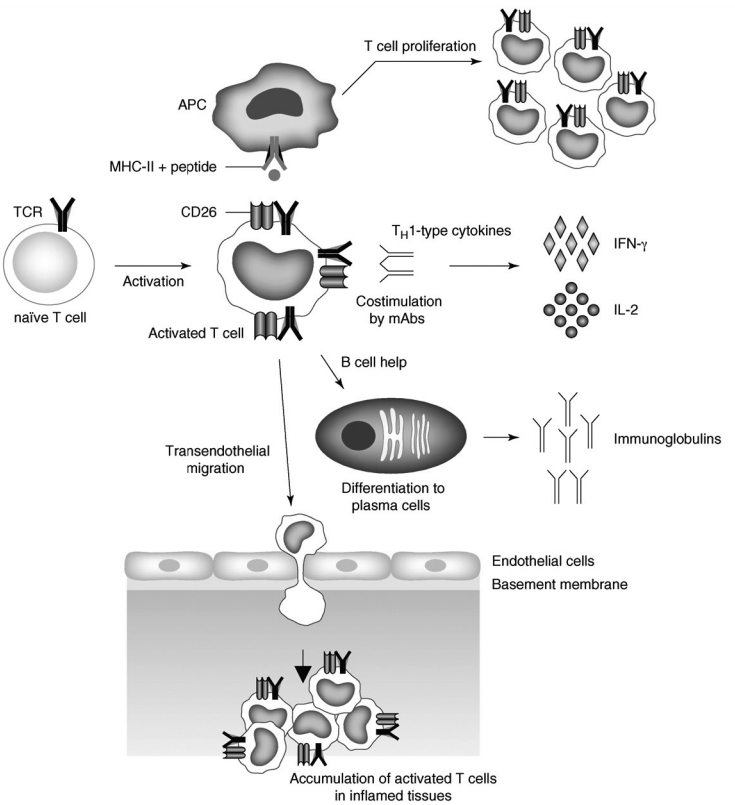

Figure 1.1. *Cellular functions of $CD26^{high}$ T-cells[21].*

The crosslinking of CD26 and CD3 with immobilized monoclonal antibodies, can enhance human T-cell co-stimulation and IL-2 production by $CD26^+$ T cells, but this is not effectuated by complexation with CD3 monoclonal antibodies alone[29,35]. Populations of $CD4^+CD26^+$ T-cells were furthermore known to support B-cell differentiation into antibody-producing plasma cells[29]. $CD4^+$ T-cells with in vitro transendothelial migratory capacity also appear to express high levels of surface CD26, indicating a role for CD26 in the migration of T cells[36]. It has been shown that CD26 assists the trans-endothelial migration of T-cells through interaction with the mannose-6P/IGFIIR receptor on the endothelial cell surface[37], which was shown to play a role in the pathophysiological development of inflammatory diseases in experimental studies[38,39].

The pathway for co-stimulation of T-cells is shown in figure 1.2. In the process of co-stimulation of T-cells, caveolin-1 on the antigen presenting cell binds specifically to CD26 in the presence of a TCR-CD3 receptor. Subsequent phosphorylation of caveolin causes up-regulation of CD86. In the T-cell, CD26 signaling initiates a signal cascade causing the co-stimulation of the T-cell, T cell proliferation, and to IL-2 production[40]. Other ligands that have binding sites on CD26, like ADA and fibronectin, could possibly also be involved in this stimulation[21]. Increased expression of CD26 on the T-cell surface leads to an increased in vitro SDF-1α mediated invasion. DPP4 inhibition in combination with alanyl aminopeptidase inhibition was shown to selectively suppress lymphocyte functions including proliferation and production of the Th1 cytokine IFN-γ, Th17 cytokine IL-17, as well as tumor necrosis factor alpha (TNF-α), and to ameliorate autoimmunity in vivo[41].

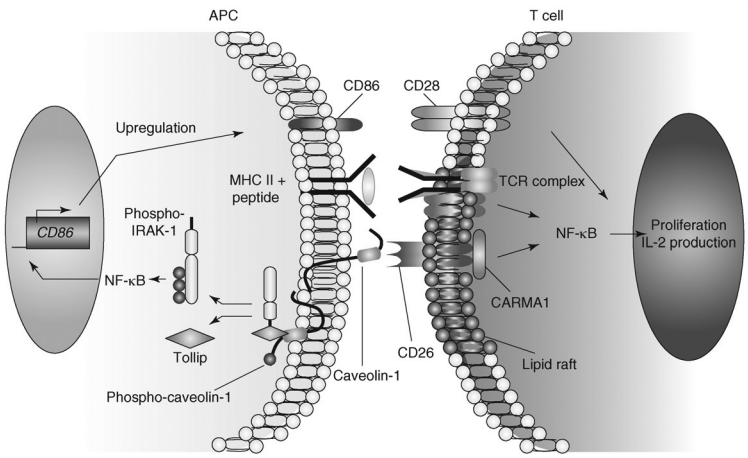

Figure 1.2. *Mechanism for co-stimulation of T-cells, with the antigen presenting cell on the left and CD-26 expressing T-cell on the right[21].*

Although DPP4 clearly plays an important regulatory role, DPP4 knockout mice are viable and have a normal phenotype under control conditions. Furthermore, they display more physical activity and an improved glucose tolerance and metabolism. At a later age, however, they display a reduced concentration of lymphocytes in the renal medulla, a decreased number of B-cells and NK-cells, and an increased number of regulatory T-cells. It is unclear whether other members of the DPP family, such as DPP8, DPP9, FAP or circulating attractin, can compensate for the absent enzyme[42].

1.2.4. Regulation of DPP4 expression

DPP4 is ubiquitously expressed, but there are high variations in the expression levels throughout the human body. In its monomeric form, DPP4 is

devoid of enzymatic activity and only becomes fully active as a homodimer. The regulation of DPP4 activity occurs at the level of gene expression, protein synthesis and substrate availability.

DPP4 expression is upregulated through the signaling pathway that involves the signal transducer and activator of transcription alpha (STAT1α) by interferons and retinoic acid. STAT1α binds specific DNA motifs to activate CD26 gene promoters[43]. At the mRNA level, adenosine down-regulates the expression of DPP4. This regulation is mediated by an increase of tyrosine phosphatase activity resulting in a decrease of phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and mitogen activated protein kinase (MAPK) leading to an alteration in DPP4 mRNA. The translocation of DDP4 towards the cellular surface is regulated by interleukin-12 (IL-12) and TNF-α[44]. The expression of the various DPP4 substrates is regulated separately and will be discussed later, but possibly occurs in close association with the regulation of DPP4 expression.

Various pathophysiological conditions have been either associated with an increase or a decrease of systemic or local DPP4 expression. DPP4 activity associated with obesity[45,46], and high fat and high cholesterol diet was shown to induce DPP4 activity in intestinal lymph[47]. DPP4 release was shown to correlate with adipocyte size, suggesting that DPP4 is strongly involved in linking adipose tissue, obesity and the metabolic syndrome[48]. A long-term increase of blood glucose in type 1 or type 2 diabetes results in an increase of DPP4 activity[49], but also in healthy subjects, an oral glucose load goes along with a transient increase of serum DPP4 levels, associated with an increased activity of adiponectin and hepatic enzymes[50]. In case of ischemic damage, a quick up-regulation of soluble DPP4 is observed in the serum[37].

In ovarian carcinoma, melanoma, endometrial adenocarcinoma, colorectal cancer, prostate cancer, non-small cell lung cancer and certain hematological malignancies, changes in DPP4 expression were reported to be manifested in the serum as well as the affected tissue[51,52]. Among other pathologies with a markedly increased DPP4 expression are chronic tonsillitis and tonsillar hyperplasia[53], psoriasis[54], cardiac dysfunction[55], colorectal cancer, rheumatoid arthritis[56,57], fibromyalgia[58] and others.

The presence of soluble DPP4 in the plasma and its expression on the cell-surface of $CD4^+$ T-lymphocytes were significantly elevated in asthmatic patients, with significant correlations found between DPP4 activity and the percentage of eosinophils and the TH2-related chemokines RANTES/CCL5 (substrate of DPP4/CD26) and CCL22[59].

Observed up-regulation of DPP4 in specific conditions has frequently led to the suggestion of using DPP4 as a potential biomarker for disease. DPP4 activity was brought forward as a potential blood-based indicator of meta-

static cancer of prostatic origin, either by itself or, by improving sensitivity and specificity of already established markers[60]. Also other DPPs have been put forward as useful biomarkers, like dipeptidyl peptidase 10 for colorectal cancer[61].

A reduction of DPP4 expression mostly reflects a decreased presence of DPP4 on the surface of T-lymphocytes and other immune cells, but may also characterized by decreased circulating levels in serum. Among pathologies with a decreased DPP4 activity are Crohn's disease[62], Chronic Obstructive Pulmonary Disease (COPD)[63], non-Hodgkin Lymphoma, leukemia and multiple myeloma[64]. In COPD patients, the decrease of DPP4 activity was suggested as a candidate biomarker for the early diagnosis of COPD[63].

1.3. Substrates of DPP4

DPP4 has a wide variety of substrates, which have important roles in metabolism, inflammation, cell migration, and cell differentiation[65]. One of the important groups of substrates are incretins, who have an important role in the secretion of insulin and have been shown to exert beneficial pleiotropic effects[66-68]. Also neuropeptides and anti-inflammatory peptides were found to be regulated by DPP4. A list of important DPP4 substrates can be found in table 1.1.

All of these substrates are produced in specific tissues and have one or different receptors in the same or other tissues throughout the human body. During their lifespan and circulation, they are truncated by DPP4, changing their receptor binding affinity and specificity and thus deactivating or modulating their activity. These active circulating substrates also have a variable half-life due to their different affinity for truncation by DPP4. DPP4 thus serves as an important and complex regulator of protein activity throughout the human body. A brief overview of the various functions of the most important DPP4 substrates will be given in this section. The most relevant DPP4 substrates in the context of kidney function and bone metabolism will be discussed further in detail.

1.3.1. Glucose-dependent Insulinogenic Peptide

Glucose-dependent Insulinogenic Peptide (GIP), also called Gastric Inhibitory Peptide, is a highly conserved 42aa peptide derived from a 153aa protein synthesized by intestinal K-cells, located in the mucosa of the duodenum and proximal jejunum, in response to nutrient ingestion, specifically glucose or fat intake, but also specific peptides[70,86]. It is also expressed in the adult hippocampus of the brain[87,88], where secretion of GIP is mediated by the activation

Table 1.1. *Important substrates of DPP4.*

Abbreviation	Name	Main origin	Receptor(s)	Half-life	Ref
Incretins					
GIP	Glucose-dependent insulinotropic peptide	Intestinal K-cells	GIPR	4’	69,70
GLP-1	Glucagon-Like Peptide 1	Intestinal L-cells	GLP-1R	1-2’	71,72
Gastrointestinal peptides					
GLP-2	Glucagon-Like Peptide 2	Intestinal L-cells	GLP-2R	5-7’	73,74
Cytokines					
SDF-1a	Stromal Derived Factor 1 alpha	Brain, thymus, heart, lung, liver, kidney, spleen and bone marrow	CXCR4	< 1’	75,76
Neuropeptides					
NPY	Neuropeptide Y	Brain, autonomous nervous system	Y1-5	2-3’*	6,77
PYY	Peptide YY	Intestinal L-cells	Y1-5	8-12’*	78,79
VIP	Vasoactive Intestinal Peptide	Nerve fibers, immune cells, pancreas, brain	VPAC1-2	2’	80,81
PACAP	Pituitary Adenylate Cyclase Activating Peptide	Brain, autonomous nervous system	VPAC1-2 PAC1	5-10’	82,83
SP	Substance P	Nerve fibers	Neurokinin-1R	~10’	84,85
Others					
Insulin-like Growth Factor 1 (IGF-1), Growth-Hormone Releasing Hormone (GRH), B-type Natriuretic Peptide (BNP), Substance P (SP), CCL5/RANTES (CCL5), Gastrin Releasing Peptide (GRP), Enterostatin, Peptide Histidine Methionin, Eotaxin (CCL11), Macrophage-Derived Chemokine, CXCR3 receptor agonists (Mig/CXCL9/IP-10/CXCL10/I-TAC)					

* Activity and/or receptor specificity of the substrate is modulated through truncation by DPP4.

of adenylyl cyclase, increases in intracellular Ca^{2+} levels, K^+-mediated depolarization, gastric releasing peptide (GRP) and β-adrenergic stimulation[70]. The basal circulating GIP-levels range up to five-fold after a meal. GIP is degraded by DPP4 to the inactive GIP(3-42) and has a relatively short half-life of about 4 minutes in the plasma[89]. GIP is cleared from the circulation by the kidney, resulting in increased GIP levels in the serum of patients with chronic kidney disease (CKD) and uremia as well as in nephrectomized rats[90].

The GIP receptor (GIPR) is a G protein coupled seven-transmembrane domain receptor predominantly expressed on pancreatic islet β-cells, but also in the stomach, small intestine, adipose tissue, adrenal cortex, pituitary, heart, testis, endothelial cells, on the surface of active osteoblasts in the bone, trachea, thymus, lung, thyroid, and several regions of the central nervous system[91]. However, GIPR expression was found to be absent in the kidney, spleen or liver[92]. Activation of GIPR has various effects of which some are very subtle[69], mediated by cyclic adenosine monophosphate (cAMP), activation of phosphatidylinositol 3-kinase (PI3K), protein kinase A (PKA), protein kinase B (PKB), MAPK and phospholipase A2. GIPR undergoes rapid and reversible desensitization after agonist binding.

In the intestine, GIP results in a reduced food transfer rate through the stomach, inhibition of gastric motility and secretion of acid (protecting the small intestine; mostly regulated by secretin). In the pancreas, it effectuates an induction of insulin secretion through receptors mainly on pancreatic β-cells. The latter process is primarily stimulated by hyperosmolarity of glucose in the duodenum[71]. This is considered the most important function of incretin hormones like GIP and GLP-1, and is referred to as the incretin effect. Intracellular cAMP and Ca^{2+}-levels increase, and K_{ATP}-channels are inhibited, resulting in insulin secretion and exocytosis in a glucose-dependent manner.

GIP acts synergistically with glucose to stimulate cell proliferation and to improve survival of pancreatic β-cell-lines after exposure to cytotoxic substances like streptozotocin (STZ). These protective effects of GIP are observed in wild-type islets, and not in the islets of GIPR knockout mice. A few in vivo studies have shown that the infusion of GIP into diabetic rats for 2 weeks significantly reduces β-cell apoptosis by activation of PI3K/Akt-PKB and subsequent phosphorylation and nuclear exclusion of FoxO1, resulting in decreased expression of the pro-apoptotic Bax gene and up-regulation of the anti-apoptotic Bcl-2 gene in β-cells[93].

The reduced incretin effect in type 2 diabetic (T2DM) patients has been stated to be more closely related to loss of insulinotropic activity of GIP, and to a lesser extent those related to GLP-1[94]. However, intravenous administration of GIP does not result in a normalization of glucose levels in diabetic patients[90]. In type 2 diabetic patients, GIP levels can be either normal or slightly increased in the serum[95]. The pancreatic GIP receptor was found to be down-regulated by hyperglycemia[96]. Due to an increased DPP4 activity, activity of intact GIP was shown to be lower after meal ingestion in obese subjects when compared to normal subjects, whereas total GIP was not affected[45]. A reduction of GIPR mRNA and protein levels in the islets of diabetic rats as well as humans was associated with the defective GIP response in diabetes[97]. The absence of GIPR in diabetic knockout mice was shown to result in resistance to obesity[98].

GIPR activation also results in stimulation of lipoprotein lipase activity, causing significant effects on fatty acid metabolism and possibly also playing a role in nutrient re-partitioning during lactation[99]. GIP was shown to link overnutrition with obesity by acting on adipocytes[98], whilst decreased expression of the GIPR gene in subcutaneous fat tissue was found to be associated with signs of insulin resistance in non-diabetic women with central obesity, demonstrating that fasting hyperinsulinemia is a possible negative regulator of GIPR gene expression in subcutaneous fat[96,100]. GIPR agonist-desensitization and down-regulation probably plays a major role in the reduction of insulin secretion. Therefore, therapeutic strategies based on stimulating the action

of GIP were proposed as alternatives for the future therapy of type 2 diabetes and/or obesity[90].

GIP was shown to affect bone turnover, which will be discussed later[101,102]. In the brain, GIP was found to be involved in the up-regulation of mRNA expression of anti-diuretic hormone, neuropeptide Y (NPY) and other hypothalamic genes[87,88]. GIP is also expressed in the hippocampus, were it induces progenitor cell proliferation[87].

1.3.2. Glucagon-like Peptide 1

Glucagon-like peptide 1 (GLP-1) is a 30aa incretin hormone derived from pre-glucagon secreted by the intestinal L-cells of the distal small intestine in response to oral uptake of glucose, fat and long-chain fatty acids[103], but also in response to non-nutrient stimulators (acetylcholine and GRP)[69]. The plasma concentrations of GLP-1 can be modulated by influencing the intestinal environment[104] and can also be increased through other gastrointestinal peptides like truncated PYY(3-36)[105]. Also the uncarboxylated form of the bone-derived protein osteocalcin binds receptors in specific enteroendocrine cells, stimulating the secretion of GLP-1 in the same cells[106]. Active GLP-1(7-36) gets truncated by DPP4 to GLP-1(9-36) in the intestinal capillaries with a half-life of 1-2 minutes, thereby losing its insulinotropic effect[107-109].

GLP-1 binds to the GLP-1 receptor (GLP-1R), a G-coupled receptor and member of the glucagon-secretin receptor superfamily, which can be found in the islet α- and β-cells (only cells secreting insulin[110]), peripheral tissue, the brain[111] including the central and peripheral nervous system, the heart, kidneys[112], lungs, bone and the gastrointestinal tract. Binding of GLP-1 to GLP-1R causes an internalization of the G-coupled receptor (which is later recycled to the cell surface), a rise of intracellular Ca^{2+}-influx and up-regulation of cAMP, which may be compartmentalized intracellularly[113]. Production of signaling molecules such as cAMP generated through binding of GLP-1 or its analogs might vary and engage different downstream signaling networks[114]. GLP-1 was shown to control glucose metabolism in extra-pancreatic tissues through receptors other than the pancreatic cAMP-linked GLP-1 receptor, for which it presents a different affinity[115]. GLP-1 binding to GLP-1R also activates anti-apoptotic proliferation and signaling pathways such as PI3K and MAPK in the gut, central nervous system, pancreatic β-cells[116], in the bone[115], but also in insulinoma cells[113] and in insulinoma cell lines[117]. Activation of PI3K leads to the phosphorylation and inactivation of the pro-apoptotic peptide Bad, a member of the Bcl-2 family, thereby preventing cell death[113,118].

In the pancreas, GLP-1 binds on pancreatic β-cells to stimulate insulin gene expression, insulin biosynthesis, glucose-dependent insulin release and also

promotes the growth of pancreatic β-cells themselves[71,117,119]. On pancreatic β-cells, GLP-1 binding inhibits the release of glucagon. Findings from clinical trials using the GLP-1R agonist exenatide, GLP-1 agonists, and later DPP4 inhibitors, led to the development of alternative treatment options for diabetic patients to improve their glucose tolerance and decrease serum triglycerides and cholesterol[94,120,121]. The protective effects of DPP4 inhibitors on β-cell function and survival are attributed to GLP-1[122]. Like GIPR, pancreatic GLP-1R was also found to be down-regulated by hyperglycemia[96].

In the gastrointestinal tract, GLP-1 binding promotes satiety and slows down gastric emptying[123]. In the liver, GLP-1 stimulates the action of glycogen synthase through a PI3K-dependent mechanism[124] and suppresses hepatic lipogenesis through a cAMP-protein kinase pathway[99]. GLP-1 also increases glucose uptake and glycogen synthesis in a PI3K-dependent mechanism in human satellite muscle cells in vitro. The latter occurs independently of the insulin signaling pathway, indicating that attenuation of both GLP-1 and insulin-induced glucose metabolism by hyperglycemia is likely to occur downstream of PI3K[125].

In obese subjects, total GLP-1 concentration is reduced after meal ingestion when compared to normal subjects. The increased DPP4 activity in obese subjects was shown to decrease the activity of intact GLP-1 even further[45]. In general, GLP-1 was found to be decreased in type 2 diabetic subjects. Acute administration of GLP-1 was found to be very effective at improving insulin secretion in patients suffering from T2DM[120,126], but it also appears that at a comparable level of circulating peptide, the sensitivity of diabetic patients to GLP-1 had been significantly reduced relative to non-diabetic individuals, indicating a down-regulation of GLP-1R[94,96].

In heart muscle strips in vitro, GLP-1 stimulates the synthesis of glycogen, glucose oxidation and utilization, and inhibits the activity of glucogen phosphorylase A[127]. Administration of GLP-1 in vivo generally induces a remarkable improvement of cardiovascular parameters, through changes of blood pressure, endothelial function, body weight, heart and fat metabolism, left ventricular function and cardiac output[113,128-130], and response to ischemia reperfusion[131-134]. In cardiomyocytes incretin hormones like GLP-1 have been recognized as immune-modulators of atherosclerosis[135] as they have a positive effect on endothelial function[136] and the development of atherosclerosis through anti-inflammatory and anti-proliferative properties[137,138]. In STZ-induced diabetic rats, GLP-1 was shown to protect against cardiac microvascular injury via a cAMP/PKA-dependent mechanism.

An up-regulation of GLP-1, and in turn GLP-1 receptors and endothelial nitric oxide synthase (eNOS), was also shown to protect against hypertensive nephropathy[139]. In the kidney, GLP-1 function was associated with natriuretic

and diuretic mechanisms[140] and in the bone, GLP-1 was shown to be an active regulator of bone turnover[141]. Furthermore, a neuroprotective effect of GLP-1 analogues was observed through neuroimaging[142]. GLP-1 agonists were also found to have an antipsychotic effect in a mouse model of psychosis[143]. The pleiotropic effects of GLP-1 have generally been found positive[144].

1.3.3. Glucagon-like Peptide 2

Glucagon-like Peptide 2 (GLP-2) is a 33aa incretin hormone which, like GLP-1, is derived from pre-glucagon from the intestinal L-cells of the distal small intestine in response to oral uptake of glucose, fat, long-chain fatty acids[103], dietary lipids, sweeteners[145], and other specific non-nutrient stimulators[69,146]. It is also produced by various neurons in the central nervous system (CNS). In the intestine, GLP-2 is co-secreted along with GLP-1 after nutrient ingestion[74]. GLP-2 is deactivated by DPP4 in the blood with a half-life of 5 to 7 minutes[73]. Truncation of GLP-2 by DPP4 occurs considerably slower than truncation of GLP-1[107]. Like GLP-1, the plasma concentrations of GLP-2 can be modulated by influencing the intestinal environment[104]. Expression of GLP-2 and GLP-2R is variable according to the developmental stage in intestinal tissues[147-149]. GLP-2 has one receptor, GLP-2R, which is expressed mostly in the stomach and bowel, but also in heart, kidney, liver, brain and the bone[150-153].

In the intestine, GLP-2 induces intestinal growth and enhancement of intestinal function[154], and also increases the intestinal secretion of HCO_3^- at the level of the intestinal L-cells[155]. GLP-2 binding to GLP-2R also induces anti-apoptotic regulation of proliferation through the same mechanism as GLP-1. GLP-2 links intestinal growth and metabolism to nutrient intake through its endocrine regulation of food intake and has multiple beneficial effects on the intestine, including expansion of the mucosal surface area through stimulation of crypt cell proliferation and enhancement of nutrient digestion and absorption[156]. It also plays a major role in duodenal contractility[157]. Exogenous administration of GLP-2 reduces food intake, except in diet-induced obese mice[158]. GLP-2 can reduce the mucosal injury on intestinal epithelial cells induced by acute pancreatitis through its anti-apoptotic effects[159]. Furthermore, GLP-2 was shown to exert a protective effect in experimental corrosive esophagitis[160]. The intestinotropic effects of GLP-2 have been found to be associated with IGF-1, IGF-2 and ErbB ligands and also to vasoactive intestinal peptide (VIP) activity[156]. Through PI3K signaling, GLP-2 induces the expression of VIP in nearby gastrointestinal neurons[161] but was also shown to induce intestinal gene expression through pathways independent of VIP[162].

GLP-2 has been proposed as a treatment for short bowel syndrome and Crohn's disease, and as adjuvant therapy during intestinal cancer chemo-

therapy. In untreated cancer cell lines, the combination of GLP-2 with DPP4 inhibitors results in an increased proliferative as well as migratory activity, thus increasing the risk of promoting an already existing intestinal tumor and possibly supporting the potential of colon cancer cells to metastasize[163].

In rat hearts, post-conditioning with GLP-2 given in early reperfusion protects against myocardial ischemic injury, limiting infarct size, and improving post-ischemic mechanical recovery[164]. GLP-2 was shown to cause coronary vasoconstriction in the heart and can effectuate positive as well as negative inotropism[151]. In the brain, GLP-2 has a neuroprotective effect and induces hypotension by modulating vagal afferent inputs and inhibiting the sympathetic nervous system[165]. It also plays a major role in the regulation of bone resorption, which will be discussed in a later section[166].

1.3.4. Stromal Cell-Derived Factor 1 alpha

The stromal cell-derived factor 1 alpha (SDF-1α) also known as C-X-C motif chemokine 12 (CXCL12) is a 89aa isoform of SDF-1, a small cytokine that belongs to the intercrine family[75,167,168]. It is highly conserved among species and expressed in many tissues including brain, thymus, heart, lung, liver, kidney, spleen and bone marrow. SDF-1α is the most efficiently processed DPP4 substrate, and is deactivated in the plasma by DPP4 with a half-life of less than one minute[169].

The SDF chemokine family is a family of which members activate leukocytes and are often induced by pro-inflammatory stimuli such as lipopolysaccharide, tumor necrosis factor (TNF), or interleukin-1 (IL-1). SDF-1α binds to its receptor CXCR4[170]. This SDF-1α/CXCR4 interaction was considered exclusive, but recent evidence suggests that SDF-1α may also bind the CXCR7 receptor[171,172]. SDF-1α is strongly chemotactic for CXCR4 expressing lymphocytes and was shown to be crucial for life[76,173-176]. SDF-1α knockout mice are not viable. During embryogenesis it directs the migration of hematopoietic stem cells (HSCs) from fetal liver to bone marrow and the formation of large blood vessels[177]. SDF-1α also has a central role in tissue repair and progenitor cell migration, is an important chemoattractant for $CD34^+$ cells and also enhances cell survival of $CD34^+$ cells[178]. The SDF-1α/CXCR4-axis was considered the central signaling axis regulating trafficking of HSCs to injured tissue, although attempts to modulate this axis by SDF-1α administration or blocking the axis were not successful[179]. CD26 and CXCR4 are co-expressed on the surface of human lymphocytes (B and T cell lines) and are co-distributed on membrane lipid rafts. When SDF-1α avoids truncation by CD26 and binds to CXCR4, both CXCR4 and associated CD26 are co-internalized, reducing the surface activity of DPP4[180]. Its important role in angiogenesis by recruiting

endothelial progenitor cells from the bone marrow also makes it a very important factor in carcinogenesis and the neo-vascularization linked to tumor progression[181,182]. SDF-1α secretion by stromal cells is known to direct the proliferation of cancer cells and angiogenesis via stimulation of the CXCR4 receptor that is upregulated by tumor cells[183].

SDF-1α was shown to reduce ischemic injury following myocardial infarction[184] and renal ischemia reperfusion[185]. In lung transplantation in mice, SDF-1α was shown to result in an increased migration of regenerative stem cells, exerting a protective effect against inflicted ischemic injury[39]. In T2DM patients, active SDF-1α was found to be correlated with the amount of circulating endothelial progenitor cells[186].

1.3.5. Neuropeptide Y

Neuropeptide Y (NPY) is a 36aa protein neurotransmitter, which is truncated by DPP4 with a half-life of 2-3 minutes[187]. It is expressed in the hypothalamus, heart, pancreatic islet cells, megakaryocytes, endothelial cells, nerve fibers in the bone, immune cells, adipose tissue, osteoblasts, osteocytes, chondrocytes and stromal cells, and it is considered the most abundant peptide in the heart as well as central and peripheral nervous system[77,188]. NPY was also shown to play an important role in the immune system, by modulating immune cell trafficking, T helper cell differentiation, cytokine secretion, natural killer cell activity, phagocytosis and production of reactive oxygen species[189,190].

NPY binds to the G protein linked Y-receptors Y1, Y2, and Y5[188]. These receptors have a variable distribution across different cell types and mediate different functions for the peptide. The Y1 receptor is the predominant vascular receptor mediating vasoconstriction and the major brain receptor involved in anxiety[191]. After truncation of NPY(1-36) to NPY(3-36) by DPP4, DPP8, DPP9 or a few other N-terminal peptidases, the protein loses the ability to bind to the Y1 receptor, and can no longer mediate Y1-mediated functions but remains capable of exerting all other non-Y1 mediated functions[7]. Further processing by other peptidases results in loss of NPY function through other Y receptors.

The Y2 receptor is the primary receptor responsible for the neuro-inhibitory effects of NPY in the central and peripheral nervous system and also exerts an anti-obesogenic role in the brain[192,193]. NPY plays a major role in the regulation of food uptake. In the hypothalamus, NPY binds on Y1 and Y5 receptors to increase appetite[194,195]. Exogenous NPY is considered to be a very potent orexigenic compound acting in the brain[196]. NPY was proposed to result in an autonomic response preparing the body for the consumption of a calorie rich

meal rather than initiating behavioral eating[197]. The Y5 receptor also has a role in the rejuvenation of bone marrow stromal cells. These cells all express NPY, Y1, Y2 and Y5. As the cell ages, the expression of NPY increases 130-fold and the expression of Y5 decreases 28-fold. NPY binding to Y5 stimulates the production of all bone marrow groups, and Y5 agonist blocks their proliferation. Stimulation of old bone marrow cells by NPY treatment rejuvenated the growth characteristics of aging bone marrow cells as a result of Y5 overexpression[198]. NPY was also shown to play an important role in the regulation of the sleep-wake cycle[199], adipogenesis and lipid accumulation[200].

The expression of NPY and its receptors are very finely regulated by activators of intracellular signaling pathways, classical neurotransmitters, steroid/peptide hormones, growth factors[201-203]. Among others, its expression is stimulated by GIP and inhibited by leptin, IL-6, insulin and retinoic acid[88,204]. Different NPY receptor subtypes may mediate an opposite effect of NPY on its particular function, making its regulatory role relatively complex. Since immune cells are capable of producing NPY upon appropriate stimulation, NPY can regulate immune cell functions in an autocrine as well as paracrine manner[190]. NPY was shown to exert an anti-inflammatory effect in dendritic cells by promoting their maturation and the production of IL-6 and TNF-α[205]. Also IL-10 expression was shown to be induced under influence of NPY, inducing a Th2 polarizing profile to dendritic cells[206].

In endothelial cells as well as immune cells, NPY, Y1, Y2, Y5 as well as DPP4 are expressed[207]. Through the Y1 receptor, NPY binding induces vasoconstriction as well as growth of vascular smooth muscle cells. NPY and DPP4 were found to be intracellularly co-localized and are both upregulated during angiogenesis[188,208]. The role of NPY in angiogenesis has been linked to its interaction with the Y2 receptor[209]. In aging animals, the reduction of Y2 receptor expression was found to result in an impaired angiogenesis[210,211]. NPY was also shown to be effective in revascularization and restoring functionality in a rat ischemic hind limb model[208,212], but not in Y2 knockout mice[210,211]. In the endothelium, vascular NPY is also known to contribute to the progression of atherosclerotic plaques and the activation of perivascular mast cells[213]. Like other angiogenetic factors, NPY also promotes vascularization of tumors[214-216].

NPY is expressed in neonatal β-cells, but this expression disappears in adults[217]. However, Y1 receptor expression remains, and through binding of NPY on Y1 receptors on pancreatic β-islets, it exerts a direct inhibitory action on the secretion of insulin[218]. NPY is also expressed at a low level in α-, δ-cells and polypeptide cells, which could be due to immunoreactivity of NPY-related peptides[219]. However, the inhibition of insulin release is followed by a post-inhibitory rebound which exceeds the control insulin levels by 3-fold[220]. T2DM

results in an increase of blood NPY levels, and although NPY was shown to be negatively regulated by insulin, NPY was shown to be positively correlated with insulin levels in T2DM patients[221]. In experimental animals, induction of diabetes through STZ was shown to result in an increased expression of NPY in the pancreatic δ-cells with concomitant decreases in NPY-positive pancreatic polypeptide cells. This may be a factor in reduced pancreatic hormone secretion during diabetes[222].

Studies involving NPY agonists and antagonists have proposed it to possibly help alleviate several cardiovascular disorders involving vascular remodeling, such as atherosclerosis, hypertension and peripheral vascular disease[223,224]. In the kidney, NPY was shown to exert natriuretic and diuretic effects, possibly mediated through the extrarenal Y5 receptor[225]. In the lung, signaling through Y1 receptors was shown to be a critical pathway for airway inflammation[226]. Through the Y2 receptor in the hypothalamus, NPY was observed to have a regulatory role in bone formation[227]. NPY and other neuropeptides were found to be involved in the central control of sexual behavior as well[228-230]. Furthermore, NPY was shown to play a major role in the pathophysiology of anxiety[231,232] as well as addiction[233,234].

1.3.6. Peptide YY

The 36aa gastro-intestinal hormone peptide YY (PYY) is secreted postprandially from the L-cells in the ileum and the colon to suppress appetite and limit meal size and calorie intake. It is also produced in small amounts in the esophagus, stomach, duodenum and jejunum, and by a discrete population of neurons in the gigantocellular reticular nucleus of the medulla oblongata. PYY was shown to be co-localized in the L-cells with GLP-1[78]. PYY has high structural homology with NPY, and PYY levels are increased in anorexia and decreased in obesity[79]. Post-prandial PYY release is stimulated by intraluminal nutrients (glucose, bile salts, lipids, short-chain fatty acids & amino acids), is regulated by cholecystokinin, VIP and GLP-1 and is inhibited by a fasting state[78].

PYY and NPY share the same receptors. DPP4 truncates PYY(1-36) to PYY(3-36) with a half-life of 8 to 12 minutes, thus altering its specificity for its receptors Y1, Y2 and Y5. Truncated PYY(3-36) loses its affinity for the receptor Y1 and Y5, and binds exclusively to Y2[78].

PYY(1-36) binds Y1 and Y5 receptors in the brain to increase appetite and stimulate weight gain[78]. Truncated PYY(3-36) inhibits gastrointestinal functions by reduction of gastric acid secretion, gastric emptying, small bowel and colonic chloride secretion, mouth to caecum transit time, pancreatic exocrine secretion and pancreatic insulin secretion[78]. It was also shown to regulate insulin sensitivity[235] and to lower glycemia after a glucose tolerance test. In

the brain, PYY(3-36) binds Y2 receptors on NPY-containing cells in the arcuate nucleus, inhibiting NPY release, thereby decreasing appetite and promoting weight loss[78]. Furthermore, PYY was found to be a potent stimulator of GLP-1 secretion, mediated exclusively through Y2R. It is however, not clear whether this effect is mediated through the capillary bloodstream at the level of the L-cells, through Y2R expressing neurons in the autonomous nervous system (which is very dense around the portal vein), or through the CNS[105]. It also promotes postprandial natriuresis and elevates systolic and diastolic blood pressure. As a result of its orexigenic activity, PYY results in a decreased bone turnover in anorexia nervosa[236]. But also an anabolic role has been attributed to PYY in the bone metabolism of premenopausal women[237], mice[238] and PYY deficient rats[239]. Like NPY, PYY was shown to play a role in addiction, as it was found to be a marker to predict craving and risk of relapse in abstinent smokers[240].

1.3.7. Vasoactive Intestinal Peptide

Vasoactive Intestinal Peptide (VIP) is a pleiotropic, highly conserved anti-inflammatory 28aa neuropeptide encoded by the VIP gene and found in many different biological systems throughout invertebrate phyla. It gets degraded in the circulation by DPP4 and has a half-life in the blood of 2 minutes. It is mostly produced by cells of the immune system in many areas of the human body but mostly in the gut (myenteric plexus and ganglion cells in the sub-mucosal plexus), the pancreas and the suprachiasmatic nuclei of the hypothalamus in the brain[80,81]. It is also present in nerve fibers in the retina, the kidney[82], the bone[241] and the female genitals[242].

VIP has two receptors. VPAC1 is expressed in nerve fibers in the CNS, the liver, lung, intestine and T-lymphocytes, monocytes and plasmacytoid dendritic cells (PDCs). Binding of VPAC1 agonists effectuate a dose-dependent reduction of IL-6 and TNF-α levels in cultured monocytes and reduced expression of IFN-γ as well as major histocompatibility complex-1 in PDCs and a decrease of infiltrating T-cells[243]. VPAC2 is expressed in the CNS, the pancreas, skeletal muscle, heart, kidney, adipose tissue, testes, and also in monocytes and PDCs. In the latter, agonist binding effectuates a dose-dependent increase of IL-6[243]. VPAC2 was also found to have an important role in circadian functions[244] and in cutaneous active vasodilatation through modulation of nitric oxide activity[245].

VIP plays an important immunoregulatory role and is involved in the regulation of Th1/Th2 balance, anti-inflammatory actions, induction of regulatory T cells and generation of tolerogenic dendritic cells. Its role has been demonstrated in several animal models of septic shock, rheumatoid arthritis,

acute pancreatitis[246] and Crohn's disease[247,248]. Furthermore, in the ischemic liver as well as in the infected cornea, VIP down-regulates pro-inflammatory Toll-like receptors (TLRs) and upregulates anti-inflammatory TLRs, inducing a beneficial immunological profile and attenuating injury[249,250]. In renal clear cell adenocarcinoma, VIP induces the formation of reactive oxygen species, increases adhesion and decreased expression of metalloproteinases MMP2 and MMP9, as well as cell migration and vascular endothelial growth factor (VEGF) expression, preventing further invasion and metastasis[251,252].

VIP seems to be an essential neuropeptide in intestinal morphology and also increases intestinal motility through smooth muscle relaxation, acting as a downstream mediator of GLP-2[162]. In a recent study, VIP was found to be an early prognostic marker of arthritis[253]. VIP was also shown to play an important role in the development of the heart. Critically-important heart failure-related genes are upregulated in VIP knockout mice associated with a spontaneous phenotype of cardiomyopathy involving both left and right ventricles, detrimental to both left and right cardiac homeostasis[254]. In the lungs, VIP is one of the most abundant neuropeptides in pulmonary parenchyma, mediating broncho- or vasodilation, exerting anti-oxidant and anti-inflammatory effects through radical scavenging, and inhibiting nitric oxide synthase[255]. In a model of experimental pulmonary hypertension, VIP was shown to prevent and revert pulmonary artery hypertension[256]. In the kidney, VIP was found to affect tubular reabsorption of sodium, chloride and potassium[82].

In the bone, receptors are expressed which are targeted by VIP expressing nerve fibers[257]. VIP was shown to stimulate alkaline phosphatase (ALP) activity in osteoblasts[258] and inhibit bone resorption in osteoclasts[259,260]. VIP has been found to play a role in the renewal of the endometrium after pregnancy, inducing RANTES (or CCL5 another DPP4 substrate) expression, decidualization and the recruitment of regulatory T-cells under stimulation of progesterone[261]. The human vagina is also known to be heavily innervated by VIP-immunoreactive nerve fibers, and VIP was shown to control physiological changes observed during sexual arousal, like genital vasodilation and vaginal lubrication[242,262].

1.3.8. Pituitary Adenylate Cyclase-Activating Peptide

Pituitary Adenylate Cyclase-Activating Peptide (PACAP) is a 38 or 27aa neuropeptide similar to VIP that binds the same receptors (VPAC1 and VPAC2) and an additional PACAP receptor PAC1. PACAP binding results in a stimulation of adenylate cyclase and an intracellular cAMP increase or the activation of the ERK signaling pathway, and is an important modulator of hypothalamus-pituitary neuroendocrine functions[263]. It has also been shown to inter-

fere with the ERK signaling pathway, inducing adipogenesis[264]. Both forms are truncated by DPP4. N-terminal modifications of PACAP confer resistance to DPP4. The active half-life of PACAP38 and PACAP27 are less than 5 and more than 10 minutes, respectively[265].

PACAP was shown to stimulate proliferation of enterochromaffin-like cells, and to play a role in cutaneous active vasodilatation through PAC1 and VPAC2, associated with NO activity[245]. Retinal expression of PACAP, VIP and receptors VPAC1 and VPAC2 were reported to be transiently up-regulated in STZ-induced diabetic rats at the onset of diabetic retinopathy[266]. Administered PACAP38 is able to promote neuron survival and restore STZ-induced changes to non-diabetic levels, suggesting a role of PACAP and its receptors in diabetic retinopathy[267]. VIP as well as PACAP were found to protect against retinal ischemic reperfusion injury, but PACAP was found to be 10 times more potent[268]. In experimental acute ileitis, PACAP also ameliorates the outcome[269].

In the kidney, both PACAP forms but mostly PACAP38, as well as the three PACAP receptors are expressed. PACAP38 was shown to exhibit tissue protective effects in ischemic models, mediated through the PAC1 receptor[83]. These cytoprotective effects are mediated through the regulation of IL-6 expression and reduction of TNF-α and TLR4 expression[270]. VPAC1 receptor binding on the proximal tubular epithelial wells was reported to modulate sodium reabsorption[82]. Like VIP, PACAP was shown to inhibit bone resorption in osteoclasts[259] and exerts a role in bone homeostasis[85], which will be extensively discussed later.

1.3.9. Substance P

Substance P (SP) is a small 11aa neurotransmitter and neuromodulator and plays a role in various physiological mechanisms. SP is expressed in nerve fibers, also in the skeleton, where it is found in the periosteum, bone marrow, the epiphysis, subchondral bone, ligaments and the synovium[271]. SP is truncated by DPP4 with a half-life of about 10 minutes. An important role of SP is nociception and it has been associated with the regulation of various psychological disorders[272]. Furthermore, it was found in high concentrations in the vomiting center of the medulla oblongata[273]. SP was shown to stimulate cell growth in culture[84], and has been found to reverse diabetes when injected into pancreatic nerve fibers[274]. SP levels were found to increase general sympathetic activity during combined angiotensin converting enzyme (ACE) and DPP4 inhibition[275].

SP binds to its receptor neurokinin-1R (nk-1R). Both SP and its receptor are widely distributed in the brain, mostly in emotion regulating regions (hypothalamus, amygdala, and the periaqueductal gray), and the central and pe-

ripheral nervous system throughout the body[276]. In the kidney, SP administration was shown to result in natriuretic and diuretic effects. The nk-1R receptor can also be found on osteoblasts as well as osteoclasts where it was found to result in an increase of bone formation as well as bone resorption[85,271].

1.3.10. Insulin-like Growth Factor 1

Insulin, a crucially important incretin hormone, produced by the pancreatic β-cells (of which secretion is induced by GIP and GLP-1), promotes the formation of insulin-like growth factor 1 (IGF-1), which is produced primarily by the liver as an endocrine hormone as well as in target tissues. IGF-1 is deactivated by DPP4 with a half-life of 10 minutes. Next to insulin, the production of IGF-1 is stimulated by growth hormone and is generally delayed by under-nutrition or disturbances in the growth hormone stimulatory pathway. IGF-1 binds to the IGF-1 receptor (IGF-1R) as well as to the insulin receptor. The IGF-1 receptor (IGF-1R) signals through multiple pathways among which PI3K.

IGF-1 can be considered a growth hormone, as it has growth-promoting effects on cells in many organs, like skeletal muscle, cartilage, bone and bone marrow, liver, kidney, nerves, skin and lungs. IGF-1 also mediates the growth effects of growth hormone and GLP-2 on intestine or linear growth in preclinical models of bowel resection, and thus exerts a central role in mediating the trophic hormone action in the small intestine[277]. IGF-1 is produced throughout the entire lifespan, but the highest rates are known to occur during the growth spurt in puberty. Lowest levels occur in infancy and old age. Deficiency of IGF-1, like deficiency of growth hormone, results in diminished stature. As a growth factor, it has also been associated with cellular growth in both prostate and breast cancer[278]. Both insulin as well as IGF-1 were found to exert an osteogenic effect in the bone[279].

1.3.11. MIG, IP10, I-TAC and RANTES

Monokine induced by gamma interferon (MIG or CXCL9), interferon gamma-induced protein 10 (IP10, also called C-X-C motif chemokine ligand 10 (CXCL10) or small-inducible cytokine B10) and interferon-inducible T-cell alpha chemoattractant (I-TAC or CXCL11) are cytokines belonging to the CXC chemokine family[280]. They are mostly produced by monocytes, endothelial cells and fibroblasts in response to IFN-γ in various organs, but also by renal epithelial cells[281]. These three cytokines share the same receptor, CXCR3, and are truncated in the plasma by DPP4[280]. The half-life of I-TAC is 2 minutes, and the truncation of IP10 and MIG is respectively 3- and 10-fold slower, resulting in reduced CXCR3-binding properties, loss of calcium-signaling capacity through CXCR3 and reduced chemotactic potency[282]. Their primary function

is the chemo-attraction of monocytes, Th1 T-cells, NK cells and dendritic cells, but was also shown to promote T-cell adhesion to endothelial cells and exert anti-tumor activity by inhibiting angiogenesis[283]. MIG and IP10 are also involved in cellular proliferation[284] and were previously proposed as a marker of various pathologies[285-287]. Another mention worthy DPP4 substrate and cytokine, CCL5 or RANTES, plays a role in the regulation of glucose-dependent GLP-1 and GLP-2 secretion. It was known to impair glucose-induced insulin secretion by reducing the expression of these incretins[288].

1.4. DPP4 substrates in the circulation

The miscellaneous functions of DPP4 substrates throughout the human body reflect its complex regulatory role. Figure 1.3 displays a schematic representation of the regions of expression of some important DPP4 substrates in the human body, clearly illustrating the complexity of their functional regulation by ubiquitously expressed DPP4. Some of the effects of DPP4 substrates are directly related to the occurrence of specific events, like elevated levels of GIP and GLP-1 inducing insulin release after food intake or the developmental variations of GLP-2 expression in the intestine. Some of the effects of DPP4 substrates are strictly local, like the release of neuropeptides from nerve fibers in the near vicinity. Other effects of DPP4 substrates are found to be systemic and influence specific organs according to their half-life and their region of expression.

Shortly after oral food intake, the glucose concentration in the intestinal lumen increases. The K-cells in the duodenum and proximal jejunum and the L-cells in the distal small intestine are stimulated to produce GIP and GLP-1, respectively. GIP and GLP-1, with a half-life of respectively 4 minutes and 1-2 minutes, are brought into circulation and arrive at the pancreatic β-cells to bind their G-coupled receptors, which stimulate the release of insulin. The incretins are not only insulinotropic, but also lower glucagon levels, increase satiety and slow gastric emptying. In general, insulin promotes the uptake of glucose by all cells, and stimulates the polymerization of glucose to glycogen in the liver. As an acute anti-lipolytic, insulin also inhibits lipolysis. Intake of a mixed meal is strongly associated with a significant decrease of markers of bone resorption. The coupling between bone resorption and bone formation results in a strong reduction of bone turnover during daytime, while bone turnover increases during night time. Circadian rhythm also greatly influences the expression of gastrointestinal peptide GLP-2.

Parenteral feeding does not influence bone turnover, which proves a definite link of incretin hormones with the bone metabolism. The relationship between feeding and the bone will be extensively discussed in a later sec-

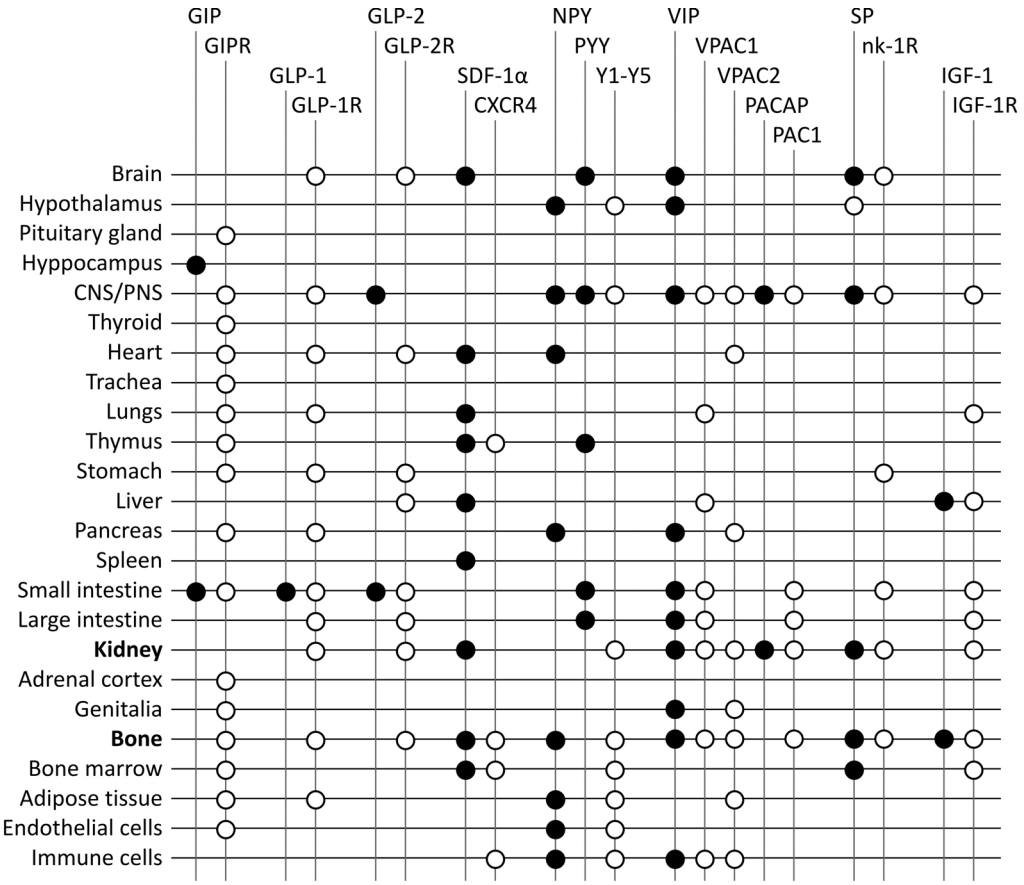

Figure 1.3. *Important regions of expression of DPP4 substrates (●) and their receptors (○).*

tion[98,166]. NPY, PYY, VIP, PACAP and SP can be partly held responsible for neuronal regulation of appetite, food intake and regulation of incretins.

As far as most substrates are concerned, theory predicts that the activity of DPP4 is correlated with a reduced insulin response to the oral uptake of glucose, an increased lipolysis in the liver, increased apoptosis in the pancreas and an increase of appetite and food intake. Furthermore, a reduction of angiogenesis and a negative influence on the bone metabolism is to be expected. Cytokines like SDF-1α, CXCL9-11 and others are continuously expressed and rather influenced by inflammatory factors or injury. These cytokines have a major influence in the signaling of immune cells and their infiltration, as well as the recruitment of stem cells to sites of injury and neo-angiogenesis. Theory predicts that in case of injury, activity of DPP4 is correlated with a reduced half-life of these chemotactic cytokines, resulting in reduced angiogenesis, a reduced inflammation and infiltration of immune cells as well as a reduced

recruitment of progenitor cells potentially able to repair inflicted damage.

1.5. DPP4 inhibitors

1.5.1. Use of DPP4 inhibitors

Studies have concluded that reducing the activity of DPP4 mainly results in the increase of the incretin effect mediated by GIP and GLP-1 (figure 1.4). Therefore, DPP4 inhibitors, first approved in 2006, have been introduced for use in the treatment of T2DM. DPP4 inhibition improves glucose tolerance by increasing the half-life of GIP and GLP-1, thereby promoting the release of insulin, improving the metabolism of pancreatic β-cells, and decreasing the release of glucagon from the pancreas. Effects of DPP4 inhibitors in T2DM patients is strongly similar to the effect of GLP-1R agonists and other incretin-based therapies[69,72,89,90,289-293].

A lot of research has been performed to understand the kinetics and pharmacology of DPP4 inhibitors and GLP-1R agonists and to adapt it for application in clinical settings[294,295]. As the reduced incretin effect in T2DM patients is not only related to increased activity of DPP4, but also to loss of activity of GLP-1 and of insulinotropic activity of GIP, targeting a protease to prolong the half-life of both incretins seemed beneficial[90,94,296]. Addon therapy of vildagliptin to insulin was shown to result in improved glycemic control and sustained efficacy in patients inadequately controlled by insulin therapy[297]. Incretin-based therapies however, may be more effective in preventing the progression of pre-diabetes, and GLP-1 receptor agonists may have more potential for use in the treatment of obesity[298].

In many cases, GLP-1 agonists are preferred to DPP4 inhibitors as they confer weight loss and lower blood pressure[299]. In contrast to DPP4 inhibitors, which are generally weight neutral, GLP-1 agonist treatment with exenatide (Byetta, Eli Lilly & Co) was found to cause a slight but significant loss in weight as well as flatulence[108,300-302]. Other GLP-1 agonists include lixisenatide, liraglutide, albiglutide and taspoglutide. Synthetically produced or stabilized GLP-1 analogues, like albiglutide, a well-tolerated albumin-fusion peptide with GLP-1[303], were shown to have an increased stability and prolonged secretive action, but still are under clinical investigation[290,304-306]. In experimental animal studies, DPP4 inhibitor treatment has been associated with weight loss, but not in T2DM patients[307].

Use of GLP-1 agonists causes side-effects like nausea and vomiting. Some reports claim these side-effects cause only a small percentage to discontinue the GLP-1 agonist treatment[299,308], whilst it has been shown in other studies that because of its poor tolerability more than half of patients discontinue

their treatment[123]. Intolerance towards GLP-1 agonists and the desirable feature of oral administration make the use of DPP4 inhibitors a valuable alternative[309]. Also for T2DM patients with CKD, the treatment with DPP4 inhibitors was found to be a tolerable, effective treatment for glucose management[310]. Recently, the use of sitagliptin was also suggested as an add-on therapy for type 1 diabetes, as it decreases insulin requirements without increasing endogenous insulin production or the occurrence of hypoglycemic events[311].

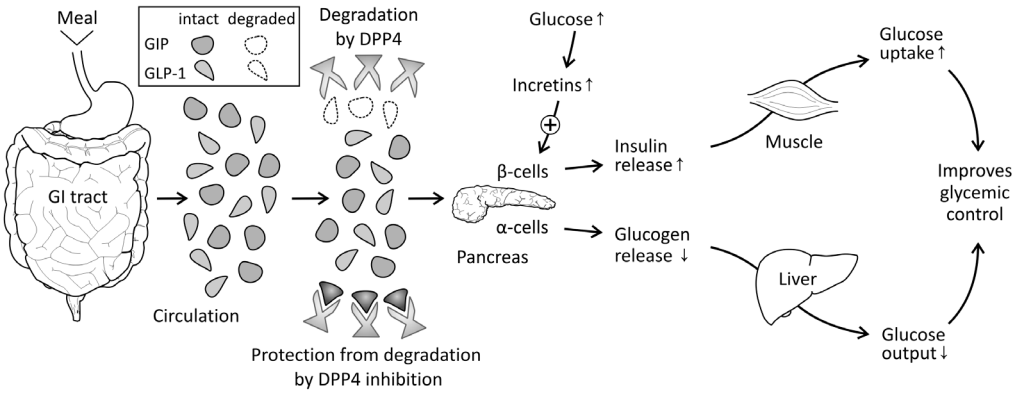

Figure 1.4. *Gastrointestinal effects of DPP4 inhibition[312].*

DPP4 inhibition was shown to result in a net increase of active GIP and GLP-1. An in vivo DPP4 inhibition of 90% results in a more than 4-fold increase in active circulating GIP during the first 90 minutes after treatment[72]. The portal concentrations of GLP-1 were shown to be more affected by DPP4 inhibition than the peripheral concentrations[313]. DPP4 inhibition results in an increase of intact GLP-1, GIP and PYY in T2DM patients. Increased activity of GIP and GLP-1 results in an increased secretion of insulin from β-cells. Concentration of total GIP and GLP-1 protein however, remains unaltered[314]. In STZ-induced diabetes, DPP4 inhibition was shown to also ameliorate GLP-1 mRNA levels and stimulate β-cell proliferation resulting in an improved glucose homeostasis[315]. NPY appears to be regulated by DPP4 derived from adipose tissue. DPP4 inhibition was shown to augment the anti-lipolytic effect of NPY locally in adipose tissue, which could explain the lack of weight management in T2DM patients treated with DPP4 inhibitors[316]. As the methods to detect active and truncated forms of DPP4 substrates are inadequate, their exact concentration profiles over time and after specific stimuli have not been completely studied.

Furthermore, it is not clear how chronic DPP4 inhibition alters the pathophysiological development of T2DM in humans. It certainly effectuates an elevation of the GLP-1 level, of which long-term animal studies have demonstrated to result in an increase in β-cell mass as well as β-cell efficiency. Markers for α- and β-cell function have been observed in some of the clinical stud-

ies, but long-term studies are needed to confirm the delay in the progression of the disease. Also, extensive tests are still to be performed with patients having diabetes for more than 10 years, who have low or no β-cell function.

1.5.2. Characteristics of DPP4 inhibitors

The first DPP4 inhibitors to be developed were sitagliptin (Januvia® 2006, Merck & Co. Inc, USA)[292,317] and vildagliptin (Galvus® 2008, Novartis International AG, Switzerland)[318], which currently are the most commonly used DPP4 inhibitors for the treatment of T2DM patients. In contrast to sitagliptin, that has been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for use in the USA and Europe, respectively, vildagliptin has only been approved for use in Europe, as the FDA rejected it because of occurrence of skin lesions in experimental studies[319,320]. The availability of these reversible inhibitors was a milestone in the study of DPP4 and related proteins, and helped to unravel DPP4-mediated mechanisms. The combination treatment of vildagliptin with metformin has also been approved by the EMA for use in Europe as Eucreas, distributed by Novartis. Several other compounds became available more recently; e.g. saxagliptin (Onglyza, Bristol-Myers Squibb/AstraZeneca) and linagliptin (Tradjenta, Boehringer Ingelheim/ Eli Lilly) or are still in (pre-)clinical development[317,321]. Recently developed inhibitors omarigliptin (Merck) and trelagliptin (Takeda) have been tailored for weekly instead of daily administration[322,323]. In recent clinical studies in T2DM patients, the highest DPP4 inhibition was found to be achieved with sitagliptin[324-326]. Linagliptin is a DPP4 inhibitor with an the extremely low IC_{50} of 1 nM, and was found to be 10000 times more specific for DPP4 compared to DPP8/9[327]. AB192, an experimental inhibitor developed in cooperation with the University of Antwerp, irreversibly and specifically inhibits DPP4 (with an IC_{50} of 0.4 µM compared to an IC_{50} of 11 µM for DPP8). Unbound AB192 half-life is short (5h in vitro) and will therefore not bind de novo synthesized DPP4[328]. Other experimental inhibitors include alogliptin[329], dutogliptin[330], DA-1229[331], berberine[332], teneligliptin[333], RBx-0128[334] and gemigliptin[335]. Over a dozen gliptins were developed over the last decade, of which the most important are displayed in table 1.2. Hybrid compound inhibitor design based on linagliptin and alogliptin was shown to yield longer and even more potent experimental DPP4 inhibitors[336].

Following oral administration in the fasting state, all DPP4 inhibitors are rapidly absorbed with peak plasma concentrations observed at 1 to 2 hours. Peak plasma concentration is slightly delayed up to 2.5 hours by food ingestion, but this does not affect the overall exposure, thus DPP4 inhibitors may be administered with or without food. The absolute bioavailability of vilda-

gliptin and sitagliptin is 85-90%. Metabolism is the major elimination pathway for vildagliptin in humans, accounting for 69% of the dose. Renal excretion of the unchanged vildagliptin accounted for 23% of the dose after oral administration. Sitagliptin is primarily eliminated unchanged in urine (79%) through active tubular secretion, and metabolism is a minor pathway. Varying degrees of renal impairment can thus result in an increased exposure to specific DPP4 inhibitors[337], which is the reason why vildagliptin as well as sitagliptin use is contra-indicated in T2DM patients with renal impairment[338]. As renal excretion of linagliptin is < 7%, renal impairment only has a minor effect on linagliptin pharmacokinetics, so this DPP4 inhibitor can be administered in uremic patients without the need to adjust the dose[339].

DPP4 inhibition by vildagliptin was found to be unaffected by gender as well as age. There seems however to be a difference in the efficacy of DPP4 inhibition according to ethnicity. The glucose-lowering effect of DPP4 inhibitors was found to be more efficient in Asians than in other ethnic groups, although variations occur dependent on body mass index (BMI)[340]. The baseline DPP4 activity predicts to what extent inhibition with sitagliptin will be efficient for the treatment of T2DM patients[341]. The apparent terminal half-life of sitagliptin following a 100-mg oral dose of sitagliptin was approximately 12.4 hours according to the EMA assessment report. Twice daily oral administration of 50 mg vildagliptin results in a permanent >80% inhibition of DPP4 in Japanese individuals. Acute overdosing with sitagliptin was considered safe by a study conducted in Japan[342].

Selectivity for a specific DPP is important when developing a DPP inhibitor, as DPPs have important and various roles. Sitagliptin selectively inhibits DPP4 with high affinity, whereas vildagliptin has a 5-fold higher IC_{50} for DPP4, and also inhibits DPP8 and DPP9. However, low concentrations of vildagliptin were shown to only inhibit DPP4. Half of available vildagliptin dissociates from DPP4 after 55 minutes, while it dissociates already within 10 seconds if bound to DPP8 or DPP9[343]. Inhibition of DPP8/9 suppresses mitogen stimulated T-cell responses and is therefore rather avoided; it was also linked to toxicity in animal studies[344]. Toxicity is logically a very important issue limiting potential use of an inhibitor. Pharmacological inhibition of DPP4 has a wide array of physiological effects. The complete pharmacological spectrum of DPP inhibitors is yet to be discovered, potentially revealing risks or side-effects, but also novel therapeutic niches[345]. Not only do they prolong the half-life of DPP4 substrates and modulate their function, many developed inhibitors are also able to inhibit DPP8 and DPP9, or have the ability to irreversibly bind to DPP4. Due to the interaction of DPP4 with CD45, the inhibition of DPP4 is associated with a reduction of CD45 tyrosine kinase activity (resulting in decreased phosphorylation)[346]. Throughout the human body, natural (endogenous) inhibitors

of DPP4 are also produced, which include PTH[347]. Small bioactive peptides also function as natural inhibitors of DPP4 in the proximal gut, by preventing the degradation of these substrates by binding to DPP4 and by stimulating the release of DPP4 substrates produced in the gut[348]. Dietary whey proteins generate such bioactive peptides, which could be the reason for the association of dairy product consumption with a reduced risk of metabolic diseases (like T2DM) and cardiovascular disease[86].

Table 1.2. *Important DPP4 inhibitors and their properties.*

Name	Dosage	Half-life	IC_{50}			El.	Ref.
			DPP4	DPP8	DPP9		
Sitagliptin – Januvia (US2006)	100 mg/d	11.8 h	18 nM	>45 µM	>45 µM	R/M	292,349
Vildagliptin – Galvus (EU2008)	100 mg/d	2.8 h	62 nM	1 µM	122 nM	M/R	318,349-353
Saxagliptin – Onglyza (US2009)	5 mg/d	2.2 h	26 nM	10.4 µM	1.95 µM	R/M	353-357
Linagliptin – Trajenta (US2011)	5 mg/d	113 h	0.77 nM	50 µM	>10 µM	B	358,359
Anagliptin – Suiny (JP2012)	100 mg/d	4.37 h	3.8 nM	68 µM	60 µM	R	360-362
Teneligliptin – Tenelia (JP2012)	20 mg/d	24.2 h	0.37 nM	260 nM	540 nM	R/M	363
Alogliptin – Nesina (US2013)	25 mg/d	5.7 h	24 nM	>240 µM	>240 µM	R/M	353,364
Trelagliptin – Zafatek (JP2015)	100 mg/w	6.2 h	4 nM	>400 µM	>400 µM	R/M	323,365
Gemigliptin – Zemiglo (KR2012)	50 mg/d	16.6 h	16 nM	169 µM	47 µM	R/M	366-368
Omarigliptin*	25 mg/w	120 h	1.6 nM	>67 µM	>67 µM	R/M	322,365,369
Dutogliptin*	-	12.2 h	2.5 nM	-	-	R/M	330,370,371
Berberine*	-	3-4 h	67 µM	-	-	R/M	332,372-374
AB192*	-	5.33 h	0.4 µM	11 µM	18 µM	-	328,375

*Name includes the name of the gliptin, the name under which it is marketed and the region and date where it was first approved for use - * not (yet) approved nor marketed, in which case dosage is based on clinical studies. The dosage represents basic orally administered dosage for a diabetic patient without complications. Half-life is the approximate half-life of DPP4 inhibitor (unbound to DPP4) in human serum; in case multiple values were found, the shortest is displayed. IC_{50} displays the half maximal inhibitory concentration of DPP4, DPP8 and DPP9 by the inhibitor. Elimination represents the elimination route from the human body (R: renal, M: metabolism, B: biliary).*

1.6. Currently knowledge about DPP4 inhibitors beyond incretin effects

Inhibition of DPP4 results in an increase of the active concentrations of their substrates. Therefore DPP4 inhibition was targeted to increase the concentration of GIP and GLP-1. The effects of the different DPP4 substrates, as presented earlier, clearly illustrate that the consequences of DPP4 inhibition throughout the human body are versatile and go beyond prediction.

A few important pathophysiological conditions are directly associated with insulin, whose secretion is regulated by DPP4 substrates. Others are directly associated with substrates regulated by DPP4 themselves and have all been shown to have an important function in the glucose metabolism, inflamma-

tion, cellular metabolism, liver detoxification pathways, blood pressure, neurotransmitters, oxidative stress, hormonal production and the development of specific pathologies. Due to the many beneficial effects of various DPP4 substrates, it is assumed that the treatment with DPP4 inhibitors results in beneficial pleiotropic effects which have been extensively studied in many organ systems[376,377]. Although it is not proven that DPP4 inhibitors have a beneficial effect on all diabetic complications, it is generally accepted that the use of DPP4 inhibitors does not increase the overall adverse effects related to T2DM[378].

In initial safety tests, the DPP4 inhibitors sitagliptin, vildagliptin and saxagliptin were shown not to impair innate and adaptive immune responses in normal control mice nor give rise to specific pathologies[379]. The initial experimental studies conducted by the FDA using vildagliptin indicated an increased risk of development of skin rashes in monkeys, resulting in a ban for the use of vildagliptin in the US[319,320]. Although there are case studies pointing to other risks, a recent review concluded that the benefits of any GLP-1 related therapy far outweigh the risks[380].

GLP-1 receptor agonists are often used for T2DM treatment as they provide adequate glycemic control as well as weight loss. However, as they are administered through injection, and give rise to unpleasant side-effects like vomiting and nausea, they are often poorly tolerated. The ease of oral administration and the lack of side-effects of DPP4 inhibitors make them a preferred treatment when weight is not a concern[309,381]. Nevertheless, it seems crucially important to further study the consequences of chronic inhibition of DPP4 in vivo. Previous studies indicating major influence of DPP4 inhibition on various processes throughout the body will be discussed below.

1.6.1. Inflammation

Preclinical data using DPP4 inhibitors in mice demonstrated their role in immunomodulation in the pathophysiology of diabetes[382]. Sitagliptin was shown to exert anti-inflammatory effects in older Japanese T2DM patients, associated with a reduction in serum low density lipoprotein (LDL), C-reactive protein (CRP) and TNF-α, an increase of serum IL-10 and plasma GLP-1, and a similar IL-10 and TNF-α expression pattern in monocytes. It also improves the phenotype of peripheral blood monocytes in T2DM patients[383]. After 6 weeks of treatment with sitagliptin, plasma markers of low-grade inflammation and cell adhesion molecules were found to be reduced in T2DM patients, associated with an increase of plasma GLP-1 levels and an improvement of glucose-insulin homeostasis[384]. In these patients reduction of glucose excursions with DPP4 inhibitors was also found to be associated with reduction of

oxidative stress and markers of systemic inflammation[385]. In diabetic ob/ob mice, linagliptin was found to attenuate inflammation and accelerate wound epithelialization[386]. In a study conducted on high-fat diet fed rats, DPP4 inhibition was found to result in a reduced inflammation in adipose tissue, which was attributed to GIP-related induction of adiponectin[387].

DPP4 inhibitors were found to suppress pathogenic effects of pro-inflammatory Th1 and Th17 cells and upregulate anti-inflammatory Th2 cells and regulatory T-cells, ameliorating the outcome in autoimmune diabetes[376]. Also, in a model of experimental autoimmune encephalomyelitis, a reduced inflammatory response was shown after inhibition of DPP4, possibly through an effect on T-cell effector function[388].

Early infiltration of inflammatory cells is a well-known phenomenon after renal ischemic injury, and inhibition of T-cell activation reduces the inflicted damage[389]. The activation of T-cells in ischemic injury could possibly be prevented through inhibition of DPP4. DPP4 deficiency was also shown to affect the neuro-immune response at systemic and local levels during the development as well as the resolution of colitis in mice[390]. On the other hand, DPP4 itself was shown to be a neutrophil chemorepellent in human as well in mice, improving the pathophysiological outcome in a model of acute respiratory distress syndrome[391].

Recent studies have suggested an increased occurrence of respiratory tract infection[392], nasopharyngitis[301] and urinary tract infection, as well as headaches after DPP4 inhibitor treatment, possibly due to the anti-inflammatory effect of DPP4[393].

The combined effect of DPP4 inhibition on inflammation is complex and mediated through different substrates. Although the exact mechanisms have not yet been elucidated, a reduction of inflammatory markers as well as an anti-inflammatory modulation of the immune cell population is mostly observed as a result of DPP4 inhibitor treatment.

1.6.2. Heart

The therapeutic application of DPP4 inhibitors for cardiovascular protection has been proven promising due to its effect on the modulation of endothelial progenitor cells, inflammatory pathways and ischemic responses and therefore has been extensively reviewed[66,134,394-399]. Already in 2005, the first articles describing a beneficial effect of GLP-1 on myocardial function were published[113].

In a model of myocardial infarction by coronary occlusion, sitagliptin treatment resulted in reduced apoptosis and reduced oxidative damage, most probably due to the activation of the PI3K/Akt signaling pathway through GLP-

1R binding[400]. In the same model, a preservation of cardiac function was seen through GLP-1 signaling in DPP4-deficient rats[401]. Furthermore, GLP-1R agonists have demonstrated beneficial effects on vascular endothelium of coronary and peripheral mouse vessels exerting anti-inflammatory and anti-atherogenic actions. The insulinotropic-inactive metabolite of GLP-1, GLP-1(9-36) was also found to have a beneficial effect on palmitate and glucose-induced cell-death in cultured cardiomyocytes and cardiac fibroblasts[402]. DPP4 inhibition with sitagliptin was shown to improve the migratory capacity of peripheral blood mononuclear cells to the infarct area, improving cardiac recovery and repair[403]. DPP4 inhibition also improves the pathophysiology of heart failure and increases the survival rate in pressure-overloaded mice[404]. In DPP4 deficient rats, cardiac performance against pressure overload was improved, an effect which was attributed to a great quantity of GLP-1 accumulation[405]. In a model of ischemic cardiomyopathy, it was shown that DPP4 inhibition with PTH leads to increased SDF-1α levels, which enhances recruitment of $CXCR4^+$ bone marrow cells into the ischemic heart[406].

Circulating DPP4 activity is known to be normal or mildly increased in T2DM patients, which was associated with subclinical left ventricular dysfunction[407]. A more recent experimental study also showed that a low dose of sitagliptin is cardioprotective in diabetic mice, whereas at a dose necessary for glycemic control, it induces left ventricular hypertrophy[408]. DPP4 inhibition with linagliptin reduces myocardial infarction and vascular injury in T2DM[409]. In obese mouse models, DPP4 inhibition with sitagliptin was shown to exert a beneficial effect on the heart in obese mouse models[410]. In cardiomyocytes of hypertensive rats, sitagliptin changed the cardiac electrophysiological characteristics and calcium regulation[411]. Vildagliptin was also shown to reduce infarct size and attenuate cardiac dysfunction in ischemic hearts by attenuating mitochondrial dysfunction and cardiomyocyte apoptosis[412]. The same effect was observed after treatment with linagliptin and experimental DPP4 inhibitors in rats[413]. In T2DM patients with coronary artery disease, administration of sitagliptin is associated with a sustained improvement in myocardial performance during dobutamine stress, and a reduction in post-ischemic stunning[414]. The myocardial infarct size-limiting effect of sitagliptin is PKA-dependent[415], whereas that of the GLP-1 agonist exenatide was found to be mediated by phosphodiesterase-3 and occur independently of insulin[416,417]. However, the reduction of myocardial infarct size was found to be inversely related to glucose levels[418]. DPP4 inhibition was shown to increase the myocardial glucose uptake in non-ischemic cardiomyopathy[419].

Recent meta-analyses of randomized clinical trials concerning the cardiovascular effect of DPP4 inhibitors are inconclusive. Some have concluded a beneficial effect[420] whereas others indicated that there is benefit nor harm

resulting from the treatment of diabetic patients with DPP4 inhibitors[421]. Another recent meta-analysis by a research team that before reported beneficial effects suggested that the use of DPP4 inhibitors might as well be associated with an increased risk of heart failure in diabetic patients, suggesting that the effect is dependent on specific subpopulations of patients[422]. The definite relationship between DPP4 inhibition and better cardiovascular outcomes in diabetic patients remains to be proven. Major prospective clinical trials involving various DPP4 inhibitors with pre-defined cardiovascular outcomes are currently still under way in T2DM patients as well as other patients with a high-risk cardiovascular profile. If these trials confirm that specific DPP4 inhibitors can reduce cardiovascular burden, this could bear potential to influence management of cardiovascular disease[423,424425].

1.6.3. Vessels & endothelial cells

DPP4 inhibitors do not only mediate favorable effects in cardiovascular pathology, but also modulate vascular tone and exert an immune-modulating role in atherosclerosis[135,426]. In an in vitro study, DPP4 inhibitors were shown to possibly have an anti-atherosclerotic effect, repressing foam cell formation by macrophages through the PKC pathway[427]. They inhibit the activation and chemotaxis of monocytes to the aorta in a model of atherosclerotic lesions[428]. The reduced carotid atherosclerotic process could also be linked to control of acute glucose fluctuations in T2DM patients treated with DPP4 inhibitors[429]. Interestingly, a recent experimental study indicates that sitagliptin also reduces the area of the atherosclerotic lesion, possibly by regulating MAPK pathways and reducing leukocyte-endothelial cell interaction and inflammation reactions. These vascular actions are independent of weight loss and glucose-reducing effects[430] and are mediated through a GLP-1 independent mechanism involving the release of NO and the activation of vascular potassium channels[394,431,432]. In another study, sitagliptin was shown to protect endothelial function in hypertension through a mechanism that is dependent of GLP-1[433]. In wild-type mice, vildagliptin was shown to increase vascularization in ischemia reperfusion-induced injury through eNOS signaling, which can occur dependent and independent of GLP-1, and through adiponectin[434].

In T2DM, vildagliptin was found to exert a protective effect against vascular injury attenuating the deleterious effect of the advanced glycation end products (AGEs)-oxidative stress axis by lowering the mRNA and protein expression of the receptor of the advanced glycation end products (RAGE)[435]. A similar effect was observed after treatment with linagliptin[436]. The protective effects on endothelial function in diabetic animals has also been attributed to an increase of $CD34^+$ cells after treatment with sitagliptin[437]. In type 1 diabetic

(T1DM) rats, alogliptin was also found to inhibit vascular complications[438]. The beneficial effect of DPP4 inhibition of vascular repair is also effectuated to an increased mobilization of stem cells in T2DM[439]. In a study with non-diabetic and diabetic apolipoprotein E-deficient ($ApoE^{-/-}$) mice treated with alogliptin, results showed that diabetes significantly increased atherosclerotic lesions, but alogliptin treatment reduced atherosclerotic lesions in diabetic mice, associated with an attenuation of diabetes-augmented TLR-4 mediated IL-6 and IL-1β expression, and a reduction of monocyte infiltration and smooth muscle cell proliferation[361,440-443]. Also with sitagliptin, diabetes-induced endothelial dysfunction was reversed[444,445].

In vitro, loss of DPP4 activity through inhibition was found to positively affect the anti-thrombogenic nature of the endothelium[446]. In vitro, sitagliptin pre-treated platelets also exhibited an inhibition of thrombin-induced platelet aggregation and a decrease of intracellular free calcium. Platelet aggregation was also shown to be inhibited by sitagliptin in T2DM as well as in healthy human subjects[447]. DPP4 inhibition was also found to facilitate the healing of chronic foot ulcers in diabetic patients[448]. The expression of high mobility group box 1 (HMGB1), having a positive influence on tissue regeneration and angiogenesis, was shown to be enhanced in diabetic patients treated with DPP4 inhibitors, and could be responsible for these effects[449].

Sitagliptin was shown to have significant concentration-dependent antiplatelet activity[447]. A potent vascular protective effect was also observed in Zucker Diabetic Fatty (ZDF) rats treated with linagliptin compared to ZDF-rats treated with sitagliptin, associated with a further reduction of serum malondialdehyde levels[450].

A 2% reduction of systolic and diastolic blood pressure was observed in T2DM patients treated with vildagliptin as well as sitagliptin as monotherapy[451,452]. Sitagliptin also produced small but statistically significant reductions of 2 to 3% acutely as well as on a longer term and was well tolerated in non-diabetic patients with mild to moderate hypertension[453]. Sitagliptin was shown to activate the sympathetic nervous system to diminish hypotension when combined with ACE inhibition, indicating that there is an interactive hemodynamic effect of DPP4 and ACE inhibition in humans[454]. Incretin-based treatments additionally have positive effects on the lipid profile and blood pressure which were shown to be GLP-1R dependent but also independent of GLP-1R and DPP4 cleavage[455].

1.6.4. Lungs

Inhibition of DPP4 was shown to attenuate the development of ischemic injury to lungs during transplantation[255,456-459]. This effect was attributed to

a modulation of the SDF-1α/CXCR4 axis increasing the intra-graft number of progenitor cells contributing to the recovery from ischemia-reperfusion lung injury. Stabilization of endogenous SDF-1α was proposed to be a promising strategy to intensify sequestration of regenerative stem cells and thus emerges as a novel therapeutic concept[456]. Also, in a rat model of experimental asthma, DPP4 inhibition was shown to exert a protective effect on airway inflammation. This beneficial effect was only present when DPP4 was inhibited topically, and aggravated the airway inflammation when orally administered[460]. DPP4/CD26 knockout results in a reduced number of recruited T-cells in an asthma rat model[38]. Markers of allergic asthma were also found to be correlated with an increase of $CD26^+$ T-cells in the lungs[461]. The beneficial effect of DPP4 inhibition on lung injury seems to be mostly mediated through inflammatory factors and SDF-1α.

1.6.5. Kidney

The effect of chronic DPP4 inhibition on kidney function after ischemic injury was evaluated by Vaghasiya et al. in STZ-induced diabetic animals, where it was shown to exert a protective effect[462]. In a model of overpacing-induced heart failure, which is associated with an increase of the glomerular filtration rate (GFR), sitagliptin was able to maintain a constant GFR[463]. DPP4 inhibition with linagliptin offers a new potential therapeutic approach for diabetic nephropathy, attenuating the increase of osteopontin and reducing albuminuria and glomerulosclerosis in diabetic animals when combined with angiotensin II receptor blockade[464]. These findings were confirmed by another study concluding that sitagliptin offers an additional protection against early-stage diabetic nephropathy via reduction of renal oxidative stress by SDF-1α-cAMP pathway activation[465].

Also in a model for hypertensive nephropathy, DPP4 inhibition through sitagliptin was shown to exert a renoprotective effect associated with increased GLP-1 levels and an up-regulation of GLP-1R[139]. In early stage of diabetic nephropathy, DPP4 inhibitors exert an anti-inflammatory effect in T1DM rat model[466]. In the Zucker obese rat, DPP4 inhibition was shown to attenuate injury of the filtration barrier and oxidant stress-related glomerulopathy and associated proteinuria[467]. In dyslipidemia-related kidney injury in the $ApoE^{-/-}$ mice, DPP4 inhibition with sitagliptin was shown to exert a renoprotective mechanism associated with a reduction of Akt levels and a restoration of MAPK activity[468].

Many studies regarding beneficial effects of DPP4 inhibitors in diabetic kidney disease were summarized in a recent review[469]. The protective effect of DPP4 inhibition on the kidney seems mediated mostly through a reduction of

glucose load, oxidative stress and apoptosis, and the DPP4 substrates GLP-1 and SDF-1α, and will be discussed extensively in the second chapter.

1.6.6. Bone

The expression of DPP4 has been shown on both osteoblasts and osteoclasts[15,16]. Multiple DPP4 substrates were shown to affect the bone metabolism, like GIP[101], GLP-1[141], GLP-2[166], NPY[470], PYY[237], and others. Most of these effects are positive, indicating the possibility that the inhibition of DPP4 might positively affect the bone[471]. In T2DM patients, treated daily with 100 mg of vildagliptin for a year, vildagliptin was found not to affect serum bone markers C-terminal telopeptide, ALP, calcium and phosphate[472]. A retrospective meta-analysis of clinical bone trials regarding fracture risk in diabetic patients showed a trend towards a reduced fracture risk in patients treated with DPP4 inhibitors[473,474]. However, another meta-analysis of randomized clinical trials with DPP4 inhibitors[473] as well as GLP-1R agonists[475] was not able to prove an effect on the bone metabolism in T2DM subjects. The effects of DPP4 substrates and DPP4 inhibition on bone metabolism will be discussed in the third chapter.

1.6.7. Gastrointestinal system and the liver

GIP, GLP-1, GLP-2 and PYY are expressed in the intestinal cells, and VIP and other neuropeptides are expressed in the intestinal nerve fibers. Although information about their active levels in the intestine after DPP4 inhibition have not been reported, some studies associate a positive effect to the use of DPP4 inhibitors. In an experimental study, DPP4 inhibition with a long-lasting experimental inhibitor was shown to prevent the formation and promote the healing of intestinal ulcers in indomethacin-fed rats[476]. An improved immunological profile was observed in inflammatory bowel disease in DPP4 deficient mice, in which VIP, NPY and IL-10 were found to be increased, suggesting neuro-immune-modulative properties of DPP4 inhibition[390]. GLP-2 was found to promote growth of intestinal tumor cell lines, indicating that it might contribute to the proliferation of existing intestinal tumors, an effect which was further amplified by administration of DPP4 inhibitors[163]. On the other hand, long term treatment with sitagliptin was associated with a reduction of colon carcinogenesis and reactive oxygen species in a model of rat colon cancer[477].

In T2DM patients, DPP4 inhibitors were found to improve liver dysfunction after a period of 6 months[478]. In rat experiments however, an increased incidence of hepatic adenomas and carcinomas was observed at systemic exposure levels to sitagliptin 58 times the human exposure level. In a porcine model of serum-induced liver fibrosis, DPP4 inhibition was found to inhibit

the development of liver fibrosis in rats via suppression of HSCs proliferation and collagen synthesis[479]. A recent case study raised concern about the possibility of linagliptin-induced liver damage and advises a closer follow-up of hepatic function in patients at high risk[480].

1.6.8. Pancreas

The pancreas is the main indirect target of DPP4 inhibition, and has been extensively studied in the context of DPP4 inhibition. In isolated human β-pancreatic islets exposed to glucose and palmitate, linagliptin was found to restore β-cell function and increase survival through stabilization of GLP-1[122]. In experimental studies with diabetic mice, it has been shown that sitagliptin significantly restores α- as well as β-cell mass[481]. Vildagliptin was shown to have an anti-apoptotic effect on pancreatic β-cells in db/db mice[482] and also enhance β-cell function in diet-induced obese mice[483]. In diabetic mice as well as in rats, this beneficial effect of vildagliptin on β-cell survival was also linked to the suppression of oxidative stress[484,485]. The same increase of β-cell mass was observed in neonatal rats treated with vildagliptin[486]. Also in human subjects, vildagliptin was found to repair pancreatic β-cell function as well as insulin sensitivity in T2DM[487]. The response of rodents as well as humans to the proliferating effect of incretin-based therapies on β-pancreatic cells was recently found to decline with age[488], an effect which was associated to the elongation of telomere length in β-pancreatic cells[489].

The Institute for Safe Medication Practices, a US non-profit organization monitoring the adverse effects of drugs reported to the FDA, concluded in a study from 1 July 2011 to 30 June 2012 that injectable GLP-1 analogues make a diabetic patient 28.5 times more likely to develop pancreatitis, while daily use of DPP4 inhibitors make a patient 20.8 times more likely to develop pancreatitis when compared to use of other diabetic treatments. After these early clinical meta-analyses, case reports about occurrences of pancreatitis were continuously reported[490,491]. The odds ratio for developing pancreatic cancer in diabetic patients calculated by the same non-profit organization was 23.3 and 13.5 for GLP-1 analogues and DPP4 inhibitors, respectively. The main reason why GLP-1 in particular is suspected to be a culprit in the development of pancreatic cancer is the fact that pre-malignant pancreatic lesions have a high expression of GLP-1 receptors. The use of GLP-1 receptor agonists as well as DPP4 inhibitors are associated with increased levels of serum lipase and amylase[492]. The post-marketing reporting to the FDA of adverse effects to the pancreas has led to a contra-indication of incretin-based therapies for patients with a history of pancreatitis[493-497]. Also, a leucine/proline or proline/proline genotype of NPY was associated with a lower risk for pancreatitis than

a leucine/leucine genotype[498]. Various case studies have been reported raising awareness for the incidence of acute pancreatitis in patients on incretin-based therapies[499-503].

However, based on experimental data, it was concluded that DPP4 inhibitor use infers no increased risk of pancreatic changes after all[504-506]. Most epidemiological studies in general report that the benefits involved with the use of DPP4 inhibitors in general far outweigh the risks[380,507,508]. The majority of pre-clinical evidence does not support the notion that GLP-1R agonists or DPP4 inhibitors cause pancreatitis[509] or even completely abolishes the potential pancreatic risks of DPP4 inhibitors[510,511]. Another recent publication exclusively associates a slightly elevated risk of pancreatitis with the use of GLP-1R agonists use, not with DPP4 inhibitors[512]. Thyroid cancer has also been associated to the use of GLP-1 analogues, while there have been no reports of thyroid cancer in T2DM patients using DPP4 inhibitors[513].

1.6.9. Brain

As DPP4 substrates are also expressed in the brain and nerve fibers throughout the central and peripheral nervous system, the effect of DPP4 inhibition has been studied in the brain of animals as well as T2DM patients. In a STZ-induced rat model of Alzheimer's disease, DPP4 inhibitor treatment results in an improved memory retention, a dose-dependent attenuation of inflammatory markers and an increased hippocampal and cortical GLP-1 level[514,515]. In high-fat diet fed T2DM mice, vildagliptin was shown to prevent brain mitochondrial dysfunction and attenuate impaired cognitive function[516,517]. In the same model, linagliptin was found to enhance neural stem cell proliferation after stroke induced by middle cerebral artery occlusion[518]. DPP4 inhibitors were also shown to improve glucose control and protects against worsening in cognitive functioning in older patients with T2DM affected by mild cognitive impairment[519].

1.6.10. Adverse effects

In general, DPP4 inhibitors are well tolerated and reported adverse effects are relatively rare considering the theoretical importance of regulation by DPP4. Vildagliptin was not approved for use in the FDA because an occurrence of skin rash in an experimental study conducted on monkeys[319,320]. The use of DPP4 inhibitors in diabetic patients has also been associated with an increased occurrence of bullous pemphigoid[520-522]. On the other hand, use of sitagliptin by a 17-year old patient was linked to the complete disappearance of psoriasis after three months without affecting his blood glucose levels[523].

There were reports of occasional cases of bilateral symmetrical seronega-

tive polyarthritis in patients treated with DPP4 inhibitors[524] as well as a report about a case treated with the GLP-1 receptor agonist liraglutide[525]. Polyarthropathy has also been reported in a Japanese group of T2DM patients treated with DPP4 inhibitors, which was associated with decreased plasma SDF-1α levels[526]. In one specific case, a patient taking sitagliptin developed severe arthralgia within weeks and a diffuse skin rash. These joint pains disappeared around six weeks after discontinuing sitagliptin[527]. Furthermore, the use of DPP4 inhibitors was associated with the occurrence of dropped head syndrome in a diabetic patient in Japan[528].

1.7. Aims of this thesis

DPP4 exerts a versatile regulatory function, and the modulation of DPP4 activity by DPP4 inhibitors and, as a consequence, the modulation of the activity of its substrates, was shown to alter physiological mechanisms as well as the development of various pathologies. Further evaluation of DPP4 inhibitors is crucial considering they are currently already being used worldwide, although the mechanisms behind the beneficial as well as detrimental effects they have been associated with are yet to be elucidated.

DPP4 inhibition in lung transplant models and acute ischemic myocardial infarction has been associated with a reduction of ischemic injury, associated with anti-apoptotic and anti-inflammatory effects. Similar beneficial effects were observed in the heart, vessels, the gastrointestinal system and the brain. Although some research regarding DPP4 inhibition in the kidney has already been published, the effect of a single bolus administration on the outcome of acute ischemic kidney injury in healthy subjects has never been studied. Acute kidney injury, a complication in hospitalized patients associated with high mortality due to ischemic injury, is, like ischemic lung and heart injury, characterized by increased inflammation, apoptosis and necrosis. A similar ischemic injury occurs in the kidney due to a transplantation procedure. The **first aim** of this thesis is to evaluate the effect of DPP4 inhibition with vildagliptin on the pathophysiological development of acute kidney injury in a unilateral model of renal ischemia reperfusion injury of the Wistar rat. The observed effect will be studied further in order to unravel the contributions of specific mechanisms.

The expression of DPP4 was found to be modified in diabetic subjects, questioning its role in the development of pathologies related to diabetes, as well as the potential of inhibition of DPP4 activity to modulate these pathologies. Diabetes is also associated with a modulation of bone turnover, shifting the balance between bone formation and bone resorption. Osteoporosis independent of diabetes was also characterized by an increase of DPP4 activity

in the serum, making the effect of DPP4 inhibition on the bone a particularly interesting subject. The **second aim** of this thesis is the assessment of the effect of DPP4 inhibition on postmenopausal osteoporosis as well as on diabetic bone pathology.

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Chapter 2

DPP4 inhibition attenuates acute kidney injury

The results of this study were published in June 2012
in the American Journal of Physiology – Renal Physiology:
Glorie, L. L. et al. DPP4 inhibition improves functional outcome
after renal ischemia-reperfusion injury.
Am J Physiol Renal Physiol 303, F681-688 (2012).

Chapter 2: Contents

2.1. Kidney function and the pathophysiology of acute ischemic injury

2.1.1. Introduction: kidney structure and function

The kidneys are bean shaped organs serving essential regulatory roles in vertebrates. They are by far the best-perfused organs in the body, receiving one quarter of the cardiac output per minute. As they are responsible for urinary excretion, they are the natural filters of the blood, but also serve homeostatic functions such as the regulation of electrolytes, blood pH and blood pressure (through salt and water balance). They effectuate the reabsorption of water, glucose, and amino acids from the excreted fluid. Through the ureters, the kidneys excrete wastes such as urea and ammonium to the urinary bladder. The kidneys also produce hormones including calcitriol, erythropoietin, and renin. These functions are regulated by the hypothalamus and the pituitary gland, and various regulatory peptides produced in different organs[1].

The kidney has a convex and concave surface. At the concave surface, the renal hilum comprises the entering renal artery and the exiting renal vein and ureter (figure 2.1B). The kidney is surrounded by tough fibrous tissue, the renal capsule, which itself is surrounded by fat and the renal fascia. The kidney can be divided into two structures: the superficial renal cortex and the deep renal medulla. These structures take the shape of about a dozen cone-shaped renal lobes, each containing renal cortex surrounding a portion of medulla called a renal pyramid or body of Malpighi. The functional filtering units found in these bodies are called nephrons, consisting of a glomerulus followed by a renal tubule passing from the cortex deep into the medullary pyramids. Medullary rays are collections of renal tubules draining from the cortex into a single collecting duct. These ducts empty into the renal pelvis which is drained by the ureter. A single human kidney contains about a million nephrons that effectuate the role of the kidney through systems of active transport and passive filtration. Nephrons are classified according to their location as cortical, cortico-medullary and medullary nephrons. The structure of the nephron is displayed in figure 2.1C. The renal tubule is divided according to function and structure into the proximal convoluted tubule, the loop of Henle (consisting of a descending limb and a thin and thick ascending limb), and the distal convoluted tubule, which drains into the collecting duct. The proximal tubule can be further divided into three segments: S1, S2 and S3. Passive filtration occurs in the glomerulus and is followed by processes of active transport between the tubular lumen and the blood in a reabsorption and secretion process. The filtrates of the glomeruli, after removal of substances through reabsorption towards the peritubular capillaries and additional secretion of substances towards the tubules arrive, in the collecting ducts, which transport the resulting

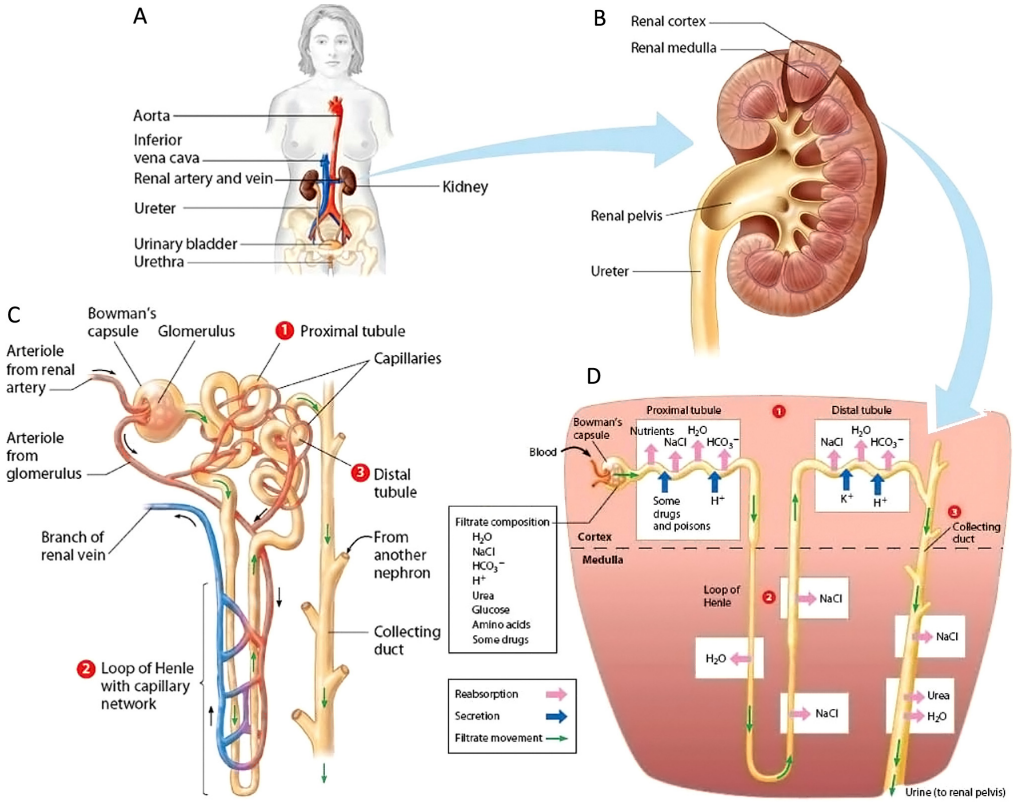

Figure 2.1. *(A) The urinary system (B) Kidney structure (C) Detailed structure of the nephron (D) Function of the nephron. Adapted from Biology, Concepts & Connections. 2014 Pearson Education Inc.*[2]

fluid towards the renal pelvis for urinary excretion through the ureter to the bladder (figure 2.1D).

Depending on the blood pressure, at the region of the glomerulus, the afferent and efferent arteriole of the blood supply to the glomerulus can be constricted, in order to ensure an efficient ultrafiltration in the glomerulus at a rate of approximately 125 mL/min, which is called the glomerular filtration rate (GFR). Of the 150-180 L of pre-urine arriving in the renal tubules, only 1-1.5 L is transported to the bladder.

Renal blood flow (RBF) (or renal plasma flow (RPF)) is set by the balance between glomerular capillary blood pressure and hydrostatic pressure in the glomerulus, and determines the total GFR. The GFR is a result of the net filtration pressure, which is a combination of the permeability of the glomerular capillaries and the glomerular capillary blood pressure (favoring filtration), the plasma-colloid osmotic pressure and the hydrostatic pressure over Bowman's capsule (both opposing filtration).

Within the auto-regulatory range of blood pressure changes (a mean arterial pressure of 70-210 mmHg), the GFR is kept constant and only diuresis can be changed acutely. Indirectly, the RBF regulates the reabsorption of salts and water in the proximal tubule, the concentration of urine, the transport of oxygen, nutrition (energy) and hormones towards the nephron, and the uptake (reabsorption) of salts and water and transport of carbon dioxide out of the nephron. The resistance of the afferent and the efferent arteriole determine the RBF and GFR. The resistance of the afferent arterioles is modulated by a myogen mechanism, through which the afferent arterioles are gently stretched, decreasing their diameter and increasing their resistance. The myogen mechanism is quick and the local vasoaction does not directly feedback on the fluid input signal. Another mechanism regulating the blood flow in the afferent arterioles is the tubulo-glomerular feedback mechanism. In case of high blood pressure in the distal tubule, the fluid delivery and salt transport at the macula densa cells of the distal tubule increase, causing the smooth muscle cells in the afferent arteriole to contract and restrict blood flow. The opposite system occurs at a low blood pressure. The latter mechanism is slower than the myogen mechanism and is auto-regulatory. Another regulatory system is the induction of the renin-angiotensin system through renin (from the kidney) and angiotensin converting enzyme derived from arterial endothelium. The reduction of the flow and calcium (Ca)-transport at the macula densa cells results in sympathetic stimulation, and renin production and release is promoted at granular cells of afferent arterioles. Angiotensin II is a potent vasoconstrictor, preferentially of the efferent arteriole, which reduces RPF whilst maintaining or even increasing filtration, stimulates sodium (Na) reabsorption, H_2O retention, potassium (K) secretion (through aldosterone), and reabsorption of $NaHCO_3$ and water. At higher plasma levels angiotensin contracts mesangial cells (decreasing GFR and filtration area) and causes generalized vasoconstriction in the afferent as well as the efferent arteriole, maintaining central arterial blood pressure at the sacrifice of RBF and filtration. Regulation of blood pressure as well as water and sodium balance is crucial for pressure throughout the nephron to assist reabsorption and secretion[3].

2.1.2. Ischemic acute kidney injury

Acute kidney injury (AKI) is defined as a rapid decline in GFR and renal function within 48 hours. It is a common clinical syndrome, in which the rate of production of metabolic waste exceeds the rate of renal excretion, and results in a number of complications including metabolic acidosis, uremia and perturbations in fluid balance. As the nitrogen-rich metabolite levels rise, this

condition is associated with azotemia[4].

The incidence of AKI is fairly high, affecting 1 – 2% of all hospitalized patients and up to 6% of patients in the intensive care unit, where it is mostly caused by a sceptic shock[5,6]. In critically ill hospitalized patients, it is associated with a mortality of about 60% as it is frequently accompanied by dysfunction of other organs[7,8]. Ischemic AKI in general has a mortality rate of 30%. Up to a certain level, AKI is reversible due to the regenerative capacity of the kidney which is able to completely restore morphology and function, but in many patients AKI ultimately progresses to end stage renal disease. These patients are in need of frequent dialysis or transplantation[9,10]. Hospitalized patients with a modest increase in serum creatinine level have a 70% greater risk of death compared to persons without increase[11]. AKI can be classified according to the etiology of the damage into pre-renal, intrinsic and post-renal AKI[4].

Pre-renal AKI (55% of AKI cases) is most often related to a decreased renal blood flow, initially without any internal parenchymal injury to the kidney. It can be considered as a functional response of structurally normal kidneys to hypo-perfusion with conservation of renal microstructure, due to volume depletion (dehydration, severe hemorrhage, nephrotic syndrome, oliguria), decreased cardiac output (cardiac failure, arrhythmia), peripheral vasodilatation (sepsis) or renal vasoconstriction (sepsis, non-steroidal anti-inflammatory drugs or angiotensin-converting-enzyme inhibitors.

Intrinsic AKI (40% of AKI cases) is the result of structural parenchymal damage to the renal tubules, glomeruli, interstitium, or renal vasculature. Most intrinsic cases of AKI are associated with acute necrosis of tubular cells (ATN) from prolonged ischemia or toxic injury. It can be caused by ischemia or sepsis, nephrotoxins, and immunological reactions (autoimmune disease, immunoglomerular disease, interstitial nephritis).

Post-renal AKI (< 5% of AKI cases) is the least common and is caused by a downstream mechanical or functional obstruction of the renal calyx, the ureter, or even the bladder or urethra, and is initially accompanied by few microscopical changes. It can be either congenital (caused by obstruction or valve defect) or acquired (caused by clots or calculi). In most AKI cases, inflammation plays a major role in the pathophysiological development[1].

ATN is caused by inadequate perfusion of renal tubuli and is a frequent complication of various surgical procedures, especially in elderly patients or patients with severe underlying diseases[12,13]. It can be caused in the clinical setting by systemic hypotension. Reperfusion occurs when the blood pressure and the perfusion of the tubuli is restored. Long-lasting surgical procedures like thoraco-abdominal aortic and abdominal aorta aneurysm surgery, as well as coronary arterial bypass graft procedures are prone to cause hypoperfusion of the renal tubuli and AKI[14,15]. Rarely, ischemic AKI can be caused by

stenosis or occlusion of renal vessels, but this occurs only if the occlusion is bilateral, occurs in patients with renal complications, or in a transplantation setting.

Renal transplantation is always accompanied by ischemia. During prelevation as well as storage and transport, the organ is subjected to cold ischemia. At the insertion of the transplanted organ, it is subjected to a period of warm ischemia. In case of cadaveric kidney transplant, the extraction of the organ is preceded by a warm ischemic event caused by hemodynamic instability of the donor. The most common complication during renal transplantation is the delayed function of a kidney transplant, which is more frequent in renal grafts from cadaveric donors compared to living donors. The duration of cold ischemia is an independent prognostic risk factor for delayed graft function of the kidney, resulting in longer hospitalization, acute rejection and long-term impaired function of the graft[16,17].

ATN was also found to be caused by exposure to imaging contrast media. This contrast-induced nephropathy is most common in patients with chronic kidney disease (CKD), diabetics, older patients and patients suffering from congestive heart failure[18]. Some of the pathophysiological mechanisms that can underlie this nephropathy have been identified, including direct toxicity to renal tubular epithelium, oxidative stress, ischemic injury, and tubular obstruction, but the complete pathophysiology is yet to be elucidated[19].

Throughout the years, no specific causative therapies have been developed to prevent the occurrence of AKI or post-ischemic ATN[20]. However, many agents were found to positively influence AKI by either attenuating kidney injury or improving the recovery of the kidney after AKI[21]. Their mild beneficial effect is mostly due to an increase of renal blood flow (dopamine, atrial natriuretic peptide, prostaglandins[22]), an increase of urinary flow, and prevention of tubular obstruction and reduction of swelling of tubular cells through anti-oxidative and anti-apoptotic mechanisms (EPO[23], mannitol[24]). IGF-1, a DPP4 substrate, was also found to accelerate recovery from ATN in experimental studies[25,26], but like all other promising treatments, they fail to influence the outcome in AKI in clinical studies[27]. Although a lot of progress has been made during the last decade using stem cells and endothelial cells to aid recovery from ischemic AKI in experimental studies[28-32], dialysis still constitutes the standard intervention for clinical AKI[21,33].

As experimental treatments fail when applied to the clinical setting, and the inflicted morphological injury in different experimental models of AKI is highly heterogeneous[34-36], the ideal approach most probably is a combination of treatments acting on different mechanical pathways. The development of animal models closely mimicking the clinical setting as well as understanding the exact cellular and molecular pathways remains a necessary challenge in

order to come up with effective therapeutic strategies to attenuate the injury and accelerate recovery in AKI.

2.1.3. Pathophysiology of ischemic acute kidney injury

2.1.3.1. Introduction

As previously stated, the kidney has some efficient auto-regulatory blood pressure mechanisms to ensure a sufficient oxygen supply. Dehydration, heart failure, and obstruction or stenosis of the aorta renalis can result in a sudden drop of glomerular pressure resulting in loss of autoregulatory function. Also drugs influencing the RBF, like ACE inhibitors and angiotensin receptor antagonists, can disrupt these mechanisms and ultimately result in acute pre-renal failure. If extra-renal factors causing hypoperfusion are reversed in time, pre-renal failure can be corrected, but persistent renal hypoperfusion will ultimately lead to ischemic ATN. When the blood supply to the kidney suddenly seems insufficient to sustain metabolic demands, a number of pathological changes immediately occur in renal tubular cells due to the decrease of oxygen, resulting in dysfunction or death when exceeding critical energy depletion. This situation occurs within the hour and is called the early reperfusion phase. The pathophysiology of ischemic AKI is determined by a complex interplay of anatomical, hemodynamic and cellular factors, in which both vascular and tubular components contribute to a decrease of the GFR. It is accompanied by activation of immune cells, endothelial dysfunction and production of reactive oxygen species, and results in apoptosis and necrosis, membrane alterations, cytoskeletal degradation, oxidant injury and protein nitrosylation (figure 2.2).

2.1.3.2. Ischemic deoxygenation of the kidney

Ischemia literally means a restriction of blood supply, mostly through obstruction or malfunction of the blood vessels, which ultimately results in organ dysfunction. Tissue hypoxia is the direct consequence of interrupted blood supply. An ischemic episode activates a sequence of pathophysiological processes which lead to further tissue damage and dysfunction, even after the blood flow has been restored. Abnormal regulation of blood flow in the renal microvasculature also plays an important role in renal ischemia reperfusion injury (IRI). Although the kidneys are among the best perfused organs, the anatomical and physiological factors inherent to their excretory function make them paradoxically sensitive to decreases in oxygen supply. Blood arriving from different arterial branches first passes through a glomerular capillary system and then through a second tubular capillary system before draining

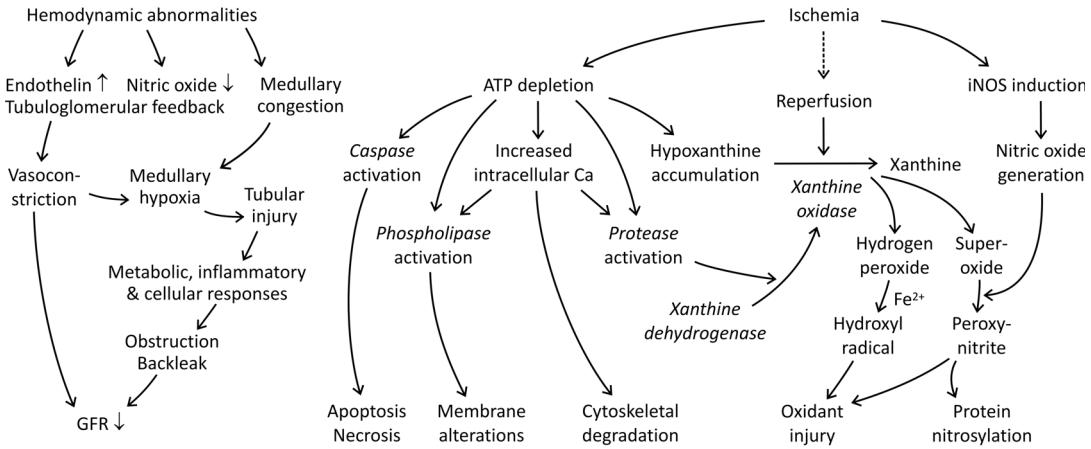

Figure 2.2. *Pathophysiological mechanisms leading to a decrease of the GFR (left), and the consequences of ischemia reperfusion on cellular integrity (right).*

into the venous capillary system. But the blood coming from the glomerular capillary system also drains into the vasa recta system, a capillary system that forms descending and ascending vascular bundles between tubules within the renal (outer and inner) medulla and which is essential for the formation of counter-current gradients. The blood flow in the vasa recta is low to preclude washing out of the solute gradient, and the counter-current configuration of the vascular bundles causes an oxygen steal phenomenon between the descending and ascending vasa recta, resulting in a gradient of decreasing oxygen tension from the renal cortex to the medulla. Due to this anatomical configuration, the oxygen extraction rate in the renal outer medulla is extremely high making a state of near oxygen deficit a normal physiological condition[37] (figure 2.3).

Although the inner medulla has an even lower oxygen tension, the outer medulla is most dependent on the oxygen supply as the region contains cells with the highest metabolic activity of the nephron. In this region, the cells of the straight part of the proximal tubule or S3 segment and thick ascending limb (TAL) are the most vulnerable to oxygen deficit as they contain many oxygen consuming basolateral Na^+/K^+-ATPase transporters. The S3 proximal tubular epithelial cells are the first to be severely affected as they are strongly dependent on oxidative phosphorylation with little or no glycolytic capacity, whereas cells of the TAL contain higher amounts of glycolytic enzymes and have a better capacity to undergo anaerobic metabolism[38-42].

2.1.3.3. Vascular and hemodynamic alterations

Ischemic AKI is associated with loss of blood pressure auto-regulation.

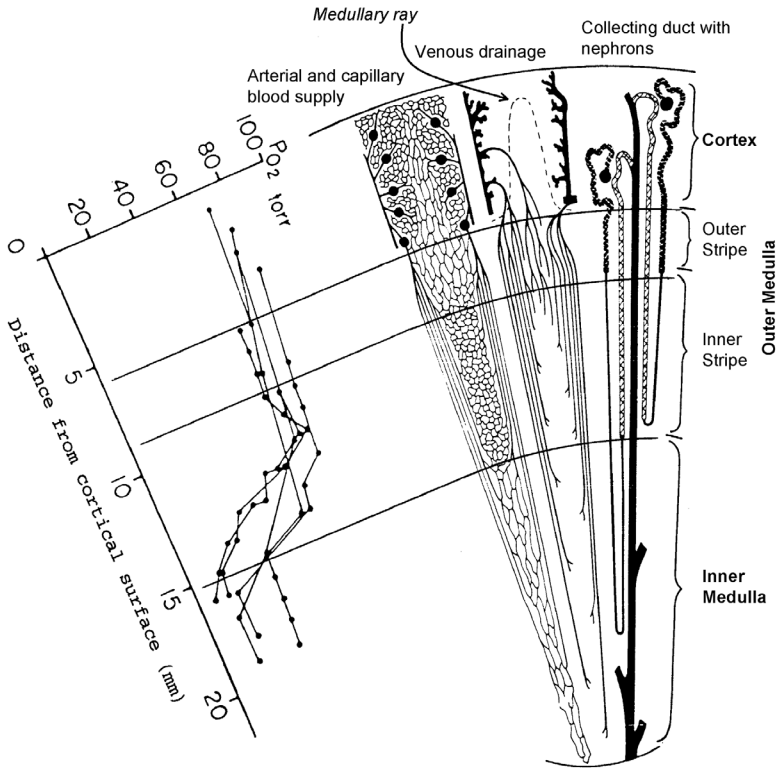

Figure 2.3. *Intrarenal oxygen tension (left) and renal vascularization (right)*[37].

Rather than the normal auto-regulatory renal vasodilatation inherent to a decrease in renal perfusion pressure, an additional vasoconstriction has been observed in the ischemic kidney in response to norepinephrine and endothelin[43-45]. Although extensive literature suggests enhanced vasoconstriction to be fundamental in the pathophysiology of ischemic AKI, no vasodilator has been found to be useful in prevention or treatment of AKI associated with ATN in humans[46,47].

Immediately at reperfusion, the massive infiltration of neutrophils, macrophages and T-cells occurs through renal capillaries. Vascular congestion in the outer medulla due to blood cell trapping is another important characteristic of ATN, which has been observed in humans[48,49] and experimental animals[50], and also specifically in the setting of renal transplantation[51,52]. During vascular congestion, red blood cells are trapped due to reduced blood flow and capillary collapse[53,54]. This congestion worsens hypoxia in the S3 segment of the proximal tubule and the TAL of Henle's loop in the outer medulla, and can be observed microscopically as well as macroscopically (darkening of the medullar tissue)[1,55]. The entrapment allows for enhanced interactions be-

tween circulating leukocytes and endothelial cells[56,57], but also in proteolytic breakdown of the perivascular matrix in the vasa recta[58]. It has been shown that specific membrane-bound proteins like intracellular adhesion molecule-1 (ICAM-1), selectins and B7-1, molecules that augment the adhesion of leukocytes to endothelial cells in AKI models, are up-regulated in endothelial cells during ischemia[59]. B7-1 plays important role in leukocyte-endothelial cell interactions along the vasa recta; anti-B7-1 blocks the adherence between CD28-expressing T-cells and B7-1 expressing endothelial cells, resulting in much less vascular congestion[60]. Blockade of T-cell CD28-B7 co-stimulation was also shown to protect against IRI in rats and to significantly inhibit T-cell and macrophage infiltration and activation in situ[61]. Also microvascular permeability was shown to be modulated by signaling molecules influencing the expression of matrix metalloproteases 2 and 9 (MMP2/9)[62]. During ischemic AKI, MMP2 and MMP9 are upregulated increasing the breakdown of collagen IV, an important structural component of the perivascular matrix, thus increasing perivascular permeability. These interactions can be reduced by genetic ablation, knockdown or pharmacological inhibition of specific proteins[63-65], resulting in a reduced vascular congestion and protection against IRI. In various studies, deactivation of ICAM-1, selectins and B7-1 were shown to be effective to reduce harmful endothelial interactions and MMP inhibitors were shown to ameliorate leakage in experimental ischemic AKI models. The processes leading to endothelial injury have not yet been fully characterized, but they are likely to play a major role in hemodynamic and inflammatory changes after renal ischemic injury[66]. Adult bone marrow-derived mesenchymal cells were also shown to influence renal activity due to vasculotrophic properties[67]. During vascular injury, an endothelial-mesenchymal transition (endo-MT) process was observed, which, similar to the epithelial-mesenchymal transition (EMT), results in a transdifferentiation of endothelial cells towards (myo)fibroblasts, stimulated by factors like TGF-β[68-70]. Interventions targeting endothelial cell function could therefore be beneficial for both short-term and long-term recovery of AKI. Although compared to the proximal tubule, there are fewer histologic changes in the medullary TAL (MTAL), ischemia was shown to modify the expression of a large number of genes in the MTAL, and the production and excretion of a number of cytokines, which in addition likely impact the microvasculature, serving as effectors for a positive feedback pathway enhancing inflammation and vascular obstruction.

2.1.3.4. Tubular epithelial alterations

Depending on the extent of cellular hypoxia and the duration of the ischemic insult, tubular epithelial cells can be sub-lethally damaged or lose their

viability and become either necrotic or apoptotic. The peak of inflicted damage in experimental animals can be observed between 12 and 24 hours of reperfusion, depending on the duration of the ischemic period. Even after a prolonged ischemic insult, the kidneys have the ability to recover the structural and functional integrity of the proximal tubule up to a certain level, which occurs at reperfusion about 48 hours after the ischemic injury, and can last up to 5 days after the insult to ensure complete tubular recovery. When the inflicted injury is too severe, kidney function will not be able to recover completely and acute injury may be followed by progressive renal injury leading to CKD. Loss of structural integrity of the tubule is associated with specific morphological changes, which have been extensively studied in animal models (figure 2.4). The clinical course of ATN may be divided into initiation, maintenance, and recovery phases. The initiation phase corresponds to the period of exposure to ischemia or nephrotoxins, in which sub-lethal renal tubule cell damage begins to evolve (but is not yet established). The GFR falls slowly and urine output decreases. During the maintenance phase, renal tubule injury is established, the GFR stabilizes at the level below normal, and the urine output is low or absent. The second phase of ATN usually lasts for 1-2 weeks in patients, but may extend to a few months and can sometimes be associated with oliguria. The recovery phase of ATN is characterized by polyuria and gradual normalization of the GFR; however, in the context of multi-organ dysfunction, regeneration of renal tissue may be severely impaired and renal function may not return.

Because of a decrease in intracellular ATP, also transport of ions through N^+/K^+-ATPase and Ca^{2+}-ATPase decreases, causing alterations in membrane potential and cell volume regulation. The cell starts to swell and Ca^{2+} starts to accumulate inside the cell[71,72]. The accumulation of Ca^{2+} in the cytosol leads to the activation of calcium sensitive enzymes, such as phospholipases[73], which is associated with the degradation of the membrane biphospholipid layer[74], changes in the bioenergetics and permeability of the mitochondrial membrane and the plasma membrane[75]. Phospholipases also induce the production of prostacyclins, which are protective against IRI due to an inhibition of leukocyte adherence in the post-capillary venules[76]. Sub-lethal injury to the renal tubular cells results in a dysfunction of the actin skeleton of the tubular cells and in loss of polarity[77]. Tubular polarity is maintained in strong association with the actin skeleton and established by targeted delivery of lipids and proteins (cell adhesion molecules and polarized membrane proteins) to the different intracellular domains[78,79]. Na^+/K^+-ATPase is re-localized from the basolateral to the apical side of the epithelial cells[80], increasing the sodium concentration further downstream at the luminal side of the distal epithelium, possibly activating a tubulo-glomerular feedback mechanism at the macula densa, resulting in vasoconstriction of pre-glomerular arterioles,

a decline in GFR, and an increased sodium concentration in the urine after AKI[81]. Redistribution of actin from apical domain and microvilli into the cytoplasm causes loss of villi and the brush border membranes, resulting in cast formation and obstruction as well as loss of junctional complexes (tight junctions and occludens junctions) and eventually back-leak of the intra-tubular fluid[82,83]. Due to the redistribution of integrins to the apical membrane, epithelial cells slowly detach from the basement membrane. The first changes on the macroscopical level can only be observed around 6 hours of reperfusion in the S3 segment of the proximal tubular cells (PTCs)[84-88]. These redistributed integrins retain their cell adhesive properties, and it was shown that

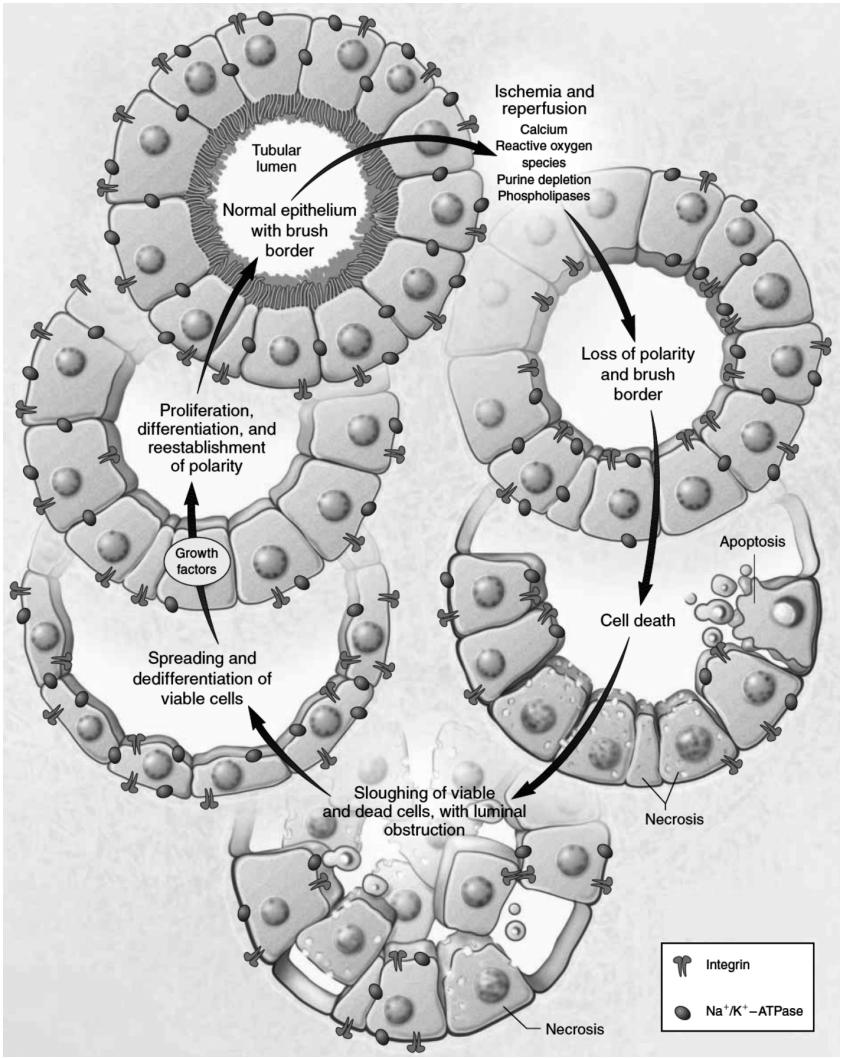

Figure 2.4. *Renal PTC injury and repair.*

administration of arginine-glycine-aspartic acid-containing peptides as well as anti-activated-b1-integrin, molecules specifically binding to these integrins, reduce the severity of inflicted injury by AKI and decrease tubular obstruction[89-93]. Naked basement membranes can be observed after 12 hours of IRI.

According to the severity of the injury, renal proximal tubular epithelial cells can undergo apoptosis or necrosis, further participating in the pathogenesis of ischemic AKI[94]. Necrosis occurs after more severe injury and in the most vulnerable PTCs, whereas apoptosis predominates in less severe injury and in distal nephron segments more resistant to ischemic damage.

Apoptosis is a regulated, active and energy-dependent form of programmed cell death following a choreographed and specific genetic expression profile, which is considered a natural and spontaneous process in cell homeostasis and differentiation. It is characterized by cytoplasmic and nuclear shrinkage, DNA fragmentation and breakdown of the cell into apoptotic bodies which are removed by phagocytosis[95]. Apoptosis is associated with an increased expression of pro-apoptotic proteins like B-cell CLL/lymphoma-2 (Bcl-2)-associated X protein (Bax) and other members of the Bcl-2 family as well as caspases[96-98]. Bax and Bcl-2 overexpression has been observed in proximal and distal tubuli during ischemic injury. The molecular pathways resulting in cell apoptosis have been studied extensively and were found to be mediated by tumor necrosis factor (TNF)-receptors, either through the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) or through Fas and Fas ligand (FasL). The mostly ATP-dependent molecular pathway causing IRI-related apoptosis involves up-regulation of Fas and FasL[99,100], increased expression of NO, TGF-β and loss of interactions between cells and cells and the matrix[101]. Following Fas activation, a balance between pro-apoptotic (e.g. Bax, Bad) and anti-apoptotic (e.g. Bcl-2) members of the Bcl-2 family is established. Caspase inhibitors were shown to restrict the inflicted damage in a model of renal IRI[102]. The apoptosis occurring during reperfusion is correlated with the duration of the ischemic period[103].

In contrast, necrosis is a rapid, traumatic and unregulated situation in which cellular homeostasis fails, associated with loss of membrane integrity, cytoplasmic swelling, nuclear pyknosis, cellular fragmentation and increased inflammatory response[90]. Necrotic cell death is often overestimated as in contrast to necrotic debris, apoptotic cell fragments are small[104] and more rapidly cleared. As ATP is required for the full execution of the apoptotic pathway, products of an incomplete pathway are often misinterpreted as necrosis. Cell necrosis occurs in case of prolonged complete ischemia, when the injury is severe and the ATP decreases below critical levels. The events leading to necrosis are irregular and involve multiple pathways[105].

Damage to the brush border causes a release of its associated proteins in the tubuli. These as well as other typical protein markers for AKI can then be measured in the urine and in the blood. Variation of expression of these markers was shown to explain the origin of renal damage as well as predict the outcome of AKI[106]. To act accordingly on a clinical level, it is necessary to determine the stage of AKI, possible antecedents and potential outcomes[107], for which the use of sequential biomarkers proves to be an excellent tool[108]. The list of early biomarkers of AKI is growing continuously. Important recent biomarkers include neutrophil gelatinase-associated lipocalin (NGAL)[108,109], liver-type fatty acid-binding protein (L-FABP), IL-18[109], interferon regulatory factor 1 (IRF-1)[110] and kidney injury molecule 1 (KIM-1). Some of these markers, like NGAL and IL-18, have been reported to hold some predictive value in the assessment of graft function after transplantation. However, it should be noted that although several of these urinary markers could be theoretically useful in the diagnosis or the early prediction of AKI, none of them has up to now proven to be relevant in the clinical setting of AKI.

The kidney has the ability to regenerate after ischemic injury. Damaged tubular epithelial cells are known to regenerate, but can also dedifferentiate and re-differentiate, or be replaced by mesenchymal stem cells. In the kidney, a delicate balance exists between renal injury and repair, cell loss and proliferation, and macrophage-dependent interstitial matrix accumulation and remodeling[111]. During the first phase of the regenerative process, the proximal tubular cells are exfoliated and expression of stress response genes increase, resulting in an accumulation of mononuclear cells, mostly macrophages. These macrophages are believed to originate from infiltrating monocytes, as resident macrophages and dendritic cells are usually considered terminally differentiated and non-proliferating. There are two distinct subtypes of macrophages, M1 and M2, which both arise from a common $CD34^+$ progenitor and are both known to play a role in tubular regeneration. Surviving tubular cells also express various molecules to enhance self-repair and self-protection[112-114] and the regional expression of paracrine growth factors could contribute to the stimulation of cell proliferation[96], which were also shown to be produced by the more injury resistant distal tubular cells[115].

Furthermore, epithelial cells have also been reported to undergo EMT in response to growth factors like TGF-β1, which is up-regulated after ischemia. These dedifferentiating cells express a set of cell cycle proteins, like cyclins, cyclin-dependent kinases[116] and proliferating cell nuclear antigen (PCNA), but also genes such as vimentin, α-smooth muscle actin and neural cell adhesion molecule[117]. Dedifferentiated cells can migrate to injured regions after occurrence of apoptosis, necrosis or cellular detachment. The contribution of dedifferentiated cells in the process of renal repair however, remains contro-

versial[111,118,119], and some growth factors of which the release was observed have proven to be effective in rodents, but fail to contribute to renal repair in humans[27,120,121]. The last re-differentiation phase consists in the expression of differentiation factors and the re-polarization of the tubular epithelium. Factors involved in the fetal development of the kidney have also been shown to play a crucial role in renal regeneration and repair processes[122-124].

Consistent evidence has been provided for bone marrow-derived cells to be able to transdifferentiate also, resulting in a switch of the phenotype in response to inflammatory cues. So, they are able to replace the renal vasculature and interstitial cells[125], renal tubular epithelial cells and cells of the glomerulus[29,30]. The majority of regenerating tubular epithelial cells are derived from an intra-renal source, bone marrow-derived stem cells only contribute to the replacement of tubular epithelial cells through a process of cell fusion[126]. This fusion occurs in post-ischemic kidneys and in cultures of bone marrow cells and renal epithelial cells in vitro[127]. Also mesenchymal stem cell-derived microvesicles were shown to exert a positive effect on renal tubular injury, because of their ability to transport various substances to damaged cells[128-130]. The persistence of renal progenitor cells in the interstitium of the kidney has been reported, which could also aid in the regeneration of damaged tubular epithelial cells[131-133]. Furthermore, adding to the complexity of tubular regeneration, renal tubular epithelial cells were shown to have the intrinsic capacity to rapidly self-duplicate[134,135].

2.1.3.5. Role of reactive oxygen and reactive nitrogen species

The formation of reactive oxygen and nitrogen metabolites is induced by a state of cellular hypoxia and subsequent regeneration. Both reactive oxygen and nitrogen species were shown to contribute to cellular damage in renal ischemic reperfusion injury.

Oxygen free radicals are naturally generated by the mitochondrion as a result of normal cellular metabolism[136]. The electron leakage from the mitochondrial oxidative respiration system produces low quantities of superoxide anion which are efficiently inactivated by mitochondrial and cytoplasmic superoxide dismutase (SOD). SODs catalyze the 'dismutation' of superoxide into oxygen and hydrogen peroxide, and is an important antioxidant defense mechanism. There are four different forms of SOD depending on their metal co-factor. The physiological importance of SODs can be illustrated by various pathologies[137-141]. SOD3 knockout mice are relatively sensitive to hyperoxic injury[140], and were shown to have increased oxidative stress and renal cast formation after ischemia reperfusion[141].

With the help of xanthine dehydrogenase, ATP is converted to hypoxan-

thine (figure 2.5). As Ca^{2+} accumulates, together with proteases, it helps the conversion of xanthine dehydrogenase to xanthine oxidase, which converts hypoxanthine to xanthine. Through oxygen deficit, cells are switching to an anaerobic metabolism and intracellular ATP starts to decrease[142]. The ischemic phase of IRI is characterized by an increased production of oxygen free radicals by mitochondria to overcome ATP depletion. During the reperfusion phase, however, the major part of tissue damage due to oxygen radicals occurs[143]. During reperfusion, the mitochondria become more active to overcome the IRI-induced lack of ATP, which is associated with the formation of oxygen free radicals like superoxide anion, hydrogen peroxide and hypochlorous acid. Furthermore, SOD is highly susceptible to nitrosylation, which causes a reduced scavenging of superoxide anion[144]. Renal ischemia reperfusion also results in a decreased gene expression of catalase, resulting in a reduced degradation of hydrogen peroxide[145,146]. During IRI, xanthine dehydrogenase is irreversibly converted to xanthine oxidase, leading to the conversion of hypoxanthine to xanthine and production of superoxide anion as well as hydrogen peroxide through conversion by superoxide dismutase (figure 2.5).

Parenchymal cells, vascular endothelial cells, and infiltrating neutrophils

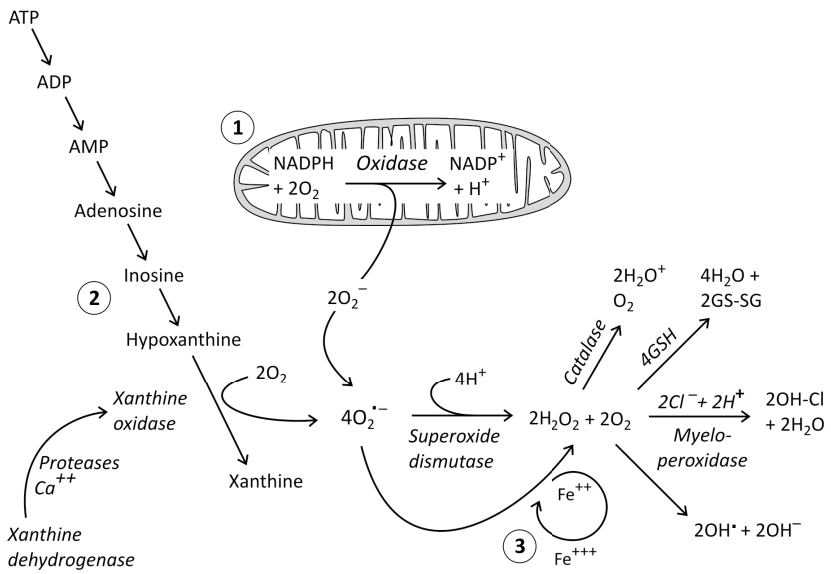

Figure 2.5. *Generation of ROS (1) Superoxide leakage from mitochondria occurs under normal physiological conditions (2) During the ischemic phase, cellular hypoxia results in ATP depletion and the accumulation of its metabolite hypoxanthine and xanthine oxidase (3) In the reperfusion phase, hypoxanthine is converted to xanthine in the presence of oxygen, resulting in the formation of oxygen radicals and hydrogen peroxide, which is further metabolized.*

and macrophages are known to be major sources of toxic oxygen free radicals during renal IRI[147]. Within 15 minutes of reperfusion of the ischemically injured kidney, the released reactive oxygen species (ROS) stimulate the S3 PTCs to produce interferon regulatory factor 1 (IRF-1), which is a transcription factor that activates pro-inflammatory genes[110].

Scavengers of ROS and other radicals have been shown to exert a protective effect against renal IRI in in vivo as well as in vitro studies[148,149]. Also a transgenic knockout of IRF-1 ameliorates the impairment of renal function, morphological injury and inflammation after acute renal injury by inhibiting secondary effects of ROS[110]. Injection of free radical scavenger SOD resulted in a complete prevention of tissue injury during reperfusion, demonstrating the devastating effects of these radicals[150], and other scavengers like edavarone[151] and ascorbic acid[152] were also shown to be effective. An SOD3 analog was also shown to attenuate IRI through an attenuation of the increase of serum creatinine and serum TNF-α, IL-2, IL-4 and IL-13 and by partially preventing the infiltration of $ED1^+$ macrophages and $CD8^+$ T-lymphocytes in the kidney[153]. Factors indirectly influencing superoxide formation like the iron transporters (apo)transferrin[154] and NGAL[155] also exert a protective effect against renal IRI, as well as exposure of experimental animals to hyperbaric oxygen, which was shown to reduce lipid peroxidation[156].

The pathophysiology of ischemic AKI is greatly influenced by the generation of nitric oxide (NO), mainly produced by NO synthase (NOS). During ischemic AKI, the activity of endothelial NOS (eNOS) was shown to decrease, with a concurrent increase of active inducible NOS (iNOS)[157]. The genetic ablation or inhibition of iNOS activity was shown to reduce renal IRI in in vitro as well as in vivo studies[158-160]. The activity of NO irreversibly inhibits mitochondrial respiration, but NO also combines with superoxide to become the reactive nitrogen species peroxynitrite, which causes lipid peroxidation, DNA damage and protein modification of tyrosine to nitrotyrosine[161]. Malondialdehyde (MDA) is a reactive aldehyde resulting from the degradation of polyunsaturated lipids through lipid peroxidation, and can be measured as a biomarker of oxidative stress. Peroxynitrite scavengers have also been shown to exert a protective effect in ischemic AKI[157,162].

Hemoxygenase 1 (HO-1) or heat shock protein 32 (Hsp32) belongs to the heat shock protein family, present in virtually all living organisms, that are up-regulated in case of increased stress, and also have a function as intracellular chaperones for other proteins, as housekeeping proteins or as antigen-binding proteins. HO-1 is an important protein in the metabolic pathway of the heme group[163]. The three metabolic products of the degradation of heme, biliverdin, Fe^{2+} and carbon monoxide have immunomodulatory[164], anti-apoptotic, and the latter also vasoactive properties. HO-1 has been shown

to be upregulated after IRI[165], and the modulation of Hsps has been shown to effectuate protective effects in renal IRI[166-168]. The potential importance of induced HO-1 in maintaining renal perfusion has been demonstrated in renal transplantation experiments[169,170]. Its protective function is effectuated through the inhibition of afferent arteriolar vasoconstriction. HO-1 has a direct anti-apoptotic function, promoting cell survival by induction of cyclin-dependent kinase inhibitor p21[171], but also through carbon monoxide, which was shown to function as an anti-apoptotic agent in physiological concentrations[172]. Studies using chemical inducers of HO-1 have shown an amelioration of experimental IRI, while genetic ablation or pharmacological inhibition of HO-1 results in an augmented injury phenotype[173].

2.1.3.6. Inflammation and leukocyte infiltration

During AKI, pro-inflammatory factors are released from damaged cells (both passive and through active synthesis). In the meantime, the expression of anti-inflammatory factors by damaged cells decreases, resulting in an inflammatory response at reperfusion[174]. Alterations in tubular cells and tubular dysfunction causes the tubular epithelia to produce increased TNF-α, IL-1β, IL-6, IL-8, TGF-β, monocyte chemoattractant protein (MCP-1), RANTES, and other chemokines following ATP-depletion[175]. An increased expression of the pro-inflammatory interleukin 1 (IL-1) also stimulates further expression of IL-6 and TNF-α, all causing further damage to epithelial cells[176]. The increased presence of damage markers and pro-inflammatory factors result in an increased infiltration of inflammatory cells.

Progression through these inflammatory phases involves infiltration of neutrophils, dendritic cells, T-cells and macrophages, whose interplay contributes to the immunopathogenesis of ATN as well as glomerulonephritis. Inflammatory mediators secreted by injured tubules induce the deceleration of intravascular leukocytes, the activation of the endothelium and leukocyte rolling on the endothelium (figure 2.6). In endothelial cells, adhesion molecules promoting the endothelial-cell-leukocyte interaction are up-regulated[177], like selectins, integrins, ICAM-1, vascular cell adhesion molecule and P-selectin. The initial interactions between leukocytes and the endothelium are mediated by selectins and their ligands. The leukocytes are triggered for infiltration through an interaction of endothelial integrins and ICAMs on the surface of leukocytes, resulting in strong adhesion. Blocking this adhesion process was shown to exert a protective effect in experimental studies[178,179]. In the process of diapedesis, the transmigration of leukocytes through the endothelial cells occurs. Vasoconstriction and vascular plugging can be caused by the activation of adherent leukocytes that remain resident in the vascular lumen, re-

sulting in an impaired blood flow in the microvasculature of the outer medulla and an aggravation of inflicted injury[54,60].

Tubular epithelia also produce fractalkines, membrane bound proteins that have the combined function of a chemokine and an adhesion molecule, facilitating the migration and adhesion of leukocytes and also facilitating monocyte-induced cell injury[181]. Infiltrating leukocytes themselves also produce TNF-α, IL-1, IL-8, MCP-1, ROS and eicosanoids. TNF-α, IL-1 and ROS upregulate more chemokines, recruiting more leukocytes to damaged sites, and activate coagulation pathways causing further injury[177]. These molecules also cause endothelial activation, endothelial cell swelling, decreased vessel patency, and also leukocyte entrapment, allowing for increased interactions between leukocytes and platelets[177]. Infiltrated macrophages and neutrophils cause an increase of myeloperoxidase activity (MPO)[178,180,182]. But also anti-inflammatory interleukins (IL-4, IL-10) are found to be increased in the circulation. The entire pathophysiological evolution of AKI is characterized by a specific mRNA signature[183].

In the pathophysiological development of renal injury, endothelial cells[28], complement activation[184-186], specific chemokines[187,188] and toll-like receptors (TLRs) have all clearly been shown to contribute. TLRs play an important role in the interaction between lymphocytes, endothelial and epithelial cells, and respond to Hsps. They activate effector cells of innate immune system in damaged tissues to generate further pro-inflammatory signals. TLR2 is constitutively expressed in the kidney. Renal proximal tubular epithelial cells express both TLR2 and TLR4, of which the expression is increased during AKI[189-192]. TLR4 is expressed on the surface of macrophages, and activation of endothelial TLR4 amplifies inflammation and tubular damage[193]. In experimental studies, genetic ablation of TLR2 and TLR4 was shown to result in a reduced production of cytokines and chemokines and a reduced leukocyte infiltration resulting in reduced kidney dysfunction and tubular damage[194,195].

Inflammatory mediators and infiltrating immune cells are depicted in figure 2.7. Neutrophils are the first inflammatory cells to massively infiltrate the kidney within the hour, although this infiltration was found to be highly species dependent[196,197]. The leukocyte infiltrate found in the post-ischemic rat kidney consists of macrophages and helper T-cells, but only poor amounts of neutrophils and cytotoxic T-cells[198]. The composition of the leukocyte infiltrate at reperfusion is still subject of debate, and many staining methods have been found to cross-react with monocytes, questioning the infiltrate compositions reported in literature[180]. Neutrophils are recruited from the vasculature to the interstitium reaching a maximum at 24 hours of reperfusion[199]. Although the total amount of infiltrating neutrophils is low, targeting molecules involved in neutrophil infiltration has been shown to be effective

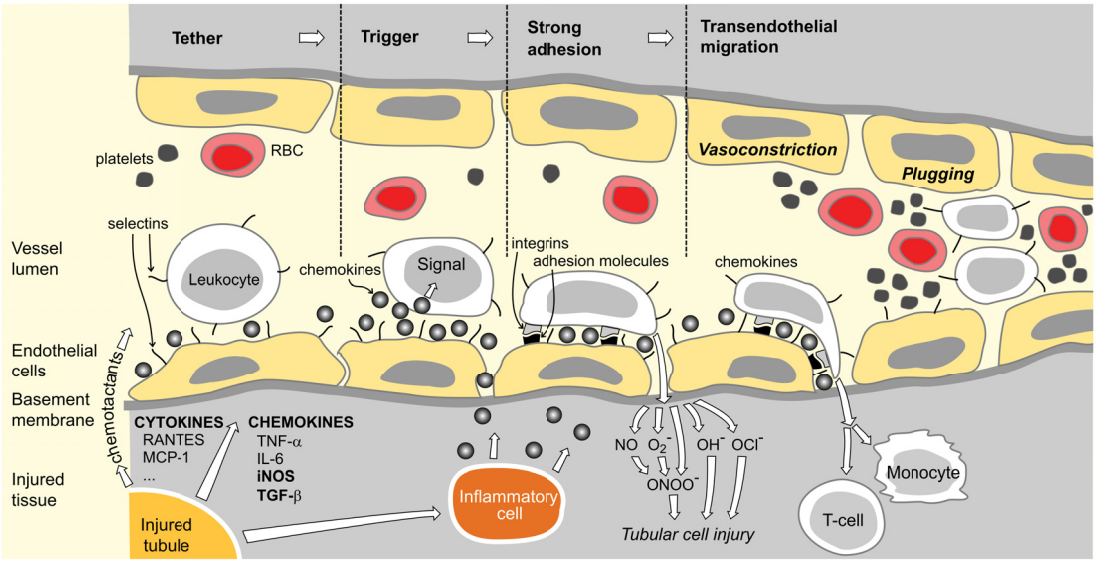

Figure 2.6. *Vascular endothelial interactions occurring during renal IRI, resulting in adhesion of leukocytes and transendothelial migration. Adapted from De Greef et al. 1998[180].*

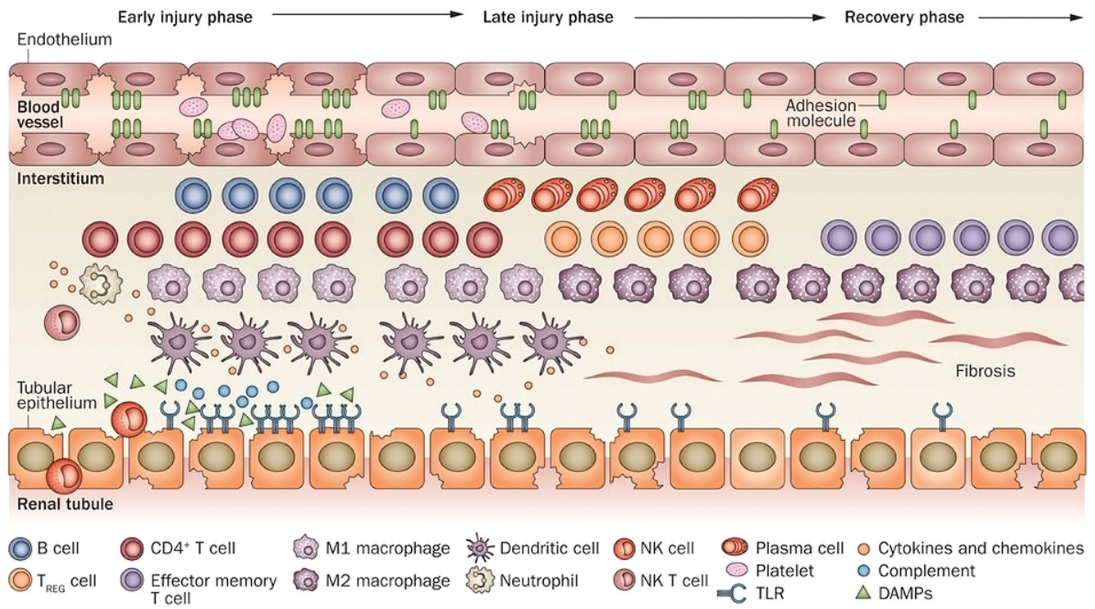

Figure 2.7. *Interaction of immune cells with the endothelial surface in the normal kidney (left) and the ischemic kidney (right). Adapted from Jang et al., 2015[204].*

in the treatment of ischemic AKI in experimental animals[200,201]. Neutrophils remain increased in the cortex as well as in the outer medulla at 3 and 10 days after IRI[202].

Monocyte-derived tissue effector cells, macrophages, are present in large numbers in all types of kidney disease with inflammation, and their roles in inflammation as well as their molecular effectors have been difficult to decipher[203,204]. Macrophages are the dominant infiltrating cells in ischemia reperfusion injury, infiltrating shortly after T-cells at about two hours of reperfusion, and seem to have a bivalent role. On one hand, they aggravate injury by enhancing the inflammatory cascade through adherence to the ascending vasa recta[60], secrete cytokines, recruit neutrophils, induce apoptosis[205], and contribute to the pathogenesis of renal fibrosis[206]. On the other hand, macrophages were shown to play a beneficial role during tissue repair and actively participate in the resolution of injury and promote tissue restoration in both immune- and non–immune-mediated renal disease. These repair processes are mediated by local release of growth factors, coagulative control and scavenging of cellular debris[119,198]. Their action is determined by their activation state. Macrophages have two activation states, a pro-inflammatory M1 phenotype and an anti-inflammatory M2 phenotype, and can change their phenotype depending on the surrounding microenvironment and inflammatory stimuli[207,208]. Infiltrating macrophages were shown to present both phenotypes, but the first of infiltrating macrophages are pro-inflammatory, and release the cytokines MCP-1 and macrophage inflammatory protein 1 alpha (MIP-1α), with a peak at 24 and 72 hours, respectively. Macrophages release anti-inflammatory signals upon phagocytosis of apoptotic cells, like IL-6 and IL-10 (with a peak at 72 hours). Macrophages are thus found to adapt their phenotype and signal to the inflammatory surroundings. On the long term, M1-macrophages induce fibrosis, which was found not to occur in tubulointerstitial depletion of macrophages. Ex vivo manipulation of macrophages, inducing an M2 phenotype, was also shown to reduce renal injury and to facilitate repair, as shown by adoptive transfer studies of M2-polarized macrophages injected into mice with chronic inflammatory renal disease[209]. Also, beneficial effects on development of renal injury have been reached by modulating macrophage phenotype and function in models of glomerulonephritis, allograft injury, and interstitial fibrosis[203,210,211]. In general, non-selective depletion of macrophages was found to reduce AKI by abrogating persistent inflammation and subsequent development of fibrosis[209].

T-cells were also shown to exert a major influence in the pathophysiological development of IRI. The infiltration of T-cells and natural killer (NK)-cells is observed at one hour of reperfusion, is at its peak at about 3 hours after ischemic injury, and is associated with an increased activation of transcrip-

tion factors NF-kB, heat shock factor protein 1 (Hsf1) and hypoxia inducible factor 1 (HIF-1). Dendritic cells residing in the kidney and originating from secondary lymphoid organs effectuate T-cell activation and also trigger naïve T-cell responses. Dendritic cells were found in increased numbers after IRI[212] and have been identified as major inflammatory mediators in early IRI[213]. Six hours after ischemic injury, a steep decrease of the chemokine receptor CXCR3 (marker for activated T-cells) is observed. The number of T-cells and NK T-cells is clearly decreased at 24 hours after ischemic injury compared to its peak. Action of T-lymphocytes is shown to be crucial in mediating induction of injury and CD4/CD8-knockout mice seem to be resistant to extensive IRI[214]. Mostly CD4-knockouts were found to be significantly protected from renal IRI, and adoptive transfer of $CD4^+$-cells into CD4 knockout mice restored early post-ischemic injury[215]. Literature shows that shortly after the induction of IRI, there is increased trafficking of $TCR\beta^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ T- regulatory T-cells, which are recruited to the sites of injury and are known to contribute to the recovery of ischemic injury. Regulatory T-cells do not belong to the most common helper 1 and 2 $CD4^+$-subsets. When activated, they secrete large amounts of immunosuppressant IL-10 to counter pathogenic lymphocyte responses, protecting renal function[202,216]. Immunosuppression by T_{reg}s was also shown to be dependent on the expression of HO-1[217]. Up to 3 days after reperfusion, the amount of regulatory T-cells is doubled in ischemic kidney when compared to the contralateral kidney[202], after which the number of activated T-cells is equal in both ischemic and contralateral kidney. At day 10 after reperfusion, more activated $CD4^+$ and $CD8^+$ T-cells are present in the ischemic kidney compared to the contralateral kidney, predominating mostly in the interstitium of the cortex and in the outer medulla. Regulatory T-cells are crucial in reducing the expression of IL-2, IL-10, TNF-α and IFN-γ , resulting in a reduction of inflicted tubular damage in an experimental mouse model of ischemic IRI[202]. They have been identified as key regulators in autoimmunity and transplant tolerance, asthmatic inflammation and airway hyperreactivity, and can suppress innate and cognate immune responses using multiple mechanisms, including the suppression of pro-inflammatory cytokines[218]. IL-16 produced by T-cells and endothelial cells was shown to induce the migration of regulatory T-cells[219]. It also plays a role in pancreatic invasion in autoimmune diabetes[220] and is increased in ischemic models, suggesting a role in post-injury inflammatory response[221]. IL-16 was also shown to be increased in the proximal and distal tubule after IRI. Inactivation of IL-16, however, was shown to reduce inflammation-mediated renal IRI, associated with a decrease of infiltration of T-cells[222].

The evolution of the infiltrate was shown to be crucial as it determines the success of kidney transplantation. Between a few days and a week after

kidney transplantation, an acute systemic reaction occurs. The risk of rejection is the highest during the first 3 months after transplantation, due to the mismatch of human leukocyte antigen in the major histocompatibility complex. In the absence of immunosuppressants acute rejection occurs in the vast majority of the cases. This process of acute rejection is mainly driven by effector T cells (CD8+), who are activated and differentiated to recognize the exogenous HLA, resulting in necrosis of cells of the transplanted tissue under the influence of endogenous cytotoxic signals and systemic hypertension. Also eosinophils and neutrophils will repeatedly infiltrate the transplanted organ. The level of IL-16 expression has also been related to a delay in graft transplant function[223]. Aside of regulatory T-cells, Th17 T-helper cells were found to play an important role in transplant rejection and tolerance. Th17 T-helper cells are a subset of T-helper cells producing IL-17 who were found to be distinct from other T-helper cells and to play a key role in autoimmune disease, inflammation and tissue injury[224]. Their production is stimulated by cytokines like TGF-β, IL-1β, IL-6, IL-21 and IL-23[225].

NK-cells and B-cells were also shown to play a role in the pathophysiology of ischemic AKI. NK-cells are able to induce apoptosis in tubular epithelial cells[226]. Also natural killer T (NKT)-cells, a heterogeneous group of lymphocytes sharing characteristics of NK-cells as well as T-cells, were known to migrate to the post-ischemic kidney as early as three hours following reperfusion[227]. Within 1 to 2 hours after activation, they produce massive amounts of Th1 (IFN-γ, TNF-α) as well as Th2 (IL-4, IL-10, IL-13) cytokines. They also induce the production of IFN-γ by neutrophils and their activation have been shown to contribute to the development of ischemic injury[228]. The role of B-cells in ischemic AKI has not been clearly established, but B-cell deficient mice seem to be protected against renal IRI in the initial phase[229], and B-cells could also be involved in tissue repair after AKI[230]. On the contrary, mice lacking T-cells as well as B-cells were found to present worsening of renal injury[231].

2.1.4. Factors influencing the outcome of AKI

The kidney can be preconditioned to change its reaction to ischemic damage. This protective mechanism can be mediated through different mechanisms. Rat kidneys who have recovered from a short ischemic attack with a duration of 10 minutes, react better after being subjected to a second (longer) ischemic attack, indicating an endogenous intrinsic mechanism for protection against IRI. Pharmacologically, the kidney can also be preconditioned by agents inducing the up-regulation of protective proteins[197].

Pharmacological preconditioning with erythropoietin (EPO) results in a decreased serum creatinine level after IRI, decreased apoptosis and necrosis,

and an increased expression of anti-apoptotic protein Bcl-2[232]. TNF-α, known to be crucially involved in the pathogenesis and progression of renal and myocardial IRI and detrimental during sustained ischemia, was also shown to contribute to ischemic preconditioning[233].

Alpha-melanocyte stimulating hormone (α-MSH) is an endogenous anti-inflammatory cytokine and protects against ischemic injury by binding directly to melanocortin receptors on renal tubules and inhibit the expression of genes that cause inflammatory and cytotoxic renal injury[234].

Proximal tubular epithelia also modulate T lymphocyte activity with membrane marker CD40 which serves as a receptor for CD154, which is typically present on T cells. Exposure to CD154 was shown to result in an increased resistance towards ischemic AKI[175]. NOS also protects the kidney against ischemic injury. Prior ischemia results in a prolonged expression of NOS as well as some heat shock proteins. Deletion of NOS as well as inhibition of NO synthesis results in increased susceptibility to ischemic damage[235]. Serine and threonine kinases which play central roles in determining the response to multiple signaling inputs of proliferation, differentiation and apoptosis[236,237].

Ischemic preconditioning of heart as well as kidney was shown to be strongly associated with the phosphorylation of Akt through PI3K. This phosphorylation increases after multiple cycles of preconditioning, and strongly reduces the infarct size. The PI3K-Akt cascade also seems to be defective in diabetic animals[238].

2.2. DPP4 and the kidney

2.2.1. DPP4 activity and inhibition in the kidney

In the kidney, DPP4 expression was observed on the luminal brush border surface of proximal tubular epithelial cells[239], but also weakly in interstitial and glomerular cells[240,241] (figure 2.8). The weak expression of DPP4 in glomerular podocytes was known to be occasionally upregulated in various lysosomal storage diseases, primary renal diseases, but also in cases with no detectable renal pathology[242]. DPP4 is expressed slightly weaker in S3 cells compared to S1 and S2 cells[241]. Although it is not expected to pass the glomerular filtration barrier, soluble DPP4 was found in the microcirculation of the kidney and has also been shown to be present in the urine[243].

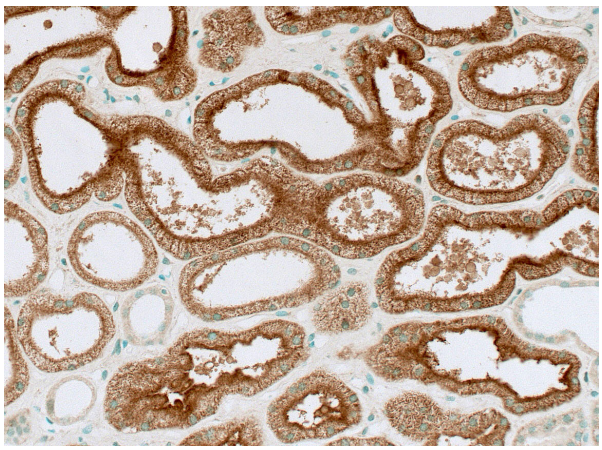

Figure 2.8. *Expression of DPP4 in the brush border of proximal tubular epithelial cells (visualized using an OX-61 anti-CD26 antibody purified at the Laboratory of Medicinal Chemistry).*

The precise function of DPP4 in kidney physiology is not known, but inhibition of DPP4 activity is associated with diuresis and natriuresis[244]. DPP4 inhibition, however, has been found to influence the evolution of various renal pathologies. In T2DM, DPP4 inhibition results in a reduced albumin-to-creatinine ratio through a decrease of both blood pressure and GFR[245]. This nephroprotective effect in diabetes has been found to be GLP-1/GLP-1R dependent as well as –independent[246]. There is evidence that incretin-based therapy also reduces albuminuria, glomerulosclerosis, oxidative stress, and fibrosis in the kidney, partially through GLP-1R-independent pathways[247]. The DPP4 inhibitor linagliptin was also shown to have a beneficial effect on kidney fibrosis in streptozotocin (STZ)-induced diabetic mice by inhibiting EMT[248]. In a mouse model of ureteral obstruction, an experimental DPP4 inhibitor

decreased the synthesis of many pro-inflammatory and pro-fibrotic molecules[249]. In the Zucker obese rat, DPP4 inhibition was shown to also attenuate filtration barrier injury and oxidant stress-related glomerulopathy and associated proteinuria[250]. DPP4 inhibition was also shown to reduce glomerular and tubular injury in obese, hypertensive insulin resistant rats which were fed a western diet, reducing serum uric acid levels, oxidative stress and IL-12, and increasing IL-10[251]. Low doses of sitagliptin results in an amelioration of diabetic nephropathy in diabetic and control ZDF rats[252]. Further studies are needed to shed light on the molecular mechanisms through which DPP4 inhibition and affected incretins or other DPP4 substrates exert their renal effects. But the above mentioned effects, as well as the observed beneficial effects of DPP4 inhibition in the context of other ischemic models, suggests a potential effect of DPP4 inhibition in renal IRI. In experimental IRI models in the heart as well as in the lungs, the concentration of serum DPP4 was found to increase after the ischemic insult. This increase has even been observed after the invasion of the peritoneum in case of sham operation. In the pathophysiological setting of IRI, soluble DPP4 was known to interact with the endothelial mannose-6-phosphate/insulin-like growth factor II receptor, thus promoting the transendothelial migration of T-cells[253,254]. As previously mentioned, the latter migration plays a major role in the pathophysiology of renal IRI. Furthermore, the expression of intracellular IL-16 is correlated with the expression of surface DPP4 in lymphoma[255]. The expression of IL-16 in the kidney was found to play a modulatory role in immune response and to be upregulated during IRI[222]. Recently, it has also been shown that human Th17 cells express high levels of DPP4[256]. During transplantation, Th17 helper cells were found to play an important role in graft rejection.

2.2.2. DPP4 substrates and the kidney

Over 70 renal substrates of DPP4 were isolated using a combined peptidomics platform and DPP4 knockout mice, expressed in different regions of the kidney[257]. Most of their functions are unknown, and it is hard to judge the relevance of the presence of these peptides in the pathophysiology of renal ischemia reperfusion injury. However, the expression of many substrates and their receptors, some more than others, as well as their function in the kidney has been described in literature and will be summarized below.

2.2.2.1. GLP-1 has a protective effect in IRI and is a potent diuretic

GLP-1 is produced in and released from the intestinal L-cells into the portal circulation. In the kidney, real time RT-PCR in microdissected rat nephron segments revealed GLP-1R mRNA expression predominantly in the proximal

convoluted tubule[258,259], but also in glomerular cells[260]. GLP-1 was shown to play an active role in the regulation of sodium hydrogen exchanger 3 (NHE3) through its G-coupled receptor at the luminal side of the nephron. NHE3 is primarily responsible for maintaining the balance of sodium by its reabsorption at the luminal side of the PTCs in the kidney[1,261] (figure 2.9). About 25-50% of NHE3 is co-localized with DPP4 on lipid rafts on the luminal side of the nephron in S3 cells[262]. Insulin also exerts a direct influence on the regulation of NHE3, stimulating sodium reabsorption[263]. GLP-1 has diuretic and natriuretic effects mediated directly through GLP-1R[264] and at least partially through downregulation of NHE3 transport function in the proximal tubulus[259]. Experiments of stationary microperfusion performed in rat renal proximal tubular epithelial cells revealed that GLP-1 significantly reduced sodium, potassium and bicarbonate reabsorption associated with reduced bicarbonate flux rate, a significant increase of NHE3 phosphorylation via a PKA-dependent mechanism, and decreased expression of the exchanger in microvillus membrane vesicles. NHE3 transport function is also up-regulated in diabetic nephropathy and heart failure, while it is reported to be down-regulated in AKI, regulated by extractable tissue factors[265,266].

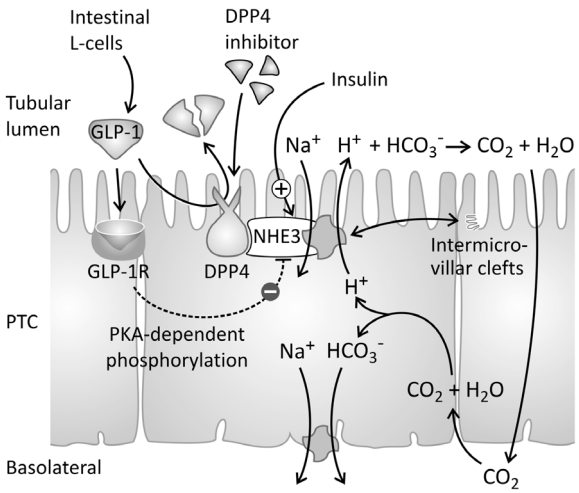

Figure 2.9. *Regulation of NHE3-mediated sodium reabsorption by GLP-1 in proximal tubular epithelial cells.*

Girardi *et al.* have shown that long-term inhibition of DPP4 activity in rats leads NHE3 to shift to megalin-enriched intermicrovillar clefts in the renal brush border, downregulating its activity[267]. As a result, there is a diminished reabsorption of sodium, a decreased re-uptake of water and an increased urinary volume[267-269]. Various acute[270,271] and chronic (mostly hypertensive) stimuli[272,273] were found to influence the distribution of NHE3 and DPP4 in microvillar domains[274]. Inhibition of NHE3 by a NHE3 blocker in a bilateral

experimental IRI model (with a ischemic duration of 40 minutes) results in a significantly lower creatinine level in the plasma[275]. GLP-1 induced diuresis and natriuresis were also accompanied by increases in RPF and GFR[260,276]. A reduced sodium reabsorption was also observed in obese subjects, but this was associated with a reduction of glomerular hyperfiltration[277]. In vitro, this natriuretic effect had no effect on the reabsorption of glucose[258]. The diuretic effect was confirmed by GLP-1 agonist exenatide in STZ-diabetic rats[276]. Although DPP4 inhibition was previously mentioned to have a natriuretic effect, this effect was shown to be mechanistically different compared to GLP-1 induced natriuresis[244]. Exenatide increased the GFR by up to 50% while suppressing proximal tubular reabsorption by up to 40% and increasing urine flow rate six-fold without altering the efficiency of tubuloglomerular balance and the responsiveness of tubuloglomerular feedback, making exenatide both a proximal diuretic and a renal vasodilator[278]. As GLP-1 is regulated by intake of fat and carbohydrate, but not by salt, the reason for the control of salt excretion by the GLP-1R can most probably only be seen in the light of a unified nutritional homeostasis, which has not been completely unraveled[279].

GLP-1 has also been shown to exert anti-apoptotic effects when binding to its receptor in various tissues, like the pancreas, gut and heart[280-282]. In the gut, but also in the central nervous system, pancreatic β-cells[281], myocardial tissue, bone[282], and cancer cell lines[280,283], binding to GLP-1R activates anti-apoptotic proliferation and signaling pathways such as PI3K and MAPK. As mentioned in the general introduction, activation of PI3K leads to the phosphorylation and inactivation of pro-apoptotic peptide Bad, preventing apoptosis[280,284]. In an experimental model of renal IRI, the GLP-1 agonist exenatide resulted in a reduction of apoptosis and necrosis, which could be based on the same pathways[285].

GLP-1 itself was shown to have a protective effect in models of cardiac ischemia reperfusion, this through receptor dependent as well as receptor independent mechanisms[280,286-288]. GLP-1 administration generally induces a remarkable improvement of cardiovascular parameters, through changes of blood pressure, endothelial function, body weight, heart and fat metabolism, left ventricular function[286], and response to ischemia reperfusion[289]. It was also shown to improve left ventricular function in patients with acute myocardial infarction (AMI) and severe systolic dysfunction after successful primary angioplasty. Furthermore, GLP-1 agonists (as well as DPP4 inhibitors) were shown to attenuate diabetic glomerular nephropathy[290]. In the kidney, the GLP-1 receptor agonist exendin-4 was found to ameliorate renal injury through anti-inflammatory action without affecting the blood glucose levels in STZ-induced diabetic rats[291,292]. A similar effect was observed in STZ-induced diabetic rats treated with the GLP-1 agonist liraglutide, which was as-

sociated with an improvement of eNOS activity and an inhibition of the NF-kB pathway, also without eliciting a glucose lowering effect[293]. In experimental renal IRI, aside from affecting apoptosis, the administration of the GLP-1 agonist exenatide in T2DM rats resulted in a decrease of glutathione in the serum as well as a reduced lipid peroxidation status as indicated by measurement of MDA. Increased levels of SOD and catalase in the serum were also observed, and a decreased MPO activity and TNF-α levels compared to untreated rats. Renal injury after IRI was attenuated in the exenatide treated T2DM rats, associated with a preservation of kidney morphology, and a decreased infiltration of monocytes and leukocytes[285].

A case study reported occurrences of sudden exenatide-associated renal failure through sudden decline of GFR in four T2DM patients treated with ACE inhibitors and diuretics in addition. The origin of these occurrences remains to be explained, but biopsies indicated ischemic glomeruli, interstitial fibrosis, tubular atrophy, glomerulonephropathy, nausea, vomiting, and a reduced fluid intake. When treatment was halted, recovery of renal failure was incomplete in three out of four patients[294].

2.2.2.2. GLP-2 has an anti-apoptotic effect in myocardial IRI

One of the main functions of GLP-2, as explained in the general introduction, is intestinal growth and proliferation, linked to anti-apoptotic activity[295]. Post-conditioning of the heart with GLP-2 in early reperfusion was found to protect against myocardial IRI, limiting infarct size and improving post-ischemic mechanical recovery[296]. As GLP-2 receptors were also found in the PTCs of the kidney[297], it might be possible that GLP-2 binding could exert the same anti-apoptotic activity during AKI which is also observed through the binding of GLP-1.

2.2.2.3. SDF-1α modulates hematopoietic stem cell (HSC) trafficking and immunological response

SDF-1α is expressed in the kidney in the glomerular endothelial cells and distal tubular cells in normal conditions, but only weakly in the PTCs. The SDF-1α receptor CXCR4 is only expressed in tubular cells, and more in distal tubular cells then in PTCs. As described in the general introduction, signaling of SDF-1α and its receptor CXCR4 is held responsible for the migration of HSCs during embryogenesis[298], but also to the attraction of $CD34^+$ cells to sites of tissue injury, as well inhibition of apoptosis of $CD34^+$ cells[299].

The role of the SDF-1α/CXCR4-axis in the kidney at IRI and kidney disease in general is controversial[300,301]. The early phase of renal IRI was shown to cause a general increase of expression of SDF-1α and its receptor CXCR4 in

the glomerular endothelial cells and (proximal and distal) tubular cells of the kidney[300,302,303]. After 1.5 hours of reperfusion, the mRNA expression of SDF-1α was found to be increased 2.5-fold. After 24 hours of reperfusion, mRNA expression was still increased 2-fold. Also the SDF-1α protein was found to be increased[300]. Surviving PTCs show an increased expression of SDF-1α as well as CXCR4. Some cells in glomeruli stain weakly after IRI induction. Necrotic tubule segments express no SDF-1α/CXCR4 at all.

Although the SDF-1α/CXCR4-axis was previously considered the central signaling axis regulating trafficking of CD34+ HSC to injured tissue, attempts to modulate this axis by SDF-1α administration or blocking the axis were not successful[304], so one cannot assume that the SDF-1α up-regulation is organized to favor tissue repair. Although not crucial for the migration of hematopoietic progenitor cells, SDF-1α was shown to directly mediate tubular epithelial protection after ischemic injury[305]. Many research teams have already shown that bone marrow stem cells migrate specifically to locations of IRI damage and possibly differentiate to renal tubular epithelial cells[29,30,306], but others have concluded only an immunoregulatory role as the CXCR4 receptor is also expressed on B- and T-lymphocytes[307,308]. On their cell surface, CXCR4 and DPP4 are co-expressed on lipid rafts. When SDF-1α binds CXCR4, both CXCR4 and associated DPP4 are co-internalized, reducing the surface activity of DPP4[309]; truncation of SDF-1α by DPP4 results in the loss of the chemotactic signal. DPP4 was also shown to exert a role in the normal mobilization of HSCs induced by granulocyte-colony stimulating factor (G-CSF). Inhibition or loss of CD26 in mice results in defect in the normal mobilization of HSCs hematopoietic stem cells induced by G-CSF[310]. Then again, inhibition of DPP4 was found to enhance the engraftment of bone marrow HSCs in a mouse model[311].

SDF-1α, of which active quantities were increased through DPP4 inhibition, has been proven to exert a protective effect in IRI models in the lung as well as the heart[312-314]. Protease-resistant SDF-1α following myocardial infarction was also shown to lead to dramatic improvement in angiogenesis and ventricular function even 3 hours after the onset of ischemia, suggesting it as a tool for prevention of heart failure[315]. SDF-1α was also shown to mediate remote ischemic preconditioning to reduce heart infarct size in an experiment blocking the CXCR4 receptor specifically[316]. Also parathyroid hormone, a known natural inhibitor of DPP4, was shown to cause an increase of SDF-1α-driven homing of $CXCR4^+$ HSCs into the ischemic heart[317].

2.2.2.4. NPY stimulates natriuresis and diuresis, angiogenesis and also exerts an anti-apoptotic effect

NPY is a co-transmitter of the sympathetic nervous system, including the renal nerves. The NPY receptors expressed in the kidney are activated by NPY released from nerve fibers, but can also be activated by PYY released from gastrointestinal cells. Through binding of the Y1 receptor, NPY produces potent renal vasoconstriction in vitro in isolated interlobar arteries and in the isolated perfused kidney as well as in vivo, upon intrarenal or systemic administration. NPY has more effect on the vas efferens than the vas afferens, resulting in only a little increase of the GFR. NPY also inhibits the release of renin via Y1-like receptors. Via Y2 receptors, NPY can stimulate Na^+/K^+-ATPase in proximal tubules and antagonize the effects of vasopressin on isolated collecting ducts. Pre-junctionally, it can act to inhibit noradrenaline release via Y2 receptors. Although these mechanisms infer a reduction of RBF, the systemic infusion of NPY causes diuresis and natriuresis, probably mediated by an extrarenal Y5 receptor[318]. Furthermore, NPY plays a major role in the excretion of potassium (mediated through Y1 and possibly through Y2)[319]. NPY was also shown to effectuate anti-inflammatory signaling on dendritic cells, increasing the expression of IL-6 as well as TNF-α[320].

In general, NPY plays a role in angiogenesis. Ischemia was shown to increase the release of NPY and to induce the expression of Y2 receptors as well as DPP4 in ischemic muscle tissue, pointing to a role of endogenous NPY and Y2/DPP4 system in the process of re-vascularization. NPY-dependent angiogenesis is impaired with aging and is associated with a decreased expression of endothelial Y2 receptors and DPP4[321]. This is mediated by neuronally derived NPY, accelerating the early response to femoral artery ligation, as well as platelet-derived NPY, critical for sustained capillary angiogenesis[322]. NPY, Y2 and DPP4 could thus have an important role in re-vascularization and tissue repair in the setting of renal IRI.

Furthermore, NPY was also found to exert an anti-apoptotic effect. NPY has been shown to improve myocardial perfusion and function in a porcine model of hypercholesterolemia and chronic myocardial ischemia through a decrease of apoptosis and fibrosis[323,324].

2.2.2.5. VIP stimulates excretion of sodium and potassium and plays an immunomodulatory and anti-apoptotic role

VIP is expressed in the PTCs of the kidney, where it is rapidly degraded by DPP4 at the luminal as well as the basolateral side of proximal tubuli[325]. Only the VPAC2 receptor was found in the kidney. Also VIP containing nerves are present in the kidney[326] and were shown to be involved in the regulation of

sodium excretion[327,328]. VIP was found to be an immunomodulator, inhibiting the production of pro-inflammatory cytokines and chemokines, and reducing the expression of co-stimulatory molecules on antigen presenting cells (reducing the stimulation of $CD4^+$-T-cells[329]. PACAP38 also results in a reduction of TLR4 expression[330]. The addition of VIP to perfused kidneys effectuated an increase of urine volume, fractional excretion of sodium, chloride, and potassium, without any significant changes in GFR or RBF, suggesting VIP affects renal tubular reabsorption[331]. In rabbits, injection of VIP results in an increase of the heart rate, coronary vasodilatation and a fall in plasma sodium, potassium and phosphate concentrations, as well as a two-fold increase of the fractional excretion of sodium, potassium and chloride and an increase of plasma renin activity[331,332]. In cultured TNF-α-activated PTCs, addition of VIP synergistically enhances the synthesis of pro-inflammatory cytokines IL-6 and IL-8.

In vivo administration of VIP before lung transplantation in a rat model attenuates IRI, associated with a reduction of TNF-α, IL-6, IL-12 and NO, and increases the titer of the anti-inflammatory cytokine IL-10. The inhibition of DPP4 was shown to increase the activity as well as the mRNA expression of VIP in those pulmonary transplants[333]. In a model of liver IRI, VIP binding results in a decrease of TLR4 mRNA and protein by macrophages in the liver, and a decrease of TNF-α and IL-6 expression (which normally increases after IRI)[334]. In mouse models of liver IRI, VIP administration was shown to reduce injury via a cAMP/PKA dependent pathway[335]. Also in a setting of hemorrhagic kidney IRI, VIP seems to be able to regulate the activity of ROS, making it a promising agent to defend against IRI as an antioxidant[336,337]. In vitro, VIP administration was shown to suppress apoptosis by the up-regulation of Bcl-2 in ovarian granulosa cells[338]. Furthermore, VIP attenuated experimental acute pancreatitis enzymatically as well as morphologically by inhibiting pro-inflammatory cytokine production from monocytes, resulting in a decreased serum amylase, IL-6, and TNF-α. Histologically, VIP and VPAC1 agonist attenuated the severity of pancreatitis[339]. VIP could thus positively influence the outcome of renal ischemic injury through a modulation of the inflammatory profile and through a reduction of apoptosis.

2.2.2.6. IP10 delays epithelial cell proliferation and increases inflammation and leukocyte infiltration

The cytokine IP10 is produced by the donor organ after transplantation, and effectuates an increased expression of cytokines and chemokines, increased infiltration of leukocytes, and increased graft injury[224]. Like IL-1β and TNF-α, IP10 is considered a pro-inflammatory marker in IRI. TNF-α and IFN-γ were shown to synergistically enhance the response of IP10[340]. Direct

anti-IP10 therapy was shown to delay graft rejection and reduce cytokine expression, leukocyte infiltration and graft injury[341]. In heart and lung transplantation, IP-10 was shown to have a potential as a predictive marker for respectively acute cardiac rejection[342] and primary graft dysfunction. Urinary CXCL9 and CXCL10 levels were also found to correlate with the extent of renal allograft rejection[343], subclinical renal tubulitis[344] and hepatitis C-associated glomerulonephritis[345]. An experimental inhibitor of IP-10 secretion by PTCs was proposed as a dose-reducing agent for conventional immune-suppressors in kidney rejection management[346]. IP-10 was also shown to play a role in development of renal IRI, as anti-IP10 antibodies resulted in an increased proliferation of tubular epithelial cells[347].

2.2.2.7. Other DPP4 substrates also influence the outcome of renal IRI

The PACAP receptor PAC1 was shown to be present in the kidney, mostly localized in cortical proximal tubular epithelial cells[348]. Like VIP, PACAP was found to be a potent immunomodulator[329]. In diabetic nephropathy, PACAP was shown to result in renal protection mediated through a reduction of apoptosis, fibrosis and an anti-inflammatory and anti-oxidant effect[349-351]. Treatment with PACAP38 was also found to result in a decrease of TGF-β and TNF-α in cyclosporin A-induced nephrotoxicity[352] as well as cisplatin-induced renal failure[353], and to prevent EMT of renal epithelial cells. PACAP was also found to exert this anti-inflammatory and anti-apoptotic effect in the setting of renal IRI[354]. PACAP-deficient mice were shown to present an increased susceptibility to in vivo renal IRI[355].

B-Type/Brain Natriuretic Peptide (BNP) was shown to protect cardiomyocytes from apoptosis after IRI by blocking the mitochondrial calcium uniporter[356]. Substance P, on the other hand, was shown to aggravate renal injury in salt-induced hypertension through an increase of oxidative stress and macrophage infiltration[357] and the neurokinin-1 receptor was found to be involved in early nephropathy in STZ-induced diabetic rats[358]. Although IFG-I was found to be a promising agent to attenuate the development of ATN in rats[25], use of recombinant IGF-I in a clinical trial seemed not to affect the recovery of renal function in AKI patients[27]. MIP-1β and RANTES, both substrates of DPP4, like IL-16, have been shown to induce a stimulation of the increase of migration of regulatory $CD25^+$ T-cells[219], but also of macrophages[224]. The possible effects of the increase of half-life of these migratory chemokines by inhibition of DPP4 could result in an immunosuppressive effect mediated by regulatory T-cells as well as a pro-inflammatory effect.

Aside from these DPP4 substrates, insulin, released through an increased half-life of GIP and GLP-1, was also shown to protect kidneys of diabetic rats

against massive cell death through apoptosis, but also protects the kidney against inflammation and fibrosis if administered before inflicting IRI. After IRI, however, insulin treatment has no protective effect[359].

2.2.3. Summary: DPP4 inhibition and IRI

DPP4 is expressed in the glomerular cells and the PTCs of the kidney. The receptors of some important DPP4 substrates are also expressed in the PTCs. An overview of their potential effect is given in figure 2.10. Although it is not exactly known what the exact effect is of the naturally occurring increase of DPP4 activity in the pathophysiological development of IRI in the lung and the heart, the inhibition of DPP4 could potentially reverse or prevent the effect of DPP4 on this development.

In various models of ischemia reperfusion of other organs, DPP4 inhibition was found to be beneficial towards the inflicted damage. In a model of cardiac ischemia reperfusion, DPP4 inhibition was shown to exert a protective effect and reduce infarct size[360]. DPP4 inhibition with the irreversible inhibitor AB192 seems to abrogate acute organ rejection in a model of lung transplantation by suppressing T-cell activation thus reducing T-cell cytotoxic capacity and humoral allospecific antibody response[361,362]. DPP4 inhibitors were also shown to exert a positive effect on diabetic glomerular nephropathy[290]. In a model of cisplatin induced AKI, alogliptin was found to ameliorate the outcome through anti-apoptotic effects[363].

It is important to note that over 70 substrates were identified in homogenized kidney tissue, but merely a few have been identified, of which the role in the kidney has been evaluated. Increased levels of circulating GLP-1 as well as GLP-2 are expected to increase anti-apoptotic activity where its receptor is expressed, which is also the case in the PTCs of the kidney. GLP-1 was shown to exert this effect through activating PI3K and MAPK anti-apoptotic pathways in various cell types[364]. DPP4 inhibition has been shown to exert an anti-apoptotic effect through the prolongation of the half-life of active incretins in many tissues, as discussed in the 'Introduction', including models of transplantation[365]. Furthermore, GLP-1 has a natriuretic and diuretic effect on the kidney, which could be beneficial in kidney failure, which is characterized by a reduced GFR. SDF-1α was shown to play a role in the chemoattraction of HSCs and the immunological response, although its role in kidney disease remains controversial[301]. In light of previous publications about lung and heart protection by DPP4 inhibition against IRI[312-314], it seems that the exact influence of DPP4 inhibition in the attraction of T-cells and in the SDF-1α/CXCR4-axis remains to be elucidated. NPY and VIP could both affect the development of IRI through their anti-apoptotic effect, in addition to the anti-inflammatory

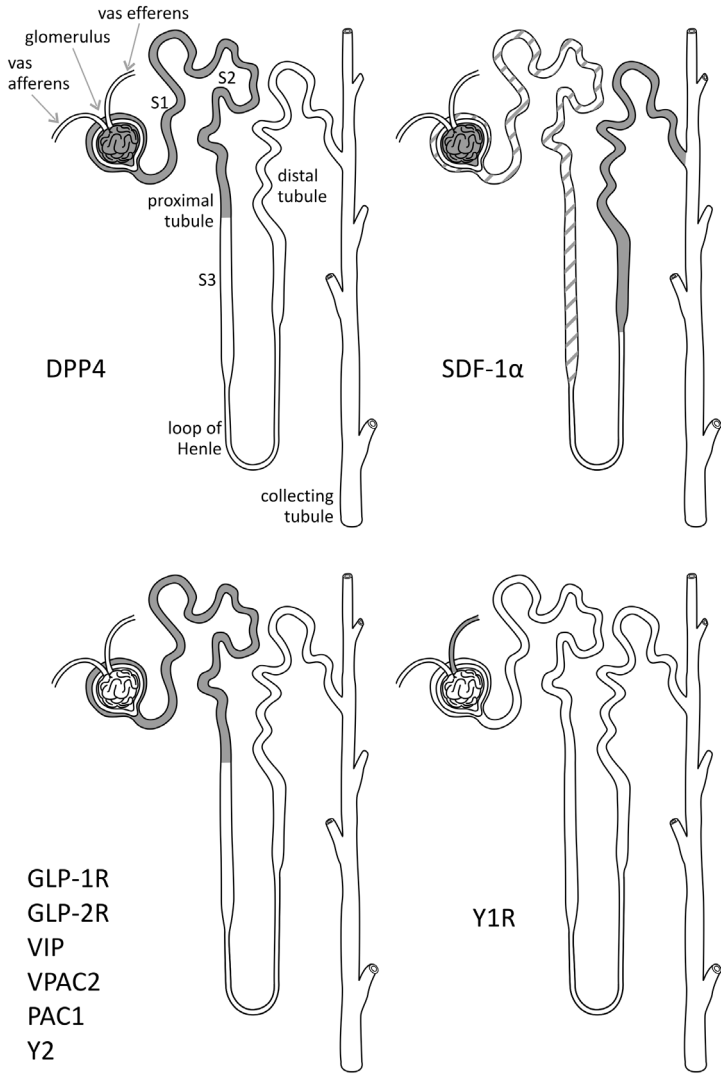

Figure 2.10. *DPP4 and DPP4 substrate expression in the nephron. DPP4 substrates, after increasing their half-life by DPP4 inhibition, are expected to exert an increased natriuretic, diuretic, anti-apoptotic, anti-inflammatory and anti-oxidant effect, reducing ischemic injury.*

effect of VIP. IP-10, on the other hand, is known to delay proliferation of renal tubular epithelial cells and can be assumed to negatively affect the development of renal IRI.

As the expression of IL-16, an immune modulator in IRI[222], was shown to be correlated with DPP4 activity in specific conditions[255], it would be interesting to know whether DPP4 inhibition affects the changes in IL-16 expression during IRI. Soluble DPP4 promotes the transendothelial migration of T-cells

through the endothelial mannose-6-phosphate/insulin-like growth factor II receptor, so the inhibition of DPP4 might result in a reduced infiltration of leukocytes[253,254]. As mentioned in the introduction, DPP9 activity was shown to play a role in the differentiation of macrophages[366]. Possibly, the reduction of DPP4 activity by the inhibitor vildagliptin, which also affects the activity of DPP8 and DPP9, has an effect on the phenotypic distribution of macrophages affecting their role in the development of ischemic injury. The fact that Th17 cells, that are known to play an important role in graft rejection, express high levels of DPP4, points towards the relation of DPP4 activity with transplantation success.

2.3. Aims of the study

The inhibition of DPP4 activity has been used in the clinical settings for many years in order to improve the glucose tolerance of diabetic patients, but has also been shown to have a beneficial outcome in pathophysiological models of ischemic injury. Although studies describe the effect of DPP4 inhibition in ischemic models of the heart in myocardial infarction and lung during transplantation, as well as a nephroprotective effect in diabetic subjects, the effect of DPP4 inhibition in renal IRI of healthy subjects still remains unknown.

In the previous section, potential mechanisms through which DPP4 inhibition could exert a beneficial effect in the setting of ischemic AKI have been discussed. This effect could be mediated by DPP4 itself, by modulation of the effect of DPP4 on inflammation, through activity of DPP4 substrates or through their indirect anti-inflammatory effects. Many of the immunomodulatory effects of DPP4 activity have been described, as have the miscellaneous direct and indirect effects of DPP4 substrates, which include inflammation, cellular metabolism, trafficking, differentiation, proliferation and apoptosis. Notwithstanding the clinical importance of renal IRI in the setting of AKI and renal transplantation, and the high expression levels of DPP4 in the proximal tubule, the effects of DPP4 inhibition are not fully understood in the renal setting.

The present study aims

- to investigate the effect of DPP4 inhibition on the functional and morphological outcome of the kidney after IRI
- to identify mechanisms and mediators involved in this potential effect.

2.4. Materials & methods

2.4.1. Unilateral ischemia-reperfusion model for AKI

The study described in this chapter is performed using the Wistar rat, an albino laboratory outbred rat strain (species *Rattus norvegicus*) first developed in 1906. Estimated life duration of a Wistar rat is about two years. As rat strains appear to present a variable susceptibility to injury, a widely used and comparable strain was opted for[367-369]. The number of animals used for each experiment is based on variability of injury, mortality and variability of treatment effects in different groups, extracted from published articles and past experience. The procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (no. 85–23, 1985) following demand approval for these experiments by the Antwerp University Ethical Committee.

In renal ischemic injury, the kidney is completely or partially deprived of blood flow, which can be caused by various renal pathologies, but also by kidney transplantation. During this ischemic period, the kidney undergoes tubular and vascular damage, mostly due to lack of oxygen, resulting in an immunological reaction. In order to minimize the inflicted damage and ensure its reversibility, ischemic periods in transplantation should be kept as short as possible. At reperfusion, the return of blood flow results in infiltration of immunological cells, upregulation of auxiliary factors, initiation of repair mechanisms and a slow recovery of kidney function. The pathophysiology of IRI is extensively discussed in the introduction of this chapter.

Various models are available to inflict ischemic damage to the kidney, involving unilateral or bilateral clamping, often combined with nephrectomy to take a healthy contralateral kidney out of the equation and assess the recovery of the damaged kidney. Either the complete renal pedicle or single renal arteries can be clamped. For the current study, the unilateral model of acute renal failure with complete pedicle clamping and combined contralateral nephrectomy is used, which closely resembles a transplantation model. In this model, the abdomen is opened via a long midline incision, and during surgery, one renal pedicle was clamped with a microvascular clamp to block the renal circulation completely. After a specified interval of oxygen deprivation, the clamp is released. After ascertaining reperfusion of the kidney, the contralateral renal pedicle is ligated and nephrectomy is performed shortly after blood flow to the ischemic kidney is restored. Abdomen is closed and rats are left to recover from anesthesia under heat producing infrared lamps before returning to their cages for a period of reperfusion before euthanasia.

In this in vivo experiment, 6 week old male Wistar rats (Iffa Credo, Brussels,

Belgium) are used, as previous published studies have shown that females are more protected against induced ischemic renal injury[370]. The outcome of experimental ischemia reperfusion injury is also dependent on age[371], rat strain[372], diabetic pathology[373] and ambient temperature. The result of the inflicted injury can be easily recognized biochemically through markers of kidney function and immunological markers, and morphologically through the depolarization and dismantling of renal PTCs and the infiltration of immunological cells. At reperfusion, the damaged kidney representing total kidney function is very similar to the condition of acute renal failure and the reperfusion of a kidney transplant after a successful transplantation.

2.4.2. Study setup

To evaluate the potential effect of DPP4 inhibition on AKI, vildagliptin was administered to healthy male Wistar rats before subjecting them to 30' of unilateral renal ischemia. Eighty-four male Wistar rats of 200 – 225 grams were randomly assigned to three treatment groups (n=28 each): saline, 1 mg/kg vildagliptin (VG)-treated (VG1) or 10 mg/kg VG-treated (VG10) animals. The doses in this study setup were chosen to be a normal and a supratherapeutic dose. Most T2DM patients are given a 50 mg tablet twice a day, which is a dose of about 1.4 mg/kg in a 70 kg subject.

The study setup is presented in figure 2.11. Prior to experiment initiation, animals were kept in a fasting state for 12 hours. Animals were intravenously injected with saline or VG (custom synthesized, GLSynthesis, Worcester, MA) in a single bolus 15 min before surgery. VG was prepared from a concentrated powder, synthesized by the Laboratory of Medicinal Chemistry for the Laboratory of Medical Biochemistry at the University of Antwerp.

Rats were weighed and anesthetized intraperitoneally with 60 mg/kg sodium pentobarbital. During surgery, the left renal pedicle of rats in the ischemia group was clamped for 30 min with a microvascular clamp, followed by a right nephrectomy, as described above. Body temperature was kept constant around 35.5°C using a heating pad. After surgery, the abdomen was closed and rats were left to recover from anesthesia under heat producing infrared lamps before returning to their cages for a period of reperfusion before euthanasia. Eight animals per experimental group were euthanized at 2, 12, or 48 hours after reperfusion as these reperfusion times can be considered specific for the early reperfusion phase, the peak in ischemic damage and full regeneration phase, respectively. Four sham-operated animals per experimental group were euthanized 48 hours after sham operation. Except for occlusion of the renal pedicle, sham-operated animals are treated in an identical manner, and thus also underwent a right nephrectomy.

Serum samples were taken from the tail vein right before saline or VG administration, from the retro-orbital sinus after the ischemic operation, and from the abdominal aorta at sacrifice. Right and left kidneys were harvested immediately after nephrectomy and exsanguination, respectively. After washing the kidney in a saline solution, both poles are removed from the right kidney, and the remaining is cut into slices of ~ 4 mm for further analysis, snap-frozen in liquid nitrogen or fixated in formaldehyde calcium/methacarn and paraffin embedded. Four-micrometer-thick sections were cut to perform (immuno)histochemical and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. (Semi)quantitative analysis of each staining was performed in a blinded way.

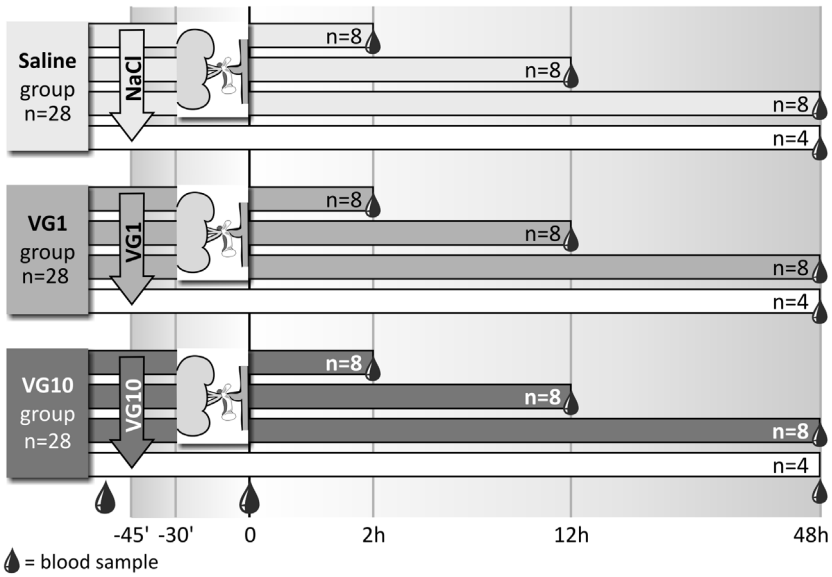

Figure 2.11. *Study setup of DIR01, evaluation of the effect of DPP4 inhibition by vildagliptin on renal ischemic reperfusion injury (n = 84).*

In a separate experiment, 36 male Wistar Han rats (200 – 225 g) were again assigned to three treatment groups (n = 12): saline, VG1 or VG10. Exactly the same procedures were followed as described above. Per treatment group, 4 sham-operated animals were included. After the operation, the left kidneys of all animals were allowed a reperfusion time of 5 days to assess the difference in regeneration and infiltration of immune cells within a longer timeframe.

To evaluate the influence of vildagliptin on systolic blood pressure, the systolic blood pressure of treated and untreated rats were monitored in a separate experiment (n=10) over a period of 6 weeks. GFR and tubular secretion based on inulin and creatinine clearance curves was also assessed in a separate experiment (n=10).

2.4.3. DPP4-activity measurement

As the presented experiments are based on the inhibition of DPP4, the activity of DPP4 was measured in cooperation with the Laboratory of Medical Biochemistry of the University of Antwerp (Prof. Dr. I. De Meester), experienced with the necessary methodology.

DPP4 enzymatic activity was measured by using 0.5 mM glycyl-prolyl-4-methoxy-β-naphthylamide (Gly-Pro-4-Me-β-NA) as a fluorogenic substrate in a 50 mM Tris buffer pH 8.3. DPP4 activity was determined kinetically over 10 minutes at 37°C by measuring the initial velocities of 4-Me-β-NA release (λ_{ex} = 340 nm, λ_{em} = 430 nm) from the substrate using an Infinite^{TM} 200 reader (Tecan Group Ltd., Switzerland). One unit of enzymatic activity is defined as the amount of enzyme that catalyzes the release of 1 µmol of 4-Me-β-NA from the substrate per minute under the assay conditions. The catalytic reaction is stopped using citrate. Since vildagliptin and sitagliptin are reversible inhibitors, the dilution of the plasma in the assay is an underestimation of the *in vivo* percentage inhibition of the DPP4 enzymatic activity in undiluted plasma, due to dissociation of the reversible inhibitor-DPP4 complex during sample dilution in the assay[374]. Matheeussen et al. developed and described a robust method to correctly estimate the in vivo inhibition of DPP4 based on in vitro measurements[375].

In lysates of sections of the ischemic as well as the contralateral kidney, the enzymatic activity of DPP4 was tested by use of pNA, a colorimetric analysis of protease activity. The substrates Ala-Pro-pNA (1 mM in 0.05 M HEPES buffer, pH 7.0, containing 10 mM EDTA, 14 µg/ml aprotinin, and 0.1% Tween 20) and Gly-Pro-pNA (0.5 mM in 0.05 M Tris buffer, pH 8.3, containing 10 mM EDTA and 14 µg/ml aprotinin) can be used to probe DPP4, DPP8/9, and/or FAPα activity. DPP4 activity is again determined kinetically in a final volume of 200 µl for 10 min at 37°C by measuring the initial velocities of pNA release (405 nm) from the substrate using a Spectramax plus microtiterplate reader (Molecular Devices, Sunnyvale, CA, USA). One unit enzyme activity is the amount of enzyme that catalyzes release of 1 µmol pNA from the substrate/min.

2.4.4. Evaluation of glycemia

Increases of blood glucose were found to negatively affect the outcome of renal IRI[359]. Decreases of blood glucose due to DPP4 inhibition could thus result in a protective effect. To assess the influence of vildagliptin administration on the evolution of blood glucose, glucose was measured in function of reperfusion time in a separate ischemic experiment with 5 animals. Whole blood glucose levels were measured using a standard automated whole blood glucometer with glucose strips (GlucoMen Lx Plus+, Menarini, Florence, Italy).

2.4.5. Evaluation of kidney function

2.4.5.1. Measurement of serum creatinine

Measuring serum creatinine is a useful and inexpensive method of evaluating renal dysfunction. Creatinine is an endogenous non-protein waste product of creatine phosphate metabolism produced by skeletal muscle tissue. Creatinine is produced at a constant level proportional to muscle mass. Serum creatinine levels are determined by the balance between endogenous production and renal excretion. Since endogenous production is constant and renal excretion mainly occurs by glomerular filtration, serum creatinine levels can be used to estimate GFR. However, next to glomerular filtration, creatinine to a much lesser extent (5-10%) is also excreted by tubular secretion and therefore GFR determined based on serum creatinine and creatinine clearance in fact is an overestimation of the actual GFR. Renal dysfunction diminishes the ability to filter creatinine causing the serum creatinine to rise. Serious renal disorders, such as glomeronephritis, pyelonephritis, and urinary obstruction, will cause abnormal elevations. The comparison of creatinine clearance of older and younger individual humans (with or without administration of cimetidine) has shown that there is a net reabsorption of creatinine in the renal tubules of healthy old persons[376]. The creatinine reabsorption rises significantly when the kidney is inflamed or chronically obstructed[377].

Other factors can also significantly influence the creatinine content of the serum and bias the evaluation of glomerular filtration. Cirrhotic patients, elderly people and vegetarians have a decreased muscle mass resulting in a decrease of muscle creatinine release[378]. The tubular secretion of creatinine can be diminished by cimetidine and trimethoprim[379].

Serum creatinine levels were either measured using an automatic cartridge reader (Vitros CREA Slides for Vitros Chemistry Systems 350, Ortho Clinical Diagnostics, Bucks, UK) at the University Hospital of Antwerp (UZA Edegem, Belgium) or determined spectrophotometrically by the Jaffé method, first described in 1886[382]. This method is based on a reaction with sodium hydroxide and picric acid. Presence of vildagliptin or sitagliptin in serum did not interfere with creatinine measurements.

2.4.5.2. Determining GFR from inulin clearance curves

Inulin is a fructose polymer of plant origin, which is, in contrast to creatinine, exclusively excreted from the blood through glomerular filtration[383-385]. It is therefore a valuable tool in the assessment of the GFR[386]. Like creatinine, it is measured to determine GFR as a diagnostic test of kidney function. Cre-

atinine clearance is almost equal to inulin clearance in a dehydrated state, indicating a reabsorption of creatinine equal to secretion[387]. Determining inulin clearance is more accurate in humans compared to rats as the bladder is more easily catheterized and water intake is more easily controlled. To avoid urine collection and related problems, many turn to the measurement of plasma clearance by using either constant infusion or single bolus injection[388-391].

Inulin concentrations in the serum were measured after a single bolus injection of inulin (Inutest 25%, Fresenius Pharma, Graz, Austria) using a D-Glucose/D-Fructose-kit (Boehringer Ingelheim, Ingelheim-am-Rhein, Germany) on a Cobas Mira Biochemistry analyzer (Roche, Basel, Switzerland) to determine inulin clearance. Based on the inulin concentrations at different time points in control rats (n=5) and rats treated with VG10 (n=5), inulin disappearance curves were plotted. The experiment was performed twice, so that each individual animal was alternately treated with VG10 and saline. The GFR was calculated based on serum inulin disappearance curves according to the formula established by Jung *et al.*, using 30 and 240 minutes as time points (t_1, t_2) for measurement of inulin concentration (C_1, C_2)[392-394].

$$GFRi = (I*\ln(C_1/C_2)) / (C_1*(t_2-t_1)*(e\text{^}((t_1/(t_2-t_1))*\ln(C_1/C_2))$$

In order to assess the effect of DPP4 inhibition on tubular secretion, serum creatinine was measured in vildagliptin treated rats with and without the administration of 100 mg/kg cimetidine (Sigma, St. Louis, USA), which is known to inhibit the tubular secretion of creatinine. Serum creatinine values were measured immediately and 6 hours after vildagliptin and cimetidine administration.

2.4.6. Blood pressure measurement

In literature, a reduction of blood pressure was described in diabetic as well as non-diabetic patients treated with DPP4 inhibitors[395,396]. Since a state of hypotension is considered to exert a protective effect on the outcome of AKI, the systolic blood pressure of treated and untreated rats was monitored in a separate experiment (n=10). Systolic blood pressure was measured in a non-invasive way once a week at 2, 12, and 48 hours after intravenous injection of saline (n=5) or VG10 (n=5) using a volume pressure-recording sensor and an occlusion tail cuff (CODA 6 Blood Pressure monitor, Kent Scientific, Torrington, CT) over a period of 6 weeks, following a 6 weeks habituation period to blood pressure measurements. Every measurement was an average of a minimum of 7 successful measurements as calculated by CODA software.

2.4.7. Evaluation of tubular morphology, apoptosis and regeneration

Kidney sections for periodic acid-Schiff and proliferating cell nuclear antigen (PAS-PCNA) staining were fixated for 4 hours in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid), a fixation method preventing morphological shrinkage. PAS is primarily used to identify glucogen in tissues, and will generally stain sugar-containing macromolecules (glycogen, glycoproteins, proteoglycans) of the cellular membrane and of fibrous and connective tissue, and reacts also with the saccharide bonds in the brush border of the proximal tubuli of the kidney. The reaction of periodic acid selectively oxidizes glucose residues, creating aldehydes that react with the Schiff reagent, resulting in a purple-magenta color.

Proximal tubules in the center of 40 fields in the outer stripe of the outer medulla (OSOM) of these sections were analyzed at a magnification of x320 using a well-established semi-quantitative double-blind scoring method[60,198,397], in which tubular appearance is categorized as either normal, with signs of sublethal injury, with signs of ATN or with signs of regeneration (figure 2.12). In general, following the unilateral model of IRI, PTCs become depolarized and display sublethal damage (loss of apical brush border) after 2 hours of reperfusion. After 12 hours, most of the PTCs are in a depolarized state, with a peak of ischemic damage between 12 and 24 hours. Following 48 hours of reperfusion, PTCs are in a regenerative phase, and the active proliferation of renal PTCs results in a positive PCNA staining in the core.

Proliferation of tubular epithelium was evaluated by counting the average number of PCNA-positive cells/mm^2 in 20 fields in the OSOM at x500 magnification, using digital image-processing software (AxioVision, Carl Zeiss). PCNA is an antigen which is expressed in the nuclei of cells during DNA synthesis phase of the cell cycle[398]. The natural function of PCNA is to help holding the DNA polymerase delta (Pol δ) to DNA. It is clamped to DNA through the action of replication factor C, and its expression is under the control of E2F transcription factor-containing complexes[399-401]. Due to the PCNA staining, regenerating nuclei of tubular cells will have a clearly recognizable brown color. PCNA has also been used as a marker for acute cellular rejection in renal allografts[402]. Methyl green was used to counterstain and reveal chromatin-rich tubular structures. PAS-PCNA does not allow the distinction between a necrotic and an apoptotic cell. Apoptotic bodies can be detected through labeling of apoptotic nicks in the DNA through the TUNEL-assay. It also labels cells having suffered from severe DNA damage. Apoptotic cells can alternatively be recognized by to their cloudy shape. DNA breaks were detected by the TUNEL staining assay using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA) according to the manufacturer's instructions[403].

Sections were counterstained with hematoxylin and apoptotic bodies were counted in 20 fields of the OSOM at x400 magnification.

2.4.8. Infiltration of immune cells

In order to assess the infiltration of immune cells, kidney sections were stained specifically to display macrophages, neutrophils and T-cells. Infiltration of these cells was evaluated in the inner as well as the outer stripe of the outer medulla (ISOM/OSOM). Infiltrating monocytes/macrophages were evaluated on ED-1-stained sections (antibody MCA341R, Serotec, Oxford, UK) at a magnification of x250. Sections were prepared from methacarn fixated tissue. ED1 recognizes a cytoplasmatic antigen in monocytes and most macrophage populations[404]. Cells were counted in 25 evenly distributed fields. A spleen section served as a positive control. To estimate the presence of polymorphonuclear cells like neutrophils infiltrating in the kidney, standard

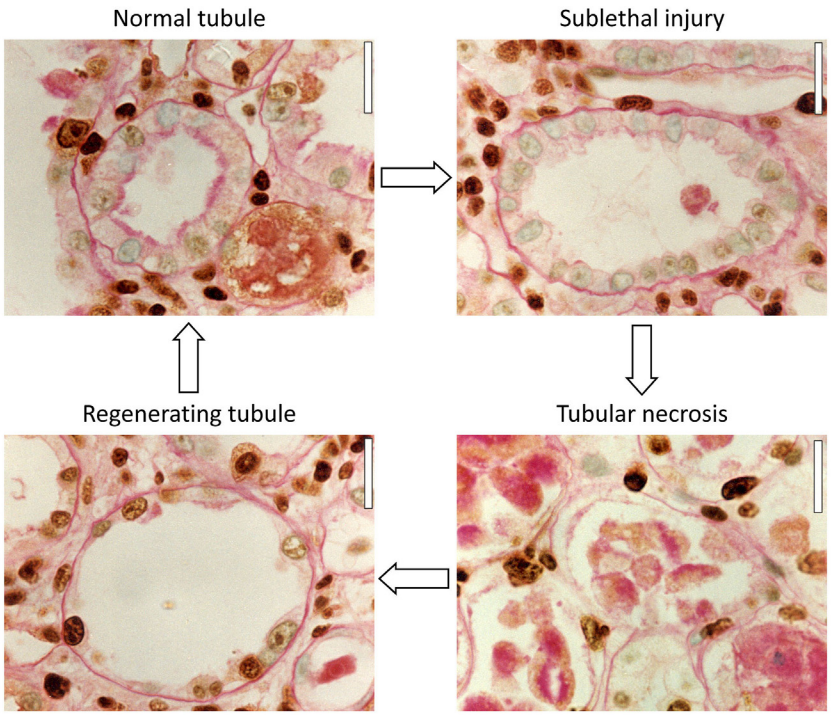

Figure 2.12. *Morphology of proximal tubular epithelial cells as visualized by PAS-PCNA staining (x320). Normal: tubular cells with normal morphology. Sublethal: tubular cells with degenerated brush border and partly detached basement membrane. Necrosis: completely depolarized and destroyed cells, naked basement membrane. Regeneration: PCNA^{+}-cells with large nuclei and small cytoplasmic border.*

hematoxylin-eosin staining was performed on methacarn fixated kidney sections and cells were counted based on the morphology of the multi-segmented nucleus in 20 evenly distributed fields (at x320 magnification). Infiltrating $CD3^+$ T-cells were evaluated (x250 magnification) using a CD3ε polyclonal goat anti-rat antibody (antibody CD3ε M-20 SC-1127, Santa Cruz Biotechnology, Santa Cruz, CA) on methacarn fixated tissue with AEC as a chromogen. All infiltrated immune cells were manually counted, averaged, and normalized over the analyzed surface.

2.4.9. Evaluation of oxidative stress

Malondialdehyde (MDA) is a naturally occurring reaction product and is a marker of oxidative stress[405]. MDA originates from the degradation of polyunsaturated lipids and causes toxic stress in cells, forming covalent protein adducts referred to as advanced *lipoxidation* end-products (ALE). Many pathologies result in increased serum MDA levels, like corneal diseases, osteoarthritis[406], but also renal experimental IRI[285]. Serum MDA baseline levels in rat vary around 4.5 µmol/L[407]. MDA and other thiobarbituric reactive substances condense with two equivalents of thiobarbituric acid (TBA) to give a fluorescent red derivative that can be assayed spectrophotometrically. MDA levels in rat serum were measured to assess in vivo lipid peroxidation status using a fully validated HPLC-fluorimetric detection method[408]. The thiobarbituric reaction was carried out by mixing 50 µL of rat serum/standard, 25 µL of 1% (w/v) butylated hydroxytoluene in ethanol, 250 µL of 1.22 M phosphoric acid, 425 µL of HPLC graded water and 250 µL of a 0.67% TBA solution in clean, sealed glass tubes. The reaction mixture was heated at 95 °C for 40 minutes and then cooled to 4 °C in ice. Proteins were precipitated by adding 360 µL methanol and 40 µL of 1 M sodium hydroxide to 200 µL sample mixture. After centrifugation (9500 g for 5 minutes), samples were analyzed on a complete Gilson HPLC system (Gilson, Inc., Middleton, Wisconsin) with a fluorescence detector (Jasco, Essex, United Kingdom). The detector was set at excitation wavelength 532 nm and emission wavelength 553 nm. As mobile phase, solvent A: potassium phosphate buffer (25 mM, pH 4) / methanol (55:45) and solvent B: potassium phosphate buffer (25 mM, pH 4) / methanol (10:90) were used. The gradient profile consisted of a 6 min isocratic elution at 0% B, after which the concentration was increased linearly up to 100 % B in 6 min. This was maintained for 10 min, after which the initial conditions were reinstalled in 5 min. A flow rate of 0.8 mL/min was used. Samples were cooled at 4°C. A Licrospher 100 C18 reversed-phase analytical column (250 x 4 mm, 5 µL) with a Licrospher guard column (4 x 4 mm, 5 µm) from Merck (Darmstadt, Germany) were used for the separation of the MDA-TBA adduct. To evaluate

protein expression of HO-1, another marker of oxidative stress which was discussed in the introduction, kidney sections were stained using a primary rabbit polyclonal antibody (H4535, Sigma-Aldrich, St. Louis, MO) and evaluated using a semiautomatic scoring system at a magnification of x320. Expression of HO-1 was assessed in the ISOM and the cortex of the kidney. Messenger RNA expression was assessed using real-time RT-PCR.

2.4.10. Real-time RT-PCR

In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal, created by a labeled gene of interest. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to exceed the background level and cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (the greater the amount of target nucleic acid in the sample, the less cycles it will take to be amplified to reach the threshold). Most real time assays undergo 40 cycles of amplification. Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample. Cts of 30-37 are reactions indicative of moderate amounts of target nucleic acid. Cts of 38-40 indicate minimal (negligible) amounts of target nucleic acid.

RNA was isolated from target tissue using the Rneasy Micro Kit from Qiagen (Qiagen Benelux BV, Venlo, The Netherlands). Purity of isolated RNA and total RNA concentration is measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). To investigate the expression of target proteins on the mRNA level, real-time quantitative reverse transcriptase PCR (RT-PCR) is used (Applied Biosystems, ABI Prism 7000, Life Technologies Europe BV, Gent, Belgium)[409]. RT-PCR is preceded by a reverse transcription step using a High Capacity cDNA Reverse Transcription Kit (Life Technologies Europe BV) in which the RNA strands are converted into cDNA by random primers. Primers used in the RT-PCR reaction were inventoried Taqman Gene Expression Assays purchased through Life Technologies BV, and are given in table 2.1.

PCR was performed with a final volume of 25 µL of a PCR mixture containing 12.5 µL of qPCR Mastermix (RT-QP2X-03, Eurogentec, Seraing, Belgium), 6.25 µL of RNAse-free water, 1.25 µL of Assay-on-Demand Gene Expression Assay Mix (Applied Biosystems), and 5 µL of sample cDNA. Amplification was performed by incubation at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 amplification cycles at 95°C for 15 seconds and at 60°C for 1 minute. mRNA quantities of target genes were analyzed in triplicate, normalized against GAPDH as a control housekeeping gene; the saline-treated sham-operated group served as a calibrator sample for the comparative Ct method,

Table 2.1. *Genes of which mRNA expression was assessed using real-time RT-PCR and their assay identification number (Taqman).*

	Name	Assay ID
DPP4 and substrates	DPP4	Rn00562910_m1
	SDF-1α	Rn00573260_m1
	CXCR4	Rn01483207_m1
	IP10/CXCL10	Rn00594648_m1
Inflammatory markers	IL-1β	Rn00580432_m1
	IL-10	Rn00563409_m1
	IL-16	Rn01477722_m1
	TLR2	Rn02133647_s1
	TLR4	Rn00569848_m1
	TNF-α	Rn00562055_m1
Apoptotic markers	Bcl-2	Rn99999125_m1
	Bax	Rn01480160_g1
Markers of oxidative stress	HO-1	Rn00561387_m1
	SOD3	Rn00563570_m1
	Catalase	Rn00560930_m1
Housekeeping gene	GADPH	Rn99999916_s1

given a gene/GAPDH mRNA expression ratio value of 1.

2.4.11. Data processing and statistical analysis

All results are given as average ± standard deviation, unless indicated otherwise. Statistics were performed with IBM SPSS Statistics 20. Comparisons between study groups were assessed using the non-parametric Kruskal-Wallis H-test, followed by a Mann-Whitney U-test in combination with the Bonferroni correction when more than two groups were compared. Data from morphological evaluation were analyzed using Pearson's χ^2-test.Values of $p < 0.05$ were considered significant.

2.5. Results

2.5.1. Animal data

During the main experiment, 3 out of 84 animals died prior to sacrifice, corresponding to a mortality of 3.6%. One animal in the VG1 group died of exsanguination through the release of the ligation of the nephrectomized pedicle, two others in the VG10 group died of unknown cause after the operation, most probably due to anesthesia. These animals were excluded from analysis. During additional experiments, there was no premature mortality prior to sacrifice. Animals were operated at a temperature of 35.5°C using a heating pad. Small decreases of temperature could unfortunately not be prevented, but the temperature was measured in all animals using a rectal thermometer during surgery, and were found to be comparable in all treatment groups (35.53 ± 0.43°C in the saline group, 35.44 ± 0.35°C in the VG1 group, and 35.43 ± 0.32°C in the VG10 group). Food and water intake was similar in animals from all treatment groups. At sacrifice, the animals showed no abnormalities when examining the heart, lungs, liver, intestines and kidneys. Contralateral as well as ischemic kidneys were found to have the same weight at sacrifice.

2.5.2. DPP4 activity

DPP4 activity was measured in blood samples taken immediately after the start of reperfusion, and at the time of sacrifice. At the start of reperfusion (time 0 = 45 min after saline or vildagliptin injection), DPP4 activity was measured in both serum and kidney homogenates to evaluate the efficiency of the DPP4 inhibition after intravenous injection of VG1 and VG10. Results of serum DPP4 activity are given in table 2.2. Table 2.3 shows the activity of DPP4 per gram kidney homogenates.

At the beginning of reperfusion and after 2 hours or reperfusion, an almost complete DPP4 inhibition was observed in serum of both VG1- and VG10-treated animals with < 10% residual DPP4 activity (figure 2.13A). At 12 hours of reperfusion, the VG10-treated group still reached an inhibition percentage of 88%. Markedly, the serum DPP4 activity values at 48 hours of reperfusion indicate a higher percentage of inhibition in the VG1 group compared to the VG10 group. A dose-dependent inhibition of DPP4 activity could also be observed in the contralateral kidney (figure 2.13B, values given in total percentage of DPP4 activity). In the ischemic kidney, no differences in DPP4 activity were observed.

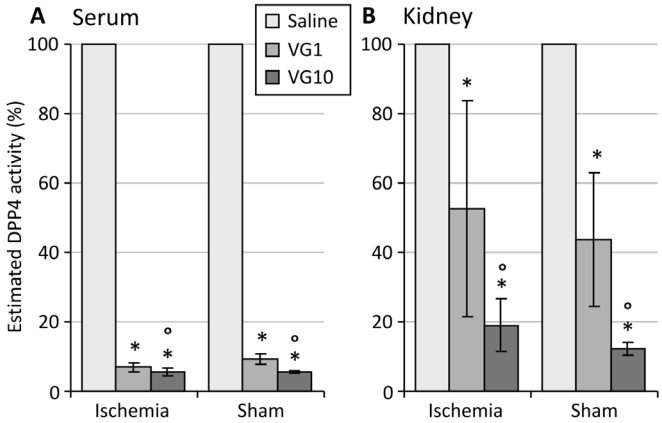

***Figure 2.13**. Estimated percentage of in vivo dipeptidyl peptidase 4 (DPP4) activity in serum (A) and in the explanted contralateral kidney (B) at the start of reperfusion (time point 0h). In serum samples, the estimation was calculated toward individual values before treatment and in the kidneys toward the values in the untreated animals. * $p < 0.05$ vs. saline-treated animals. ° $p < 0.05$ vs. VG1-treated animals.*

***Table 2.2.** DPP4 activity measured in the serum, in U/L of measured activity and estimated inhibition percentage. * Average of serum of all animals within treatment group, sacrificed with different reperfusion times.*

DPP4 activity (U/L)	Start of reperfusion*	2h	12h	48h	Sham (48h)
Saline	12.20 ± 1.88	13.90 ± 2.15	14.36 ± 2.59	11.77 ± 1.94	9.97 ± 0.95
VG1	3.54 ± 0.49	4.37 ± 0.73	13.52 ± 1.70	12.92 ± 1.58	11.64 ± 1.03
VG10	2.61 ± 0.42	2.99 ± 0.80	4.23 ± 0.86	13.17 ± 1.45	11.54 ± 0.76
In vivo inhibition (%)	**Start of reperfusion***	**2h**	**12h**	**48h**	**Sham (48h)**
Saline	-	-	-	-	-
VG1	92.5 ± 1.7	90.9 ± 2.2	30.0 ± 11.3	50.8 ± 8.3	29.4 ± 23.7
VG10	94.6 ± 4.0	94.2 ± 1.7	88.3 ± 3.6	23.9 ± 16.4	43.9 ± 6.6

***Table 2.3.** DPP4 activity measured in ischemic kidney homogenates. * Average of the contralateral kidneys of all animals within treatment group, sacrificed with different reperfusion times.*

DPP4 activity (U/g)	Contralateral kidney*	2h	12h	48h	Sham (48h)
Saline	168.0 ± 41.7	113.6 ± 23.2	100.8 ± 20.5	125.4 ± 34.5	99.6 ± 14.6
VG1	126.7 ± 38.5	90.3 ± 20.2	89.1 ± 38.1	113.2 ± 40.6	85.4 ± 12.1
VG10	72.7 ± 20.4	89.7 ± 42.3	120.6 ± 26.7	135.2 ± 35.4	127.3 ± 18.8

2.5.3. Evaluation of kidney function

DPP4 inhibition clearly prevented renal dysfunction in a dose-dependent way as reflected by lower serum creatinine levels in both VG1- and VG10-treated animals compared with saline-treated animals. DPP4 inhibition with VG10 resulted in a significantly lower serum creatinine at all reperfusion times (figure 2.14). The effects were most pronounced 12 h after the start of reperfusion (1.31 ± 0.32 and 0.70 ± 0.19 vs. 1.91 ± 0.28 mg/dL; $P < 0.05$ in VG1 and VG10-treated vs. control animals). After 5 days of reperfusion, a difference in serum creatinine could no longer be observed (0.40 ± 0.03 and 0.38 ± 0.05 vs. 0.40 ± 0.08 mg/dL in VG1 and VG10-treated vs. control animals) (not shown on figure).

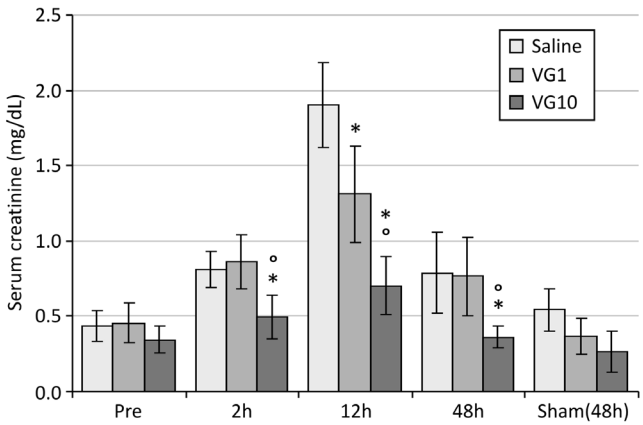

Figure 2.14. *Serum creatinine levels (mg/dL). DPP4 inhibition resulted in a significant dose-dependent reduction of renal dysfunction after 12 h of reperfusion.*
** p < 0.05 vs. saline-treated animals. ° p < 0.05 vs. VG1-treated animals.*

2.5.4. GFR and blood pressure

Treatment with VG clearly resulted in a significant decrease of serum creatinine levels. As serum creatinine levels could also have been affected by changes in GFR, tubular secretion of creatinine, or blood pressure, potential effects on these parameters were evaluated in a separate experiment (n=10). As an increase in the GFR can result in a decrease of serum creatinine levels, the GFR of experimental animals was assessed. Although literature was found to report a small decrease rather than an increase in systolic blood pressure after treatment with DPP4 inhibitors, an increase in blood pressure could also affect the GFR. DPP4 was found not to interfere with the GFR. GFR was calculated according to the formula by Jung (see 'Materials and methods' section, and was found to be similar in both treatment groups (1.19 ± 0.16 vs. 1.25 ±

0.25 mL/min/100g in VG10-treated and control animals, respectively). Also, the administration of cimetidine in VG10-treated rats did not result in an increase of the serum creatinine at 6 hours after administration, indicating that the treatment with VG10 did not affect tubular secretion of creatinine (0.64 ± 0.20 vs. 0.55 ± 0.07 in VG10 and VG10+cimetidine treated animals, respectively). Systolic blood pressure was noninvasively measured 2, 12, and 48 h after injection of saline or VG10 using a volume pressure-recording sensor and an occlusion tail cuff. Two hours after treatment, an apparent increase of systolic blood pressure was observed in all animals, which is most probably due to stress resulting from the injection and handling. At 12 and 48 hours after treatment, systolic blood pressure was variable but equal to the control values (figure 2.15). No differences in systolic blood pressure were observed 2, 12, or 48 h after a single saline or VG10 injection.

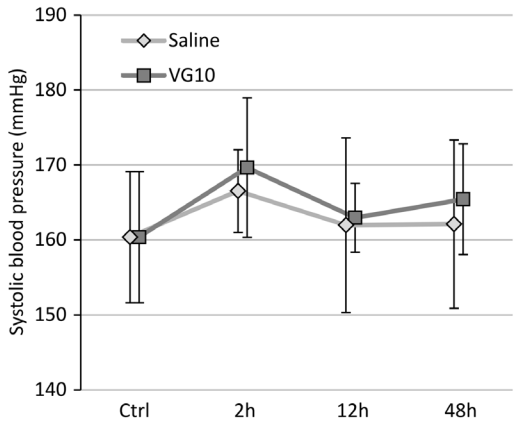

Figure 2.15. *Systolic blood pressure (mmHg) measured in animals treated with either saline (n=5) or VG10 (n=5). No significant differences were observed.*

2.5.5. Blood glucose

To assess changes in blood glucose which could affect the outcome of renal IRI, glucose was measured in whole blood in a separate ischemic experiment. Glycemia of VG10-treated (n=3) and saline-treated (n=2) animals was measured at different time points. Blood glucose did not differ significantly in control animals vs. VG10-treated animals at the beginning of ischemia (113.0 ± 11.3 vs. 110.0 ± 10.6 mg/dl), the beginning of reperfusion (128.5 ± 6.4 vs. 133.0 ± 26.9 mg/dl), and after 2 h (113.0 ± 0.0 vs. 120.3 ± 11.2 mg/dl), 12 h (113.0 ± 14.1 vs. 119.7 ± 2.1 mg/dl), and 48 h of reperfusion (116.5 ± 7.8 vs. 118.3 ± 3.2 mg/dl). Results are depicted in figure 2.16.

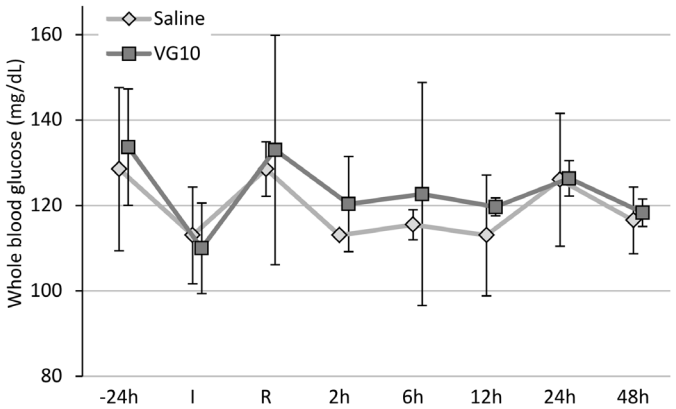

Figure 2.16. *Whole blood glucose measured in experimental animals undergoing IRI after saline (n=2) and VG10 (n=3) treatment. '-24h' indicates basal glucose levels measured the day before the operation. 'I' indicates blood glucose measured at the beginning of ischemia. 'R' indicates blood glucose measured at the beginning of reperfusion. '+xh' indicates blood glucose measured at x hours of reperfusion.*

2.5.6. Tubular morphology and regeneration

DPP4 inhibition attenuated histological damage after renal IRI as shown by significantly reduced necrosis. After 2 h of reperfusion, tubular necrosis was nearly absent in VG1- and VG10-treated animals while clearly present in saline-treated animals (0.3 and 0.0 vs. 5.3%, $P < 0.05$). After 12 h of reperfusion, tubular necrosis was still significantly reduced in vildagliptin-treated animals compared with saline-treated animals (48.2 and 62.1 vs. 77.5%, $P < 0.05$) (figure 2.17). Surprisingly, tubular necrosis was higher in VG10-treated animals compared with VG1-treated animals. As this figure leaves the presumption that DPP4 inhibition has no effect on regeneration, this was confirmed by counting the number of PCNA+ cells, where indeed no significant differences were observed among saline-, VG1-, and VG10-treated animals at 48 h of reperfusion (1,777 ± 340, 1,748 ± 161, and 1,810 ± 338 PCNA+ cells/ mm^2, respectively) (figure 2.18).

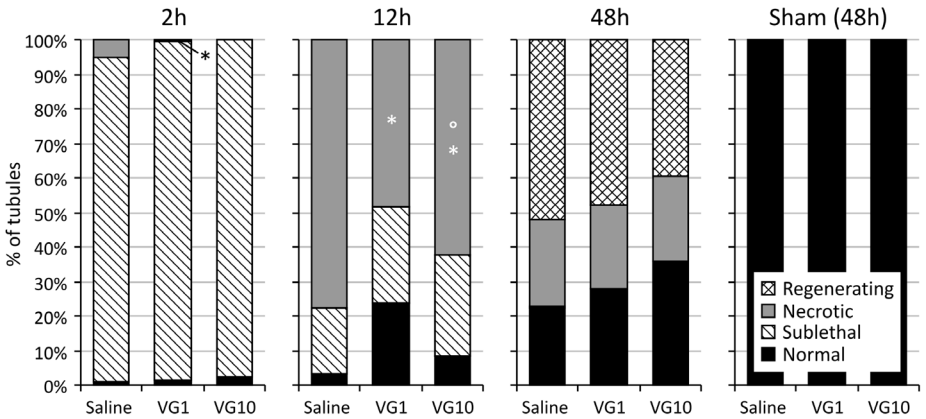

Figure 2.17. *Tubular morphology of S3 PTCs at 2, 12, and 48 h of reperfusion. DPP4 inhibition attenuated the histological damage after renal IRI as shown by significant less necrosis after 2 and 12 h of reperfusion in vildagliptin-treated animals compared with saline-treated animals. * p < 0.05 vs. saline treated animals. ° p < 0.05 vs. VG1-treated animals.*

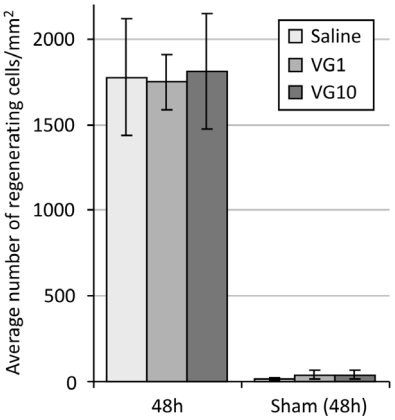

Figure 2.18. *Average number of PAS-PCNA+ cells/mm^2 in the outer strip of the outer medulla (OSOM). No differences were observed among treatment groups.*

2.5.7. Evaluation of apoptosis

IRI is associated with ischemia-induced apoptosis, which might contribute to renal dysfunction. To evaluate the effect of DPP4 inhibition on the development of apoptosis in ischemic kidneys, the mRNA expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 was investigated by quantitative real-time RT-PCR. The number of apoptotic bodies was analyzed using a TUNEL assay in the saline- and VG10-treated group. As presented in figure 2.19A, the administration of VG10 significantly reduced the Bax/Bcl-2 mRNA expression ratio at a reperfusion time of 48 h, suggesting a potential anti-apoptotic effect of DPP4

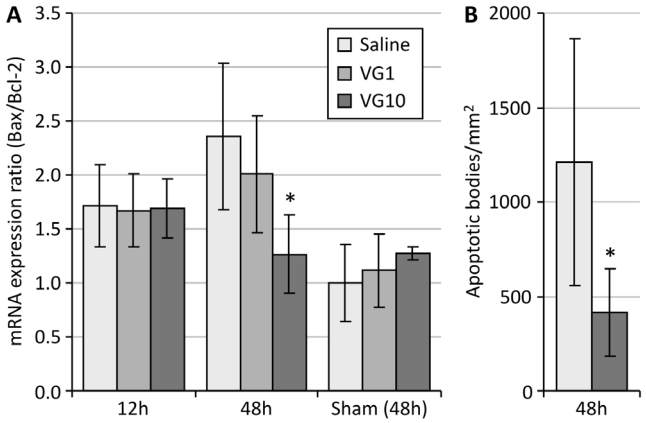

Figure 2.19. *(A) Ratio of mRNA expression of Bax and Bcl-2. Values are average ± SE. * p < 0.05 vs. saline- and VG1-treated animals. (B) Number of apoptotic bodies per mm² shown by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. * p < 0.05 vs. saline-treated animals. The Bax/Bcl-2 mRNA ratio was significantly decreased in VG10-treated animals after 48 h of reperfusion. A 3-fold increased number of apoptotic bodies were observed in the OSOM of saline-treated animals compared with VG10-treated animals. These results indicate a reduction of apoptosis in tubular cells after DPP4 inhibition.*

inhibition. This was confirmed by a threefold decreased number of apoptotic bodies in the OSOM of VG10 vs. saline-treated animals after 48 h of reperfusion (414.1 ± 231.7 vs. 1,212.0 ± 650.8 apoptotic bodies/mm^2; $P < 0.05$) (figure 2.19B).

2.5.8. Infiltration of immune cells and evaluation of immunological markers

The infiltration of monocytes, neutrophils, and T cells was assessed through staining of tissue sections. Results of infiltrating cells were highly variable and difficult to interpret. A significant increase in infiltration of macrophages was reported at 2 and 12 hours, as well as in sham animals sacrificed 48 hours after reperfusion (figure 2.20). The number of infiltrated neutrophils was low and similar in all treatment groups (data not shown). The infiltration of T-lymphocytes in vildagliptin treated animals compared to untreated animals was found to be very variable (figure 2.21). Number of infiltrated cells at 5 days of reperfusion was similar in all groups, independent of former ischemic surgery or vildagliptin treatment (data not shown).

The mRNA expression of a selection of immunological parameters was evaluated in kidney homogenates. DPP4 inhibition did not result in any alteration of the evolution of expression of IL-1β mRNA, which was found to increase after the start of reperfusion with the highest values found at 12

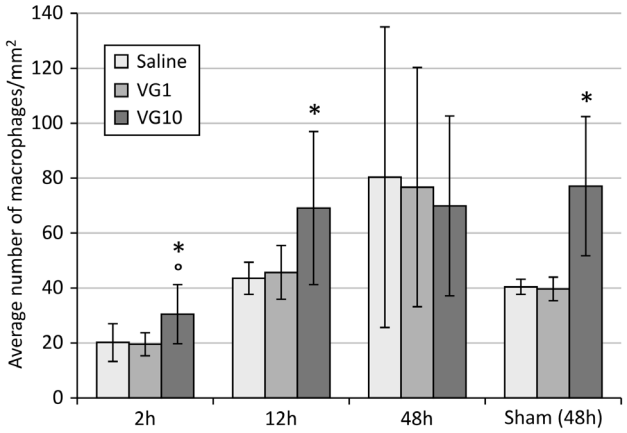

Figure 2.20. *Infiltration of macrophages in the OSOM measured in 25 evenly distributed fields at a magnification of x250. * p < 0.05 vs saline-treated animals (p < 0.05), ° p < 0.05 vs VG1-treated animals.*

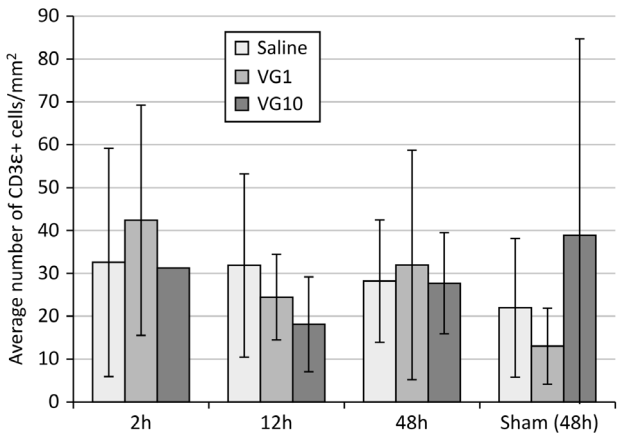

Figure 2.21. *Infiltration of CD3⁺ T-lymphocytes measured in 25 evenly distributed fields at a magnification of x250. No significant differences were observed.*

hours or reperfusion (figure 2.22A). Administration of vildagliptin however did result in an early and significant decrease of the pro-inflammatory CXCL10 mRNA expression in the VG1 and VG10-treated groups compared with saline-treated animals at 2 h of reperfusion (Figure 2.22B). An anti-inflammatory trend through DPP4 inhibition was observed in a comparison of relative mRNA expressions of IL-10 (figure 2.22C), IL-16 (figure 2.22D), and TNF-α (figure 2.22E). At 2 hours or reperfusion, vildagliptin resulted in an upregulation of IL-10 expression and a downregulation of IL-16 and TNF-α expression. These effects were however not significant and seemed to be only present at 2 hours of reperfusion. The anti-inflammatory effect of VG treatment was

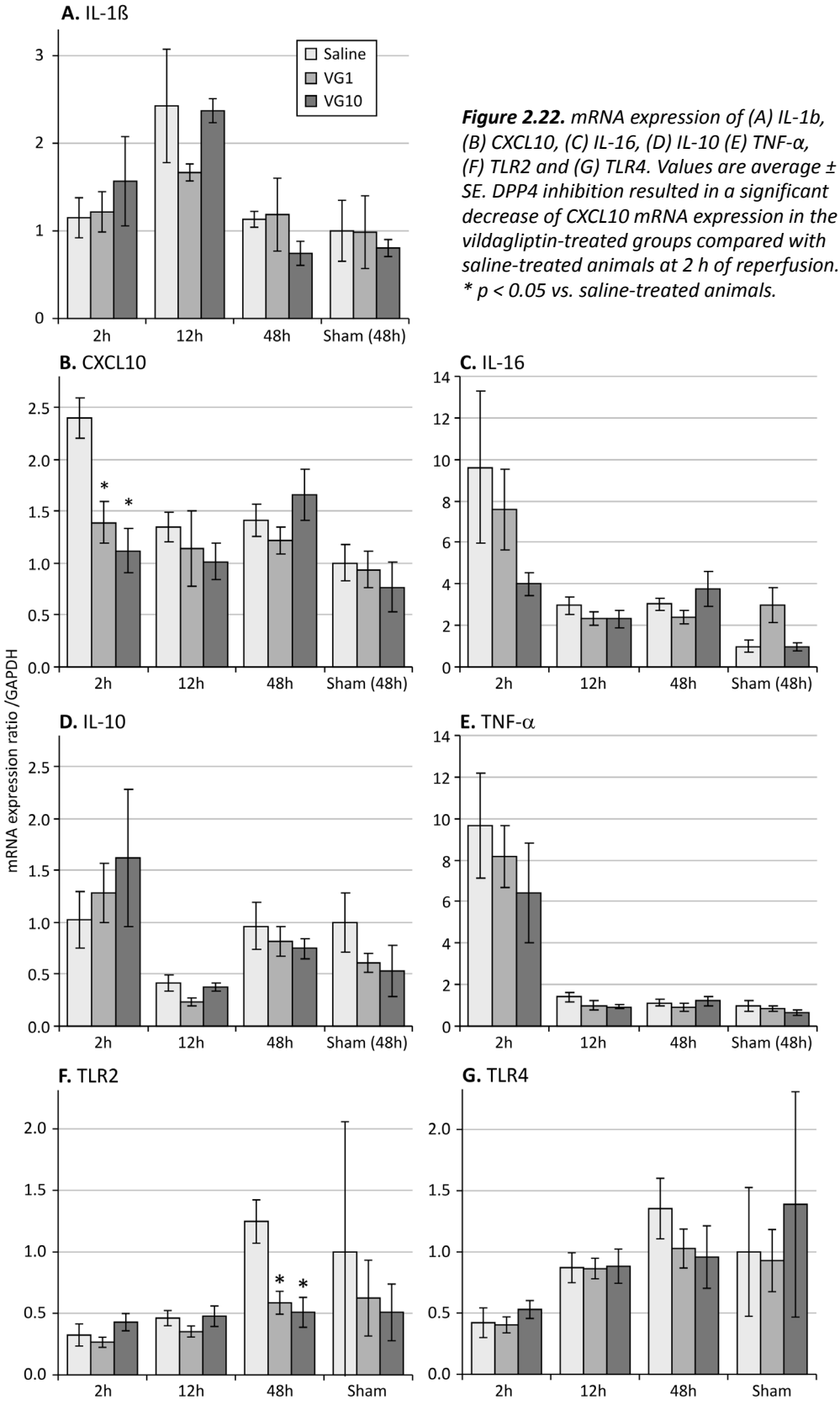

Figure 2.22. *mRNA expression of (A) IL-1b, (B) CXCL10, (C) IL-16, (D) IL-10 (E) TNF-α, (F) TLR2 and (G) TLR4. Values are average ± SE. DPP4 inhibition resulted in a significant decrease of CXCL10 mRNA expression in the vildagliptin-treated groups compared with saline-treated animals at 2 h of reperfusion. * p < 0.05 vs. saline-treated animals.*

further confirmed by the expression of TLR2 and TLR4, which seemed to be slightly and dose-dependently decreased in VG1 and VG10-treated animals at 48 hours of reperfusion, but this decrease was only significant for TLR2 expression (figure 2.22F and 2.22G).

2.5.9. Oxidative stress

At 48 h of reperfusion, the in vivo lipid peroxidation status was determined by measuring MDA in serum. Protein expression of HO-1 was quantified using a semi-automatic scoring. DPP4 inhibition significantly reduced MDA levels in both VG1- and VG10-treated animals compared with saline-treated animals in both ischemic (0.54 ± 0.17 and 0.56 ± 0.21 vs. 1.00 ± 0.35 μM; $P < 0.05$) and sham-operated rats (0.48 ± 0.09 and 0.59 ± 0.12 vs. 0.84 ± 0.16 μM; $P < 0.05$) at 48 h of reperfusion (Figure 2.23).

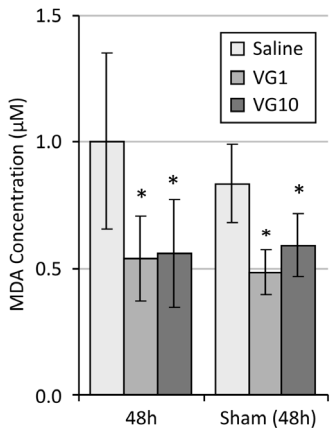

Figure 2.23. *Concentrations of malondialdehyde (MDA) measured in serum at 48 h of reperfusion. Lipid peroxidation status was significantly decreased in vildagliptin-treated animals compared with saline-treated animals.*
** P < 0.05 vs. saline-treated animals.*

The expression of HO-1, a marker of oxidative stress, was assessed at both the mRNA and protein level. The amount of HO-1 mRNA was highly variable and difficult to interpret (figure 2.24A). A decrease in protein expression was also observed in the vildagliptin-treated groups at 12 and 48 hours of reperfusion, which, however, was not significant compared with saline-treated animals due to high variation (figure 2.24B).

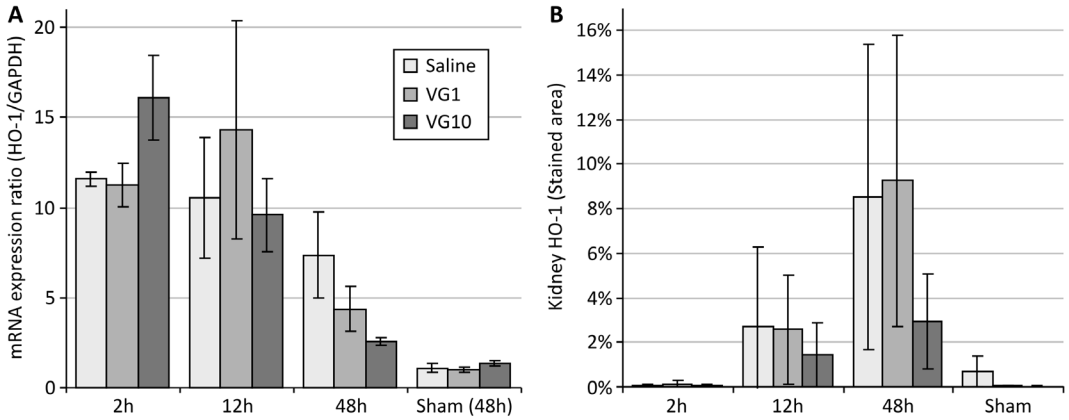

Figure 2.24. (*A) mRNA expression of heme oxygenase (HO)-1 (average ± SE). (B) Area% of HO-1 immunostaining in cortex and ISOM of the kidney. No significant differences were observed among treatment groups.*

Expression of SOD3 and catalase mRNA was also evaluated. The expression of SOD3 decreased at the beginning of reperfusion and was the lowest at two hours of reperfusion, where it was significantly lower in VG10-treated rats compared to saline treated controls. At 48 hours of reperfusion, the SOD3 mRNA expression in both VG1 and VG10-treated rats was found to be significantly lower compared to saline-treated animals. mRNA expression of SOD3 in sham-operated animals remained high and was variable (figure 2.25A). Catalase mRNA expression was also found to decrease at reperfusion and to be the lowest at 12 hours or reperfusion. At 48 hours, the mRNA expression of catalase in VG1-treated animals was significantly lower than that of saline-treated animals (figure 2.25B).

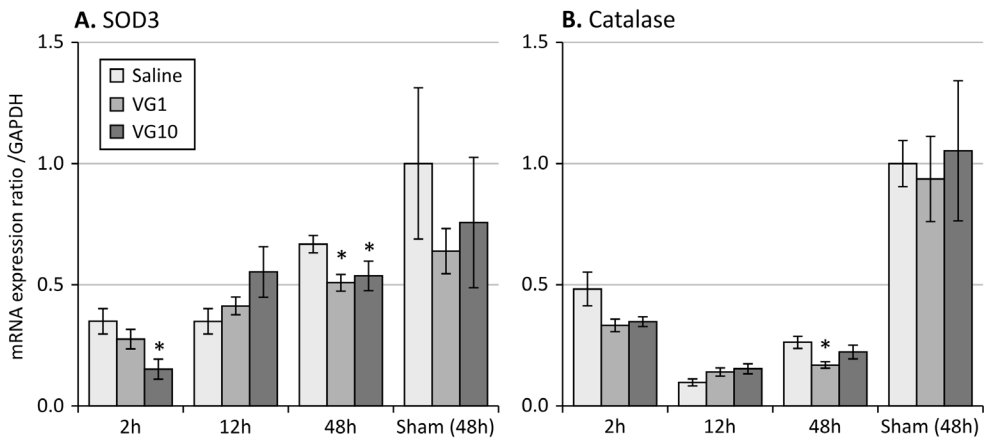

Figure 2.25. *mRNA expression of (A) SOD3 and (B) catalase. Values are average ± SE. * p < 0.05 vs. saline-treated animals.*

2.5.10. mRNA expression of DPP4, SDF-1α and CXCR4

DPP4 expression was found to be down-regulated during the course of ischemia and expression halved at 12 and 48 hours of reperfusion compared to 2 hours of reperfusion. At 12 hours of reperfusion, the DPP4 mRNA expression was found to be significantly increased in VG1-treated animals compared to saline-treated animals. Remarkably, this increase was also observed in sham-operated animals, sacrificed 48 hours after sham operation (figure 2.26A). Although literature reports an increase of mRNA expression of SDF-1α as well as its receptor CXCR4 during IRI, this increase was not observed in our study. An increase of SDF-1α mRNA expression was found in VG1 and VG-10 treated sham-operated animals compared to saline-treated animals, but not in different reperfusion groups (figure 2.26B). An apparently dose-dependent decrease of CXCR4 expression was observed in VG1 and VG10-treated animals compared to saline-treated animals, but the observed differences were not significant. However, CXCR4 mRNA expression in VG1-treated animals was found to be significantly increased compared to saline controls (figure 2.26C).

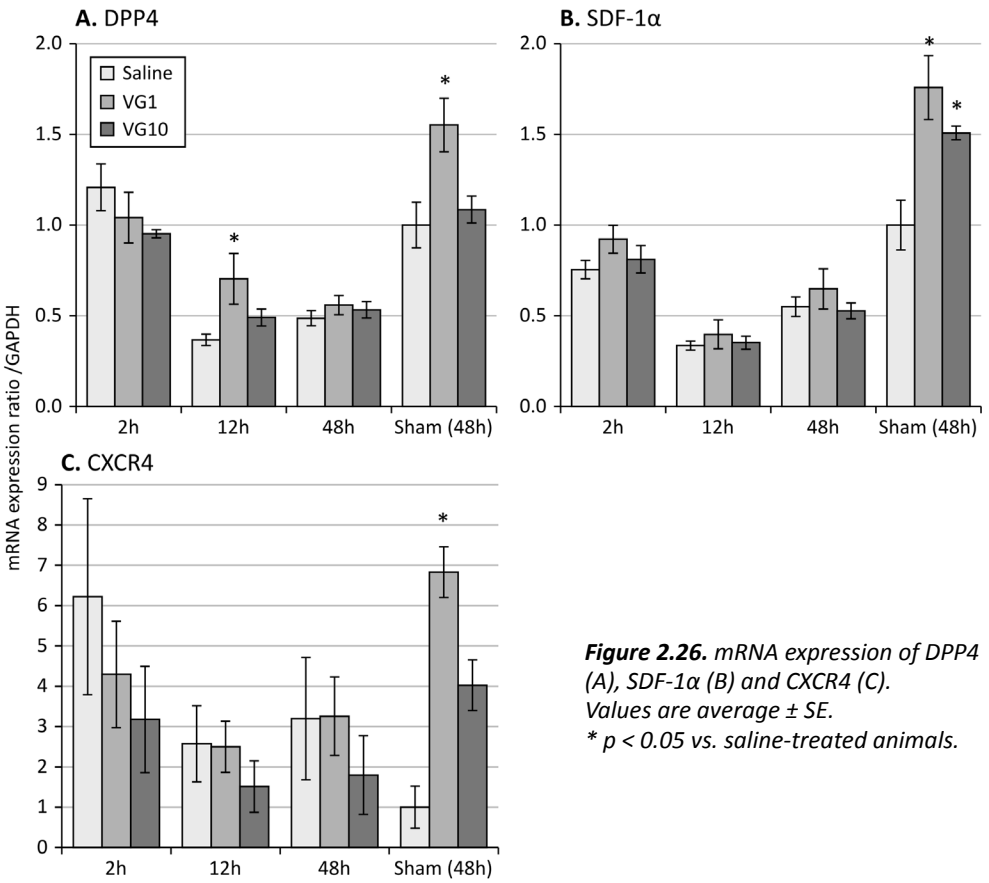

Figure 2.26. *mRNA expression of DPP4 (A), SDF-1α (B) and CXCR4 (C). Values are average ± SE. * p < 0.05 vs. saline-treated animals.*

2.6. Discussion

This experimental study confirmed the hypothesis of a protective effect of DPP4 inhibitor treatment on the kidney in ischemic AKI, observing a significant, dose-dependent protective effect of vildagliptin on kidney function after IRI. Furthermore, this study attempted to identify some of the mediators of this renoprotective effect.

An analysis of DPP4 activity in the serum clearly showed a successful inhibition of DPP4, with over 90% inhibition in both dose groups at 2 hours of reperfusion, and 88% in the VG10 group after 12 hours. Serum creatinine levels revealed a significant and dose-dependent protective effect of vildagliptin on kidney function, which at a dose of 10 mg/kg resulted in a creatinine level less than half of the control animals at 12 hours of reperfusion.

This remarkable effect on renal function as reflected by the serum creatinine levels was shown not to be caused by interference of vildagliptin with creatinine measurement, blood pressure, GFR or an increase of tubular secretion of creatinine. Melin *et al.* reported that the functional and morphological outcome of renal IRI is worse in hyperglycemic conditions[359]. However, since vildagliptin did not influence blood glucose in fasting animals undergoing ischemia, differences in blood glucose levels could not have affected kidney function.

The observed effect was accompanied by morphological protection indicated by a decrease in tubular necrosis and inhibition of the apoptotic pathway in renal proximal epithelial cells. Although a reduction of necrosis was observed in both treated groups, the reason why less necrosis occurred in the VG1-treated group compared with the VG10-treated group remains to be clarified. The decrease in the Bax/Bcl-2 mRNA expression ratio and a reduced number of TUNEL-stained apoptotic bodies in the OSOM (at 48 h of reperfusion), can further clarify the observed functional protection of the kidney. Our results are in line with those of Vaghasiya *et al.*, who reported a decrease in DNA fragmentation and apoptosis in chronic sitagliptin-treated T2DM rats[410].

An increased infiltration of macrophages was observed in VG10 treated animals at 2 and 12 hours of reperfusion, which was surprisingly also observed in sham-operated animals. Macrophages are known to be able to switch phenotype according to their inflammatory surroundings, and recent publications attribute a major role to these cells in kidney recovery after ischemic injury[211,409,410]. Possibly, an anti-inflammatory macrophage phenotype contributed to renal recovery in the VG10-treated group of this study. Neutrophils were almost absent, which has been previously observed in animal studies[197,411]. DPP4 was shown to play a role in T-cell activation, and also leukocyte-endothelial interactions have been shown to be enhanced through

DPP4[253,254]. In this study, a reduced number of T-cells was observed in the VG10 treated animals at 12 hours of reperfusion, which could have been caused by reduced leukocyte-endothelial interactions, reduced chemotaxis of T-cells or a decrease of T-cell activation. Aside from the infiltration of immune cells as a result of vildagliptin treatment, the inflammatory profile and expression of cytokines could have been altered. In the current study mRNA expression of the pro-inflammatory marker CXCL10 was significantly decreased in vildagliptin-treated animals, which is favorable since CXCL10 expression correlates with graft rejection/tissue injury following kidney transplantation[341,343]. Furthermore, mRNA expression of IL-10, IL-16, TNF-α and TLR2 indicated an anti-inflammatory cytokine production in the VG10-treated animals.

Analysis of serum MDA levels revealed a significantly reduced lipid peroxidation status, reflecting a reduction of oxidative stress in VG1 and VG10 treated animals at 48 hours of reperfusion, an effect which was also observed in sham operated animals. HO-1, as a marker of oxidative stress produced by interstitial inflammatory cells after IRI in quantities, reflecting the extent of the causal insult[168], seemed to display a decreased mRNA and protein expression as a result of vildagliptin treatment, but this difference was not significant.

Although literature suggests otherwise[254], DPP4 activity was not increased in the saline treated animals after IRI. The intravenous administration of vildagliptin resulted in a very efficient inhibition of DPP4 activity in the circulation. Furthermore, DPP4 mRNA expression was shown to be decreased after IRI in all animals, probably due to tubular damage. DPP4 activity in serum greatly influences the half-life of circulating DPP4 substrates. Prolongation of their half-life could have protected the kidney from IRI.

GLP-1 is one of the best-known and -studied of the DDP4 substrates. The cardioprotective effects of GLP-1 have been extensively studied in ischemic heart models and involve an up-regulation of cardioprotective genes, resulting in a reduction of the infarct size[280,412]. Its importance in renal IRI was recently demonstrated by Vaghasiya et al.[285], who reported a protective effect of the GLP-1 agonist exenatide on the outcome of IRI in NAD/STZ-diabetic rats. GLP-1 has been assigned an antihypertensive effect due to its diuretic and natriuretic actions. The mechanisms behind these actions were recently attributed to an increased GFR and a down-regulation of NHE3 in the PTC[260,269]. The same down-regulation of NHE3 is seen when a DPP4 inhibitor is administered [267] and is associated with an attenuation of blood pressure rise in young hypertensive rats[413]. NHE3, generally down-regulated during IRI[266], reabsorbs filtered sodium out of the proximal tubuli, which is energetically very demanding; DPP4 inhibition might lessen the severity of the ischemic insult by moving NHE3 to the intermicrovillar domain, thus reducing the metabolic needs of the kidney. However, while DPP4 inhibition in animal models results

in increased diuresis and natriuresis[410,413], there does not seem to be an effect on the GFR[413], which is in line with our study where neither systolic blood pressure nor the GFR differed significantly in vildagliptin-treated animals vs. saline-treated animals. Furthermore, GLP-1 as well as GLP-2 were found to have anti-apoptotic effects when binding to their receptors[295], which were also found in renal tubular epithelial cells[285,297]. An anti-oxidative effect of GLP-1 has been observed to occur in diabetic animals, characterized by increased SOD and catalase activity, as well as a reduction of lipid peroxidation status[285]. Only the latter could be confirmed in this study through serum MDA analysis.

Ischemic injury of the kidney was reported to result in a fast up-regulation of mRNA expression of SDF-1α as well as its receptor[300]. Contrarily to the latter publication, only CXCR4 mRNA expression was found increased in all animals. The mRNA expression of CXCR4 was found to be increased the least in VG10-treated animals. Although the chemotactic signal of the SDF-1α/CXCR4 axis is considered essential for migration of CXCR4+ progenitor cells towards the kidney during reperfusion[300], it has been shown that modulating the SDF-1α/CXCR4 axis does not result in alteration of trafficking of mesenchymal cells toward the injured kidney[304]. Nevertheless, SDF-1α was shown to play a crucial role in the functional and morphological protection of ischemically injured organs (the heart[317,414] and kidney[305]) as well as in angiogenesis[298]. Prolongation of the half-life of endogenous SDF-1α might have contributed to the protective effect of vildagliptin in this study.

VIP, a circulating neuropeptide with receptors (PAC1, PAC1, and VPAC2) in the kidney[348], was suggested to have a tissue-protective function in ischemic models, through its anti-inflammatory and anti-oxidative properties[333]. The modulatory function of the VPAC1 receptor on sodium reabsorption was previously reported in proximal tubular epithelial cells[331]. The anti-inflammatory effects of vildagliptin treatment observed in the present study are in line with previous publications concerning the anti-inflammatory effect of VIP on monocytes (reduction of TNF-α and increase of IL-10 expression)[415]. The anti-oxidative effects (reduced MDA and HO-1 levels) of vildagliptin treatment could, next to GLP-1, also point to a possible implication of VIP in the induced protection.

The report by Vaghasiya et al.[410] on IRI outcome in diabetic rats after sitagliptin administration supports the hypothesis that the functional protection by vildagliptin is related to DPP4 inhibition. In their study, sitagliptin treatment results in a decrease of serum aspartate aminotransferase, urea nitrogen and creatinine, and a decrease of lipid peroxidation, xanthine oxidase activity, MPO activity and NO level in renal tissue, a decrease in DNA fragmentation and apoptosis and an increase of antioxidant enzymes glutathione, glutathi-

one peroxidase, superoxide dismutase and catalase, an effect which is very much in line with the effect observed in this study. As the same group came to similar findings in rats treated with GLP-1 agonist exenatide[285], this DPP4 substrate could be an important factor in the observed effect. In a different study, an attenuation of the decrease of GLP-1 levels in the kidney of Zucker diabetic fatty rats was held responsible for a decrease of IL-1β and TNF-α, and a prevention of the increase of the Bax/Bcl-2 ratio and TUNEL positive cells, as well as an increased co-localization of GLP-1 and its receptor GLP-1R in the kidney, an effect which was effectuated through DPP4 inhibition[416]. Another recent article summarizing results of renoprotection with incretin-based drugs also identify GLP-1 and SDF-1α as the protagonists in the protective effect of DPP4 inhibition[417]. In diabetic nephropathy, the DPP4 activity was found to be increased in endothelial cells and tubular epithelial cells[418]. In such model, the importance of the interaction of DPP4 with integrins in the development of fibrosis was recently emphasized. The DPP4-integrin interaction appears crucial for TGF-β dimerization and start of endothelial mesenchymal cell transition[418,419]. DPP4 inhibition was found to affect this interaction and could thus also have affected the endo-MT in the development of ischemic injury in this model.

The present study is the first to assess the effect of a single dose of DPP4 inhibitor in the setting of renal IRI in non-diabetic rats and supports further studies towards the potential use of DPP4 inhibitors in pathologies other than type 2 diabetes[420]. Inhibition of DPP4 with vildagliptin results in a functional protection of the kidney against IRI, reflected by a dose-dependent decrease in serum creatinine levels and a reduction of tubular necrosis and apoptosis. This protection by vildagliptin is associated with anti-inflammatory and anti-oxidative changes. The therapeutic potential of perfusion with DPP4 inhibitors in a transplantation setting is yet to be studied. Also the complete mechanism, which is without a doubt a well concerted and multi-causal phenomenon, remains to be elucidated.

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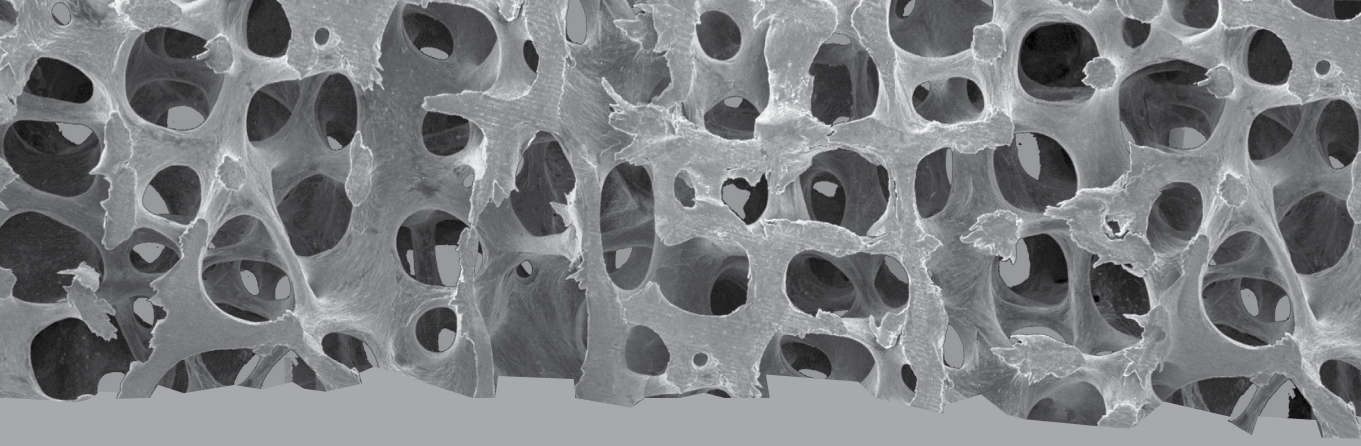

Chapter 3

DPP4 inhibition attenuates the development of bone pathology

3

The results of the study in section 3.5 were published in September 2014 in the American Journal of Physiology – Endocrinology & Metabolism:
Glorie, L. et al. DPP IV inhibitor treatment attenuates bone loss and improves mechanical bone strength in male diabetic rats. Am J Physiol Endocrinol Metab 307, E447-455 (2014)

Material obtained from this experiment was used to generate the results published in:
Baerts, L. et al. Potential impact of sitagliptin on collagen-derived dipeptides in diabetic osteoporosis. Pharmacol Res 100; 336-340 (2015)

Parts of the introduction have been included in:
Glorie, L. et al. Boning up on DPP4 and its substrates - Function, Expression and Effects on Bone Health. J Bone Miner Res (Invited Review - under review)

Chapter 3: Contents

3.1. Bone and the pathophysiology of postmenopausal and diabetic osteoporosis

3.1.1. Bone structure and function

3.1.1.1. Introduction

Bones are rigid organs forming the endoskeleton of vertebrates. The skeleton has a structural function in the support and protection of soft tissues as well as a mineral function, which can be drawn on when dietary function is inadequate to meet the body's needs. Furthermore, it produces blood cells and specific tissue progenitor cells. Bones have a variety of shapes, and consist of hard tissue, the mineralized and non-mineralized matrix, and soft tissue, the bone marrow, endosteum, periosteum, nerves, blood vessels and cartilage (figure 3.1). The non-mineralized matrix is composed of mostly collagen type I and other proteins. Hydroxyapatite is the most important component of the mineralized matrix, and has a structural role as well as a functional role in the calcium and phosphate balance and storage.

The outer layer of bones, the cortical bone, is composed of compact bone tissue with very small spaces and can have a variable porosity of 5 to 30%. The interior of the bone is trabecular bone (also called cancellous or spongy bone), an open cell porous network of rod- and plate-like mineralized ele-

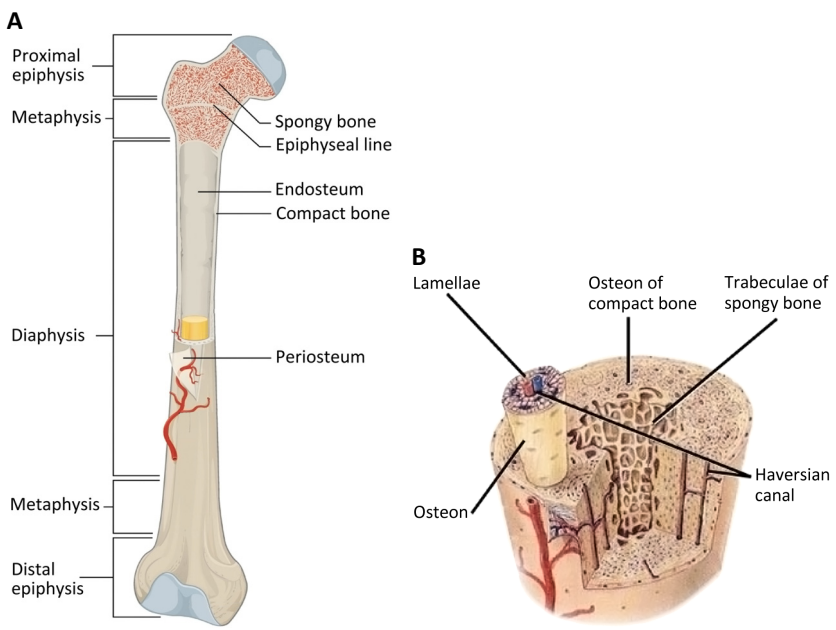

Figure 3.1. *Structure of the bone. (A) Macroscopic regions of the long bone (B) Bone diagram displaying microscopic structure of cortical and trabecular bone.*

ments, the trabeculae, allowing room for blood vessels and the bone marrow (figure 3.1B). In regular long bones, 80% of the total bone mass is cortical bone, while the remaining 20% of trabecular bone mass forms nearly ten times the surface of cortical bone with a porosity of 30 to 90%. A long bone is divided in three regions: the epiphysis, the metaphysis, and the diaphysis. The epiphysis consists of the rounded edges of the long bone, at the joints of adjacent bones. The actual growth plate, which gives rise to longitudinal bone growth, is located in the metaphysis, and is characterized by large quantities of trabecular bone which form the rugged and dynamic connection between the epiphysis and the bone shaft. The bone shaft consists solely of cortical bone and is called the diaphysis (figure 3.1A).

Alterations in strain applied to the bone result in structural rearrangement of trabeculae in the metaphysis, the most metabolically active part of the bone. From a structural perspective, the architecture of the skeleton is constantly slowly adapted to support the mass of any individual in long-term posture changes or activities, which can be very variable at the level of cortical bone mass as well as trabecular bone microstructure. The mineralized trabecular matrix is a shape-shifting dynamic matrix which undergoes constant remodeling by osteoblasts and osteoclasts. The physical properties of bone depend on factors such as the concentration of collagen, elastin, proteoglycans, and minerals, as well as bone structure, bone density, and the interactions between matrix components of bone. In healthy bones, the bone strength and integrity is maintained by an efficient balance between bone formation and bone resorption. Different cell types are involved in the process of bone formation and bone resorption: osteoblasts (OB), osteoclasts (OC), chrondroblasts, osteocytes and chondrocytes, which are the product of a differentiation of mesenchymal stem cells. At older age, the number of osteoblast cells as well as the natural renovation of the bone tissue is decreasing.

3.1.1.2. Bone homeostasis, bone turnover and the bone remodeling cycle

Bone homeostasis is the balance between bone formation and bone resorption, and determined by the number and activity of osteoblasts and osteoclasts, as well as the mineralization process of osteoid. The continuous resorption and replacement of bone tissue is called bone turnover, and is constant in normal physiological conditions to maintain bone mass, structure and integrity. As soft tissue mass is highly regulated by nutritional status, skeletal homeostasis was also found to be directly influenced by a complex interaction of nutritional and regulatory factors[1]. It has even been shown recently that the bone itself also plays a role as an endocrine organ. Imbalances in the many factors influencing bone turnover gives rise to various bone pathologies,

which will be discussed later. The bone remodeling cycle describes the active process of bone turnover, which is the sequential process of bone resorption and bone formation[2], and is summarized in figure 3.2. Bone remodeling occurs in discrete regions, which are called basic multicellular units or bone remodeling units (BMU)[3]. The BMU moves through the cortical bone replacing a tunnel of mineralized bone, while in the trabecular bone, the BMU travels the surface of the trabeculae creating grooves of mineralized bone. The bone turnover and associated metabolic activity is much higher in trabecular bone compared to cortical bone.

Phase 1 of the cycle depicts a quiescent bone surface. Osteoclasts are multinucleated cells responsible for bone resorption, and are derived from HSCs, through the stimulation of monocytes by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB-ligand (RANKL). Although their primary function is to resorb mineralized bone tissue, this function is critical for bone maintenance, remodeling and repair. Differentiation of osteoclasts occurs following stimulation by M-CSF produced by roaming osteoblasts and receptor activator for RANKL expressed on their surface. Osteo-

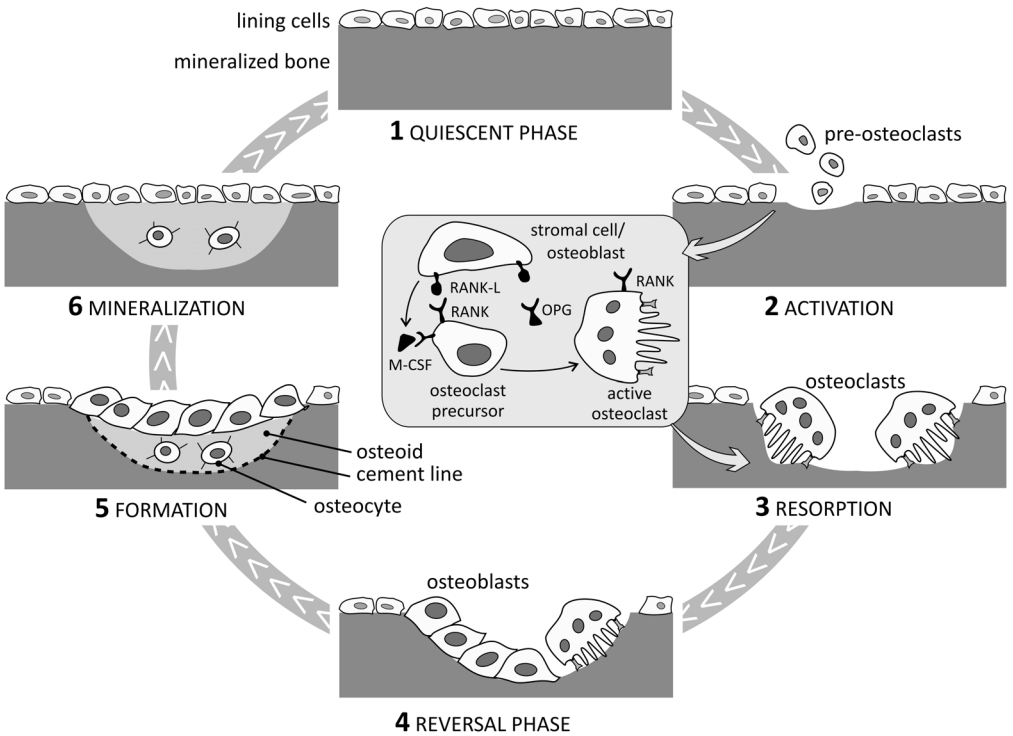

Figure 3.2. *Bone remodeling: the bone remodeling cycle and activation of osteoclasts by RANKL on the surface of osteoblasts. Adapted from PhD thesis of Tineke De Schutter, 2012[7].*

protegerin (OPG), a protein synthesized in osteoblasts and stromal cells, acts as a decoy receptor for RANKL and prevents the binding of RANKL to RANK, thus preventing the differentiation of osteoclasts. In phase 2, activation of osteoclasts occurs through TNF-α, IL-1 and IL-6 pro-inflammatory signaling. Active osteoclasts tightly bind to the bone and create a sealed resorption zone bordered by osteopontin and actin filaments[4], which functions as an extracellular micro-environment between the osteoclast and the bone surface. The osteoclast membrane at the side of the resorption zone increases in surface, becomes ruffled, hosts many proton channels and excretes lysosomes containing tartrate resistant acid phosphatase (TRAP), cathepsin K (CatK) and collagenase. These enzymes dissolve hydroxyapatite crystals and degrade the organic matrix of the bone during phase 3, the resorption phase.

In the reversal phase (phase 4), osteoclasts make way for osteoblasts, mononucleate cells responsible for bone formation. They originate from mesenchymal stem cells (MSCs), differentiating in either osteoprogenitor cells (OPCs), adipocytes, smooth muscle cells, chondrocytes or fibroblasts. Mesenchymal OPCs from the bone marrow or from the periosteum differentiate into osteoblasts by the influence of growth factors like fibroblast growth factor (FGF), platelet derived growth factor (PDGF), transforming growth factor β (TGF-β) and bone morphogenetic proteins (BMPs). Once OPCs start to differentiate into osteoblasts, they begin to express a range of genetic markers including osterix, collagen type 1 (Coll1), alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), osteonectin and bone sialoprotein. Osteoblasts migrate to the resorptive lacunae to produce osteoid, an organic matrix consisting of Coll1, glycoproteins and proteoglycans (phase 5, bone formation). The osteoid matrix acts as a scaffold for the deposition and formation of minerals, mainly hydroxyapatite crystals. ALP, present on the surface of osteoblasts, removes phosphate from various sources such as pyrophosphate, to augment the local phosphate concentration, and is considered a crucial factor for the formation and mineralization of the bone. Osteoblasts contain membrane-bound extracellular matrix vesicles filled with high concentrations of calcium and phosphate ions. Upon release, local ion concentrations reach saturation limits and favor the formation of hydroxyapatite crystals (phase 6, mineralization). Osteoblasts trapped in the bone tissue become osteocytes. They cease to generate osteoid and mineralized matrix, but are believed to act in a mechanosensory manner and exert a paracrine influence on remaining active osteoblasts, playing an important role in the local response to mechanical stress and microfractures[5]. They were also shown to undergo apoptosis around microfractures to stimulate local resorption by osteoclasts and subsequent rebuilding by osteoblasts[6]. Osteoblasts may remain present on the quiescent bone surface between bone lining cells surfacing mineralized

bone. The first resorptive phases of the bone take place in weeks, the subsequent bone formation phases during months. As the lifespan of the BMU is a lot higher than that of its active bone cells, a continuous supply of bone cells from bone marrow progenitor cells is crucial for correct functioning.

3.1.1.3. Mineral homeostasis

Calcium homeostasis

The bone serves as the main storage for calcium and phosphate ions, the homeostasis of these ions throughout the body has a crucial influence on bone formation and resorption (Figure 3.3). Calcium and phosphate are stored in the bone as hydroxyapatite or $Ca_5(PO_4)_3(OH)$. During the process of bone aging, the Ca:P ratio gradually increases from 1:1 to 1.67. More than 99% of the total amount of calcium in the body is stored in the bone. Calcium is involved in many processes such as signal transduction pathways, cell division, muscle contraction and blood clotting. In the serum, half of the total calcium content circulates as free ionized Ca^{2+}, whilst the other half is bound to plasma proteins. A small quantity is bound to circulating anions like phosphate. The concentrations of calcium and phosphate are tightly regulated through a sophisticated mechanism involving gastrointestinal absorption, uptake and release in the bone, and renal excretion. These functions are mostly regulated by three main hormones: parathyroid hormone (PTH), $1,25(OH)_2$-vitamin D_3 (calcitriol) and calcitonin.

Parathyroid hormone (PTH)

Calcium concentrations in the circulation are assessed by the parathyroid gland. When calcium concentration in the serum drops, the parathyroid gland releases PTH, which increases the concentration of calcium by acting on the bone, as well as on renal and gastrointestinal function. PTH stimulates bone resorption, increasing both calcium and phosphate concentrations in the serum. In the kidney, PTH increases calcium reabsorption in the distal tubule and the production of calcitriol in the proximal tubular cells, which in turn stimulates the gastrointestinal uptake of calcium and phosphate, and further stimulates the reabsorption of calcium through the distal tubular cells. PTH also inhibits the phosphate reabsorption in the proximal tubule in order to compensate for the increased release of phosphate from the bone and for the increased gastrointestinal uptake. In case of high extracellular calcium concentrations, the release of PTH from the parathyroid gland is inhibited, blocking all previously discussed mechanisms, as well as the production of calcitriol from the proximal tubular cell. Furthermore, a high blood concentration of calcium results in a reduction of calcium reabsorption in the thick

ascending limb, and the production of calcitonin from C-cells of the parathyroid gland, which promotes bone formation and inhibits bone resorption. The PTH hormone is secreted very quickly in response to extracellular free ionized calcium concentrations, and is regulated by calcium through the calcium sensing receptor (CaSR) at the transcriptional as well as posttranscriptional level. Secretion of PTH occurs in seconds to minutes, the degradation of PTH within the parathyroid gland is inhibited in minutes to hours, and the gene expression of PTH is stimulated over hours to days to correct for a sustained hypocalcemia[8]. High phosphate concentrations in the serum are also known to stimulate the secretion of PTH, causing hyperphosphatemia to occur often together with hypocalcemia[9]. PTH stimulates the production of calcitriol in the kidney, which in turn regulates the secretion of PTH by inhibiting its expression at the gene level, and by increasing calcium in the circulation through gastrointestinal absorption, thus creating a regulative feedback loop for PTH secretion. In the bone, PTH binds its receptor on osteoblasts, increasing the expression of RANKL and decreasing the expression of OPG. As a result, increased RANKL will bind the RANK receptor on premature osteoclasts without being disturbed by the OPG decoy, resulting in an increased activation of osteoclasts, releasing calcium, phosphate and matrix components from the bone into the circulation. Although a continuous activity of PTH results in an increase of bone turnover, mostly through increasing osteoclast activity resulting in cortical osteopenia[10], the intermittent administration of PTH results in an increased bone mineral density (BMD), and a better bone architecture with higher trabecular bone mass[11].

Calcitriol

Vitamin D, also called calciferol, is a hormone which is endogenously produced and can also originate from oral food intake. The endogenously produced calciferol needs several steps of conversion in different organs, involving exposure of skin to UV light, hydroxylation in the liver and further hydroxylation in the kidney under influence of PTH to become the biologically active $1,25(OH)_2$-vitamin D_3 or calcitriol. A deficiency of calcitriol results in an increase of activity of the kidney hydroxylase in a direct feedback loop[12].

Besides the stimulation of active gastrointestinal uptake of calcium and phosphate and of tubular reabsorption of calcium in the distal tubule, calcitriol also directly binds to a calcitriol receptor in osteoblasts, upregulating the expression of RANKL and down-regulating the expression of OPG. Like PTH, calcitriol thus promotes the differentiation of osteoclasts by increasing the binding capacity of RANKL, thus increasing bone resorption. As previously mentioned, calcitriol inhibits the secretion of PTH, and thus indirectly has a negative effect on bone turnover. In vitro vitamin D treatment was shown to

increase the expression of a transcriptional regulator of osteoblast differentiation[13], and overexpression of the calcitriol receptor increases bone formation parameters[14].

Calcitonin

High serum calcium concentrations induce the expression and secretion of calcitonin in thyroid C-cells through stimulation of the calcium sensing receptor. Calcitonin is a hormone which was shown to stimulate the incorporation of calcium in the extracellular bone matrix through osteocytes[15]. It

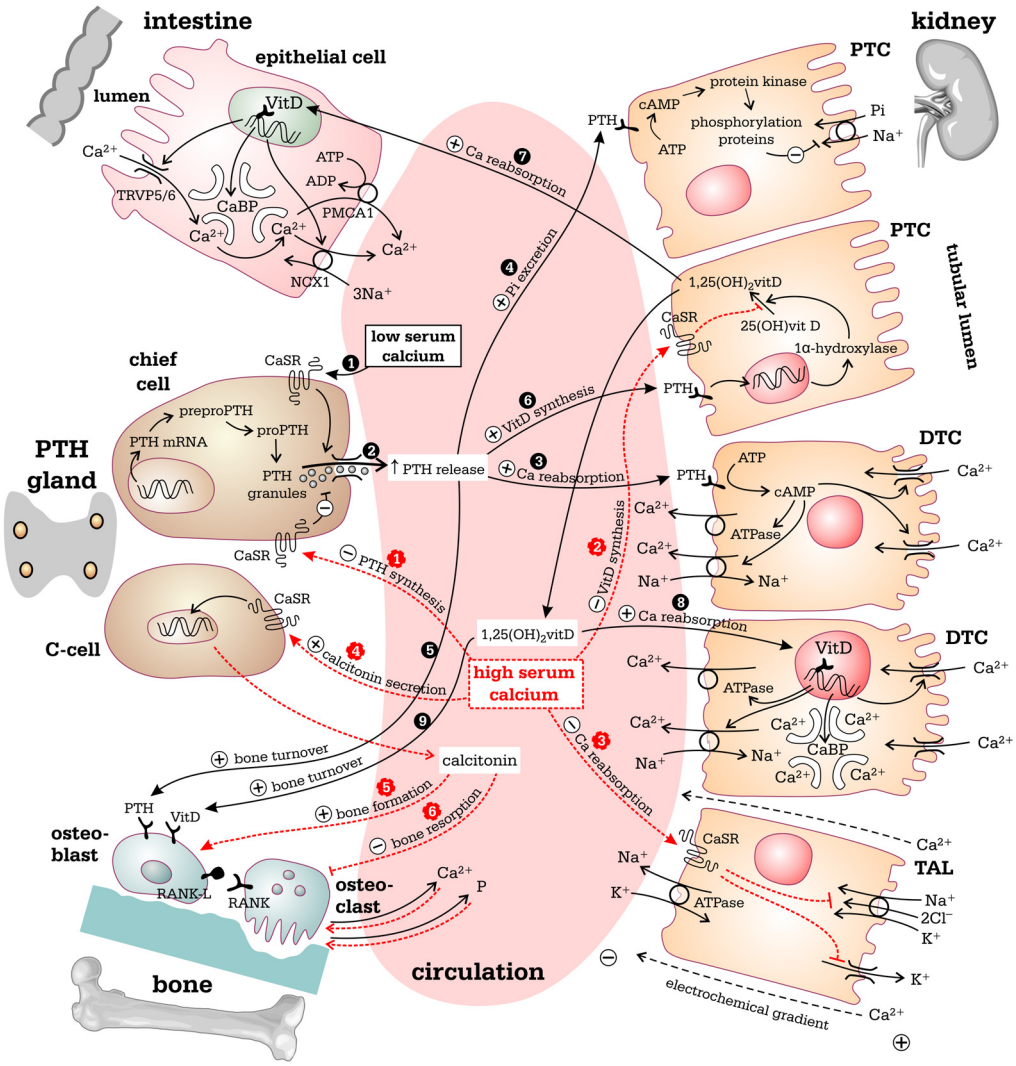

Figure 3.3. *Mineral metabolism of calcium and phosphate, and the effects of its regulation on the bone.*

also inhibits bone resorption by inactivating osteoclasts, creating a reduced release of calcium towards the circulation[16]. Nevertheless, calcitonin receptor knockouts results in an increased bone formation[17], which seems to be due to signaling between osteoblasts and osteoclasts through sphingosine-1-phosphate[18].

Phosphate and FGF23

About 85% of total body phosphorus is stored in phosphate (PO_4^{3-}) in hydroxyapatite in the bone. Phosphate is also a crucial constituent of DNA and RNA and phospholipids in the cellular membrane, and plays an important role in cellular signaling pathways and the cellular energy metabolism. Serum phosphate concentrations are, similar to calcium, a result of the balance between gastrointestinal uptake, storage and release from the bone and phosphate reabsorption in the kidney. A decreased gastrointestinal uptake of phosphate results in an increased reabsorption of phosphate in the renal proximal tubule through regulation of apical Na^+-phosphate transporters. Tubular reabsorption of phosphate reaches its maximum rates in situations of chronically reduced phosphate intake[19]. Equally, the increase of phosphate uptake through the intestine results in a reduction of activity of these transporters. These mechanisms are independent of PTH and have been shown to be controlled by intestinal phosphate concentrations before influencing the actual concentrations of phosphate in the serum[20]. PTH directly inhibits the reabsorption of phosphate in the proximal tubular cells. The reabsorption of phosphate in the proximal tubular cells is also controlled by fibroblast growth factor 23 (FGF23), a factor produced by osteocytes in the bone[21] but also in the brain[22], when phosphate or calcitriol levels are increased in the serum[23]. Calcitriol was found to promote the gene expression of FGF23 in the bone[23]. FGF23 binds its receptor on the basolateral side of proximal tubular cells, where it is in strong association with klotho, a transmembrane protein whose local regulation determines the response of the FGF23 receptor to FGF23 stimulation[24]. FGF23 signaling results in an inhibition of phosphate reabsorption[25] as well as a reduction of the hydroxylation reaction producing calcitriol[26]. FGF23, binding to its klotho-associated receptor at the thyroid gland[27], has been shown to directly inhibit the secretion of PTH in vitro as well as in vivo[28]. PTH, on the other hand, promotes the expression of FGF23[29,30]. As previously mentioned, phosphate is also directly regulated by calcitonin, whose activity effectuates a reduction of phosphate in the circulation.

3.1.1.4. Bone and nutrition

There is clear evidence of a complex interplay between nutrition and the bone metabolism, which is immediately relevant to clinical assessment of bone turnover, which is substantially different in fed and fasted state and accords to nutritional status. Parenteral feeding is associated with a reduced bone mass, clearly suggesting that hormones released after nutrient ingestion have a role in bone turnover. Generally, as nutritional status increases, it results in denser bones with greater skeletal strength. Then again, an increased body weight results in an increased load on the skeleton, and lengthening of bones may infer mechanical disadvantages, so the impact of changing nutrition on fracture rates may not always be easily predictable[1].

Adipocytes play a major role in the bone metabolism

Leptin (LP), a 16kDa cytokine-like peptide hormone predominantly secreted by adipocytes, regulates appetite and reproductive function through its hypothalamic receptors and also has direct and indirect actions on the bone[31,32]. Although leptin is not recognized as an important DPP4 substrate in literature, it has a putative DPP4 truncation site[414]. Furthermore, a strong association was found between the secretion of leptin and DPP4 by adipocytes [415,416]. LP concentrations in the plasma were found to be proportional with body weight[33]. The leptin receptor (LPR) is expressed in osteoblasts and chondrocytes, and LP was shown to promote proliferation and differentiation of osteoblasts in vitro and chrondrocytes in vitro and in vivo[34], and to also inhibit adipocyte differentiation as well as to induce apoptosis of adipocytes[35]. LP also reduces the production of RANK and RANKL[34] and increases OPG expression, regulating osteoclastic development and reducing osteoclastogenesis[36]. LP deficiency results in uncontrolled food intake and profound obesity, reduced linear bone growth, reduced cortical bone mass and increased trabecular bone in the spine but reduced trabecular bone in the femora, where most of the bone marrow space is occupied by adipocytes[37]. LP also affects the bone metabolism indirectly through the central nervous system, stimulating bone formation[38,39] but also bone resorption[40-42]. LP significantly increases oestradiol levels, IGF-1, bone ALP and OCN and thus indirectly also significantly impacts the bone metabolism. Circulating LP concentrations were shown to be inversely related to bone resorption in postmenopausal women[43]. LP also increases growth plate thickness and stimulates linear growth of long bones[34]. It can thus be concluded that LP exerts a positive influence on the bone. However, whereas initial increases in LP stimulate bone formation, higher levels of LP result in its inhibition[44]. Resistance to endogenous LP increases both with age and BMI[33,45]. Hence, the majority of postmenopausal women with nor-

mal food intake would be probably insensitive to LP treatment. The potential utility of LP as a therapy for bone loss would be preserved in conditions of LP deficiency and energy deprivation, such as food restriction, exercise-induced hypothalamic amenorrhea, anorexia nervosa, and perhaps weight loss[33]. Furthermore, LP exerts pleiotropic effects in such areas as hematopoiesis, blood pressure, T-lymphocyte function, reproduction and bone mass regulation.

Adiponectin (APN), another adipocyte-specific secreted peptide, increases insulin sensitivity, and its circulating levels are reduced in obesity and diabetes[46]. APN receptors (APNR) are found on osteoblasts, which also secrete adiponectin themselves. APN was also shown to inhibit NF-kB signaling, the pathway regulating osteoclastogenesis in pre-osteoclasts[47]. The stimulatory effects of APN on osteoblasts and the inhibitory effects of APN on osteoclasts have also been shown in vivo[48]. DPP4 inhibition was shown to attenuate the decrease of APNR expression in type 2 diabetic Sprague-Dawley rats, influencing their cognitive functions[49].

Resistin is also produced in adipocytes and increase the proliferation of osteoblasts as well as the formation of osteoclasts. Although they are downregulated by diabetic treatment with thiazolidinediones, it is not known whether their counterbalancing effect has an actual net effect on the bone[50].

Pancreatic enzymes: insulin, amylin and preptin

Insulin, amylin and preptin are co-secreted by β-pancreatic cells and also strongly influence bone mass, as summarized in figure 3.4. Excretion of insulin is stimulated by glucose, amino acids, fatty acids and ketones, by neurostimulation, and by various gastro-intestinal hormones. Glucose is the most important stimulant of insulin secretion. The secretion process is biphasic: a first short phase is due to the secretion of already synthesized insulin, and is characterized by a quick response, one or two minutes after stimulation by glucose. The second longer-lasting phase is the slow release of freshly produced insulin. Insulin is mitogenic to osteoblasts in vitro and increases osteocalcin expression and bone formation in vivo, which could be mediated by insulin receptors on osteoblasts as well as IGF-1 receptors[51]. BMD was found to be directly correlated with circulating insulin levels and inversely correlated with insulin sensitivity[52,53]. Insulin sensitivity of osteoblasts is promoted by vitamin D[54]. Hyperinsulinemia is the reason why high BMD levels can be found in T2DM, as hypoinsulemia is held accountable for a low BMD in T1DM[55]. In experimental studies, an insulin receptor knockout develops early diabetes and growth retardation[56].

Amylin is a peptide which directly stimulates the proliferation of osteoblasts in vivo and acts as calcitonin to inhibit osteoclast development and activity and thus bone resorption[57]. It was shown to positively affect bone

mass in rodents[58]. At the same time it also effectuates calciuria through the calcitonin receptor[59]. Adrenomedullin is a strongly related peptide, also secreted by the β-pancreatic cells, which also has a strongly anabolic effect on the bone[60]. Both amylin and adrenomedullin are dependent on the presence of the IGF-1 receptor[61]. Amylin effectuates the same effect on the growth plate and the linear growth of long bones as LP[62].

Preptin is anabolic to osteoblasts in cell culture but do not influence osteoclastic activity. In vivo, it was shown to enhance bone formation in mice[63].

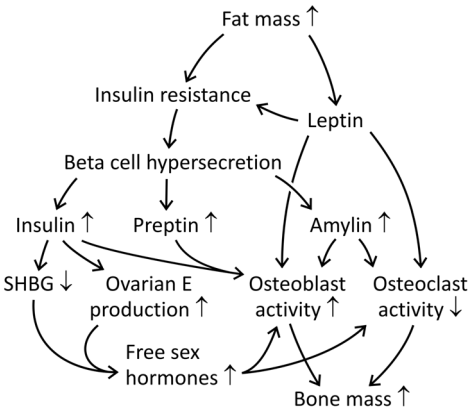

Figure 3.4. *Influence of fat mass on bone cell function (adapted from Reid et al., 2006[1]). Initial stages of diabetes type 2 result in pancreatic β-cell hypersecretion due to insulin resistance, causing a phenotype of increased bone mass. SHBG: sex hormone-binding globulin; Ovarian E: ovarian estrogen.*

Other peptides responsive to feeding

An oral glucose load results in a transient reduction of circulating serum calcium and an increase of calcium secretion[64]. These changes are a concerted result of a reduced PTH secretion[64], and an increased amylin[59] and calcitonin[65] secretion. There is also evidence of reduced bone resorption after feeding of glucose, fat or protein in humans[66]. Ingested nutrients also stimulate the release of GIP, GLP-1 and GLP-2 in the bowel. DPP4 substrates affecting the bone through mechanisms described in the next section. Ingested calcium acutely results in an increase of calcitonin secretion, a decrease of PTH secretion and a reduction of bone resorption[67-69]. Long term feeding of protein supplements also results in an increase of IGF-1, whose effect on the bone will also be described in the next section (figure 3.5).

Ghrelin is an appetite-stimulating hormone produced by the stomach, which increases in a fasting state and decreases after a meal. Although ghrelin is not a DPP4 substrate, it was shown that DPP4 inhibition with sitagliptin results in a suppression of active ghrelin[70]. Receptors for ghrelin are found in

osteoblasts, and ghrelin stimulates osteoblast proliferation and differentiation, but also osteoclastogenesis and mostly the bone-resorbing activity of mature osteoclasts[71]. There is also a close interaction of neuropeptide Y (NPY) with ghrelin as it was shown to mediate its anticonvulsant effect through its Y1, Y2 and Y5 receptors[72].

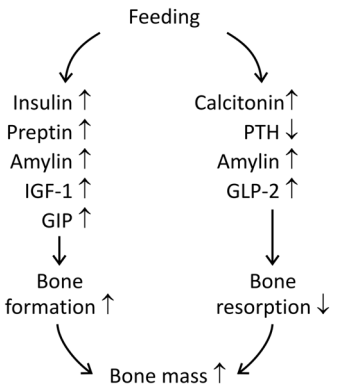

Figure 3.5. *Specific circulating peptides are known to increase after feeding, positively affecting bone mass (from Reid et al., 2006)[1].*

Bone as an endocrine organ

The skeleton itself also directly influences the endocrine system by producing hormones (figure 3.6). As previously mentioned, osteocytes in the bone produce FGF23, which acts on the kidney to inhibit hydroxylation of vitamin D and to promote the excretion of phosphate. The bone thus regulates phosphate metabolism independently of PTH and vitamin D, and the excess or deficiency in FGF23 can result in severe abnormalities. Furthermore, osteoblasts express OCN, which is produced in an uncarboxylated form (UcOCN). It can be post-translationally carboxylated by osteotesticular protein tyrosine phosphatase (OST-PTP), which is expressed in testes and osteoblasts[73]. This carboxylation occurs under negative control of the Esp gene in a vitamin K dependent process, producing active carboxylated OCN (also called bone Gla-protein). After carboxylation, OCN becomes a very efficient chelator of calcium. Carboxylated OCN is a marker of bone formation, as it plays a major role in mineralization and Ca^{2+} homeostasis[74], but was also suggested to be a potential marker for physical activity[75]. Weight loss in combination with exercise training results in an increase of serum OCN levels and a reduction of visceral fat mass, as well as increased energy expenditure, but diet-induced slight weight loss does not necessarily result in an increase of OCN levels[76]. Body weight was also shown to have an important influence on bone mass, as well as exercise. Although weight-bearing exercise during adolescence was

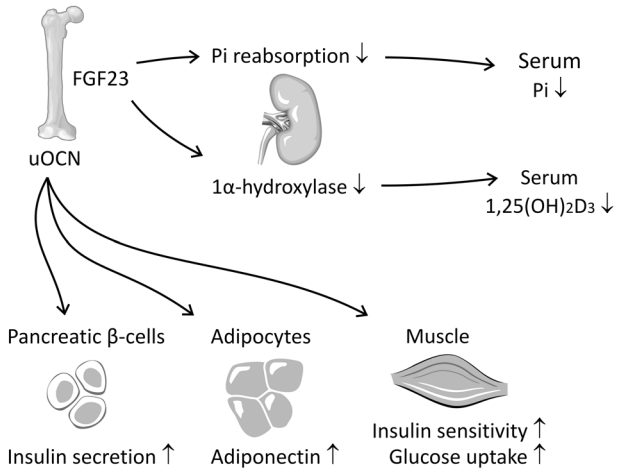

Figure 3.6. *The bone as an endocrine organ. FGF23, produced by osteocytes, decreases the reabsorption of phosphate in the renal proximal tubular cells and thus decreases serum phosphate levels. It also decreases kidney hydrolase activity to decrease the production of calcitriol from the kidney. UcOCN produced in osteoblasts increases insulin secretion in the pancreatic β-cells, the expression of APN in adipocytes and the insulin sensitivity in muscle tissue*[75]*.*

shown to increase affect peak bone mass[77], endurance exercise was found to result in a decrease of bone mass, mostly in female endurance athletes[78]. For male endurance athletes, studies evaluating the effect of exercise on the bone have been controversial[79-81]. The lipid prostaglandin E2 has also been identified as one of the mediators of exercise-induced bone formation[82,83].

UcOCN has been suggested to be useful as a marker of vitamin K deficiency and carotid calcification in hypertensive patients[84]. Increases of serum OCN levels are well correlated with increases of osteoid and BMD during osteoporosis treatment using pharmaceuticals inducing bone formation. In the pancreas, UcOCN but not carboxylated OCN was also found to induce the release of GLP-1 and thus indirectly stimulate insulin secretion[85]. OCN knockouts display decreased β-cell proliferation, decreased insulin and APN secretion, glucose intolerance, insulin resistance and have an abnormal amount of visceral fat[76]. UcOCN induces the release of APN from adipocytes, which increases the sensitivity to insulin[86-89]. In an experimental study, mice that lacked UcOCN also showed glucose intolerance and obesity, while mice with UcOCN overexpression were protected against obesity and T2DM[86]. Low levels of UcOCN have also been associated with obesity and insulin resistance[90]. Daily injections of carboxylated OCN results in an improved glucose tolerance through increase of β-cell mass and increased insulin secretion. In high-fat diet-fed

(HFD) mice, it results in an improved glucose tolerance and protection from obesity through increased energy expenditure and additional mitochondria in skeletal muscle cells. OCN-injected HFD mice are also completely protected against hepatic steatosis, although they eat as much as regular HFD mice[91]. General serum OCN levels were found to be reduced in T2DM patients.

3.1.1.5. Other factors influencing bone homeostasis

Specific cell surface receptors have been found to play an auxiliary role in the transmission of signals from outside bone cells into the bone forming cell nucleus. Among others, the receptors for BMPs, expressed on the surface of osteoblast precursor cells, were found to be potent inducers of bone formation[92]. The low density lipoprotein (LDL)-related protein 5 receptor involved in the β-catenin pathway, was also shown to be important for bone formation, and there might be an interplay between this receptor and BMP-receptors, enhancing each other's effect[93].

A complementary cell signaling system has been identified that drives the formation and activation of osteoclasts. Next to the RANK/RANKL-mediated pathway, another set of surface receptors, DNAX-activating protein 12 (DAP12) and the common γ-chain of the Fc receptor, were shown to play a role in bone resorption causing an increase of intracellular calcium, as DAP12 knockout mice were shown to display a dramatic increase in bone density (also called osteopetrosis)[94]. Both pathways, regulating osteoclast maturity, are crucial for bone resorption[95,96]. Other factors, which have been identified mostly through the study of pathophysiological development of bone disease, will be discussed later.

3.1.2. Postmenopausal osteoporosis

3.1.2.1. Introduction

Osteoporosis ("porous bones", from Greek) is a progressive bone disease that is characterized by a gradual decrease in bone mass and density, and a degradation of bone microstructure, which leads to an increased bone fragility and risk of fracture[97]. Osteoporosis affects approximately one third of the postmenopausal women, one fifth of the men aged above fifty[98], and also T1DM as well as progressed T2DM population and women with premature ovarian failure. Often, postmenopausal osteoporosis is referred to as type 1 osteoporosis, whilst the type affecting both men and women at a later age is mostly labelled type 2 or senile osteoporosis. In the general population, the prevalence of osteoporotic fractures is 40 to 50% in women and 13 to 22% in men, which is fairly high[99]. Unfortunately, the degradation of bone qual-

ity often remains unnoticed until the actual occurrence of fracture[100]. These fractures mostly occur at the hip, forearm or as vertebral compression and are associated with high morbidity, loss of self-confidence and autonomy, and mortality[97]. The condition has been extensively researched to determine possible risk factors and to assess potential treatments.

3.1.2.2. Pathophysiology

The pathophysiology of osteoporosis is a complex multifactorial mechanism involving genetic, systemic, hormonal, inflammatory and surrounding factors. Osteoporosis implies a reduction of bone mass which is directly associated to abnormalities in the bone remodeling cycle, due to changes in bone formation and resorption. Risk factors for the development of osteoporosis can be classified according to non-modifiable and modifiable risk factors. Non-modifiable risk factors most importantly include a deficiency of estrogen (menopause)[5], but also a deficiency of testosterone[101]. Furthermore, it includes European or Asian origin[102], family history of osteoporosis[103], earlier fractures[104] and small stature. Modifiable factors include many of the previously discussed influencing factors such as vitamin D (influencing bone resorption through PTH) and nutrition. The latter includes the uptake of calcium and phosphorus, but also normal feeding, which was shown to greatly affect the bone. Uptake of heavy metals[105], alcohol[106] as well as smoking of tobacco[107] has also been shown to increase the risk of fracture. Intake of medication, like non-steroidal anti-inflammatory drugs (NSAIDs)[108], could also increase fracture risk.

Hormones are crucial modulators of bone formation. The essential roles of estrogen[109], PTH[110] and also to a lesser extent testosterone[111,112], have been clearly established in the bone. The onset of menopause results in a decreased production of estrogen, which is one of the main factors managing the balance between bone formation and bone resorption through direct interaction on bone cells as well as osteoblast-osteoclast communication. Receptors for estrogen and androgens are present on bone marrow stromal cells, osteoblasts, osteoclasts and their progenitors. They express estrogen receptor alpha, which binds and transports estrogen into the nucleus of the cell where the receptor-hormone complex acts as a switch to turn on specific genes. Estrogen receptor-related receptor alpha was also found on osteoblasts, which may play an auxiliary role in the regulation of bone metabolism[113]. Circulating sex hormone binding globulin (SHBG), which facilitates the entry of estrogen into cells, could also be an influencing factor[114].

Activation by estrogen induces apoptosis of osteoclasts and inhibition of the activity of mature osteoclasts, and furthermore inhibits the apoptosis

in osteoblasts and osteocytes. Estrogen depletion results in a significant increase of osteoclast lifespan (by decrease of osteoclast apoptosis) and thus bone resorption, which quickly starts to exceed bone formation, which is lowered through an accelerated apoptosis of osteoblasts[115]. Estrogen was also shown to exert a regulatory effect on the release of cytokines which were shown to modify the bone[116]. Menopause was associated with an increased serum concentration of IL-1 and IL-6, which were shown to increase the differentiation of osteoclast precursors. An increase of IL-7 was also associated with postmenopausal bone loss[115]. Lack of estrogen also has significant effects on the fat metabolism. Estrogen deficiency causes a central insensitivity for LP and an increased concentration of hypothalamic NPY[117], which in turn regulates LPR in the hypothalamus and pituitary[118], causing increased adipogenesis from bone marrow MSCs[119]. Postmenopausal women have 20 to 28% more body fat. In experimental studies, a 22% increase of body fat was observed in rats 10 weeks after ovariectomy.

As mentioned in the beginning of this chapter, the metabolic activity of trabecular bone is a lot higher than in cortical bone, which is the main reason why bone pathologies are mostly known to affect the trabecular bone before they affect the cortical bone, as is the case in postmenopausal osteoporosis[120]. Particularly in the metaphysis of the bone, trabeculae are lost from the trabecular bone, which are insufficiently rebuilt due to the balance shift in bone remodeling, resulting in bigger trabecular size and trabecular alignment[121]. A bone phenotype displaying aligned trabeculae and loss of trabecular microstructures was shown to be more prone to injury and fracture.

3.1.2.3. Diagnosis of osteoporosis

Osteoporosis tends to develop in the elderly (primarily in women) as well as in different pathologies, and needs to be closely monitored. Osteoporosis is diagnosed as a decrease of BMD measured by dual-energy X-ray absorptiometry (DEXA), the most widely used and thoroughly studied bone density measurement technology, most frequently used for the diagnosis of osteoporosis. Measured BMD is not a true physical density, which would be measured in mass per cubic volume. Measurements are most commonly made over the lumbar spine and over the upper part of the hip and are either reported as measured areal density, or as Z- or T-score. The Z-score is defined as the number of standard deviations above or below the mean for the patient's age, sex and ethnicity, whilst the T-score is the number of standard deviations above or below the mean BMD for a healthy 30 year old adult of the same sex and ethnicity as the patient. The latter value is used in post-menopausal women and men over the age of 50 because it better predicts risk of any future frac-

ture. Z-scores are used to assess the bone in premenopausal women, men under the age of 50, and children. The criteria of the World Health Organization (WHO) are a T-score of -1.0 or higher for normal bone, a score between -1.0 and -2.5 for osteopenia, and a score lower than -2.5 in case of osteoporosis. Osteoporosis is thus defined as a condition with a bone density that is 2.5 standard deviations below the mean of a 30 year old man/woman. Bone density is used as a proxy measurement for bone strength, which is the resistance to fracture and the truly significant characteristic. A reduction of BMD as measured by DEXA is associated with an increased risk of hip fractures and osteoporosis[122], as well as an increased soft tissue mass[123]. Although BMD and bone strength are usually related, there are some circumstances in which bone density is a poorer indicator of bone strength.

3.1.2.4. Treatment of osteoporosis

Primarily, osteoporosis is treated non-pharmaceutically through the promotion of a healthy diet and lifestyle with sufficient exercise. Exercise is key to reinforce muscles and balance, which indirectly prevents falling. Often, recommendations include the reduction of alcohol intake and smoking. Alcohol intake also comprises an additional risk due to increased propensity of falling through impaired balance. The National Osteoporosis Foundation furthermore advises to watch calcium and vitamin D intake, especially in older women. Calcium and vitamin D supplements are often advised to replenish pools in the circulation, but these treatments are considered to be controversial when it comes to the actual reduction of fracture risk. Different drugs have been developed for the pharmaceutical treatment of osteoporosis, which are mostly prescribed to patients suffering fragility fractures or having a low T-score[100]. These drugs are mostly targeting the prevention of further bone loss.

Bisphosphonates are currently the most prominent treatment strategy for osteoporosis. As an anti-resorbing drugs, they are used to induce apoptosis in active osteoclasts therewith inhibiting bone resorption. These pyrophosphate analogues have a high affinity for hydroxyapatite in vitro as well as in vivo. During bone resorption they are taken up by osteoclasts to induce apoptosis, and also reduce the recruitment of osteoclasts to bone[97]. As a result, the lifespan of the bone remodeling unit increases, allowing for secondary mineralization in every resorption pit and a general increase in bone mass[124]. However, long term inhibition of bone turnover using bisphosphonates results in gastric disturbances, inflammation and fever, but mostly microfractures of the bone whose unification results in larger atypical bone fractures[125].

Specifically in postmenopausal women, estrogen therapy (or hormone

replacement therapy, HRT) can be administered to compensate the loss of estrogen production through menopause. Estrogen therapy results in the natural inhibition of osteoclast function and induction of apoptosis, and also inhibits osteoblast and osteocyte apoptosis, resulting in a net increase of BMD. This treatment, however, can result in an increased risk of developing breast cancer and cardiovascular disease, and is only recommended to prevent osteoporosis in postmenopausal women when other therapies are inadequate or not applicable[124]. Selective estrogen receptor modulators (SERMs) were developed that induce the positive effects of estrogen therapy without producing side-effects. These non-steroid treatments are either agonist or antagonist of estrogen, depending on the targeted tissue. They induce the effects of estrogen in the bone and have an anti-antagonistic effect in the breasts and uterus[97]. SERMs were found to effectively reduce bone resorption, although they are considered less effective than HRT or bisphosphonates and can cause deep venous thrombosis[124].

Strontium ranelate is an approved treatment for postmenopausal osteoporosis, and as a dual action bone agent both influencing osteoblasts and osteoclasts. It stimulates osteoblasts to form bone and inhibits osteoclasts to resorb bone[126]. The complete mechanism has not yet been elucidated, but strontium ranelate is able to influence the process of bone resorption and bone formation independently[97,127]. As strontium closely resembles calcium, it can also be integrated into hydroxyapatite[128]. In vitro studies have shown that strontium ranelate promotes the osteoblastic cell replication through multiple mechanisms and also dose-dependently reduces pre-osteoclastic differentiation and bone resorption, resulting in an increased production of bone matrix[129]. This double mechanism and dose dependency make strontium ranelate suitable to treat osteoporosis with various severity[130]. As the kidney is the main excretion route for strontium ranelate, CKD is a contraindication for its treatment, as it results in osteomalacia through insufficient mineralization of osteoid. These findings have been confirmed in in vitro studies, and high concentration of strontium were found in dialysis patients with osteomalacia[131,132]. The positive effect of strontium on the bone density is established but controversial, as it causes the incorporation of strontium in the bone, which has a higher atomic mass than calcium and thus strongly influences the result of a BMD scan[133,134].

Fluoride was previously used in combination with calcium salts and vitamin D, as it leads to the retention of calcium in the skeleton, but induces a lag in the bone mineralization causing a reduced quantity of mineralized bone and a large quantity of non-mineralized osteoid tissue, more vulnerable to microfractures and stress fractures, which disappears a few months after the start of the treatment[135]. The fluoride treatment however, was shown to positively

nor negatively affect the actual fracture outcome in osteoporotic bone[136].

Calcitonin is sometimes administered to specifically inhibit the activity of osteoclasts, as it can result in a quick and reversible inhibition of bone resorption[97,124]. Teriparatide, a recombinant fragment of human PTH that binds the PTH receptor, increases the BMD and has an anabolic effect on the bone, stimulating bone formation at quiescent locations[124].

Although many of these therapies have been proven effective in reducing the incidence of new fractures up to 50%, there is a permanent need of anabolic agents without side-effects that result in an efficient re-building of lost bone tissue[137].

3.1.3. Diabetic bone pathology

3.1.3.1. Diabetes mellitus

Diabetes mellitus (DM) is a group of metabolic diseases which are characterized by high levels of blood glucose (hyperglycemia) in a fasting state, caused by an inability to oxidize carbohydrate due to disturbances in insulin function. This condition results in episodic ketoacidosis and an osmotic effect in the kidney leading to excessive urine excretion.

According to data of the WHO, 347 million people have diabetes worldwide and the disease is rising in the list of leading causes of death because of the consequences related to chronic hyperglycemia. Criteria which clinically establish an individual as suffering from diabetes mellitus include having a fasting plasma glucose level in excess of 126 mg/dL (while normal levels should be less than 100 mg/dL) or having plasma glucose levels in excess of 200 mg/dL at two time points during an oral glucose tolerance test (OGTT), one of which must be within 2 hours of ingestion of glucose. The American Diabetes Association recommends that physicians consider patients to be pre-diabetic if their fasting blood glucose level is above 100 mg/dL but less than 125 mg/dL and whose fasting glucose levels are at least 140 mg/dL but less than 200 mg/dL following an OGTT. This hyperglycemia and glucose intolerance can either be caused by an insufficient production of insulin (T1DM) or a progressively reduced insulin receptor sensitivity (T2DM). The amount of circulating glucose increases because it greatly exceeds the glucose secretion ability of the renal proximal convoluted tubule. The prevalence of T1DM and T2DM among the diabetic population varies around 5-10% and 90-95% respectively[138]. Obesity and T2DM have revealed themselves to become serious epidemics of the 21st century in developed countries, and are held responsible for 5% of total mortality on earth. Aside from these two types, a transient diabetic state can occur during late stages of pregnancy, caused by an influence of hormonal production on insulin sensitivity. This gestational

diabetes is also characterized by high glucose levels, and increases the risk for development of obesity and T2DM at a later age[139]. Some other intermediate forms of impaired glucose tolerance or impaired fasting glycemia have been identified, which have not been fully characterized[140].

Type 1, insulin-dependent or juvenile onset diabetes (T1DM) is diagnosed in children and young adults, and is caused by the auto-immune destruction of insulin-producing β-cells of the Islets of Langerhans in the pancreas, leading to the inability to synthesize insulin. As a result, fasting blood glucose levels drastically increase. This autoimmune response may be triggered by an infection but the presence of a genetic vulnerability has previously been suggested[141], and there is indeed an observed inherited tendency to develop T1DM which has been traced to particular HLA genotypes, though the connection between them and the triggering of an auto-immune reaction is still poorly understood. Therefore T1DM is not exclusively a childhood problem; many adults that suffer from T1DM are initially misdiagnosed as T2DM. General symptoms include polyuria, polydipsia, constant hunger, weight loss, vision changes and fatigue. T1DM require a daily administration of insulin to replace endogenous production. Left untreated, T1DM can lead to fatal ketoacidosis.

T2DM is a more common and widely spread pathology, characterized by glucose intolerance due to desensitization of the insulin receptor, through which an almost ten-fold insulin signal is needed to achieve the same glucose uptake. Although it is uncertain what gives rise to this pathology, it seems largely the result of excess nutrition and physical inactivity resulting in excess weight. Insulin resistance can be caused by genetic predisposition, aging, obesity and a sedentary lifestyle, but is influenced by various metabolic stress factors ranging from psychological disorders like bipolar disorder[142] to environmental pollutes like polychlorinated biphenyls (PCBs)[143]. Through a complex mechanism insulin resistance results in hyperglycemia and permanent overstimulation of the insulin secretory machinery of the β-cells, leading to hyperinsulinemia fluently changing to hypoinsulinemia[144]. Pancreatic β-cells are initially forced to overproduce insulin to compensate the reduced sensitivity for insulin. After a period which can extend to years, the pancreatic β-cells start to burn out and are no longer able to overcome the insulin resistance. T2DM is characterized by an impaired insulin secretion, insulin resistance of the liver, muscle cells, pancreatic islet cells and adipocytes, and abnormalities in splanchnic glucose uptake, hypertension, dyslipidemia, high triglyceride levels and low levels of high-density lipoprotein cholesterol. Until recently, this type of diabetes was seen only in adults but it is now also occurring at a younger age. The earlier a person is diagnosed with diabetes the better chance the person has of staving off the primary negative consequences. Patients having T2DM are at high risk of complications in other compartments

such as cardiovascular disease[145], end-stage renal disease[146], hypertension[147], stroke, heart attack or heart failure[148], negative effects on bone metabolism[149,150], nephropathy (acute and chronic renal failure and inter-capillary glomerulonephritis)[146], peripheral vascular disease, neuropathy (loss of sensation, especially in the feet)[151], retinopathy[152], hormonal changes[153] and erectile dysfunction[154], gangrene and slowed emptying of the stomach[155]. An increased risk of developing cardiovascular diseases is caused by increased BMI, decreased level of LDL cholesterol and variations of blood pressure in the systole[145]. Among cardiovascular diseases, T2DM has also been associated with high risk of arteriosclerosis (hardening of the tunica media through calcification)[156]. For every percentage reduction in measured glycated hemoglobin, a frequently used marker for long-term control of diabetes, there was a 35% reduction in the risk of complications in patients with T2DM.

3.1.3.2. Pathophysiology of diabetes and diabetic complications

Although considerable debate prevails as to the relative contributions of β-cell dysfunction and reduced insulin sensitivity to the pathogenesis of diabetes, it is generally accepted that both these factors play important roles[157]. Adipose tissue was found to play an essential role in the pathogenesis of T2DM, as many of these patients are obese and present with central visceral adiposity. T2DM is always characterized by elevated non-esterified fatty acid concentrations, but also features the presentation of ectopic fat storage.

Adipose tissue has also been discovered as an important endocrine organ, which secretes various adipocytokines, like LP, TNF-α, resistin, APN, and others… These cytokines are key actors in the interplay between insulin resistance and β-cell dysfunction, and, as previously mentioned, they also play an important role in the bone metabolism[157]. Insulin is a strong antilipolytic, resulting in a decrease of circulating free fatty acids and inhibition of glucose production in the liver. Free fatty acids are a stress factor for adipocytes and endothelial cells, activating the aspecific immune system and causing further production of inflammatory factors like TNF-α, IL-1 and IL-6 by macrophages, adipocytes and endothelial cells, which in turn promote the production of acute phase proteins like C-reactive protein (CRP) in the liver. The result is a state of subclinical inflammation without associated physical symptoms. The occurrence of these acute phase proteins as well as high levels of γ-globulins or white blood cells can be used to identify the development of T2DM. Changes in insulinemia are an important risk factor for coronary heart disease as insulin resistance has a direct influence on vessel reactions to changes in blood pressure.

Most diabetic complications arise from the result of these changes in insu-

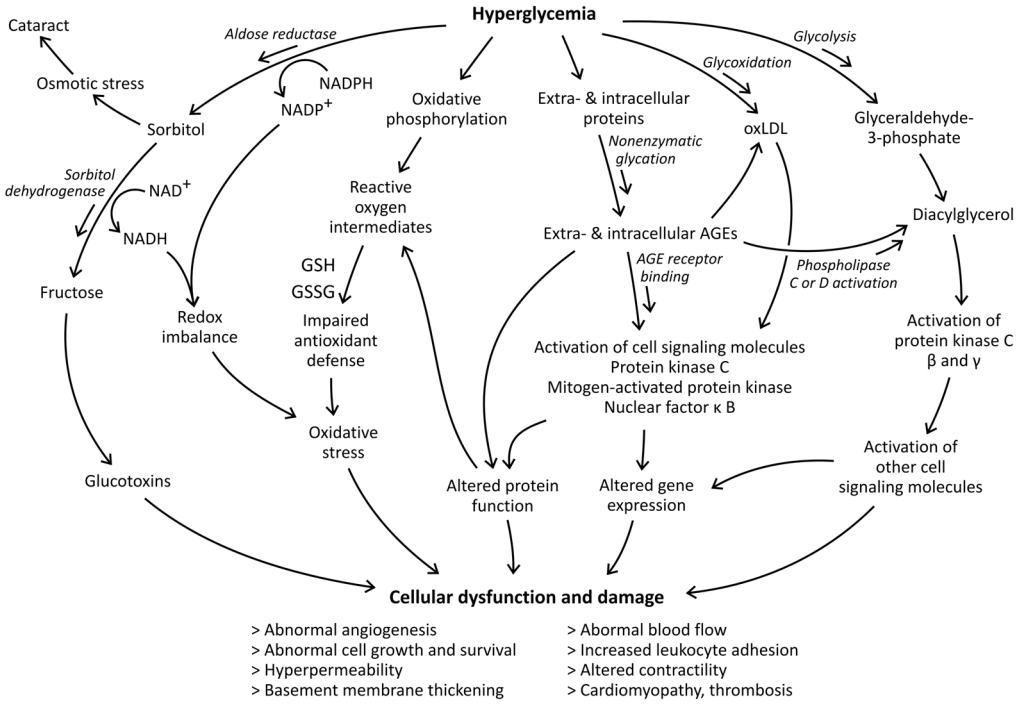

Figure 3.7. *Pathways contributing to the translation of hyperglycemia into diabetic complications (adapted from Sheetz et al., 2002[158]).*

linemia, which is primarily the increase of glycemia in the circulation. There are a number of different pathways by which hyperglycemia can translate into cellular dysfunction and damage (figure 3.7): the aldose reductase pathway, advanced glycation end-product pathway, reactive oxygen intermediate pathway, and the protein kinase C (PKC) pathway[158]. Most probably other pathways also contribute to cellular damage and the evolution of diabetic complications.

Processing of glucose to sorbitol by aldose reductase gives rise to production of reactive glycotoxins and increased oxidative stress, leading to cellular dysfunction and damage. Sorbitol also effectuates osmotic stress contributing to cataract [158] and retinopathy development[159]. Advanced glycation end-products (AGEs) were shown to be involved in the development of capillary issues, neuropathy, delayed wound healing, as well as infections and ulcers of the foot[160]. Reducing sugars can react non-enzymatically with intra- and extracellular protein aminogroups to form AGEs as a result of glycation in a multi-step mechanism, involving the formation of imines by reaction of glucose with a lysine amino group and the irreversible oxidation of the resulting product. The formation of these AGEs is promoted by the increase of circulating glucose, and typically progresses under diabetic conditions[161,162]. The

stimulation of the receptor for AGEs (RAGE) also stimulates oxidative stress generation and subsequently evokes inflammatory, thrombogenic and fibrogenic reactions, playing a role in the accelerated pathophysiology of atherosclerosis in diabetes. AGE binding is also known to alter gene expression as well as protein function[158]. Binding of AGEs to their receptor RAGE on monocytes and endothelial cells induces activation, resulting in the production of cytokines, adhesion molecules like vascular cell adhesion molecule 1 (VCAM-1) and tissue factor[163]. Therefore, the AGEs-RAGE-induced oxidative stress and inflammatory responses in the vessels could be targeted for preventing vascular damage due to atherosclerosis as well as medial vascular calcification in diabetes[162]. AGEs were also shown to inhibit the secretion of PTH. Administration of a recombinant decoy of RAGE to diabetic animals prevents hyperpermeability and vascular lesions in the kidney and improves diabetic kidney function, suggesting their central role in the development of chronic complications of diabetes[164].

Next to an increase of glycoxidation, the increase of glucose in the circulation also results in an increase of cellular oxidative phosphorylation. As a result, more ROS are formed which impairs the antioxidant defense, causing oxidative stress. AGEs, ROS and oxidized LDL cholesterol results in the activation of cell signaling molecules and three important pathways: the PKC pathway, the MAPK-pathway and the nuclear factor κB pathway. The glycolysis pathway, in combination with ROS and AGEs cause an increase of diacylglycerol, which activates members of the PKC pathway.

All of these pathways contribute to the altered gene expression in diabetes causing an aberrant cytokine and growth factor expression, as well as to the alteration of protein function. The resulting complications affect angiogenesis, cell growth and survival, permeability, kidney basement matrix thickening, blood flow, leukocyte adhesion and infiltration, cardiac contractility and cardiomyopathy, and thrombosis. Hyperglycemia was proven to increase the risk of heart disease and stroke, and also cause accumulated damage in capillaries and small vessels, leading to circulatory disorders like reduced blood flow, altered vascularization, atherosclerosis and vascular calcification. The link between AGEs and diabetic nephropathy has also been clearly established. Diabetic nephropathy (glomerulonephritis)[165], renal complications due to hyperglycemia, vessel anomalies in the glomeruli and resulting hypertension put diabetes among the leading causes of progressive kidney failure[166]. Diabetic nephropathy occurs in one third of all diabetic patients. Increased insulin levels stimulate the kidney to produce more angiotensin, causing systemic hypertension and initially, an increased GFR. Histologically, diabetic nephropathy is characterized by the thickening of the glomerular basement membrane, mesangial expansion and the reduction of podocyte number (renal hypertrophy).

This increases capillary pressure, impairs the GFR and leads to progressive albuminuria as increasing numbers of glomeruli are destroyed[167]. However, 30% of T2DM patients with a GFR below 60 mL/min had neither albuminuria nor retinopathy[168]. On the long term, the GFR decreases and risk of developing CKD increases slowly[169]. Glomerular and tubule-interstitial injury results in progressive loss of renal function, accompanied by increased expression of collagen, increased oxidative stress and infiltration of immune cells in the kidney[170]. Observed renal injury can be attenuated by treatments preventing the accumulation of AGEs in the kidney[171]. Many diabetic treatments are not kidney friendly, as they result in further proteinuria. Furthermore, diabetes in turn can cause bone disease (reduction of bone turnover or adynamic bone), which will be discussed extensively in the next section.

3.1.3.3. Diabetic bone pathology

The incidence of fractures was found to be increased in T1DM as well as in T2DM patients, indicating a diminished bone quality in the entire diabetic population[55,172]. T1DM affects the skeleton more severely than T2DM, probably because of the lack of the bone anabolic actions of insulin and other pancreatic hormones[55]. In the general population, BMD is inversely correlated with fracture risk. This association is also seen in T1DM patients, which are osteopenic as reflected by a low femoral or spinal BMD[173,174], however, no association exists in T2DM patients[175,176], who have either a normal or slightly increased BMD[173]. In untreated T1DM subjects the bone loss can occur already early in life and effectuate a reduction of peak bone mass in adolescence resulting in a lower BMD and increased risk of osteoporosis and fracture at a later age.

Diabetic osteopathy is the collection of bone abnormalities, including osteopenia, osteoporosis and a general increased incidence of low-stress fractures[177]. Section area and cortical thickness of diaphyses in T2DM patients were found narrower and thinner than those in control subjects, suggesting that bone formation as well as mineralization in T2DM group are suppressed as compared to normal subjects. High-resolution peripheral quantitative computed tomography revealed that T2DM patients had higher cortical pore volume in distal radius and also a lower bone strength as compared to control subjects[178]. Most diabetic patients carry biochemical and histological evidence of adynamic bone, a result of a general decrease of bone turnover[179,180]. Histologically, this is noticeable as a reduced osteoid area and a reduced bone formation rate (BFR). There are many mechanisms through which the pathophysiology as well as the treatment of diabetes may affect the bone metabolism. The changes in the BMD in diabetes can be caused by hypoinsu-

linemia, low peak bone mass, changes in transcriptional regulation, increased production of AGEs, negative calcium balance, deteriorated renal function, increased production of inflammatory cytokines, and others[181].

As described in the introduction, insulin plays a major role in bone formation through its action on osteoblasts as well as IGF-1 receptors[51]. BMD is directly correlated with circulating insulin levels and inversely correlated with insulin sensitivity[52,53]. Initial hyperinsulinemia is the reason why high BMD levels can be found in type 2 diabetics, as hypoinsulemia is held responsible for a low BMD in type 1 diabetics[55]. Although hyperinsulinemia can result in an increased BMD, it also results in mineralization defects undermining the physical strength of bone. Evidence suggests insulinemia cannot be interpreted without evaluation of insulin resistance, which mostly has to be held accountable for the loss of BMD in diabetic patients[182]. It is also worth mentioning that insulin sensitivity of osteoblasts was found to be promoted by vitamin D, which tends to vary among individuals[54]. Furthermore, insulin was found to play an important role in the biosynthesis of growth plate proteoglycans, which are crucial for the longitudinal growth of skeletal bones[183]. In untreated streptozotocin (STZ)-induced diabetic rats, the rapid dynamic state of biosynthesis and degradation of proteoglycans undergoes a shift towards less synthesis and more degradation, resulting in a decreased growth[184,185].

A chronic hyperglycemic state impairs osteoblast function which is considered the onset of diabetic osteopenia. It also diminishes the response of osteoblasts and osteoclasts to vitamin D, which is important as the diabetic population tends to have lower levels of vitamin D compared to a non-diabetic population[186]. Hyperglycemia is also associated with reduced serum levels of IGF-1, which can result in growth retardation. As previously mentioned, T1DM as well as T2DM subjects have an increased level of AGEs, which correlates with their blood glucose levels[161]. Accelerated formation of advanced glycation/lipoxidation and endproducts has been implicated in the pathogenesis of various diabetic complications, and AGEs also tend to deposit in the collagen bone matrix, resulting in impairment of bone mineralization, diminished bone complexity and decreased bone strength[160,187]. In the bone AGEs form cross-links between the arginine and lysine residues in collagen, weakening collagen structure as well as mineralized bone structure[188,189].

Cross-linking in the organic bone matrix by AGEs could adversely affect the fracture resistance of bone[190]. So although in diabetes BMD may increase, it goes along with loss of bone strength[55,149]. Higher BMD, but increased risk of fractures left scientists puzzled for years and made studies of development of bone pathology in T2DM produce paradoxical results[191]. Subjects with low femoral BMD are mostly hypoinsulinemic, while people with normal or increased BMD may be at high risk of fracture without being discovered

through ordinary BMD screening[192]. Accumulation of AGEs in the bone was also shown to result in osteoblastic dysfunction through an inhibition of Coll1 and OCN synthesis and a reduction of bone nodule formation, but also to increase the activity of osteoclasts[164]. Furthermore, AGEs were also found to inhibit PTH secretion, which decreases steadily in T2DM patients and is very low in T1DM patients[193]. It more specifically inhibits the secretion of PTH in response to hypocalcemia[194]. Studies have revealed that plasma content of pentosidine, a biomarker for AGEs, shows a significant linear correlation with that in cortical bone, suggesting that serum pentosidine level could be used as a surrogate marker for its content in bone and could evaluate bone strength. Serum pentosidine levels have also been associated with the prevalence of vertebral fractures in postmenopausal T2DM women[164,190].

Diabetes results in the generation of oxidative stress and increased inflammatory responses like TNF-α, IL-1, IL-6 and acute phase proteins[195]. The inflammatory state caused by diabetes results in a decreased osteoblast as well as osteoclast activity. Upon biochemical examination of the serum, decreased concentrations of bone formation as well as resorption markers are observed. Nevertheless, increased inflammatory factors like TNF-α, IL-1 and IL-6 are known to activate pre-osteoclasts into active osteoclasts, and could therefore be involved in the development of osteoporosis in diabetes[190]. The metabolism of OPG and BMPs is also altered.

Obesity and diabetes, like menopause, was also shown to result in a decreased sensitivity of LPR. As mentioned in the section about nutrition and the bone, loss of LP signaling results in uncontrolled food intake, reduced bone growth and reduced cortical as well as trabecular bone mass, through the diminished effect of LP on osteoblasts, osteoclasts, chondrocytes and adipocytes[34-36]. In the bone of T2DM patients, OCN, OPG and RANKL mRNA were found to be lower compared to controls, and there was an increased degree of bone anisotropy[196]. Serum concentration of sclerostin, an inhibitor of bone formation, is significantly higher in T2DM patients compared to control patients, and is correlated with the duration of T2DM, glycosylation of hemoglobin, markers of bone turnover, and BMD in T2DM patients[197]. Sclerostin is negatively correlated with cortical BMD and IGF-1.

The clinical onset of T2DM, apart from a reduced osteoblast activity, seems to be marked by a reduction in the amount of circulating progenitor cells, partly attributable to a bone marrow defect. This effect is initially compensated with the bone marrow reserve, until the bone marrow reserve seems exhausted at some point, which is associated with an increase of bone marrow fat[198]. This reduction of progenitor cells ultimately has an effect on their osteoblastic descendants[199].

Hypercalciuria and renal failure were also found to result in a demineral-

zation of the bone. The development of diabetes-associated angiopathy was also shown to be associated with generalized osteopenia. Adynamic bone is also characterized by the inability to handle increased calcium loads, resulting in increased artery calcification[200].

Glycated hemoglobin is not specifically inversely related to BMD. In patients with complications associated to diabetes, however, the fracture risk clearly is inversely correlated with BMD[173]. Other diabetes-related pathologies like diabetic retinopathy can cause an increased propensity to fall due to limited eyesight, further aggravated by instability due to old age, which also indirectly increases the risk of fracture[174]. With age, genetic expression profile also tends to change. The calcitonin receptor on osteocytes for example, was shown to decline with age[201], and also the sensitivity of the endogenous LPR was found to decrease with age as well as BMI[33,45].

3.1.3.5. Treatment of diabetes

A physically active, healthy lifestyle and prevention of diabetic complications, along with calcium and vitamin D repletion, represent the mainstay of prevention of osteoporosis in T1DM and T2DM patients[55]. Early lifestyle modification is most effective at controlling the insulin resistance syndrome. A healthy lifestyle involves the achievement of the ideal body weight and performing sufficient aerobic exercise. Furthermore, glycemic control is considered crucial for bone health in diabetics. Hip BMD of diabetic subjects at risk should be closely followed up. In elder population, treating neuropathy and retinopathy was also proven to be effective in the reduction of fracture incidence[174]. Drugs used to treat T2DM are primarily based on the management of blood glucose. Many pharmaceutical classes of anti-hyperglycemic agents are currently available for the treatment of T2DM and many others are still in development.

Either exogenous insulin can be administered or substances directly influencing the working mechanism of insulin, which can be classified as either secretagogues (e.g. sulphonylureas (SU), DPP4 inhibitors), which stimulate the β-pancreatic cells to increase the production and release of insulin, or sensitizers (e.g. metformin), which improve the uptake of glucose into the individual cell. Other treatments, like α-glucosidase inhibitors which are used to delay the digestion of starch in the small bowel, can also be effective to lower glucose levels. Recent research also led to the development of gliflozins, pharmaceuticals inhibiting the renal reabsorption of glucose.

Data has shown that more than 50% of patients require a second medication to control diabetes after being on monotherapy for three years[202,203]. A combination of treatments is often used[204], at the risk of causing hypo-

glycemia, a common complication inferring physical, social and psychological harm[205]. Many of the available pharmacological treatments of diabetes also have a secondary effect on bone quality. At this moment there is still a serious need of effective drugs for T2DM, as many of the developed antidiabetic therapeutic agents (mostly oral hypoglycemic drugs) that have proven to have beneficial effects, are also associated with undesirable side-effects, as can be seen in the summary below (table 3.1). Each of the candidate compounds has to be tested extensively as T2DM patients suffer chronic exposure, and the influence of most treatments in specific organs or in specific populations is not yet fully understood[206]. An overview of current available pharmaceuticals to treat T2DM, as well as their molecular targets and adverse effects, is given in the table below.

Table 3.1. *Current antidiabetic therapeutic agents, their mechanisms of action and their adverse effects (T2DM).*

	Drug class	Molecular target	Mechanism/actions	Adverse effects
Direct influence	Insulin	Insulin receptor	Correct insulin deficiency	Hypoglycemia, weight gain
Secretagogue	Sulphonylureas	SU receptor/ ATP-K^+-channel	Stimulate insulin secretion	Hypoglycemia, Weight gain
	Meglitinide	SU receptor/ ATP-K^+-channel	Stimulate insulin secretion	Gastrointestinal disturbances, lactoacidosis
	GLP-1 analogues	GLP-1 receptor	Stimulate insulin secretion	Gastrointestinal disturbances, nausea, abdominal pain, weight loss
	DPP4 inhibitors	GIP, GLP-1	Stimulate insulin secretion	Nausea, diarrhea, loss of appetite
Sensitizer	Metformin (Biguanides)	AMP kinase (activator)	Inhibition of hepatic glucose output	Gastrointestinal disturbances, lactic acidosis
	Thiazolidinediones (Pio- & Rosiglitazone)	PPAR-γ	Increase insulin sensitivity	Weight gain, edema, anemia, increased fracture risk
	(Dual) PPAR agonists			
α-glucosidase inhibitors	Acarbose, miglitol	α-glucosidase (inhibitor)	Delay the digestion of starch in small intestine	Gastrointestinal disturbances
SGLT-2 inhibitors	Gliflozins	SGLT-2 (inhibitor)	Inhibition of glucose reabsorption in kidney	Ketoacidosis, urinary tract infections, hypoglycemia

Insulin is very effective at lowering glucose concentrations in the blood, but insulin treatment can cause hypoglycemia as well as weight gain. In the bone, it was found to have an anabolic effect, which was described in the previous section. Surprisingly, insulin treatment has been shown to increase the risk of bone fractures in T2DM men[207].

Secretagogues are substances that increase the secretion of insulin from

the β-pancreatic cells, and also generally influence body weight. Among them are ATP-dependent K^+-channels (e.g. sulfonylureas (SUs) and meglitinides), GLP-1 analogues and DPP4 inhibitors. SUs and meglitinides bind SU receptors on ATP-dependent K^+-channels on the surface of β-pancreatic cells to increase the intracellular concentration of potassium, which causes the electric potential over the membrane to change. The resulting rise in intracellular calcium leads to increased fusion of insulin granulae with the cell membrane, and therefore increased secretion of insulin. Although some unpublished studies show a mild increase of fracture risk in T2DM patients treated with SUs compared to patients treated with metformin, a clear relationship between the treatment with SUs and fracture risk has not been established[208]. A relationship between meglitinide treatment and fracture risk has also not been reported. Synthetically produced GLP-1 and GLP-1 analogues are injected in T2DM patients because of their proliferative action on the pancreatic β-cells, promoting the secretion of insulin thus increasing the glucose tolerance by compensating the presented insulin resistance by a higher insulin concentration. GLP-1 agonists have been proven to have a positive effect on bone metabolism in experimental studies[196], although this has not been confirmed in clinical studies[209]. DPP4 inhibitors were introduced as a diabetic treatment since they were proven to increase the half-life of GIP and GLP-1 together[210,211]. The effect of GLP-1 as well as other substrates on the bone will be discussed extensively in a later section.

Sensitizers are substances that effectuate a more efficient transport of glucose towards the individual cells, increasing insulin sensitivity, and are mostly not associated with any weight gain. Among them are biguanides, thiazolidinediones or glitazones and dual peroxisome proliferator-activated receptor (PPAR) agonists. Metformin belongs to the biguanide class of antidiabetic drugs, is absorbed slowly and improves hyperglycemia through suppression of hepatic glucose production. The average T2DM patient has three times the normal rate of gluconeogenesis. Metformin is an AMP kinase activator in hepatocytes, reducing their glucose production, and also mediates the secretion of GLP-1 from the intestinal L-cells, an effect which is independently enhanced by the combination with incretin treatment[212]. This drug is often administered in combination with other drugs. A combination of metformin with thiazolidinediones shows in vitro and in vivo osteogenic effects and prevents the in vivo anti-osteogenic actions of the latter[213]. Thiazolidinediones or glitazones bind to peroxisome proliferator-activated receptor gamma (PPARγ), a type of nuclear regulatory protein involved in transcription of genes regulating glucose and fat metabolism. PPARs act on peroxisome proliferator responsive elements, influencing sensitive genes, which enhance production of mRNAs of insulin dependent enzymes, resulting in a better use of

glucose by the cells. Additionally, PPARγ has been implicated in the pathology of numerous diseases including obesity, diabetes, atherosclerosis and cancer. Thiazolidinediones are in the meantime known to reduce bone formation and promote bone loss and fractures as well as bone marrow adiposity through PPARγ dependent mechanisms[150,214]. These antidiabetics should therefore be avoided in postmenopausal women or in case osteoporosis is diagnosed[55,215].

Among other diabetic treatments, α-glucosidase inhibitors reduce the digestion of carbohydrates and thus decrease intestinal glucose absorption, resulting in a reduction of glycemia. These compounds have also been shown to increase calcium absorption from the gastrointestinal tract, making it a potential treatment for osteoporosis as well as diabetes[216]. Recently, a new class of antidiabetic agents was introduced, based on the inhibition of glucose reabsorption in the kidney. These gliflozins inhibit the activity of the SGLT-2 transporter in the renal tubule, and prevent the reabsorption of glucose from the tubular lumen back to the serum, causing glycosuria.

Beneficial side-effects commonly included with diabetic treatments are the increase of high-density lipoprotein (HDL) cholesterol and the decrease of triglycerides, uric acid, blood pressure and body weight. Most oral treatments against T2DM unfortunately also result in an increase of proteinuria, with the exception of DPP4 inhibitors. Another advantage of metformin is that it does not lower glucose in non-diabetics. In addition to non-pharmaceutical recommendations, mostly metformin is chosen as a first-line pharmaceutical treatment. However, metformin can cause a number of gastrointestinal side-effects, like diarrhea, lactoacidosis, cramps, nausea and increased flatulence. In many cases, the second choice is treatment with sulfonylureas, which unfortunately cause weight gain. GLP-1 agonist treatment offers additional non-glycemic benefits, like clinically relevant improvements in body weight and systolic blood pressure, as well as a potentially beneficial effect on β-cell function[217]. GLP-1 agonists are often favored over DPP4 inhibitors as they have a better outcome with regard to weight and blood pressure[218]. Then again, GLP-1 agonists are not always well tolerated and oral administration is also a desirable feature, making the use of DPP4 inhibitors a valuable alternative[219].

3.1.3.4. Diabetes and DPP4

In T1DM patients, fasting serum DPP4 activity is increased and the lymphocyte-bound DPP4 is decreased[220]. In children with T1DM, also the serum IGF-1 and APN was found to be decreased, and modulation of DPP4 activity cannot be achieved through glycemic control[221]. In T2DM, DPP4 activity was also found to be correlated with hyperglycemia as well as glycated hemoglobin[222-224]. It has been shown that the circulating AGEs in the serum

are also independently correlated with the circulating levels of DPP4 in T2DM patients, possibly due to an upregulation of cellular DPP4 expression[225]. Microvesicle-bound DPP4, the major form of DPP4 in urine, was positively correlated with the urinary albumin-creatinine ratio in patients with T2DM, suggesting that urinary level of DPP4 is associated with the severity of diabetic kidney disease[226]. A higher level of DPP4 activity was also observed in obese subjects.

In obese subjects as well as diabetic patients, lower peak concentrations of DPP4 substrate GLP-1 but not GIP were observed. However, obesity also results in an increased GIP but not GLP-1 metabolism[227,228]. A decreased level of active GLP-1 could not be confirmed in every clinical study[229]. T2DM is also associated with a reduced suppression of glucagon after meal intake, which was suggested to be caused by a reduced activity of GIP, GLP-1 and GLP-2. Remaining activity of GIP is mostly considered responsible for the remaining glucagon expression in T2DM patients after meal intake[230]. A reduction of active VIP has also been found to contribute to a reduced post-prandial insulin response[231]. The VIP/VPAC1 signaling pathway was found to be highly relevant in the pathogenesis of T2DM, and the differences in expression of VPAC1, which seem to be also gender-dependent, were found to be important in the genetic predisposition to T2DM[232].

Although the roles of DPP4 and substrates in the pathogenesis of T2DM are not known, the increase of DPP4 in diabetes as well as the alteration of activity of some of the substrates during the development of hyperglycemia indicates that DPP4 and its substrates are either involved in the pathogenesis of T2DM or directly affected by its pathogenic evolution.

3.2. DPP4 and the bone

3.2.1. DPP4 activity in the bone

DPP4 expression has already been shown on the surface of osteoblasts[233] as well as osteoclasts[234], but the function of this expression in bone metabolism has never been elucidated. The effects of GLP-1 based therapies and gliptins on the bone have until now not clearly been established. Many substrates of DPP4 as well as their receptors have been shown to be expressed in osteoblasts, osteoclasts and osteocytes, as well as nerve fibers throughout the bone. Degradation of collagen is effectuated through a complex interaction of various proteins, of which matrix metalloproteinases (MMPs), prolidase and DPP4 are key players[235-237]. Matrix metalloproteases 2 and 9 (MMP2/9) in the serum can be used as a determinant of bone turnover as they are expressed by both active osteoclasts and osteoblasts[238]. MMP9 was also found to play a

role in osteoclast recruitment[239]. Reduced fragments of alpha collagen chains containing glycine-proline sequences are also DPP4 substrates, which are recognized by DPP4 and are slowly broken down to small dipeptides[236,240].

The expression of DPP4 has been shown to vary according to pathologies affecting the bone. Although its role in diabetes is unclear, DPP4 expression was known to increase in hyperglycemic conditions. It has also been shown that the DPP4 activity measured in the serum of osteoporotic patients is associated with the severity of osteoporosis[241], indicating that the regulation of DPP4, if not involved in the pathophysiology of these bone diseases, is affected by their development. Deletion of the androgen receptor, crucial for bone accrual during puberty and bone maintenance during adulthood, was also found to result in an increase of DPP4 mRNA expression on the surface of mineralizing osteoblasts[242]. The use of DPP4 inhibitors, although this could not be confirmed by previously mentioned experimental findings, was also associated with a reduction of blood pressure. In general, it has been shown that treatment with blood pressure lowering agents is associated with a decrease of the occurrence of fractures[243]. Interestingly, on the other hand, different authors have found that DPP4 activity is essential for growth factor-induced cell proliferation and the production of extracellular matrix in other cell types[244,245].

3.2.2. Role of DPP4 substrates in the bone

3.2.2.1. GIP increases bone formation and reduces bone resorption

In the bone, GIPR mRNA and protein was observed in normal osteoblast-like cell-lines and bone marrow stem cells[246]. GIP binding to GIPR results in an increase of osteoblast proliferation, intracellular Ca^{2+} and cAMP increases coupled with the increased presence of markers of bone formation, ALP activity and Coll1 mRNA[247]. In different osteoblastic cell lines, the expression of GIPR was found to vary, suggesting a variable expression during osteoblastic differentiation[248].

The mRNA as well as protein expression of GIPR was also observed in osteoclasts. Binding of GIP to its receptor on osteoclasts results in an inhibition of bone resorption[249]. In control rats, there is a correlation between the GIPR expression level and BMD. Intravenous GIP administration apparently has inhibitory effects on bone resorption, and prevents bone loss in ovariectomized rats[249]. In some studies with ovariectomized rats, GIP was even found to result in an increase of BMD[250]. GIPR was also found on the surface of osteocytes, where it is known to increase the synthesis of collagen[247]. The expression of functional GIPR was shown to decrease on bone marrow stem cells with age, and GIP administration was also shown to normalize age-induced bone

loss in mice[251]. In GIPR knockout mice, a reduced bone mineral density was observed[250], as well as an altered bone microarchitecture and bone turnover[252]. Adult GIPR$^{-/-}$ mice show reductions in parameters for bone formation as well as increases in plasma Ca^{2+}-levels after meal ingestion[253], so GIP may provide a direct link between Ca^{2+} from an ingested meal and calcium deposition in the bone. GIPR knockouts also develop a low bone mass phenotype, increased bone resorption and cortical osteopenia, resulting in a significant reduction of bone strength and quality[254], translated in a significant reduction in ultimate load and stiffness in three-point bending tests on the femoral bone. A pronounced reduction in the degree of mineralization of bone matrix and a reduced amount of mature cross-links in the collagen matrix were also observed in GIPR$^{-/-}$ animals[255]. In a recent study, significant augmentations in ultimate load and stiffness were demonstrated in cortical bone of rats undergoing a four week treatment with an N-terminally modified GIP (resistant to degradation by DPP4), associated with a significantly increased mineralization and collagen maturity of the bone matrix[256]. Bone quality was also seen reduced in a nano-indentation experiment, indicating a reduced mineralization profile in GIPR deficient animals[257].

In conclusion, GIP has an anabolic effect on the bone, but whether there is any possibility to alter bone turnover by long-term administration of GIP in the clinical setting, is not yet known[258]. In T2DM patients, GIP levels are normal or slightly increased in the serum, and GIPR agonist-desensitization and down-regulation probably plays a major role in the reduction of insulin secretion. T2DM also results in a reduction of GIPR mRNA and protein levels in the islets of diabetic rats, consistent with the observation of defective GIP response in diabetic animals as well as humans[259]. GIPR was shown to be downregulated with hyperglycemia[260]. Experiments have shown that the absence of GIPR in diabetic mice correlates with a resistance to obesity[261]. GIPR is present on the surface of osteoblasts and osteoclasts, and deactivation by DPP4 will prevent circulating GIP to bind on its receptors in osteoblasts and osteoclasts. It is thus probable that the systemic inhibition of DPP4 will result in an increase of circulating GIP levels, and thus an increase of bone formation and a reduction of bone resorption.

3.2.2.2. GLP-1 has an anabolic effect on the bone in experimental studies

GLP-1 was found to be an active regulator of bone turnover. It also indirectly influences the bone metabolism through regulation of nutrition uptake. Two different types of GLP-1 receptors can be found on active osteoblasts with different affinities, one in osteoclasts[262].

In osteoblasts, GLP-1 exerts an insulin- and PTH-independent bone anabol-

ic action, which is independent of changes in the intracellular cAMP content, and has been shown to occur in control as well as insulin resistant and T2DM rats. GLP-1 was found to decrease Runt-related transcription factor 2 (Runx2) but increase osteocalcin as well as OPG gene expression[262] in osteoblasts of healthy rodents. In diabetic subjects, GLP-1 also effectuated an increase of RANKL expression[196]. In streptozotocin-induced diabetic rats and insulin-resistant rats, GLP-1 administration was also found to result in an improvement of bone architecture[196]. Also, the administration of GLP-1 was found to increase the activities of intracellular PI3K and MAPK, linked to the proliferative action of GLP-1[262].

In osteoclasts, GLP-1R binding results in postprandial suppression of bone resorption through a calcitonin-dependent pathway[254]. The GLP-1R agonist exendin-4 was shown to prevent osteopenia in aged ovariectomized rats through the promotion of bone formation as well as suppression of bone resorption[263]. GLP-1R was also found on osteocytes, where it was shown to colocalize with sclerostin and reduce its expression in normal and diabetic animals[264,265]. In hyperlipidemic rats, GLP-1 agonists were shown to reverse hyperlipidemic osteopenia, by effectuating an increased OCN and OPG expression causing an increased femoral as well as vertebral bone mass and a normalization of bone remodeling[266]. In diabetic subjects, hyperglycemia was found to be correlated with a down-regulation of GIPR as well as GLP-1R, most probably resulting in a decrease of their beneficial effect[259,260]. In case of hyponatremia (low serum levels of sodium), trabecular bone was found to dissolve slowly[267]. As GLP-1 induces an increase of Na-excretion, a longer half-life of GLP-1 may have an indirect negative effect on bone metabolism through a decrease of serum sodium. Sodium was also found to influence the bone metabolism through a Na-sensing mechanism on osteoclasts, and also influences serum osteocalcin[268].

GLP-1 thus clearly has an anabolic effect on the bone, both promoting bone formation through binding on its receptor on osteoblasts and inhibiting bone resorption through binding on osteoclasts. Although this effect has been shown in various studies comprising healthy as well as diabetic experimental animals, a recent clinical meta-analysis of fracture outcome in T2DM did not show any beneficial effects of GLP-1 receptor agonist use on the bone metabolism[209].

3.2.2.3. GLP-2 effectuates postprandial decrease and inhibits nocturnal rise of bone resorption markers

GLP-2 is also an important mediator of bone turnover. In the bone, GLP-2 receptors were found exclusively on osteoclasts, where GLP-2 binding induces

an increase of intracellular cAMP[262]. It is, like GIP and GLP-1, responsible for the decrease in bone resorption after meal intake[254,258].

In an experiment by Henriksen in 2003, parenteral administration of GIP and GLP-1 did not result in a reduction of bone resorption markers serum C-terminal telopeptide (CTX-1) and deoxypyridinoline in postmenopausal women, whereas GLP-2 caused a statistically significant and dose-dependent reduction of these resorption markers compared with placebo. The serum osteocalcin level was unaffected by the GLP-2 treatment[258]. During the night, subcutaneous administration of GLP-2 results in a reduced presence of bone resorption markers in healthy postmenopausal women[269]. GLP-2 treatment is clearly only associated with a reduction of bone resorption and is independent of any changes in bone formation[270]. Over a study period of four months, treatment with GLP-2 was found to result in a significant dose-dependent increase of total hip BMD[271]. Long term exposure to GLP-2 is more effective than administration of high GLP-2 concentrations in the reduction of the nocturnal rise in bone resorption markers[272]. In a clinical study with short bowel patients, administration of GLP-2 over a 5-week period resulted in a decrease of bone resorption and an increased BMD[273]. GLP-2 is essential for the reduction of bone resorption after feeding, which was shown in Roux-en-Y gastric bypass patients who display a severely increased GLP-2 response after feeding and an increased bone turnover[274]. The effect of GLP-2 administration on nocturnal as well as postprandial bone resorption markers in the serum has been clearly shown in experimental as well as clinical studies.

3.2.2.4. The SDF-1α/CXCR4-axis is a strong chemotactic for active bone cells

Another important target for DPP4, SDF-1α, is expressed in mature osteoblasts. SDF-1α as well as its receptor CXCR4 are also expressed in osteoblast progenitor cells that migrate from the bone marrow towards sites of new bone formation under the influence of the SDF-1α chemotactic signal. SDF-1α and its receptor CXCR4 were found to play a major autocrine role in the regulation of osteoblast activity and as well as the migration and transdifferentiation of bone marrow progenitor cells. Perturbing the SDF-1α signaling affected the differentiation of MSCs towards osteoblastic cells in response to BMP2 stimulation[275]. Blocking of SDF-1α signaling inhibits the BMP2-induced early expression of Runx2 and Osx, two master regulators of osteogenesis. SDF-1α also directly promotes early stages of osteoclast differentiation resulting in a stimulation of precursor cell numbers and multinucleated cell fusion and TRAP activity. SDF-1α also prevents osteoclast apoptosis induced by cytokine withdrawal. High levels of SDF-1α produced by bone endothelium, bone marrow stem cells and osteoblasts may selectively target circulating os-

teoclast precursors into bone and stimulate their marrow migration into suitable perivascular stromal sites for their early development[276]. Furthermore, lipopolysaccharides were shown to upregulate the expression of CXCR4 in pre-osteoclasts through TLR4, but not in osteoblasts. The SDF-1α/CXCR4-axis thus plays a role in lipopolysaccharide induced inflammatory bone resorption[277]. SDF-1α is also expressed in cortical and trabecular osteocytes, playing a role in the recruitment of CXCR4-bearing bone marrow cells. Furthermore, SDF-1 and CXCR4 expression were enhanced in response to mechanical stimulation. Adaptive changes in bone formation can be inhibited using a CXCR4 receptor antagonist[278].

SDF-1α and its expression by osteoblasts were shown to be required for the bone mineralization process and could be an interesting target for bone tissue repair, regeneration and modulation of osteogenesis[279,280]. But, as the CXCR4 receptor is also expressed on (pre)osteoclasts, the SDF-1α/CXCR4-axis also plays a role in bone resorption and needs to be studied further to evaluate its precise role in the bone metabolism[281].

3.2.2.5. NPY regulates bone turnover on a central and peripheral level

The skeleton is widely innervated and neuropeptides like NPY and PYY have been shown to be expressed by skeletal nerve fibers, which are present in the periosteum, cortical bone, bone marrow and the growth plate[282]. These neuropeptides are known to exert paracrine biological effects on bone cells present close to the nerve endings releasing the peptides. This interplay has been convincingly proven by the hypothalamic control of bone formation, LP stimulation of hypothalamic nuclei and the presence of peptide receptors on osteoblasts and osteoclasts. But NPY is also expressed in osteoblasts, osteocytes, osteoclasts and chondrocytes, in embryonic stages as well as in the adult[137,283,284]. In osteocytes, expression of NPY is more elevated than in osteoblasts and is able to inhibit osteoblast activity through the Y1 receptor expressed on osteoblasts[285-287]. Nevertheless, increased NPY activity has also been related to increased bone mass[283].

Mice lacking NPY, have significantly increased bone mass in association with enhanced osteoblast activity and elevated expression of bone osteogenic transcription factors, Runx2 and osterix. In contrast, wild type and NPY knockout mice in which NPY is specifically overexpressed in the hypothalamus show a significant reduction in bone mass despite developing an obese phenotype. This induced loss of bone mass is consistent with models known to mimic the central effects of fasting, which also show increased hypothalamic NPY levels. NPY is thus a critical integrator of bone homeostatic signals, increasing bone mass during times of obesity when hypothalamic NPY expression levels

are low and reducing bone formation to conserve energy under conditions of low energy when hypothalamic NPY expression levels are high[288]. Specific knockout mice lacking the Y1 receptor in osteoblasts only have a severely altered glucose homeostasis with an increased serum glucose and a decreased glucose-mediated insulin response. Osteoblast-specific Y1 stimulation was shown to improve glucose tolerance and glucose-mediated insulin response, indicating yet again the importance of the bone-brain axis in feeding and bone turnover[289]. Bone and adipose tissue mass are elevated in $Y1^{-/-}$ mice with a generalized increase in bone formation on cortical and cancellous surfaces. Inhibitory effects of NPY on bone marrow stromal cells in vitro are absent in cells derived from $Y1^{-/-}$ mice, indicating a direct action of NPY on bone cells via this Y receptor. Conditional deletion of hypothalamic Y1 receptors in adult mice did not alter bone homeostasis, food intake, or adiposity. Treatment with NPY of a calvaria-derived osteoblast culture expressing Y1 results in a reduced expression of markers of osteoblast differentiation, indicating that NPY might locally mediate the down-regulation of osteoblast differentiation[285]. The hypothalamic neuropeptide Y2 receptors were also found to be essential for control of bone metabolism by a peripheral mechanism not yet known[284,290]. Y2 receptors were shown to exhibit an anti-osteogenic effect on trabecular but not on cortical bone, through hypothalamic NPY-expressing neurons[291]. The effects of NPY on the hypothalamus were found to be counteracted by effects of LP binding. Thus Y1 receptor pathways act powerfully to inhibit bone production by non-hypothalamic pathways, with potentially direct effects on bone tissue through a single pathway with Y2 receptors[288,290,292]. For the direct osteoblast inhibitory action of Y1 receptor binding, Y1 was proposed as a potential drug target candidate for prevention and treatment of bone loss[286]. An increase of bone mass was also observed in mice lacking Y1 as well as Y2 receptor genes. Furthermore, deletion of both Y1 and Y2 receptors did not produce additive effects in bone or adiposity. Chronically elevated levels of NPY lead to transient down-regulation of the Y1 as well as Y2 receptor expression, according to the stage of osteoblast differentiation. Stimulation of osteoprogenitor cells by NPY results in a decreased expression of Y1, which increases osteoblast phenotype markers. Nevertheless, these cells exhibit a reduced calcium deposition in the extracellular matrix, which is most likely mediated by Y2 receptor signaling. NPY inhibits the transcriptional activity of the RANKL promotor in osteoprogenitor cells and enhances OPG expression in osteoblasts at early stages of differentiation. Then again, this effect of NPY on OPG seemed to be unrelated to Y2 receptor activation[293]. NPY was also recently shown to protect against excessive stress-induced bone loss through Y2 receptor-mediated modulation of central and peripheral noradrenergic neurons[294]. Altogether, the bone turnover is regulated by NPY on a central as

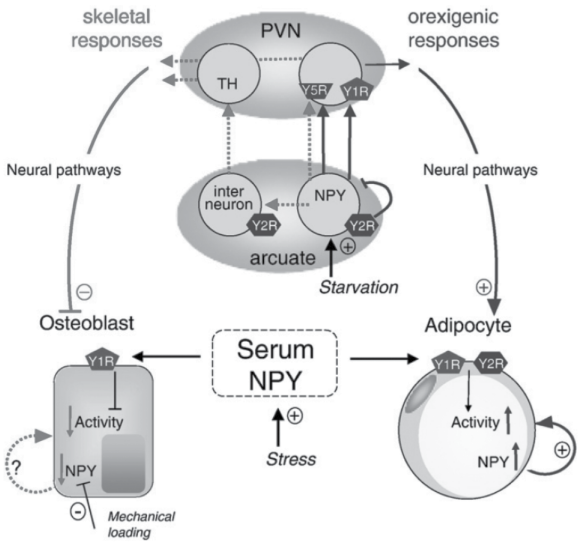

Figure 3.8. *Central and peripheral regulation of bone and fat mass by NPY and its receptors Y1, Y2 and Y5. Centrally, increased NPY expression in neurons in the arcuate nucleus propagate orexigenic signals (blue arrows) via Y1 and Y5 receptors. These neurons are also known to induce skeletal responses (red arrows) through an unknown mechanism (dotted lines). NPY is also produced by peripheral sources (most notably during stress) and can signal directly to Y1 receptors in bone cells and Y1 and Y2 receptors in fat cells. NPY is also expressed in adipocytes and can stimulate lipid accumulation, while the Y1 binding in osteoblasts reduces the response of the bone to mechanical load*[291]*.*

well as the peripheral level as shown in figure 3.8, with NPY originating from the sympathetic nervous system, osteoblasts and adipocytes[287,291].

3.2.2.6. PYY levels are negatively correlated with osteoblastic activity

PYY shares the same receptors with NPY, giving the peptide about the same effect on the bone metabolism on Y1 receptors in osteoblasts, and through the hypothalamic Y2 receptor. PYY is not expressed in the osteoblast, but was also shown to exhibit a negative relationship with osteoblast activity. PYY acts immediately on the osteoblast through Y1 signaling. PYY knockouts have an increased bone mass and an increased osteoblastic activity, like NPY knockout animals. Inducing an overexpression of PYY results in an increased osteoclast surface in the femoral metaphysis, despite the lack of PYY receptor expression in osteoclasts[295]. Reduced PYY levels are typical in obese subjects, and administration of PYY results in a reduced appetite and decreased food intake in obese adults[296]. A negative correlation was established between PYY levels and BMD, indicating a detrimental effect of PYY on bone mass[295]. In contrast,

previous studies also revealed that PYY administration resulted in an increase of BMD and PYY knockout mice had a reduced trabecular bone volume[297]. However, this could be attributed to the difficulty of producing mice with genetically altered levels of PYY, which can have lethal consequences during embryonic development in case of overexpression[298]. PYY and body weight are associated with BMD in premenopausal exercising women. Elevated PYY and suppressed estrogen concentrations are associated with, and could be directly contributing to, low BMD in exercising women with amenorrhea[299].

3.2.2.7. IGF-1 promotes osteoblast proliferation

Although primarily produced by the liver, IGF-1 is also known to be secreted by osteoblasts and osteocytes, and is an important regulator of bone growth[300]. The IGF-1 receptor is expressed on the surface of osteoblasts and osteocytes[301]. The binding of IGF-1 on its receptor on osteoblasts stimulates the expression of ALP and OCN[303]. IGF-1 also stimulates the proliferation and chemotaxis of osteoblasts[302]. Half of the basal bone cell proliferation can be blocked by inhibiting the actions of osteocyte-derived IGF-1[164]. This endogenously produced IGF-1 was found to play a role in bone mechanosensitivity[304] as well as in developmental growth and bone remodeling[305]. IGF-1 receptor expression decreases whereas insulin receptor expression increases during osteoblast differentiation. Both insulin and IGF-1 promote osteoblast differentiation and mineralization in vitro. Furthermore, IGF-1 reduces bone resorption through OPG and RANKL[303]. IGF-1, like insulin, is considered an anabolic agent in osteoblasts and bone development through the activation of protein kinase B (Akt) and extracellular-regulated kinase (ERK) signaling pathways[306]. It is also an important mediator for the actions of PTH on the bone[307]. The expression of IGF-1 is reduced in a state of poor nutrition. Long term feeding with protein supplements results in an increase of IGF-1 causing an increase of hip BMD as shown in clinical studies with patients with previous hip fracture[308]. Same observation was made in adolescent girls in a milk supplementation study[309]. A knockout in the IGF-1 receptor on the other hand results in growth retardation and a reduced osteoblastic activity, and also a reduced insulinemia. Generally, serum IGF-1 levels are positively associated with BMD and inversely with the risk of vertebral fractures in postmenopausal non-diabetic women. Also in dialysis patients, a positive correlation was found between serum IGF-1 levels and bone formation, suggesting that serum IGF-1 levels could be clinically useful for assessment of bone mass and fracture risk. Low levels of insulin or IGF-1, or loss of the sensitivity to their action, have already been associated with diabetes-associated impairment of bone quality[310].

3.2.2.8. VIP & PACAP stimulate osteoblasts and transiently modulate osteoclast activity

VIP and PACAP both exert a role in bone homeostasis and are another example of hypothalamic control of bone formation[311]. The peptidergic nerve fibers in the bone marrow were shown to express VIP and PACAP, and osteoblasts and osteoclasts express receptors for VIP as well as PACAP on their surface. Neuropeptides VIP and SP are released from skeletal nerve fibers and exert paracrine effects on nearby bone cells, in close relay with LP stimulation of hypothalamic nuclei mediated by the sympathetic nervous system and inhibitory and adrenergic receptors on osteoblasts[312].

In calvaria osteoblasts in vitro, the VPAC2 receptor is expressed, with a higher affinity binding for PACAP than for VIP. The expression of VPAC1 is induced during osteoblastic differentiation[313]. VIP, without affecting the cell proliferation, stimulates osteoblastic ALP biosynthesis and bone noduli formation by a mechanism mediated by cyclic AMP[282]. In osteoclasts, the expression of VPAC1 and PAC1 has been shown[314]. Binding of VIP as well as PACAP to receptors on osteoclasts is linked to a transient inhibition of osteoclast activity[315]. In addition, the VIP/PACAP receptors in osteoblasts are coupled to a delayed stimulation of osteoclastic resorption[316], which was found similar to the effect of mechanical loading[317]. VIP also has an inhibitory effect on osteoclast recruitment, due to upregulation of OPG and down-regulation of RANK and RANKL in late stages of osteoclast differentiation[318]. Adding VIP tot bone marrow cultures was found to result in a decreased formation of TRAP-positive multinuclear cells, an increase of in vitro osteoblast activity and a modulation of in vitro osteoclast recruitment[318]. In rat osteoblast cultures, PACAP38 signaling was shown to enhance osteogenesis increasing the expression of Coll1, osterix and ALP[319].

3.2.2.9. Substance P stimulates osteoblasts but also increases osteoclast activity

As is the case with previously discussed neuropeptides, SP is released in the bone from skeletal nerve fibers to bone cells in the vicinity. SP binds to its receptor nk-1R in the bone, which can be found on osteoblasts as well as osteoclasts[282,311]. SP stimulates the osteoblastic bone formation through the receptor in osteoblasts[320] and also plays a role in osteoblastic differentiation[321]. However, the effect of SP binding on nk-1R has a controversial effect: it results in an increase of RANKL-induced osteoclast formation in bone marrow macrophages and an increase of resorption pit size in in vivo experiments. Also, in vertebral injury models, an osteoporotic phenotype was found containing more SP-immunoreactive nerve fibers[282].

3.3. Summary and aims of the study

DPP4 expression has been observed on the surface of osteoblasts as well as osteoclasts. Osteoblasts also express DPP4 substrates, like SDF-1α, NPY and IGF-1. Many of DPP4 substrate receptors are also found on the surface of osteoblasts, like GIPR, GLP-1R, Y1, IGF-1R, VPAC1, VPAC2, and nk-1R. Aside from DPP4, osteoclasts were only shown to express the DPP4 substrate NPY. Osteoclasts also express the receptors of other DPP4 substrates, like GIPR, GLP-1R, GLP-2R, CXCR4, VPAC1, PAC1 and nk-1R. Osteocytes produce SDF-1α, NPY and IGF-1, and carry the receptors GIPR and GLP-1R. An overview of their effects on osteoblast and osteoclast activity is given in table 3.2.

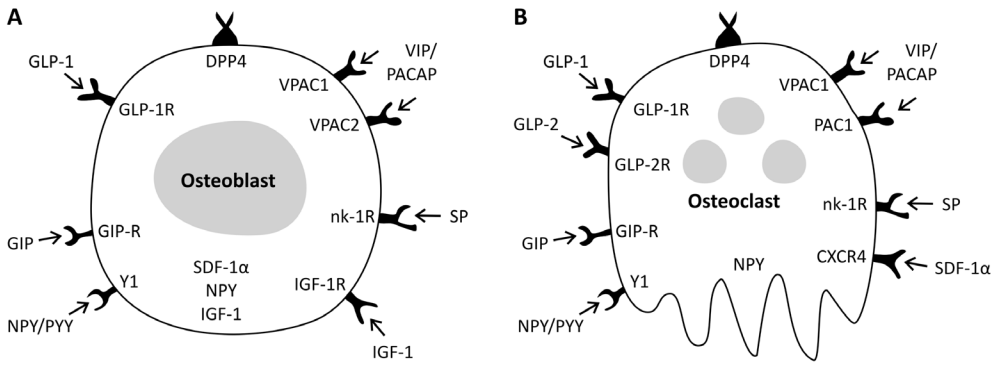

Figure 3.9. *Osteoblasts, osteoclasts and their relation to DPP4 activity and substrates.*

Many DPP4 substrates and receptors are highly dependent on the differentiation of its expressing cells and have a function in proliferation, differentiation or cell migration itself. The expression of various substrate receptors tends to change according to the differentiation state of the bone cell. The SDF-1α signal for example, was shown to play an important role in osteoblastic and osteoclastic differentiation, as well as osteogenesis. Furthermore, it is known to control the migration of osteoblast precursor cells and osteoclasts. GIPR was found to change in various osteoblastic cell lines. VIP and PACAP bind on the VPAC2 receptor in osteoblasts, but also VPAC1 increases during the osteoblastic cell differentiation. Other DPP4 substrates like NPY and PYY have a direct effect on bone metabolism, but also indirectly exert an effect through the hypothalamic control of feeding, bone formation and adiposity. Osteocytes are clearly also responsible for regulation of bone growth through the expression of DPP4 substrates. Some other substrates like VIP, PACAP and SP are exclusively produced in nerve fibers.

DPP4 substrates thus play a crucial role in the regulation of bone homeostasis, by influencing the effect of feeding as well as hypothalamic control of the bone. DPP4 and its substrates thus link bone metabolism to the gastro-

Table 3.2. DPP4 substrates with receptors on surface of bone cells and the result of their binding activity.

Receptor	Ligand	Effect of binding
Osteoblasts		
GIPR	Circulating GIP (intestinal K-cells)*	Increase of bone formation marker ALP & Coll1 and increased BMD
GLP-1R (2)	Circulating GLP-1 (intestinal L-cells)*	Decrease of Runx2, increase of OCN, OPG and RANKL expression
Y1	NPY and PYY from OBs, OCs, osteocytes and nerve fibers	Decrease of BFR
IGF-1R	IGF-1 from OBs, osteocytes and circulation	Inhibition of OCN expression, stimulation of OB proliferation and chemotaxis
VPAC1 VPAC2	VIP/PACAP released from nerve fibers	Stimulation of ALP & Coll1 synthesis as well as bone noduli formation
nk-1R	SP released from nerve fibers	Stimulation of bone formation
Osteoclasts		
GIPR	Circulating GIP (intestinal K-cells)*	Inhibition of bone resorption
GLP-1R	Circulating GLP-1 (intestinal L-cells)*	Postprandial inhibition of bone resorption
GLP-2R	Circulating GLP-2 (intestinal L-cells)*	Inhibition of postprandial and nocturnal bone resorption
CXCR4	SDF-1α from OBs and osteocytes, OPCs and circulation	Inhibition of osteoclast apoptosis
VPAC1 PAC1	VIP/PACAP released from nerve fibers	Transient inhibition of osteoclastic activity
nk-1R	SP released from nerve fibers	Increase of osteoclast activity and osteoclastogenesis

** Dependent on postprandial release of these peptides.*

intestinal system as well as the brain. In conclusion, many DPP4 substrates have been shown to exert an effect on bone metabolism which can be considered positive for BMD and bone strength. These substrates are GIP, GLP-1, GLP-2, IGF-1, VIP, PACAP and SP. Other substrates like SDF-1α, NPY and PYY have a more complicated, indirect effects on bone metabolism, resulting in controversial findings. Furthermore, it is imperative to repeat that the activity of DPP4 as well as the expression of its substrates is affected by the development of specific pathologies like diabetes. But also osteoblasts and osteoclasts have been shown to influence the pathophysiology of diabetes themselves by affecting glucose tolerance. Vildagliptin as well as linagliptin were shown to affect the AGE-RAGE interaction, meaning that other DPP4 inhibitors have similar effects, including the prevention of the previously mentioned, diabetes-related collagen maturation defect[162,322]. A summary of these complex interactions is given in figure 3.10.

Considering the activities of DPP4 substrates in the bone, it would be of particular interest to investigate whether modulation of DPP4 activity might affect the osteoblastic collagen deposition, osteoclastic collagen resorption, as well as BMD and bone strength. DPP4 inhibitors, through increase of the

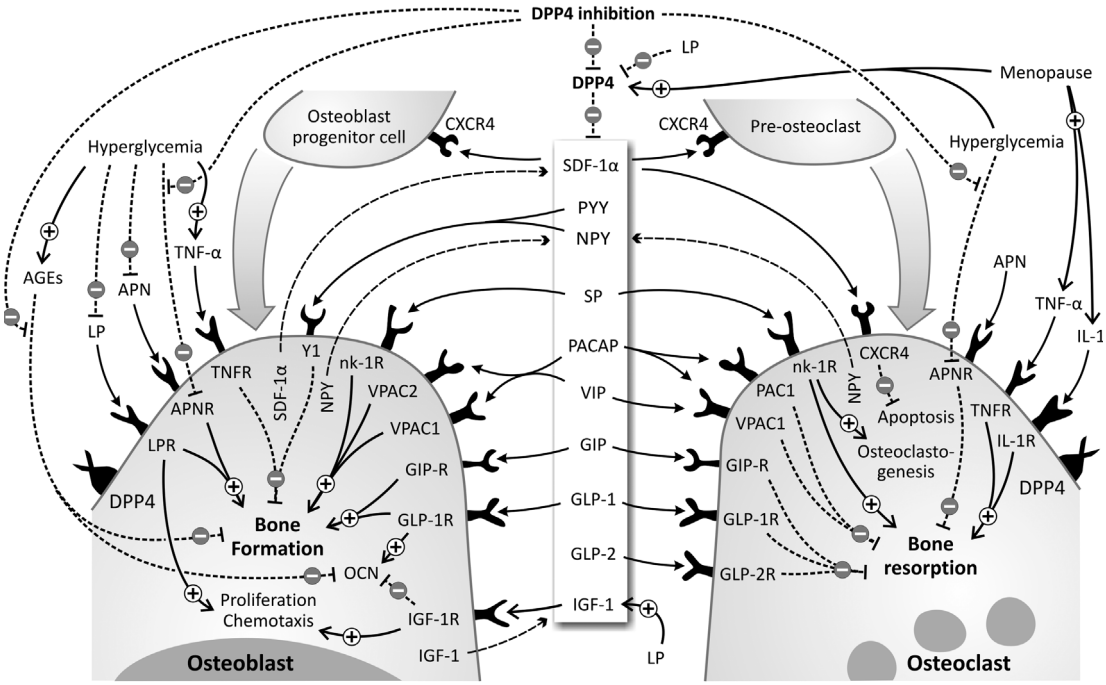

Figure 3.10. *DPP4, DPP4 substrates and active bone cells, and their interactions with bone metabolism.*
Conditions like hyperglycemia and menopause result in an increase of DPP4 activity and other alterations directly affecting osteoblasts and osteoclasts. DPP4 activity results in the truncation of its substrates resulting in decreased substrate binding on osteoblasts and osteoclasts. SDF-1α also induces the migration of OPCs and the maturation of osteoclasts. Diabetes affects insulin and LP sensitivity, as well as APN expression. Furthermore, APN sensitivity and APNR expression is reduced, and the AGE-RAGE-interaction increased in hyperglycemia, pathways which were shown to be affected by DPP4 inhibition.

half-life of DPP4 substrates, could possibly be applied here for prevention as well as recovery of decreased BMD, working through various mechanisms including feeding, insulin response and glucose tolerance, the AGE-RAGE axis, and hypothalamic as well as direct control of bone cell activity. In the following studies, the effect of DPP4 inhibition using sitagliptin will be assessed in two models associated with severe bone pathology. In the first study, the effect of sitagliptin administration will be evaluated in ovariectomy-induced osteoporosis characterized by trabecular bone loss. The effect of sitagliptin administration on diabetic bone loss in nicotinamide/streptozotocin-induced diabetic animals will be evaluated in a second study. The bone of these experimental animals will be followed up by in vivo micro-CT, and examined by bone histomorphometric analysis after sacrifice. The origin of observed effects will be further investigated with mechanical and biochemical analyses.

3.4. Materials & methods

3.4.1. Animal models

3.4.1.1. General introduction

The experiments presented are again performed using the Wistar rat, an albino laboratory outbred rat strain described in the previous chapter. All procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (no. 85–23, 1985) following demand approval for these experiments by the Antwerp University Ethical Committee.

3.4.1.2. The ovariectomized rat model for postmenopausal osteoporosis

Many techniques are available to simulate the human pathogenesis of osteoporosis: immobilization of hind limbs, orchiectomy[323] or ovariectomy[313,314], administration of glucocorticoids or retinoic acid[324], or aging[325].

Ovariectomy, the removal of both ovaries from sexually matured female animals, is a technique with very little variability and good reproducibility, recommended by the US Food and Drug Administration (FDA) for preclinical testing. In this model, the primary source of endogenous estrogen production (riping ovarian follicles) is eliminated, which induces a state of postmenopausal osteoporosis[325,326]. The bone loss displayed shortly after the procedure is characterized by a significant loss of trabecular bone and microstructure in the metaphysis of the tibia after 14 days, as can be detected by in vivo micro-CT (disappearance of trabeculae and their connections, increase of trabecular spacing)[2], and after a few months in other less metabolically active sites (like the cortical bone).

In this procedure, the female Wistar rat aged 12 weeks (Iffa Credo) is weighed and anesthetized. Ovaries are approached in a sterile manner through two separate flank incisions which are kept as small as possible, with the animal lying on its side. The skin is separated from the underlying muscle before incising the muscle. Muscle fibers are separated at the location of the ovaries in a dorsal ventral direction. The fat pad surrounding the ovary is grasped with a blunt forceps and gently pulled through the incision. A ligature is placed near the uterus at the height of the oviduct to interrupt the ovarian blood supply and a cut is made close to the ovary. With ovary and part of the oviduct removed, hemostasis is verified and the fat pad returned to the abdomen. Muscle layer and skin are closed with absorbable suture, after which the animal is turned over to remove the second ovary[327]. Uterus slowly atrophies after ovariectomy, as it does after menopause. The weight of the uterus at

sacrifice of the experimental animal confirms the success of the surgical procedure of ovariectomy. Trabecular bone loss in ovariectomized animals has been extensively described in literature (figure 3.11) and will not be taken into account in the results and discussion sections of this chapter.

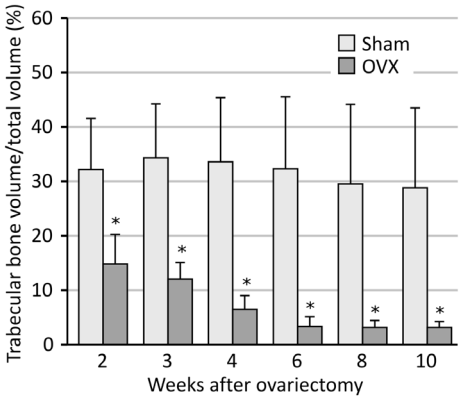

Figure 3.11. *Trabecular bone loss after ovariectomy (OVX) determined by in vivo micro-CT[7]. * p < 0.05 in OVX compared to sham operated animals*

3.4.1.3. The nicotinamide-streptozotocin-induced diabetic rat

Diabetes can be induced in healthy experimental animals through different methods: through chemical substances affecting the pancreas (alloxan[328], STZ[329] – at age or neonatally), through alteration of diet (high fat, e.g. in sandrat) or surgically[330]. Also, genetically modified, spontaneously diabetic animal models exist, like the Goto-Kakizaki T2DM rat[331], the T2DM Zucker diabetic fatty (ZDF) rat[332-334], the Cohen type 2 diabetic rat[335], the non-obese spontaneously diabetic Torii rat[336], leptin- and leptin-receptor-deficient mice (ob/ob and db/db, respectively). A specific diabetic model is used in relation to the pathology studied. Many diabetic models result in unstable insulin producing β-cells. Unstable pancreatic cells will result in a transient secretion of insulin until the pancreatic β-cells are degranulized and undergo apoptosis, resulting in a hyperglycemic condition. They lose their gained weight and become dependent of exogenous insulin, like the db/db mouse, ZDF-rat, sand rat and rhesus monkey model.

STZ is a naturally occurring cytotoxic substance which accumulates in the insulin-producing β-cells of the pancreas via the glucose transporter 2 (GLUT2) transporter, and destroys them through mechanisms involving ROS and free radicals resulting in DNA fragmentation and modification of biological macromolecules, causing insulin-dependent diabetes[337]. It can also be used medicinally to treat pancreatic cancers[338]. In experimental animals, it is used to in-

duce a insulinopenic, hyperglycemic diabetic state which can be used to study various pathologies related to diabetes mellitus like diabetic nephropathy[339], neuropathy, retinopathy, and bone pathology[340]. The STZ-induced diabetic model induces a state which closely resembles the human T1DM state, devoid of insulin production. The STZ treatment can be supplemented with nicotinamide adenine dinucleotide (NAD), a vitamin B derivative which has a proliferative and differentiating effect on β-pancreatic cells[341] and also protects them against the cytotoxic effect of STZ, to preserve a minimal number of active insulin-producing cells and a stable high-glucose hypoinsulinaemic diabetes[342]. In the NAD/STZ model, a reduced amount of insulin production resembles a state of a reduced sensitivity to insulin. The treatment with NAD/STZ results in a decrease of produced insulin stabilizing after three to four days, which was found to be exponentially related to the plasma glucose levels in non-fasting rats at four weeks after NAD/STZ administration[343]. At the lower dosage (100 mg/kg), NAD exerted only minor protection against the dramatic effect of STZ on body weight, glycemia, and insulinemia[344]. Highest NAD dosage (350 mg/kg) fully prevented STZ-induced alterations[342]. Many dosages have been used in experiments to achieve different glycemia in experimental animals[345].

Rats treated with NAD/STZ have significantly reduced quantities of urinary hydroxyproline and tibial alkaline phosphatase, are hyperphosphatemic and markedly hypercalciuric. The NAD/STZ-induced diabetes is associated with an increase of corticosterone, and decrements in both circulating PTH and $1,25(OH)_2D$, which together with decreased insulin levels are responsible for STZ-induced osteoporosis[346-348]. Induction of diabetes results in osteopenia with reduced biomechanical properties of the bone caused by a defective formation of the extracellular matrix network due to reduced chondriogenesis and calcification of the bone. Diminished bone formation and mineralization and increased bone resorption was found to be at the basis of this STZ-induced experimental diabetic osteopenia[349]. The NAD/STZ-rat has lower osteoid, osteoblasts and osteoclasts in the bone, and are osteopenic with a reduced bone turnover resulting in failure to acquire a tetracyclin label[350]. PTH is able to partly reverse these effects by suppressing osteoblast apoptosis[351]. In NAD/STZ-induced osteoporosis, the collagen of the extracellular matrix is modified by an increased concentration of AGEs in the circulation and a decreased alkaline phosphatase activity[352] and OCN secretion[58]. The bone of NAD/STZ-diabetic rats is characterized by decreases in femoral length, bone width, cortical bone thickness and bone density[353] due to stagnating cortical bone growth, and a loss of trabecular bone as measured by peripheral quantitative computed tomography[354]. The decreased thickness and density results in a decreased biomechanical integrity, a decreased fracture load and toughness, and an increased bone stiffness of the tibia as well as the femur[355]. In

some experiments the induction of diabetes did not result in a reduction of BMD, but did result in changes of femoral weight, maximum load, ultimate stress and Young's modulus at sacrifice, eight weeks after induction of diabetes[340]. Fourier transform infrared spectroscopy measured in ground cranial bone also revealed an altered bone composition with a larger mineral density but a reduced collagen maturation and carbonate content in NAD/STZ-induced diabetic animals, which was attributed to AGEs[356].

3.4.2. Biochemical analyses

3.4.2.1. Evaluation of DPP4 inhibition

DPP4 activity was measured and inhibition verified using the same measurement techniques as described in chapter 2.

3.4.2.2. Evaluation of diabetes: glucose, pentosidine and β-pancreatic islets

In diabetic subjects, basal whole blood glucose levels are increased at all times, not only after food ingestion. Whole blood glucose levels were measured using a standard automated whole blood glucometer with glucose strips (GlucoMen Lx Plus+, Menarini, Florence, Italy) in order to verify induction of diabetes as well as to provide follow-up. Pentosidine is derived from ribose, and is measured as a biomarker for AGEs. Pentosidine was measured in the serum to verify changes due to hyperglycemia as well as modulation of the AGE-RAGE axis by DPP4 inhibition, using a kit by USCNK (CEA264Ge, USCN Life Science Inc., Wuhan, Hubei, China). To assess morphological appearance of β-pancreatic islets, H&E-stained pancreatic islets were visualized under the microscope (x200).

3.4.2.3. Evaluation of bone metabolic parameters

Calcium & phosphate

Circulating calcium levels as well as urine calcium levels are increased in the presence of an elevated bone resorption, increased glomerular filtration or reduced tubular reabsorption. Calcium was determined in the serum and urine by flame atomic absorption spectrophotometry using a Perkin-Elmer AutoAnalyst 800. In the abdominal aorta, calcium deposits were assessed by Von Kossa staining. Increased levels of phosphate in the serum are indicative for an impaired kidney function or an increased bone resorption. Serum and urinary phosphate were determined using the Phosphate FS* kit by DiaSys (Diagnostic Systems GmbH, Holzheim, Germany).

OCN, CTX-1, PTH, MMP2, MMP9, prolidase and dipeptides

To assess osteoblast activity, OCN expression was measured in the serum (ng/mL) using Immunotopics Osteocalcin kit 60-1505 (ELITech Group, Puteaux, France). The serum level of CTX-1 was measured (ng/mL) to evaluate changes in bone resorption, using a kit by USCNK (CEA665ra, USCN Life Science Inc.). Due to the importance of PTH in bone turnover and calcium metabolism, serum PTH was assessed (pg/mL) using the rat PTH-IRMA kit (Immunotopics Inc., San Clemente, CA, USA). MMP2/9, a determinant of bone turnover expressed by both osteoclasts and osteoblasts[238] was measured in the serum by gelatin zymography, an extremely sensitive method based on digestion of a gelatin substrate by renaturated MMPs after a standard gel electrophoresis[357]. Prolidase activity was determined by measuring the production of proline from glycylproline[235]. The latter analyses were performed at the Laboratory of Medicinal Biochemistry of the University of Antwerp (Prof. Dr. I. De Meester). Dipeptide concentrations were determined in cooperation with the Laboratory of Toxicology of the University of Antwerp, using the liquid chromatography - tandem mass spectrometry hybrid technique, based on the EZ-FAAST kit from Phenomenex[358].

3.4.2.4. Pancreatic & liver enzymes

As many publications raised concerns about the influence of high concentrations of DPP4 inhibitors on the development of pancreatitis and hepatitis, some pancreatic and liver enzymes were measured in the serum of treated animals.

Amylase and lipase are enzymes produced by the pancreas that help to digest carbohydrates and fat. When pancreatic cells are damaged, high levels of these intracellular enzymes are released and can be detected in the bloodstream. Amylase as well as lipase can be increased because of the occurrence of acute or chronic pancreatitis, pancreatic cancer, cholecystitis, chronic kidney disease, ulcers, and others. Increased lipase levels can also be caused by coeliac disease (gluten allergy). The lipase test is considered more accurate than the amylase test for diagnosis of pancreatitis. To evaluate the effect of chronical exposure to high doses of DPP4 inhibitors on the liver, the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are measured in the serum, which are elevated in liver failure. The aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio can also be useful in differentiating between causes of liver damage (hepatotoxicity). Serum amylase, lipase, AST and ALT were determined in the serum obtained at sacrifice using an automatic cartridge reader (Vitros 5.1 Fusion auto-analyzer, Ortho Clinical Diagnostics, Rochester, NY, USA) at the Antwerp University Hospital

(UZA Edegem, Belgium).

3.4.3. Evaluation of bone quality

Different methods are available to assess bone health, of which each has its important benefits and drawbacks. Bone metabolic markers in the serum are a good indication of bone turnover but often provide insufficient information about the effect of the underlying bone pathology on bone structure and integrity. Therefore, further methods have been developed throughout the years to assess bone structure, density and activity.

Bone microstructure can be evaluated completely with in vivo X-ray micro-computed tomography, a non-invasive in vivo measurement technique allowing to determine trabecular as well as cortical bone parameters like fractional mineralized bone volume, trabecular and cortical thickness, trabecular number, trabecular separation. Bone structure can be entirely visualized by reconstructing virtual cross sections from X-ray shadow images. Using standardized hydroxyapatite phantom bones, an estimate of trabecular BMD can also be calculated. The golden standard to assess bone metabolism and disease is quantitative bone histomorphometry, which allows to determine bone volume of nonmineralized bone as well as number and activity of active bone cells, whereas micro-CT is only able to measure mineralized bone. Also dynamic parameters like bone turnover, mineralization lag time and mineral apposition rate can be calculated. Overall bone strength can be estimated using a three-point bending setup, in which the bone is bent with a stepping motor and physical properties like bone stiffness, ultimate load, ultimate displacement, energy to failure and flexural modulus of elasticity can be determined. Unfortunately, the latter two techniques are quite invasive and require the sacrifice of experimental animals. In humans, the non-invasive technique DEXA is used, which was described in the section about diagnosis of osteoporosis.

3.4.3.1. Micro-CT

Although no single method is able to completely characterize bone quality, the in vivo follow up of the bone through non-invasive imaging combined with ex vivo mechanical and compositional techniques provides a comprehensive understanding of bone quality[359]. Micro-CT uses X-ray scanning to obtain 2D-images that are reconstructed into a 3D structure and analyzed to calculate bone structural parameters[360]. Micro-Ct techniques can also be used to make an estimate of the BMD[361].

X-rays are highly energetic and ionizing electromagnetic rays, which can be used to visualize tissue structure, and are generated in a vacuum X-ray tube,

consisting of a cathode and an anode. Electric heating of the cathode causes the release of electrons, which are accelerated through the tube by applying voltage tension between the anode and the cathode. The electrode collides with the anode made of tungsten or molybdenum, generating X-rays with a polychromatic character and an energy proportional to the applied voltage. Filters like titanium or aluminum of various thicknesses can be used to filter out rays with specific energies. Aluminum is used as a filter to preserve high-energy photons to study hard tissue like mineralized hydroxyapatite while discarding low-energy photons which are mostly used to study soft tissue in detail. Impact of X-rays on a specific sample results in an attenuation of the X-rays in function of atomic and structural properties of the sample, and the energy of the rays. The attenuation of X-rays is correlated with the atomic number of elements. In calcified tissue, calcium will absorb more X-rays as compared to elements like hydrogen, oxygen or nitrogen, the main components of organic soft tissues.

Ionizing radiation may generate chemical reactions in the DNA of living tissues leading to mutations and cancer, even in non-radiated parts. For this reason, the use of this non-invasive technique in hospitals and research centers is restricted, to a minimal radiation exposure time allowing to obtain sufficient structural information. The short wavelength of the X-rays allows for the visualization of structures in detail with a resolution of micrometers by the creation of virtual cross-sections, and is commonly used in ex vivo as well as in vivo biomedical research and in material sciences. Virtual cross-sections are created by reconstructing two dimensional images of the object at various angles, captured by an X-ray film detector. Smaller rotational angles result in a higher image quality but longer exposure time. Considering the divergence of the emitted X-rays, a rebinning algorithm is applied to correct cone-beam data to parallel-beam data. Images are registered as a raw floating point matrix, which can be recalibrated to grey values between 0 and 255. The resolution of the scan determines the distance between cross sections, which is expressed in cubic voxels.

In cooperation with the Department of Microtomography of the University of Antwerp (Prof. Dr. N. De Clerck), bones of experimental animals were structurally analyzed in vivo using a SkyScan 1076 (SkyScan, Kontich, Belgium). In this in vivo micro-CT scanner, the X-ray source and the detector rotate around the object (Figure 3.12). Animals were injected with pentobarbital to ensure their immobility during scanning. Animals were scanned before the start of the experiment and served as their own controls. Right tibia was immobilized in the sample tray using a soft cloth and clamp to avoid wounding the skin tissue. A low resolution (large voxel size) relative to the size of the structure of interest can cause an underestimation of BMD and aberrational structural

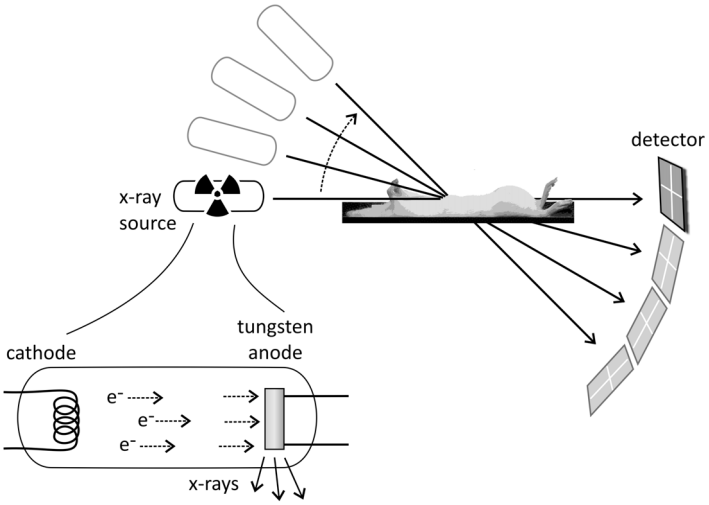

Figure 3.12. *Schematic of the SkyScan 1076 Micro-CT setup.*

properties owing to partial-volume effects and overestimation of object thickness. The measurement error increases proportionally with the ratio of voxel size to object size, which should not be lower than 2[362,363]. A high resolution results in longer scanning times and increases the discomfort and radiation time for the experimental animal. Practically, it becomes unfeasible to follow and compare large groups of animals due to the increased time interval. As a compromise between scanning time and precision of bone structure, we used a voxel resolution of 34.96 µm. For all bone scans in the following experiments, the X-ray source was set at 80 kV and 100 µA with a 0.5 mm aluminum filter. At each rotational step of 0.6°, three frames of an exposure time of 474 msec were averaged. In vivo scanning time of tibial metaphyses with the micro-CT scanner was approximately 15 min, which in our experience does not have adverse effects on animal health[364]. The proximal tibia was targeted as it has a high metabolic activity. To allow accurate comparison of density and volume in function of time, a reference point is chosen in the trabecular bone of the proximal metaphysis, on the border of the primary spongiosa with the epiphysis. About 1.75 mm above this reference point, in a range of 3.5 mm (100 slices) in the secondary spongiosa, an area in the metaphyseal region is defined which will be analyzed in each scan. A three-dimensional bone structure was reconstructed from the bone scans and analyzed with specialized software CtAn from SkyScan. A cut-off threshold is chosen for the binary interpretation of grayscale values, interpreting them as either soft tissue or bone, which is important for consequent relative analysis. From these binary reconstructions, bone parameters such as bone volume, trabecular thickness, trabecular number, trabecular spacing, and others can be calculated.

The definitions of these parameters are given in the glossary (3.6). Assessing trabecular bone requires a suitable region of interest (ROI) in the metaphysis, and particular consideration has to be paid to the extension of the ROI into the diaphysis of long bones (which is primarily cortical bone), which will decrease the mean bone volume fraction relative to an ROI that is contained in the metaphyseal region. To represent trabecular bone architecture accurately, the ROI should contain at least three to five intertrabecular lengths. For post-mortem ex-vivo BMD analysis of the trabecular bone (tBMD) of the femoral necks of experimental animals, bones were scanned in saline in a standardized vial to correct for background absorption. Phantom bones were obtained from SkyScan, with a diameter of 2mm (mouse) or 4mm (rat), with a standard BMD of 250 and 750 g/cm³. The region of the femoral neck was chosen as it is the region studied for the diagnosis of osteoporosis in humans. In order to calculate cortical transversal bone surface, the length of the femur was measured, and after cone-beam correction and binary conversion using the Otsu's algorithm[365], the transversal bone surface was measured by counting the pixels of a slice in the middle of the diaphysis of the bone using MathLab.

The micro-CT parameters which were determined using the CtAnalyser software have been precisely defined by SkyScan and are repeated below, tailored to the use in previously mentioned experiments. The precise calculation of these parameters is based on complicated algorithms and can be retrieved on the website of SkyScan/Bruker micro-CT, www.skyscan.be.

Calculations are based on a selected region, labelled 'region of interest' (ROI). Analyses within two dimensions are based on the ROI. Analyses in three dimensions are the integration of ROI selected within cross-sections, which form a three-dimensional volume of interest (VOI).

- Total volume of the volume-of-interest (VOI)/Tissue volume (TV) [mm³]: tissue volume refers to the volume of interest. It does not mean any kind of recognition of any particular density range as biological tissue, soft, hard or otherwise. Unless mentioned otherwise, the VOI is the volume of the bone without the surrounding cortical layer.
- Object/Bone volume (BV) [mm³]: total volume of binarized objects within the VOI. This volume, cortical or trabecular, refers to the total volume of cortical or trabecular bone.
- Ratio of bone volume/tissue volume (BV/TV) [%]: the proportion of the VOI occupied by binarized solid objects. This parameter is only relevant if the studied volume is fully contained within a well-defined biphasic region of solid and space, such as a trabecular bone region, and does not for example extend into or beyond the boundary of the object – such as the cortical boundary of a bone sample. The meaningfulness of measured

percent volume depends on the criteria applied in selecting the volume of interest.

- VOI surface/Tissue surface (TS) [mm²]: the surface area of the volume of interest, measured in 3D according to the marching cubes method.
- Object/Bone surface (BS) [mm²]: the surface area of all the solid objects within the VOI, measured in 3D according to the marching cubes method.
- Object/Bone surface to volume ratio (BS/BV) [1/mm]: the ratio of solid surface to volume measured in 3D within the VOI. Surface to volume ratio or "specific surface" is a useful basic parameter for characterizing the thickness and complexity of structures.
- Structure model index (SMI) [-]: indicates the relative prevalence of rods and plates in a 3D structure such as trabecular bone. SMI involves a measurement of surface convexity. This parameter is of importance in osteoporotic degradation of trabecular bone which is characterized by a transition from plate-like to rod-like architecture. An ideal plate, cylinder and sphere have SMI values of 0, 3 and 4 respectively.
- Trabecular thickness (TbTh) [mm]: the structure or trabecular thickness is the diameter of the largest sphere which encloses a hollow point and is entirely bounded within the solid trabecular surface.
- Trabecular number (TbN) [1/mm]: implies the number of traversals across a trabecular or solid structure made per unit length on a random linear path through the VOI.
- Trabecular spacing (TbSp) [mm]: essentially the thickness of the spaces as defined by binarisation within the VOI. SkyScan CT-analyzer software measures TbSp directly and model-independently in 3D by the same method used to measure trabecular thickness, just applied to the space rather than the solid voxels.
- Total (trabecular) porosity [%]: defined as the volume of all open plus closed pores as a percentage of the total VOI volume. A closed pore in 3D is a connected assemblage of space (black) voxels that is fully surrounded on all sides in 3D by solid (white) voxels. An open pore is defined as any space located within a solid object or between solid objects, which as any connection in 3D to the space outside the object or objects.

3.4.3.2. Bone histomorphometry

Although a lot of information regarding bone structure can be derived from the non-invasive in vivo micro-CT analysis, the golden standard to investigate bone health still remains bone histomorphometry as it allows to visualize mineralized as well as non-mineralized bone, bone cells and their activity on the

bone surface. Aside from these static parameters, tetracyclin labelling allows for measurement of dynamic parameters such as BFR, mineralization lag time, mineral apposition rate, and others, thus providing additional information to the non-invasive in vivo micro-CT.

Bone samples are dehydrated in increasing ethanol concentrations and impregnated in methyl methacrylate for one week according to the technique described by Goodman *et al.*[366], which allows embedding of the bone sample without decalcification by EDTA (as is necessary for paraffin embedding), thus preserving the mineralized and nonmineralised bone structure. After sacrifice and removal of the tibia, it is fixed overnight in 70% ethanol. Subsequently, the bone sample, placed in a molding tray, is embedded in a catalyst containing a monomer solution of methyl methacrylate. Under a N_2 atmosphere, the solution is allowed to polymerize for 24 hours in the dark at 4°C. The polymerization reaction is exothermic and oxygen sensitive. From the resulting blocks, 5 µm sections are cut which are stained according to Goldner's procedure. Goldner staining consists of hematoxylin nuclear staining followed by trichrome staining (Ponceau de xylidine, acid fushin, fast green and orange G combined with molybdato-phosphonophoric acid). The result is blue-colored mineralized bone, red-colored nonmineralised bone, pink-colored cellular cytoplasm and blue-black nuclei.

The Goldner-stained cross sections are used for histomorphometric measurement of static bone parameters in the metaphysis: mineralized bone area, osteoid dimensions, regions of erosion, and active osteoclasts and osteoblasts (figure 3.13A). A custom made macro program was written for the computerized semiautomatic image analysis system AxioVision (Release 4.5; Carl-Zeiss). Image pixel size is calibrated and Goldner stained cross sections are captured under the microscope at a magnification of x200 using a digital camera. Bone area, osteoid area, osteoid perimeter, eroded perimeter, and quiescent perimeter were manually traced by the operator in a minimum of 5 consecutive fields with a total surface of 1.5–2 mm² at a distance of 0.66 mm from the growth plate in the secondary spongiosa. Cuboidal cells covering the osteoid seams were labelled as active osteoblasts, whereas large multinucleated cells laying within resorption lacunae were labelled active osteoclasts. Secondary parameters were calculated according to standardized procedures[367]. These primary and secondary bone parameters as well as their definition and calculation is given in the glossary at the end of this section.

To evaluate the structural parameters of the growth plate, consecutive fields of Goldner-stained bone sections were analyzed over the entire length of the growth plate at a magnification of 200x. Thickness of the proliferative as well as the hypertrophic zone in the middle of each field was measured after image pixel calibration.

Tetracyclin labelling allows for measurement of dynamic parameters, as they become incorporated in the bone after forming complexes with calcium. These labels are injected intraperitoneally at a specific time interval (mostly 7 and 3 days) before sacrifice. Different labels, like tetracyclin, demeclocyclin and others, can be used to assure visual distinction. These antibiotic labels are also manually traced on the screen by the operator, after which the length of the labels and distance between the labels is calculated (figure 3.13B).

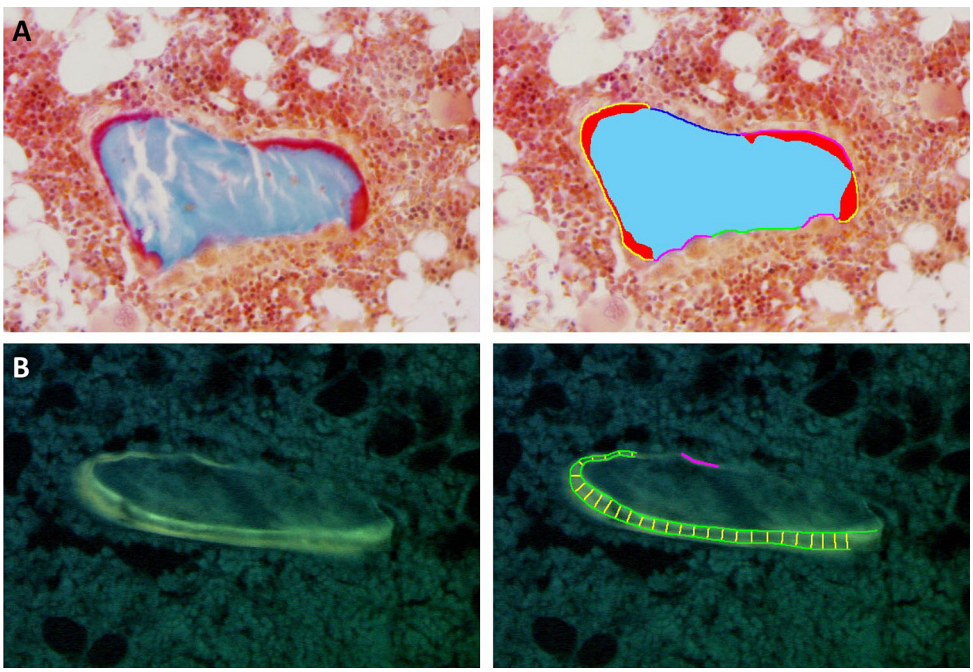

***Figure 3.13**. (A) Goldner stained bone. Right image shows the same bone section displaying manual tracing used for calculation of static bone parameters (cyan: mineralized bone surface, red: osteoid surface, blue: quiescent bone perimeter, yellow: osteoid perimeter, green: erosion perimeter, purple: osteoblast or osteoclast perimeter) (B) Unstained bone section exposed to UV light used for the measurement of dynamic bone parameters (green: tetracyclin label, yellow: demeclocyclin label). Right image shows manually traced labels, including yellow lines computer drawn perpendicular to the labels to represent the distance between the labels.*

The most common parameters calculated by bone histomorphometric analysis are explained below. Their detailed calculation can be found in table 3.3.

Static parameters

- Bone area (BAr) [%]: ratio of the trabecular bone area (mineralized bone and osteoid) to the total visualized area

- Mineralized area (MdAr) [%]: percentage of tissue area covered by mineralized, trabecular bone tissue
- Osteoid area (OAr) [%]: ratio of the area of osteoid to the total visualized area
- Osteoid perimeter (OPm) [%]: coverage of the total bone perimeter by osteoid
- Eroded perimeter (EPm) [%]: percentage of total bone perimeter displaying osteoclast erosion (Howship's lacunae)
- Osteoblast perimeter (ObPm) [%]: percentage of osteoid perimeter covered by active, cuboid osteoblasts
- Osteoclast perimeter (OcPm) [%]:percentage of eroded perimeter covered by active multinuclear bone resorbing osteoclasts
- Trabecular thickness (TbTh) [mm]: average thickness of trabeculae measured in total area
- Trabecular number (TbN) [-]: average number of trabeculae counted in total area
- Trabecular spacing (TbSp) [mm]: average distance between trabeculae

Dynamic parameters

- Double-labelled perimeter (dlPm) [%]: percentage of total bone perimeter covered by double fluorescent labelling
- Mineral apposition rate (MAR) [µm/day]: rate of osteoid mineralization = average distance between tetracycline labels divided by administration time interval
- Adjusted mineral apposition rate (AjMAR) [µm/day]: apposition rate of mineral normalized over osteoid surface
- Bone formation rate (BFR): surface bone production rate normalized over total bone surface
- Osteoid maturation time (OMT): average time interval between initiation of matrix deposition and mineralization into osteoid at each site of bone formation
- Mineralization lag time (MLT): average time interval between osteoid maturation and resulting mineralization

3.4.3.3. Three-point bending

In order to evaluate mechanical properties of bone, the femoral bone is subjected to a three-point bending test. Destructive mechanical testing of

Table 3.3. *Calculated bone histomorphometric parameters, derived from Parfitt et al.[368] Results and measurements are reported in two-dimensional units.*

Parameter		Formula	Unit
B.Ar	*Bone area (see note 1)*	$\frac{Ab.B.Ar}{Ab.Tt.Ar}$	*%*
O.Ar	*Osteoid area*	$\frac{Ab.O.Ar}{Ab.B.Ar}$	*%*
O.Wi	*Osteoid width*	$\frac{Ab.O.Ar}{Ab.O.Pm} * 1000$	*µm*
O.Pm	*Osteoid perimeter*	$\frac{Ab.O.Pm}{Ab.O.Pm + Ab.E.Pm + Ab.Q.Pm}$	*%*
E.Pm	*Eroded perimeter*	$\frac{Ab.E.Pm}{Ab.O.Pm + Ab.E.Pm + Ab.Q.Pm}$	*%*
sL.Pm	*Single-labelled perimeter*	$\frac{Ab.sL.Pm}{Ab.Tt.Pm}$	*%*
dL.Pm	*Double-labelled perimeter*	$\frac{Ab.dL.Pm}{Ab.Tt.Pm}$	*%*
Ob.Pm(O)	*Osteoblast perimeter (relative to osteoid perimeter)*	$\frac{Ab.Ob.Pm}{Ab.O.Pm}$	*%*
Ob.Pm(T)	*Osteoblast perimeter (relative to total perimeter)*	$\frac{Ab.Ob.Pm}{Ab.O.Pm + Ab.E.Pm + Ab.Q.Pm}$	*%*
Oc.Pm(E)	*Osteoclast perimeter (relative to eroded perimeter)*	$\frac{Ab.Oc.Pm}{Ab.E.Pm}$	*%*
Oc.Pm(T)	*Osteoclast perimeter (relative to total perimeter)*	$\frac{Ab.Oc.Pm}{Ab.O.Pm + Ab.E.Pm + Ab.Q.Pm}$	*%*
MAR	*Mineral apposition rate*	$\frac{Ir.L.Di}{Ir.L.t}$	*µm/day*
AjAr	*Adjusted apposition rate (see note 2)*	$\frac{\left(Ab.dL.Pm + \frac{1}{2} Ab.sL.Pm\right)}{MAR * (O.Pm * Ab.Tt.Pm)} / 1000$	*µm/day*
BFR	*Bone formation rate (see notes 2 and 3)*	$MAR * \frac{Ab.dL.Pm + \frac{1}{2} Ab.sL.Pm}{Ab.Tt.Ar}$	µm²/mm²/day
Mlt	*Mineralisation lag time*	$\frac{O.Wi}{AjAr}$	*days*
Omt	*Osteoid maturation time*	$\frac{O.Wi}{MAR}$	*days*
Tb.Th	*Trabecular thickness*	$\frac{Ab.B.Ar}{Ab.O.Pm + Ab.E.Pm + Ab.Q.Pm} * 2000$	*µm*
Tb.N	*Trabecular number (see note 1)*	$\frac{Ab.O.Pm + Ab.E.Pm + Ab.Q.Pm}{Ab.Tt.Ar} * \frac{2}{\pi}$	*mm⁻¹*
Tb.Sp	*Trabecular spacing*	$\frac{1000}{Tb.N} - Tb.Th$	*µm*

Ar: area, Pm: perimeter, Ab: absolute, Tt: total
Note 1: Ab.Tt.Ar derived from the Goldner measurements.
Note 2: For human biopsies. For rat samples, the factor ½Ab.sL.Pm is omitted. See text for further details.
Note 3: Ab.Tt.Ar derived from the fluorescence measurements.

whole bone is an effective way to determine overall skeletal fragility. The bone is placed on a support with a specific distance between points, and a middle rod is lowered onto the middle at a specific speed and a constantly measured load to establish a load-displacement curve. This allows to calculate bone attributes like the ultimate load (N) and the ultimate displacement (mm), which is the applied force and the displacement of the middle rod, respectively, at which failure of the bone occurs (cortical breakage). Further determination of cortical geometric properties allow for the determination of stress and strain and other mechanical parameters.

Three-point bending experiments were performed with a 100N load cell and a pressure transducer. At sacrifice, a last micro-CT measurement was made to determine bone transversal cortical surface, after which right femora were frozen at -20°C before measurement of mechanic properties. In our experimental setup, the distance between both resting points was 40 mm and the bending point was positioned in the middle of the diaphysis. After a preload of about 1 N, the displacement (coordinated at a speed of 5 µm/second) and applied force were measured and communicated to Mathlab software. Movement of the displacement rod occurred in the anterio-posterior axis. Stiffness (N/mm), ultimate or fracture load (N), ultimate or failure displacement (mm), energy to failure (J) and flexural modulus of elasticity (N/mm²) were determined from force-displacement curves registered by Mathlab (figure 3.14). Using the geometrical properties determined by micro-CT, further parameters were determined, like ultimate stress (mPa), failure strain (%), toughness (J/mm³) and the Young's modulus (MPa)[369].

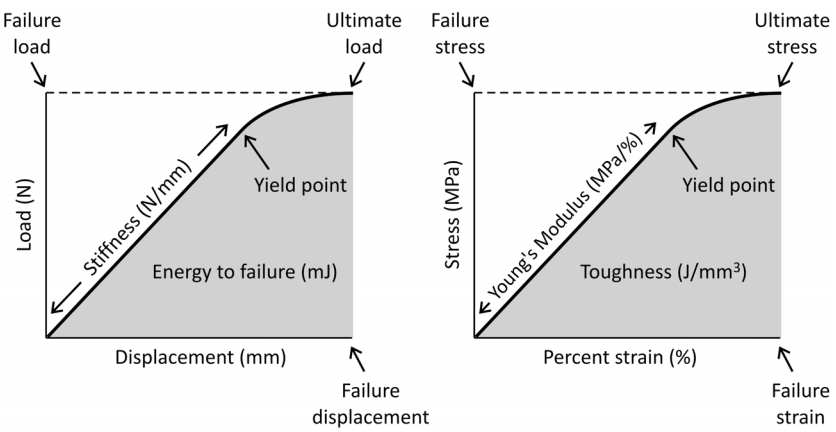

Figure 3.14. *Typical load-displacement (left) and stress-strain curve (right). Adapted from Kyle et al.[369]*

The linear region of the load-displacement curve represents elastic behavior, meaning the specimen should return to its original shape if the load is

removed within this region. The slope of this region represents the stiffness of the bone and is reflective of rigidity. The yield point defines the transition between the elastic and plastic behavior of the long bone. In the plastic region, bone is permanently deformed by the applied load. The mechanical properties derived from load-displacement curves are largely influenced by the structure and geometry of the long bone. A formula using geometric values measured by micro-CT allows to normalize the load-displacement curve into a stress-strain curve. The conversion assumes a structurally homogenous specimen over the entire length, which is not entirely accurate but allows for an approximation to compare bone material properties. The following formulas were applied to convert load (F, in N) into stress (σ, in MPa) and displacement (d, in mm) into strain (e, in %). The material properties ultimate stress (MPa), Young's modulus (MPa/%), failure strain (%), and toughness (MPa.%) were determined from individual analysis of each stress-strain curve.

Stress (MPa)	Strain (%)
$\sigma = (F * D * Ø_{AP}) / (8 * I_{AP})$	$e = (6 * d * Ø_{AP}) / D^2 * 100$

F	ultimate load (N)
D	distance between lower supports (mm)
$Ø_{AP}$	anteroposterior diameter (mm)
I_{AP}	anteroposterior moment of inertia (mm^4)
d	displacement (mm)

3.4.4. Data processing and statistical analysis

All results are given as average ± standard deviation, unless indicated otherwise. Bone histomorphometric data are presented as individual data and median. Statistics were performed with IBM SPSS Statistics 20. Comparisons between study groups were assessed using the non-parametric Kruskal-Wallis H-test, followed by a Mann-Whitney U-test in combination with the Bonferroni correction when more than two groups were compared. Data from morphological evaluation were analyzed using Pearson's χ^2-test.Values of $p < 0.05$ were considered significant.

3.5. Effects of DPP4 inhibition on postmenopausal osteoporosis

3.5.1. Introduction

As extensively discussed in the previous chapters, many substrates of DPP4 have a clearly established effect on bone metabolism and turnover. DPP4 was also found to exert an increased activity in osteoporotic patients. The inhibition of DPP4 and the prolongation of the half-life of its substrates could result in an increase of anabolic DPP4-afflicted proteins on osteoporotic bone. In this experiment, the effect of DPP4 inhibition by sitagliptin will be assessed on the development of osteoporotic bone in the ovariectomized (female) Wistar Han rat.

3.5.2. Study setup

Preceding the actual experiment, a pilot experiment was setup to establish a dosage of sitagliptin through which sufficient inhibition is achieved. Sitagliptin was administered through oral gavage either once daily at dosages of 50, 100 and 200 mg/kg, and twice daily with an interval of 12 hours at dosages of 50 and 100 mg/kg. Sitagliptin tablets were ground and dissolved in 0.1% carboxymethylcellulose to increase viscosity and avoid sedimentation of undissolved tablet film. DPP4 activity was measured at 2, 4, 6, 12 and 24 hours after oral gavage. As the most efficient inhibition was achieved with an oral gavage of 100 mg/kg sitagliptin twice per day (results given later), this dosage was used over the course of the entire experiment. The study setup of this experiment is displayed in figure 3.15.

Forty female Wistar Han rats (10 weeks old) were included in this experiment, divided into four groups (n=10 each). Half of the experimental animals underwent ovariectomy (OVX), the others served as controls (C). Half of controls and half of ovariectomized animals were treated with sitagliptin (SG), the other half was treated with vehicle (V). In the following sections, the animal groups will be abbreviated as follows: C/V for control animals treated with vehicle, C/SG for control animals treated with sitagliptin, OVX/V for ovariectomized animals treated with vehicle and OVX/SG for ovariectomized animals treated with sitagliptin. Before induction of osteoporosis, the right tibia of each animal was scanned by in vivo micro-CT after peritoneal anesthesia. Immediately after the initial scanning procedure, the ovaries of OVX animals were removed. The day after the initial bone scan and ovariectomy, treatment was started with either 100 mg/kg sitagliptin or vehicle twice daily. Every two weeks, the bone was scanned in vivo to follow-up the evolution of bone density in all animals, and a serum sample was collected. As ovariectomy results

in a phase of hyperfagia[370], animals were pair-fed during the course of the experiment. Food consumption of the untreated control group was weighed daily and determined the food quantity for the other groups for the next day. One week before the start of the experiment, right after the operation, in week 2 and right before sacrifice, the animals were kept in metabolic cages to allow a 24-hours urine collection. The proximal part of the right tibia was used for histomorphometric analysis. Seven and four days before sacrifice, respectively, tetracyclin (30 mg/kg) and demeclocyclin (25 mg/kg) were injected intraperitoneally to form a complex in the mineralizing bone for analysis of dynamic bone parameters. After six weeks, animals were sacrificed after the last micro-CT scan. Weight of the uterus, which was known to become atrophied after a successful ovariectomy, was verified at sacrifice.

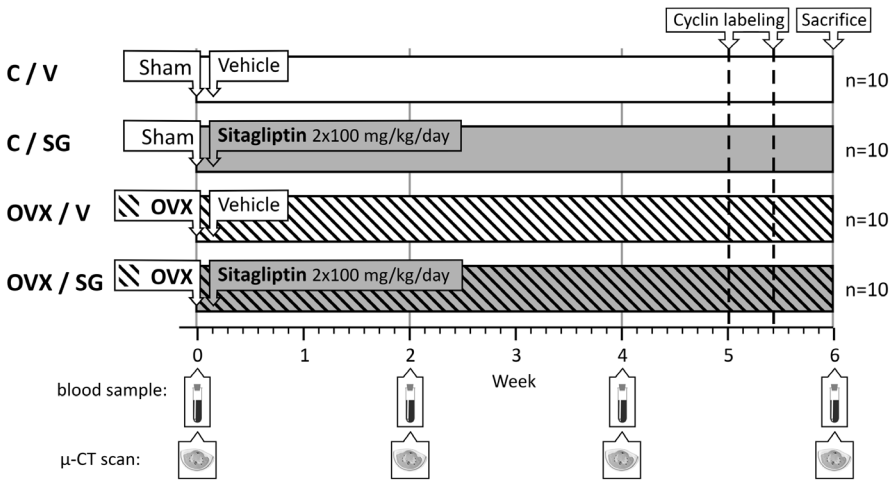

Figure 3.15. *Study setup. Forty 10 week old female Wistar Han rats were divided into four groups. (C/V) Control group treated with vehicle (C/SG) Control group treated with sitagliptin (OVX/V) Ovariectomized group treated with vehicle (OVX/SG) Ovariectomized group treated with sitagliptin. SG: gavage with 100 mg/kg twice daily. V: gavage with vehicle, or 0.1% carboxymethylcellulose.*

3.5.3. Results

3.5.3.1. Animal data

During the experiment, three out of 40 animals died prior to sacrifice, corresponding to a mortality of 7.5%. Two untreated control animals died during the micro-CT scan following anesthesia in week 0 and week 4. One SG-treated ovariectomized animal died in week 2 due to a perforation of the esophagus following gavage. These animals were excluded from the analysis. All experimental animals were weighed weekly, and started at an average of 216.7 ± 7.4

g. At week 2, a significant weight increase was observed in the ovariectomized animals compared to the untreated control group (figure 3.16A). From week 3 on, their body weight is also significantly increased compared to the SG-treated control group. The body weight of the ovariectomized animals kept increasing steadily until the end of the experiment at week 6. Weight of the uteri was found to be effectively reduced in all ovariectomized animals, indicating atrophy and a successful ovariectomy (figure 3.16B).

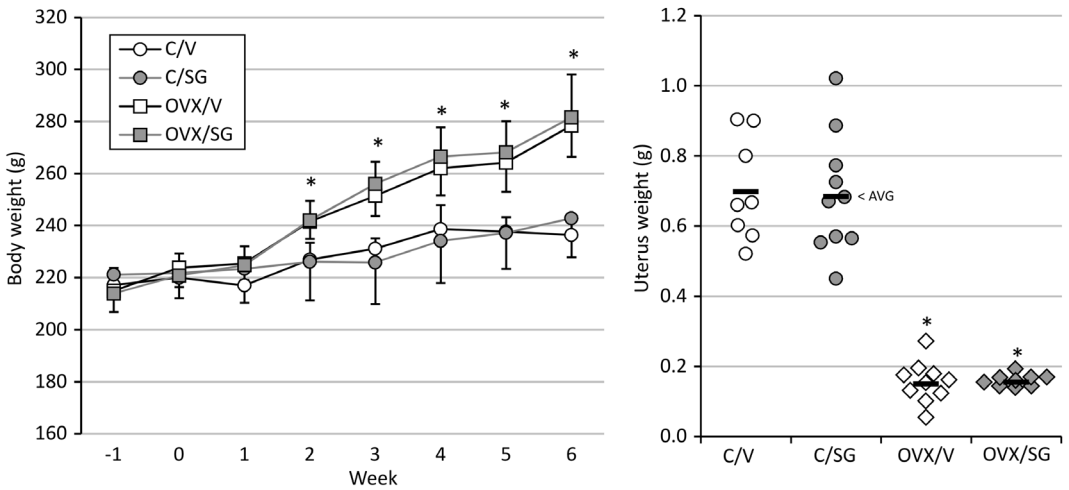

Figure 3.16. *(A) Body weight of animals (g) as measured weekly. (B) Uterus weight (g) at sacrifice. * p < 0.05 compared to controls after Bonferroni-correction.*

3.5.3.2. Biochemical analyses

DPP4 inhibitor dosage test and DPP4 activity

In order to establish a dose that achieves almost complete DPP4 inhibition, a separate inhibition experiment was organized with 7 female Wistar Han rats. At a dose of 50 mg/kg sitagliptin, DPP4 inhibition sinks below 75% within 8 hours (figure 3.17, hollow round marker). Repeated administration of 50 mg/kg also results in a decrease to 60% inhibition at 12 hours (hollow square marker). Single doses of 100 and 200 mg/kg result in an efficient inhibition at 12 hours after administration, but a decrease below 50% after 24 hours (black and grey round marker). A repeated dose of 100 mg/kg twice per day with a 12 hour interval, however, guarantees an inhibition above 75% at all times (black square marker).

DPP4 activity was measured in the serum of experimental animals a week before the start of the experiment for basal values, and in week 0, 2 and 6, at 12 hours after the last oral gavage with sitagliptin. The DPP4 activity of the SG-

treated controls was shown to be significantly lower compared to untreated controls at week 0 and at week 6 (figure 3.18). In untreated ovariectomized animals, the DPP4 activity was increased at week 2 compared to both control groups, an effect that persists until the end of the experiment. The DPP4 activity in the SG-treated OVX animals was significantly decreased compared

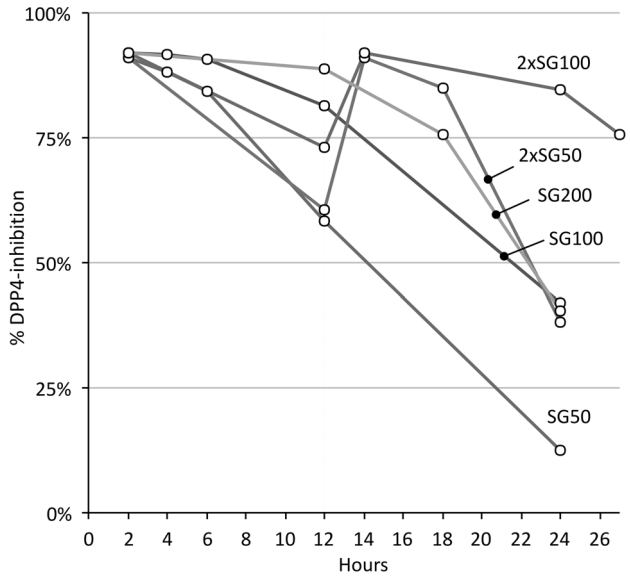

Figure 3.17. *Sitagliptin dose-optimization experiment. An inhibition twice daily with 100 mg/kg sitagliptin through oral gavage resulted in the best inhibition*

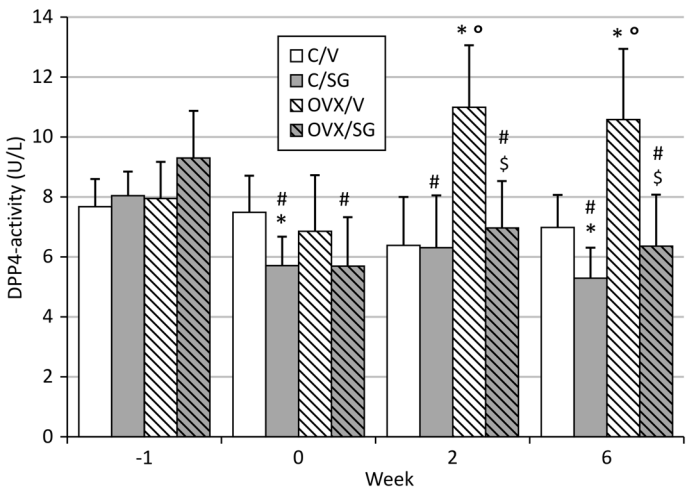

*Figure 3.18. DPP4 activity measured in the serum (U/L). * p < 0.05 compared to C/V, ° p < 0.05 vs. C/SG, § p < 0.05 vs OVX/V, # p < 0.05 vs basal value at wk-1, after Bonferroni correction.*

to the untreated OVX animals from week 2 on. Throughout the experiment, the DPP4 activity of both SG-treated groups was found to be significantly decreased compared to the basal values of each group.

Pancreas and liver enzymes

The weight of the pancreas was found to be significantly elevated in SG-treated as well as untreated OVX animals compared to untreated controls. The concentration of serum amylase was found to be increased in the SG-treated OVX animals compared to controls. No other significant differences were observed.

Table 3.4. *Weight of the pancreas, pancreatic (amylase, lipase) and liver (AST, ALT) enzymes in the serum.*

	C/V	C/SG	OVX/V	OVX/SG
Pancreas weight (g)	0.642 ± 0.105	0.794 ± 0.189	0.945 ± 0.166*	0.891 ± 0.180*
Amylase (U/L)	394.3 ± 86.4	486.2 ±53.9	448.2 ± 67.5	501.0 ± 34.8*
Lipase (U/L)	34.08 ± 1.79	34.87 ± 2.93	37.02 ± 1.78	39.24 ± 4.14
AST (U/L)	53.66 ± 7.48	53.86 ± 14.58	50.92 ± 9.94	59.54 ± 15.88
ALT (U/L)	16.14 ± 5.39	18.29 ± 3.25	23.20 ± 9.53	17.28 ± 4.45

** p < 0.05 compared to C/V after Bonferroni correction.*

Calcium and phosphate metabolism

Calcium concentrations in serum and urine were highly variable and inconclusive (data not shown). Serum phosphate and urine levels are shown in figure 3.19. Although some significant differences were found, no real conclusions could be drawn.

Renal function

Renal function was evaluated by measuring serum creatinine and creatinine clearance (figure 3.20). In general, no clear effect of OVX nor of SG treatment on renal function was observed. For unknown reasons, a significant and transient increase in creatinine clearance was observed 2 weeks in OVX animals compared to their baseline.

3.5.3.4. Evaluation of bone microstructure

Micro-CT reveals an improved trabecular microstructure following SG treatment in controls and in the early phase after ovariectomy

The results of the trabecular bone analysis are given below. The effect of ovariectomy already becomes clear at week 2 when evaluating both trabecu-

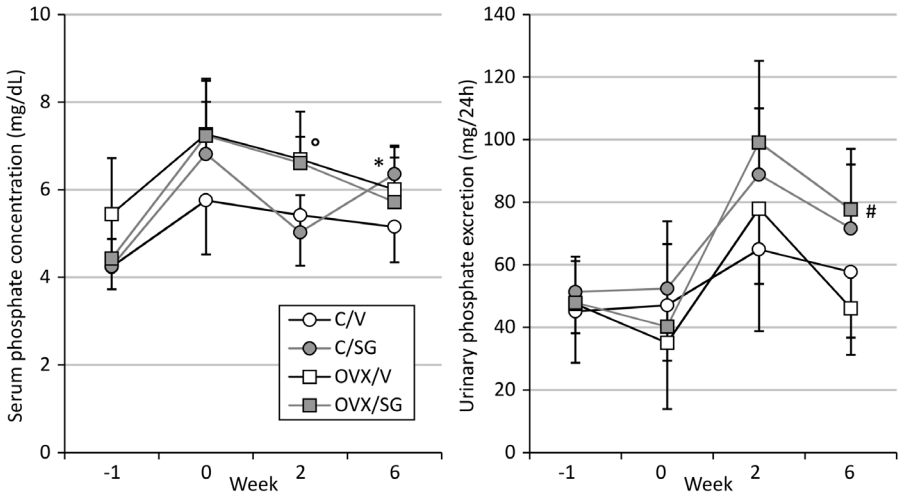

Figure 3.19. *Phosphate concentration in the serum (left, mg/dL) and urinary excretion (right, mg/24h). * p < 0.05 vs C/V, ° p < 0.05 vs C/SG, # p < 0.05 vs OVX/V after Bonferroni correction.*

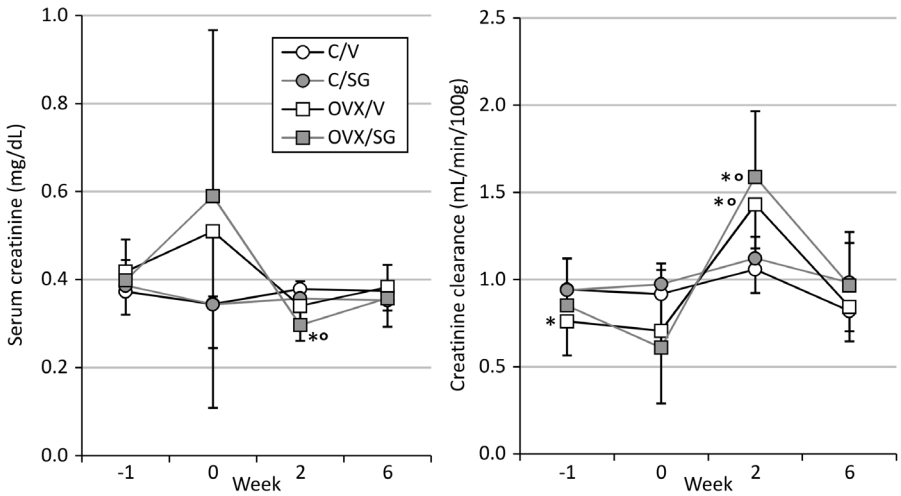

Figure 3.20. *Creatinine concentration in the serum (left, mg/dL) and creatinine clearance (mL/min/100g). * p < 0.05 vs C/V, ° p < 0.05 vs C/SG after Bonferroni correction.*

lar bone volume and surface. However, absolute trabecular bone volume nor trabecular bone surface were found to differ significantly between treated and untreated groups, either ovariectomized or controls. Recalculating the results in relation to the baseline of individual animals, however, a significantly increased bone volume was observed in SG-treated control animals compared to untreated controls in week 2 and week 6 (figure 3.21A). The surface of the trabecular bone was also found to display significant differences between

groups when calculated to individual baselines. The SG-treated OVX animals displayed a higher bone surface compared to the untreated OVX group at 2 weeks after the start of treatment. At week 4 and week 6, an increased bone surface was also observed in SG-treated control animals compared to untreated controls (figure 3.21B). Results are shown relative to their individual baselines (displayed as 100%). The ratio of the trabecular bone surface over

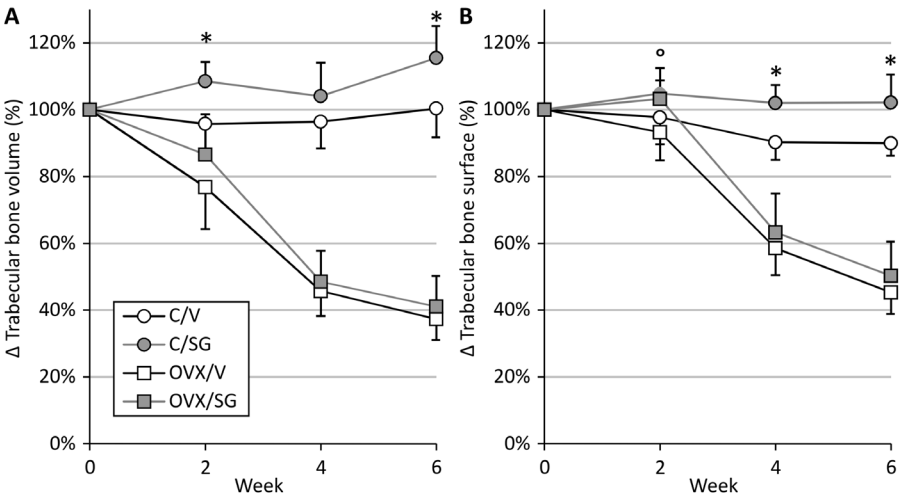

Figure 3.21. *(a) Trabecular bone volume and (b) trabecular bone surface (%) as measured by micro-CT. * p < 0.05 in C/SG vs C/V animals. ° p < 0.05 in OVX/SG vs OVX/V animals.*

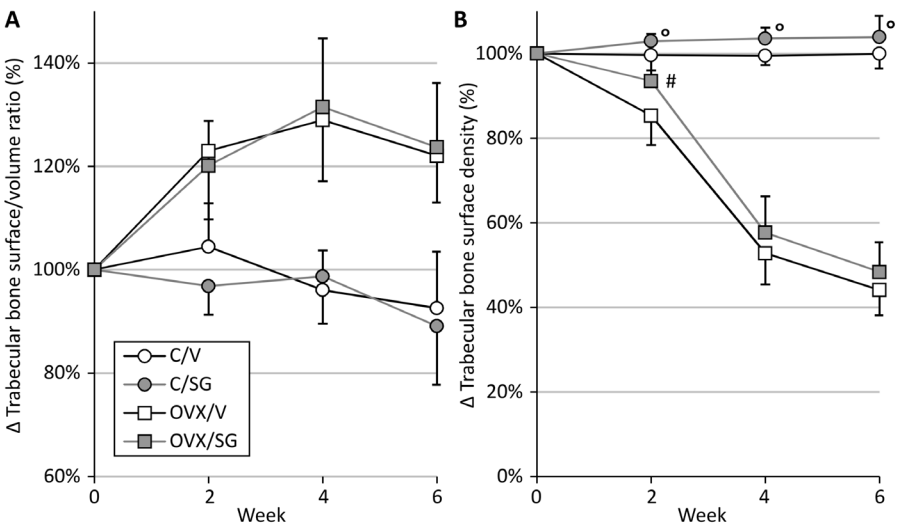

Figure 3.22. *(A) Trabecular bone surface to volume ratio and (B) trabecular surface density (%) as measured by micro-CT. ° p < 0.05 vs C/V. # p < 0.05 vs OVX/V.*

trabecular bone volume increased quickly in ovariectomized groups and was found increased compared to control groups from week 2 until the end of the experiment (figure 3.22A). No significant differences were observed between SG-treated groups and untreated groups. The trabecular bone surface density is defined as the ratio of the trabecular bone surface to the total volume of interest within the cortical bone (figure 3.22B). It was also found to be significantly decreased compared to controls in all ovariectomized animals from week 2 until the end of the experiment. Differences were calculated relatively to the individual baseline, and were only significant in SG-treated control animals compared to untreated control animals from week 2 until week 6. Only in week 2, the SG-treated OVX animals also had a significantly increased trabecular bone surface density compared to the untreated OVX group.

Ovariectomy resulted in a decrease of trabecular thickness and number, and an increase of the trabecular spacing. SG treatment, however, had no effect on trabecular thickness, number and spacing throughout the experiment (table 3.5). The untreated control group presented with a significantly increased number of trabeculae compared to the other groups at the start of the experiment. In week 2, the trabeculae of the SG-treated OVX group were found to be significantly more plate-like compared to the untreated OVX group, comparing the SMI values. Total trabecular porosity is defined as the volume of all open and closed pores as a percentage of the total volume of interest within the cortical bone. A closed pore is an intertrabecular space which is surrounded in all dimensions by bone. Contrarily, an open pore is still connected through an intertrabecular space outside of the pore. The total porosity was significantly increased in ovariectomized groups compared to controls from week 2 on. No differences were observed between the total porosity of SG-treated and untreated animal groups.

Micro-CT analysis shows that cortical bone remains unaffected by both OVX and SG-treatment

Cortical bone volume, surface and surface density were found to be highly variable, already at the start of the experiment. Therefore, values were compared to their individual basal values. No significant differences can be observed because of ovariectomy or SG treatment (figure 3.23). Only one difference was observed between the cortical bone surface of SG-treated animals and that of untreated animals at week 2 (figure 3.23B).

Bone histomorphometry reveals no significant differences following SG-treatment

As expected, the mineralized bone area was lower in ovariectomized animals compared to controls. However, no significant differences were observed between the mineralized bone area of SG-treated and untreated ani-

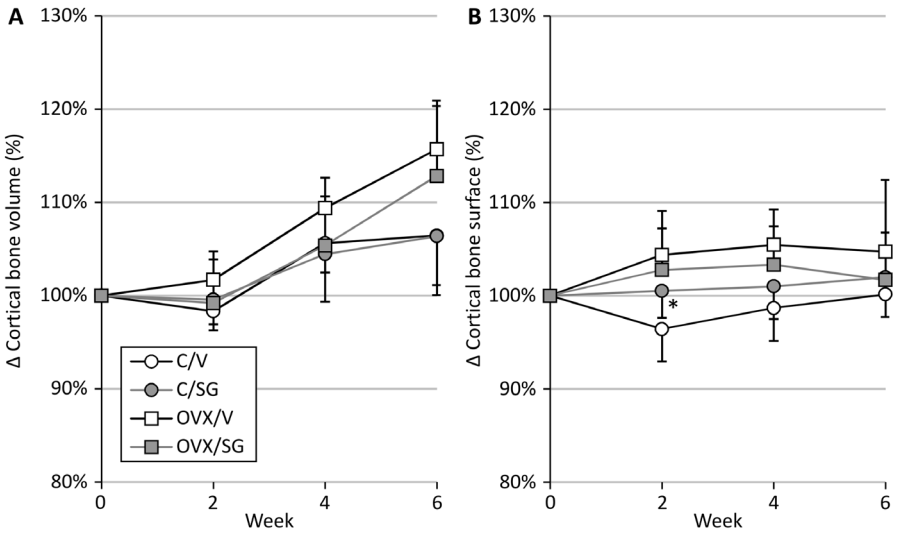

Figure 3.23. *Cortical bone volume and surface (%) compared to individual base values, as measured by micro-CT in the metaphysis. * p < 0.05 vs C/V.*

Table 3.5. *Further trabecular parameters determined by micro-CT. Trabecular thickness, number and spacing, SMI and total trabecular porosity.*

		C/V	C/SG	OVX/V	OVX/SG
Trabecular thickness (μm)	Wk 0	167.35 ± 12.28	168.17 ± 8.79	159.80 ± 5.19	166.25 ± 11.63
	Wk 2	165.15 ± 9.35	168.98 ± 6.56	151.77 ± 7.71	156.43 ± 4.85
	Wk 4	171.35 ± 9.40	166.76 ± 6.38	149.81 ± 3.52	154.80 ± 6.52
	Wk 6	174.87 ± 8.90	173.84 ± 10.54	155.29 ± 3.27	157.71 ± 5.72
Trabecular number (1/mm)	Wk 0	2.97 ± 0.47	2.74 ± 0.27*	2.60 ± 0.32	2.58 ± 0.29
	Wk 2	2.89 ± 0.51	2.89 ± 0.24	1.91 ± 0.28	2.13 ± 0.26
	Wk 4	3.00 ± 0.42	2.91 ± 0.29	1.14 ± 0.21	1.21 ± 0.22
	Wk 6	3.08 ± 0.44	3.11 ± 0.34	0.97 ± 0.16	1.06 ± 0.16
Trabecular separation (μm)	Wk 0	218.6 ± 57.5	246.1 ± 61.8	253.9 ± 50.2	270.8 ± 60.2
	Wk 2	218.3 ± 62.2	228.6 ± 54.2	282.4 ± 42.9	268.1 ± 48.2
	Wk 4	212.7 ± 58.2	225.1 ± 49.5	608.5 ± 185.6	511.4 ± 106.4
	Wk 6	211.3 ± 64.4	206.0 ± 34.5	978.6 ± 257.6	860.7 ± 146.6
SMI	Wk 0	0.071 ± 0.890	0.478 ± 0.329	0.788 ± 0.394	0.655 ± 0.374
	Wk 2	0.299 ± 0.859	0.253 ± 0.273	1.634 ± 0.229	1.394 ± 0.233#
	Wk 4	0.060 ± 0.798	0.311 ± 0.367	1.967 ± 0.105	2.007 ± 0.225
	Wk 6	-0.166 ± 0.858	-0.244 ± 0.611	1.872 ± 0.125	1.808 ± 0.124
Trabecular porosity (%)	Wk 0	0.50 ± 0.11	0.54 ± 0.05	0.58 ± 0.06	0.57 ± 0.07
	Wk 2	0.52 ± 0.10	0.51 ± 0.04	0.71 ± 0.05	0.67 ± 0.05
	Wk 4	0.48 ± 0.09	0.52 ± 0.06	0.83 ± 0.03	0.81 ± 0.03
	Wk 6	0.46 ± 0.09	0.46 ± 0.08	0.85 ± 0.03	0.83 ± 0.03

** p < 0.05 vs C/V, # p < 0.05 vs OVX/V.*

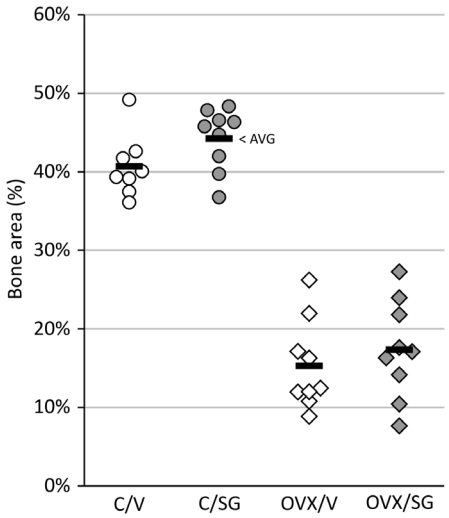

Figure 3.24. *Mineralized bone area as measured by bone histomorphometry.*

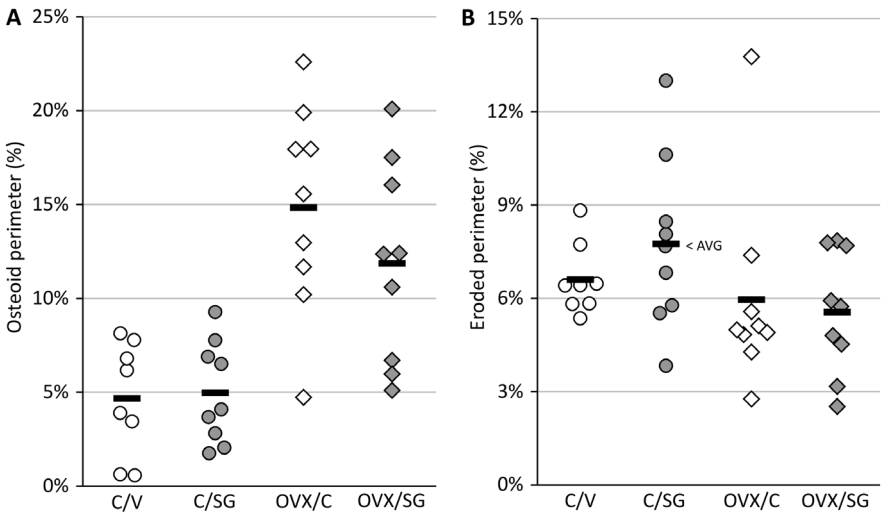

Figure 3.25. *Osteoid and eroded perimeter as measured by bone histomorphometry.*

mals (figure 3.24).

The perimeter of the mineralized bone covered with osteoid, as well as the eroded mineralized bone perimeter displaying resorption pits were also shown to be similar between the treated and untreated groups (figure 3.25).

The osteoblast perimeter, the ratio of the osteoid perimeter covered by osteoblasts to the total osteoid perimeter, is shown in figure 3.26. Similarly, the osteoclast perimeter is the percentage of resorbing osteoclast coverage

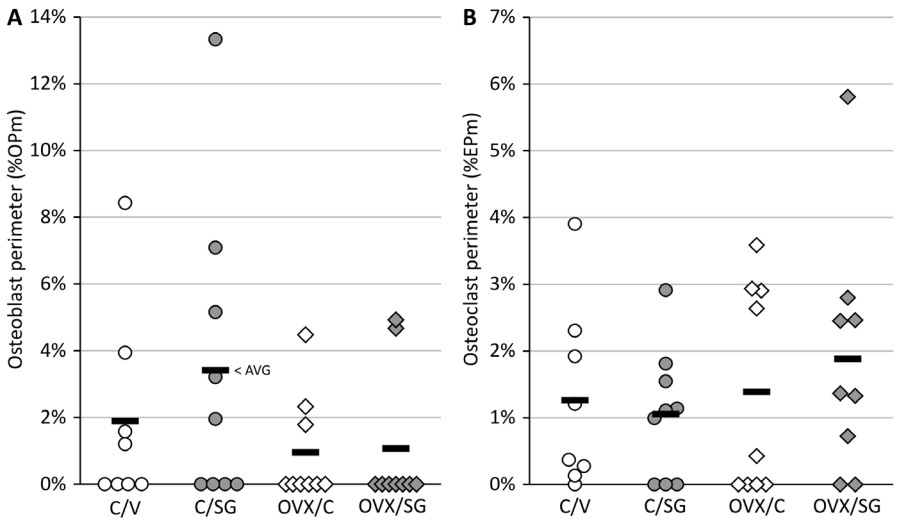

Figure 3.26. *Osteoblast perimeter (% of osteoid perimeter) and osteoclast perimeter (% of eroded perimeter) as measured by bone histomorphometry.*

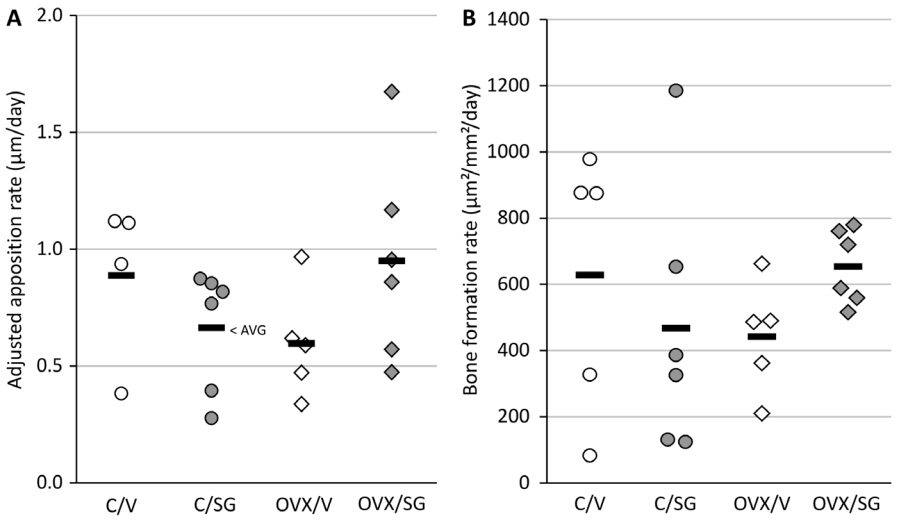

Figure 3.27. *AjAR (µm/day) and BFR (µm²/mm²/day) as measured by bone histomorphometry.*

of the eroded perimeter. No significant differences were observed comparing the osteoblast and osteoclast perimeter of all animal groups.

Further determined static bone parameters are given in table 3.6. The BAr as well as the OAr, as well as trabecular parameters determined by bone histomorphometry, were found not to be affected by SG treatment. Tetracycline labelling allowed for the calculation of some dynamic bone parameters. Again, both AjARs and BFRs were found not to differ between different treatment groups (figure 3.27).

The results of the other dynamic bone parameters are displayed in table 6. Of all calculated dynamic perimeters, only the double labelled perimeter was found to be significantly higher in the SG-treated OVX rats compared to the untreated OVX rats.

Table 3.6. *Static and dynamic bone histomorphometric parameters. * p < 0.05 vs OVX/V*

	C/V	C/SG	OVX/V	OVX/SG
BAr (%)	40.68 ± 4.01	44.21 ± 3.93	15.28 ± 5.70	17.35 ± 6.27
OAr (%)	0.57 ± 0.39	0.56 ± 0.33	2.21 ± 0.95	1.62 ± 0.80
TbTh (µm)	63.99 ± 6.71	63.48 ± 7.43	53.84 ± 3.92	55.33 ± 5.20
TbN (1/mm)	8.15 ± 0.93	8.94 ± 0.99	3.56 ± 1.08	3.93 ± 1.19
TbSp (µm)	60.21 ± 11.44	49.66 ± 9.33	249.16 ± 86.83	226.42 ± 110.09
dlPm (%)	1.93 ± 1.42	1.58 ± 1.16	2.54 ± 0.35	4.36 ± 1.58*
OMT (days)	1.43 ± 0.36	1.68 ± 0.32	1.56 ± 0.30	1.44 ± 0.36
MLT (days)	4.30 ± 3.89	6.48 ± 3.64	7.35 ± 3.02	4.64 ± 2.24

3.5.4. Discussion

Weight of the uteri at sacrifice confirmed atrophy of the uterus due to lack of estrogen production and thus a successful ovariectomy procedure. The induction of menopause through ovariectomy is associated with an initial phase of hyperphagia due to LP insensitivity[117]. Food intake was controlled through pair-feeding, in order to avoid the influence of mechanical loading through additional body weight on the BMD, which can give rise to a falsely increased BMD in ovariectomized animals in comparison to their controls[371]. Also the expression of important DPP4 substrates are dependent on food intake. These include GIP, GLP-1, GLP-2 and neuropeptides like PYY, which are increased after food intake and associated with an inhibition of bone resorption[372]. An increase of food intake after ovariectomy could result in increased concentrations of these substrates in the circulation. Regardless of the restriction of food intake through pair feeding, the ovariectomized animals displayed a significantly higher body weight compared to the control animals from week 2 on. Sitagliptin treatment did not affect body weight in sitagliptin treated OVX

mice, as was already published in previous experimental studies[214]. Obesity in general has been linked to an increased DPP4 activity, and also a decreased expression of GLP-1[228].

At the start of the experiment, no significant differences were observed as to the DPP4 activity measured in the serum of the experimental animals. Ovariectomy results in a clear increase of DPP4 activity which, similar to the body weight, increases at 2 weeks after surgery and remains increased throughout the entire experiment. The ovariectomized animals treated with sitagliptin have a clearly decreased DPP4 activity compared to the untreated OVX animals from week 2 on. In the control animals, DPP4 activity was found to be variable throughout the course of the experiment, which could be due to a variation according to the phase of the menstrual cycle[373]. At week 0 and week 6, the DPP4 activity of the sitagliptin-treated controls was found to be significantly decreased compared to the untreated controls.

As the dosing experiment was performed with animals that did not undergo ovariectomy, the DPP4 activity was underestimated, causing an insufficient inhibition during the experiment. As gavage frequency cannot be increased, an efficient inhibition could only be achieved by the use of osmotic pumps or through drinking water.

The ovariectomized animals display a strongly decreased sensitivity to LP due to estrogen deficiency, decreasing their satiety signaling and rendering the animals voracious. Although the food intake was controlled through pair feeding, body weight increased steadily in OVX animals and remained significantly higher. This could be due to NPY, known to be increased in correlation with LP resistance, as a downstream effector of LP in the energy metabolism[117]. Ovariectomy as well as food deprivation were shown to result in an increased NPY expression in the hypothalamus, hippocampus and other brain areas, altering the energy metabolism[291,374]. A similar increase in NPY levels could have been caused by pair feeding. Variations of circulating estrogen levels throughout the menstrual cycle are known to cause variations of the Y1-receptors in the hypothalamus in female rats, regulating NPY sensitivity[375]. NPY is critical for the effect of estrogen deficiency on the reduction of the energy metabolism, as well as the early OVX-induced obesity[376]. The animal model of ovariectomy-induced bone loss might therefore not have been the most ideal to assess the effect of DPP4 inhibition on the bone. Ovariectomy has an effect on DPP4 activity as well as LP sensitivity, which makes alternative osteoporotic models like streptozotocin-induced diabetes, retinoic acid or corticosteroids potentially more promising models. A male orchiectomy model could circumvent the effect of estrogen on the bone metabolism, but could also be associated with an increase of DPP4 activity.

As expected, ovariectomy resulted in a quick and steep decrease of the tra-

becular bone volume and a loss of trabecular bone micro-architecture, which was observed by in vivo micro-CT. Considering the absolute values measured by micro-CT as well as bone histomorphometry, no significant differences were observed between sitagliptin treated and untreated groups. However, a few trends were noticeable. When trabecular parameters are evaluated in relation to their basal values, it becomes clear that sitagliptin treatment results in a slightly elevated bone formation, in SG-treated control animals compared to untreated control animals: bone volume, surface and surface density were found to be significantly increased in the treated controls compared to the untreated controls. In the sitagliptin treated ovariectomized animals, the same effect could be observed, although less pronounced and only at 2 weeks after OVX.

Static bone parameters as determined by bone histomorphometry clearly show a difference between the ovariectomized and control group, but do not show any differences between sitagliptin treated and untreated groups. A reduction of bone resorption, as recently observed in postmenopausal diabetic women after a 12-week treatment with sitagliptin[377], could not be confirmed. Evaluation of fluorescent labels in bone sections reveals a significantly increased perimeter percentage of double labels in sitagliptin treated ovariectomized animals when compared to untreated ovariectomized animals. Furthermore, a tendency was observed towards an increased mineral apposition rate and bone formation rate in sitagliptin treated ovariectomized rats. The lowest value of bone formation rate of these rats exceeds the average of the untreated ovariectomized group. Mineralization lag time was also found to be shorter in sitagliptin treated ovariectomized animals. The combination of measured dynamic parameters suggests an increased formation as well as mineralization of osteoid through sitagliptin treatment. However, osteoid perimeter and osteoid area were found similar in treated and untreated groups.

In conclusion, effects of sitagliptin treatment on bone were only minimal and most pronounced in control animals and early after ovariectomy in OVX rats. This could be due to the radical effect of ovariectomy, involving rapid metabolic changes in the trabecular bone which could not be altered by DPP4 inhibition.

Cortical bone parameters reflect the expected biovariability between groups. The metabolic activity of trabecular bone is much higher than cortical bone, and the observation period of the experimental animals was most probably too short to affect the cortical bone in ovariectomized groups. In a study conducted with postmenopausal women, the effect on the cortical bone was found to manifest itself only after five years[378]. Cortical bone volume increased steadily in all groups, maintaining a constant ratio between groups, indicating a normal growth of the cortical bone. Also the cortical bone surface

and cortical bone surface density are a reflection of regular bone growth and variability. Although the effect of ovariectomy might be too drastic in the trabecular bone to cause significant changes through sitagliptin-treatment, the duration of the study did not allow for any changes in the cortical bone following ovariectomy.

Earlier experimental studies observed occurrences of necrotic pancreatitis in diabetic rats treated with 200 mg/kg of sitagliptin after a period of 12 weeks[379]. Acute pancreatitis is diagnosed in case of a three-fold increase of amylase and lipase values measured in the serum, in combination with an increase of liver AST and ALT[380]. An only mild increase of serum amylase activity in sitagliptin treated ovariectomized animals suggest none of the animals in this study had developed acute pancreatitis due to sitagliptin treatment. Fluctuations of serum creatinine in ovariectomized groups are most probably due to the ovariectomy operation itself. Variations of urinary creatinine, creatinine clearance, and serum calcium and phosphate are within normal ranges of biovariability.

The results of this experiment indicate that the inhibition of DPP4 activity in the serum indeed affects the bone metabolism, although the results became only significant in control animals and in the early phase after ovariectomy. The fact that no effect of DPP4 inhibition could be observed in ovariectomized animals at later time-points could be due to the intensity of the effect of ovariectomy on the trabecular bone, and due to its complicated effects on DPP4 activity and feeding.

3.6. DPP4 inhibition attenuates bone loss in NAD/STZ-diabetic rats

3.6.1. Introduction: DPP4 inhibition with sitagliptin results in a reduced osteoclast activity in db/db-mice

In an introductory experiment, the femora of leptin-receptor-deficient mice (db/db$^{-/-}$-mice) treated with sitagliptin were analyzed using bone histomorphometry. Experimental animals were participants in a study conducted by the Laboratory of Physiology of the University of Antwerp to evaluate the effect of DPP4 inhibition with sitagliptin on heart function. The bone histomorphometry was performed to evaluate the potential effect of DPP4 inhibition on bone metabolism in diabetic animals. The db/db mouse is a mouse which lacks activity of LPR due to a homozygous point mutation in the LPR gene, through which they are unable to reach a sensation of satiety and become obese and diabetic, as well as dyslipidaemic[381]. This genetic mouse model is very similar to the ob/ob model, in which the LP gene itself is disabled. LPR deficient mice were found to display a moderately reduced trabecular volume and a strongly reduced cortical bone volume in tibiae as well as vertebrae measured by micro-CT, as well as an increased bone fragility measured by three-point bending[41], which was associated with a reduction of trabecular thickness and trabecular number[382].

In this study, 20 mice (age 8 weeks) were divided into three groups. One group of mice were left untreated and served as non-diabetic controls (C/V, n=4). Sixteen diabetic db/db$^{-/-}$ mice were either treated with 300 mg/kg/day sitagliptin (DM/SG, n=8) or with saline vehicle (DM/V, n=8). Sitagliptin was administered through the drinking water. In this study, 10 tablets of 100 mg sitagliptin were dissolved per liter of drinking water, resulting in a concentration of 1 g/L and an average dose of 300 mg/kg/day. At an age of 16 weeks (after 8 weeks of daily treatment), mice were sacrificed and the femora were evaluated with bone histomorphometry.

The activity of DPP4 was found to be significantly decreased in the db/db-mice treated with sitagliptin when compared to control animals (figure 3.28). Analysis of the right femora using bone histomorphometry revealed no significant differences. Nor LP deficiency nor DPP4 inhibition were found to significantly affect bone area, eroded perimeter, osteoclast perimeter or trabecular thickness. However an interesting trend can be observed when looking at figure 3.29: SG treatment seems to normalize bone parameters affected by deficient LP signaling.

In conclusion, the administration of sitagliptin through the drinking water resulted in an efficient and significant inhibition of DPP4 activity in the treated animals. Kidney function as measured by serum creatinine remained

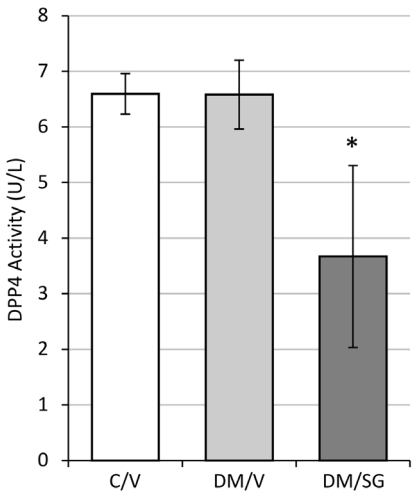

Figure 3.28. *DPP4 activity measured in controls (C/V), untreated (DM/V) and SG-treated (DM/SG) db/db$^{-/-}$ animals. * $p < 0.05$ vs db/db.*

unaffected by SG-treatment, and was also found to be similar in db/db-animals compared to controls. The analysis of ventricular function indicated an improvement in db/db-animals treated with sitagliptin, which led to further research and an article published by the Laboratory of Physiology[383]. Results of bone histomorphometry did not reveal any significant result of DPP4 inhibition, however allowed to suggest a protective effect of sitagliptin treatment: DPP4 treated db/db-mice approached the values of control animals.

These results led to the establishment of a new study setup, in which the effect of DPP4 inhibition through administration of sitagliptin through the drinking water was tested in diabetic osteoporosis in NAD/STZ-diabetic rats. The NAD/STZ-induced diabetic rat model, resulting in partial destruction of pancreatic β-cells, was chosen as it induces rapid onset of hyperglycemia, and resulting diabetic osteopenia. STZ-diabetic rats show quick metabolic changes such as disturbances in insulin and insulin-like growth factor production, causing a chronic hyperglycemic state[329]. As described earlier, the induction of diabetes results in osteopenia associated with a reduction of osteoid area, number of osteoblasts and osteoclasts, and general bone turnover[350]. Also a defective formation of extracellular matrix due to calcification abnormalities occurs in presence of increased AGE concentrations, resulting in reduced biomechanical properties[349,355]. The evaluation of bone microstructure and quality in rat models has also been proven to yield in more significant results in comparison to mouse models. These reduced structural and biomechanical properties that arise relatively quickly and significantly make the NAD/STZ-diabetic model suitable for the assessment of the effect of DPP4 inhibition on

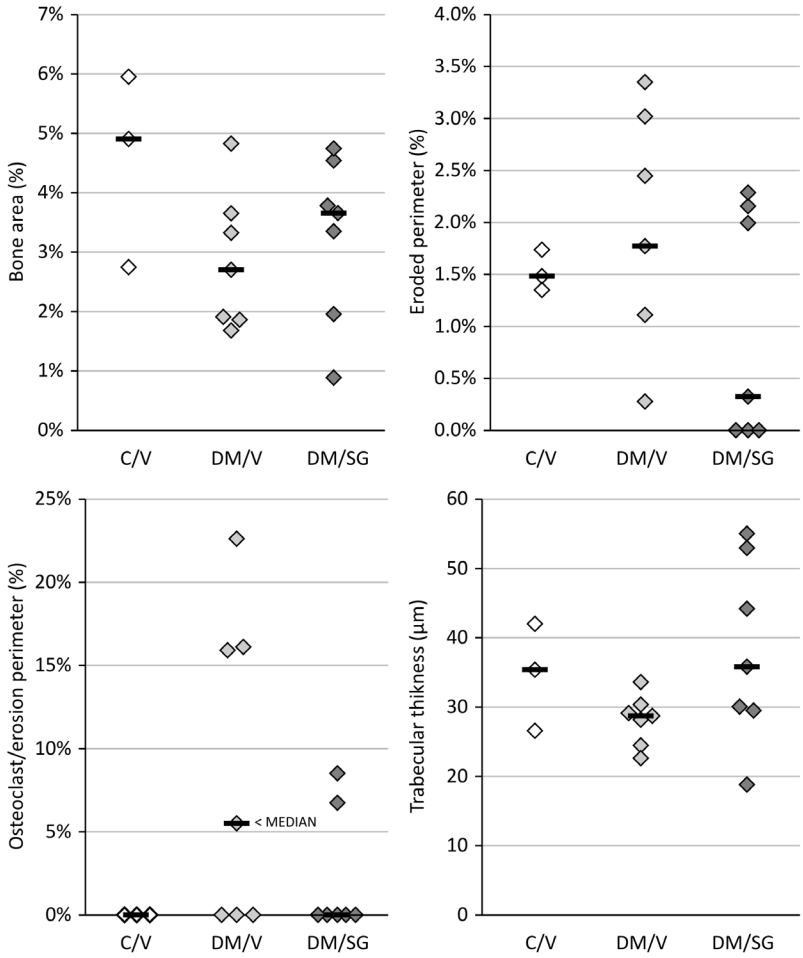

***Figure 3.29**. Bone histomorphometric parameters of the right femur. No significant differences were observed.*

bone metabolism. Furthermore, diabetic models based on STZ are characterized by a high glucose level, in contrast to db/db-mice which is expected to remain unaffected through DPP4 inhibition. This would allow to investigate the effect of DPP4 inhibition on the development of diabetic induced bone loss, without the need to take into account the effect of reduced glycemia on the bone.

3.6.2. Study setup

In this experiment, 64 male Wistar rats (10 wk of age, 300–325 g; Iffa Credo, Brussels, Belgium) were randomly assigned to four experimental groups, two control groups and two diabetic groups, each one treated orally with 2

g/L sitagliptin dissolved in drinking water (figure 3.30). Diabetes was induced chemically through administration of an intravenous injection of 65 mg/kg STZ (Sigma-Aldrich, St. Louis, MO) 15 min after an intraperitoneal injection of 230 mg/kg NAD (Sigma-Aldrich). Sitagliptin treatment through the drinking water was initiated immediately following STZ injection in the diabetic SG-treated group over the course of the entire experiment. Four days after NAD/STZ administration, glucose was measured using a GlucoMen Lx Plus^{+} automated whole blood glucometer with glucose strips (Menarini, Florence, Italy) to verify induction of diabetes. No diabetic animals met the exclusion criterium of a blood glucose level < 200 mg/dL. Animal groups are abbreviated as follows: C/V (controls treated with vehicle), and C/SG (controls treated with sitagliptin), DM/V (diabetic animals treated with vehicle), DM/SG (diabetic animals treated with sitagliptin).

During the course of this study, body weight and non-fasting blood glucose were recorded every 3 wk. To collect urine and register consumption and excretion, rats were placed in metabolic cages at weeks 6 and 12. The proximal ends of the right tibiae of all animals were evaluated by in vivo micro-CT every 3 weeks as described in the materials & methods section, after an intravenous injection of 35 mg/kg sodium pentobarbital (Nembutal(R), Ceva Santé Animale, Brussels, Belgium). Half of the animals were euthanized 6 weeks after the induction of diabetes by exsanguination through the abdominal aorta after pentobarbital sodium anesthesia. The remaining animals were euthanized after 12 weeks. Pancreatic tissue was also isolated for H&E staining to assess the morphology of β-pancreatic islets, and thoracic aorta was removed to measure calcification by Von Kossa staining. Right tibiae were processed as previously described for bone histomorphometric analysis. Right femora were harvested and frozen for mechanical testing by three-point bending.

3.6.3. Results

3.6.3.1. Animal data

During the course of the experiment, two out of 64 animals died prior to sacrifice, corresponding to a mortality of 3.125%. One SG-treated control animal died during the micro-CT scan following anesthesia in week 1. One animal from the SG-treated diabetic group died following induction of diabetes before SG-treatment started, displaying an initial glucose value of over 600 mg/dL. These animals were excluded from the analysis. Results are presented as means ± standard deviation. Statistics were performed with IBM SPSS Statistics 20. Comparisons between study groups were assessed using the Kruskal-Wallis H-test, followed by a Mann-Whitney U-test in combination with the Bonferroni correction when more than two groups were compared. Values of

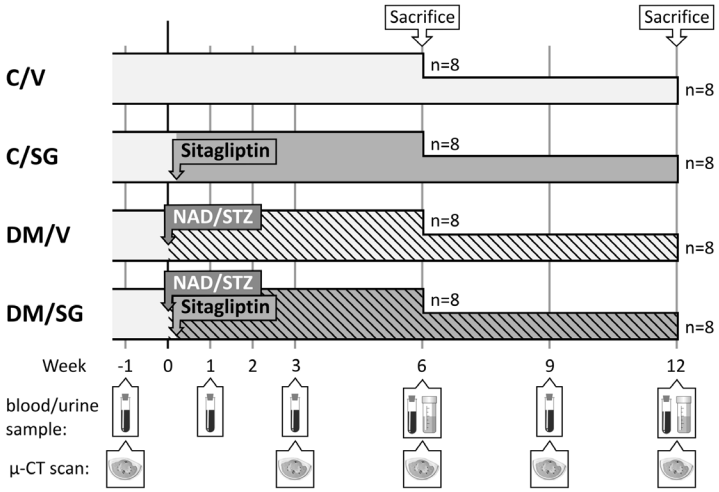

Figure 3.30. *Study setup (n=64) divided in four equal groups: C/V (n=16, controls treated with vehicle), C/SG (n=16, controls treated with sitagliptin), DM/V (n=16, diabetic animals treated with vehicle), DM/SG (n=16, diabetic animals treated with sitagliptin). Half of the animals were euthanized after 6 weeks.*

$p < 0.05$ were considered significant.

3.6.3.2. SG-treatment affects femoral length, but not blood glucose levels in diabetic animals

Results evaluating the body weight, femoral growth and blood glucose levels are presented in table 3.7. No significant differences were observed between the initial weight of the control group and the diabetic group. After the induction of diabetes, the growth of the diabetic animals stagnated in comparison to the control groups, resulting in a significantly decreased body weight from week 3 on (figure 3.31A). The SG-treated control group was found to display a lower body weight compared to the untreated control group, however the difference was not significant. Glucose levels increased steeply in diabetic animals after NAD/STZ administration, rising to significant levels > 200 mg/dL from week 3 on (figure 3.31B).

Growth stagnation was reflected by femoral length, which was found to be significantly lower in diabetic animals compared with nondiabetic animals at weeks 6 and 12, except for the sitagliptin-treated diabetic animals at week 12, of which the femoral length did not differ from the control groups.

Morphological assessment of H&E-stained pancreatic sections revealed a slightly reduced size of β-pancreatic islets in diabetic animals, which however remained unaffected through SG treatment (not shown).

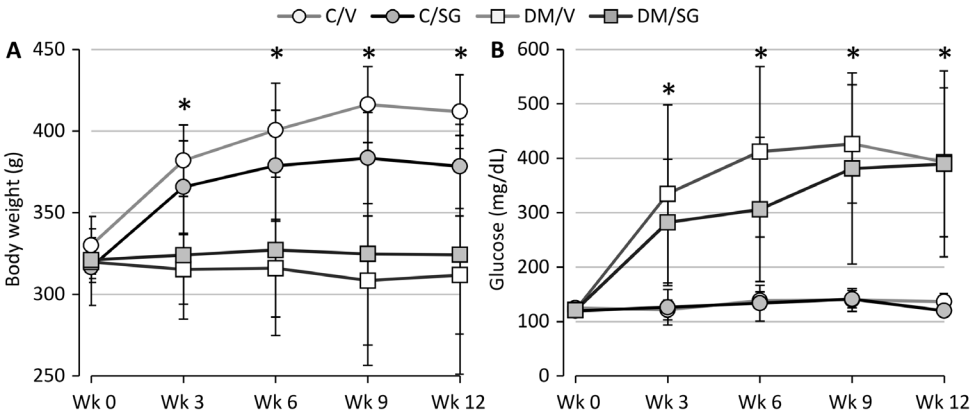

Figure 3.31. *Body weight (A) and blood glucose (B) of experimental animals (g).*
** p < 0.05 vs C/V.*

Table 3.7. Weight, femoral length and glycemia of animals at the start and the end of the experiment.

		C/V (n=16)	C/SG (n=16)	DM/V (n=16)	DM/SG (n=16)
Initial weight (g)		330 ± 18	317 ± 24	320 ± 10	321 ± 14
Final weight (g)		412 ± 23	378 ± 26	312 ± 36*	324 ± 73*
Femoral length (mm)	wk6	38.1 ± 0.8	38.1 ± 1.1	36.9 ± 0.4*	36.7 ± 1.3*
	wk12	38.6 ± 1.1	38.5 ± 1.4	36.9 ± 0.7*	37.1 ± 2.0
Initial glycemia (mg/dL)		125 ± 6	120 ± 7	121 ± 7	121 ± 7
Final glycemia (mg/dL)		136 ± 15	120 ± 6*	392 ± 137*	390 ± 171*

** p < 0.05 compared to untreated control animals (C/V).*

3.6.3.3. Sitagliptin reduces food and water consumption but does not affect creatinine clearance

NAD/STZ-induced diabetic animals displayed a significantly increased food consumption, drinking volume, and urine volume compared with control animals (table 3.8). Treatment with sitagliptin lowered each one of these parameters, with a significant reduction in food consumption at week 12 and drinking volume at weeks 6 and 12. A similar but nonsignificant trend was observed for the urine volume. Surprisingly, drinking and urine volume were also significantly lowered by sitagliptin treatment in controls. Renal function as assessed by serum creatinine or creatinine clearance did not differ between groups.

Table 3.8. *Food consumption, drinking volume, urine volume and creatinine clearance as measured in metabolic cages at week 6 and 12.*

		C/V (n=16)	C/SG (n=16)	DM/V (n=16)	DM/SG (n=16)
Food consumption (g)	wk6	11.5 ± 9.7	9.3 ± 6.1	28.8 ± 11.9†	19.1 ± 12.6
	wk12	14.6 ± 9.0	10.0 ± 10.4	32.0 ± 17.4†	14.5 ± 11.9*
Drinking volume (mL)	wk6	18 ± 6	9 ± 6†	110 ± 53†	60 ± 46†*
	wk12	22 ± 11	10 ± 9†	127 ± 93†	39 ± 40*
Urine volume (mL)	wk6	17 ± 6	8 ± 4†	102 ± 57†	67 ± 53†
	wk12	14 ± 7	7 ± 4†	92 ± 53†	51 ± 40†
Creatinine clearance (mL/min/100g)	wk6	0.33 ± 0.04	0.26 ± 0.09	0.36 ± 0.11	0.33 ± 0.13
	wk12	0.35 ± 0.08	0.32 ± 0.09	0.39 ± 0.10	0.38 ± 0.12

** p < 0.05 compared to untreated diabetic animals (DM/V).*
† p < 0.05 compared to untreated control animals (C/V).

3.6.3.4. Variable sitagliptin dosage resulted in efficient DPP4 inhibition

Treatment with sitagliptin in the drinking water resulted in an average inhibition of serum DPP4 activity of over 85% in diabetic as well as control animals despite variable dosage due to increased water consumption in the diabetic groups. The dosage was 23.4 ± 16.2 mg/kg and 153.3 ± 136.3 in control animals and diabetic animals, respectively. Although DPP4 activity was recorded to be the highest in the diabetic control group, increase of DPP4 activity was never significant compared to basal values during the course of the experiment. Detailed percentages of DPP4 activities and inhibition are given in table 3.9.

Table 3.9. *DPP4 activity during the course of the experiment (U/L) and DPP4 inhibition in SG-treated groups (%).*

	Week 0	Week 3	Week 6	Week 9	Week 12
C/V (U/L)	16.0 ± 3.0	12.6 ± 1.6	16.4 ± 3.3	16.7 ± 3.7	16.0 ± 2.9
C/SG (U/L)	16.0 ± 1.1	5.3 ± 2.2	8.9 ± 5.1	6.4 ± 3.4	10.3 ± 3.1
DM/V (U/L)	15.4 ± 2.6	14.9 ± 3.4	21.2 ± 4.1	19.0 ± 2.0	17.1 ± 3.1
DM/SG (U/L)	16.0 ± 1.7	4.9 ± 2.9	8.3 ± 4.0	7.6 ± 6.7	7.5 ± 5.6
C/SG (%)	-	88.7 ± 2.8	84.8 ± 6.4	86.8 ± 6.6	82.3 ± 5.9
DM/SG (%)	-	89.1 ± 4.0	83.1 ± 9.4	68.7 ± 33.8	87.0 ± 5.8

3.6.3.5. Evaluation of bone quality

Micro-CT analysis reveals an attenuation of trabecular bone loss in SG-treated diabetic animals

Micro-CT analysis of the trabecular bone structure in the metaphysis of the tibiae revealed distinct structural differences between diabetic animals and controls, as can be seen in 3D images of the metaphysis reconstructed by CtAnalyser (figure 3.32F). The ratio of the trabecular bone volume over the total cancellous tissue volume (BV/TV) was found to be significantly lower in diabetic animals at weeks 6, 9, and 12 (figure 3.32A). The loss of trabecular bone in diabetic animals was completely attenuated by sitagliptin treatment. BV/TV of these animals was preserved compared with untreated diabetic animals and at no point differed significantly from the nondiabetic controls. The ratio of bone surface to bone volume was significantly increased at week 6 in untreated diabetic animals compared to control animals and SG treated diabetic animals (figure 3.32B). No significant difference in trabecular thickness was observed between animal groups (figure 3.32C). Trabecular spacing increased in all groups over time, which was significantly more pronounced in diabetic animals, as evidenced by a threefold increase compared with an only twofold increase in control animals. This difference became significant at weeks 9 and 12. In sitagliptin-treated diabetic animals, the effect of diabetes on trabecular spacing was completely abolished and at no time point differed significantly from the control animals. Compared with the untreated diabetic animals, the trabecular spacing was significantly lower at week 12 (figure 3.32D). The number of trabeculae decreased steadily in all groups. In untreated diabetic animals, the trabecular number decreased by 60% compared with almost 40% in controls, resulting in a significant difference between both groups from week 3 until the end of the experiment. The trabecular number of diabetic animals treated with sitagliptin at no time point differed from the untreated nondiabetic controls and was significantly higher compared with untreated diabetic animals at week 9 (figure 3.32E).

Micro-CT analysis reveals a delayed stagnation of cortical bone growth in SG-treated diabetic animals

The cortical bone volume of the tibiae as measured with micro-CT in the metaphysis increased steadily with time in the control animals, whereas the cortical bone growth was significantly lower in diabetic animals and stagnated, resulting in a significant difference vs. control animals from week 3 onward. The cortical bone volume of the SG-treated diabetic animals stagnated at week 6 only, at a higher level compared with the untreated diabetic animals, and was at no point significantly different from the control animals

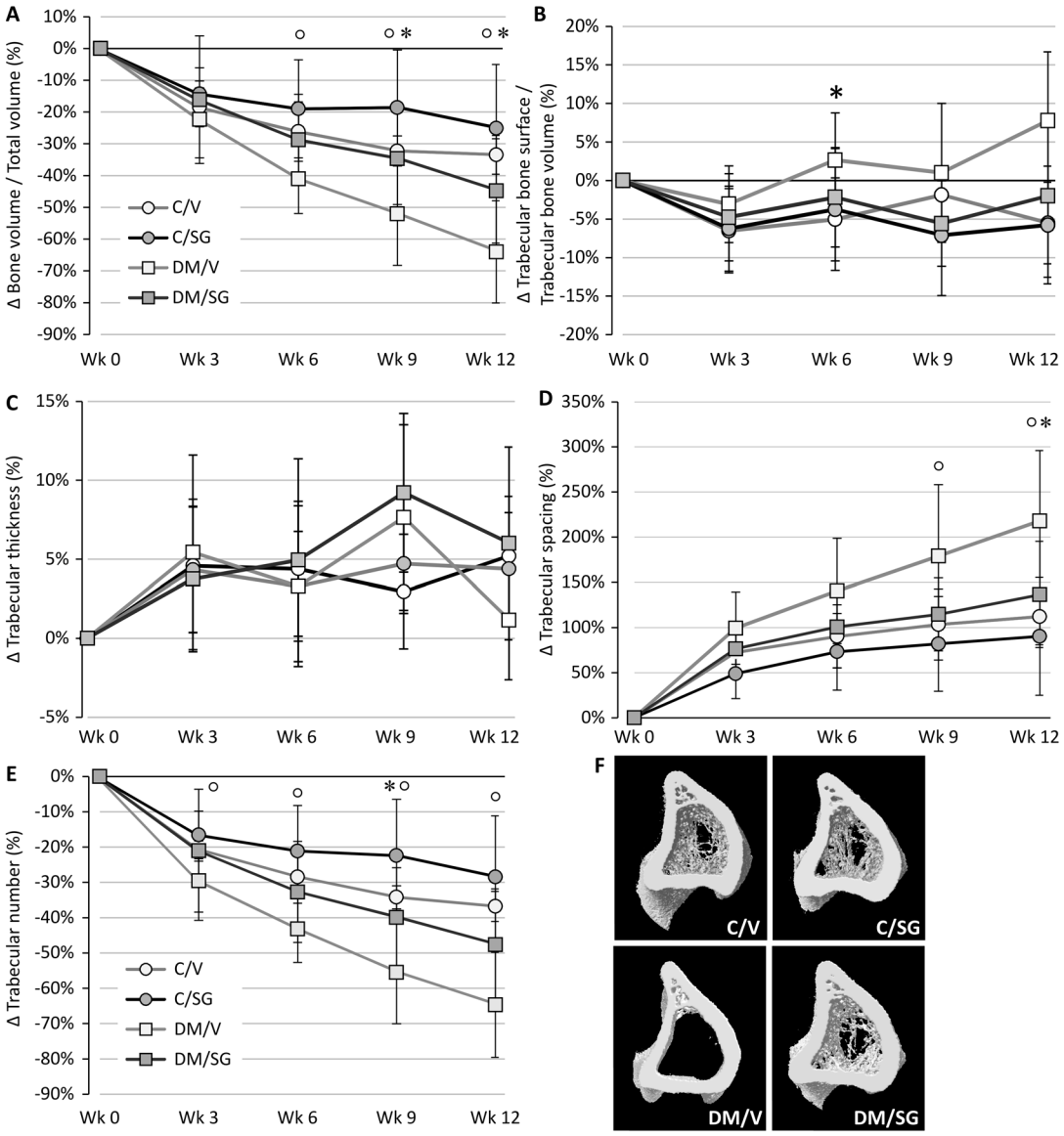

Figure 3.32. *(A) Ratio of trabecular bone volume over tissue volume, (B) ratio of trabecular bone surface over bone volume, (C) trabecular thickness, (D) trabecular spacing and (E) trabecular number in the metaphysis as assessed by micro-CT analysis. Change relative to individual baseline value (%) ° p < 0.05 in DM/V compared to C/V. * p < 0.05 DM/SG compared to DM/V. (F) 3D images of the metaphysis reconstructed by CtAnalyser.*

(figure 3.33A). The ratio of cortical bone surface over bone volume reveals thinning of the cortical bone in diabetic control animals, which is less pronounced in SG-treated diabetic animals (figure 3.33B).

Sitagliptin treatment results in a conservation of femoral neck BMD in diabetic animals

An approximation of BMD of the trabecular bone within the femoral neck was made analyzing the femur of sacrificed animals with micro-CT at week 6 and week 12, and comparing them with standardized phantom bones. This analysis revealed similar BMDs measured at week 6, but a significantly reduced tBMD of the femoral necks of diabetic animals at week 12. In SG-treated diabetic animals, this tBMD remained similar to control animals and was significantly higher than the untreated diabetic animals (figure 3.34).

Three-point bending shows an increased resistance to fractures in SG-treated diabetic bone

In diabetic animals, a significantly lower ultimate load and thus mechanical strength were observed compared with controls in week 6 as well as in week 12. Sitagliptin treatment prevented the loss of strength in diabetic bone, as the ultimate load measured was significantly higher than that in untreated diabetic animals at week 12. When the load was normalized over the transversal cortical bone surface, this difference remained significant (figure 3.35). Neither diabetes nor sitagliptin had any significant effect on bone stiffness, ultimate displacement, energy to failure, Young's modulus, ultimate strain, ultimate stress, or toughness (table 3.10).

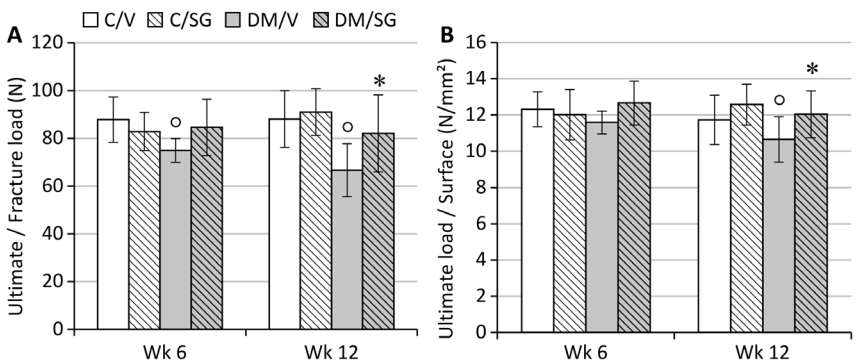

Figure 3.35. *Ultimate load measured in femora by three-point bending (left), normalized over transversal cortical bone surface (right). ° $p < 0.05$ in DM/V compared to C/V. * $p < 0.05$ DM/SG compared to DM/V.*

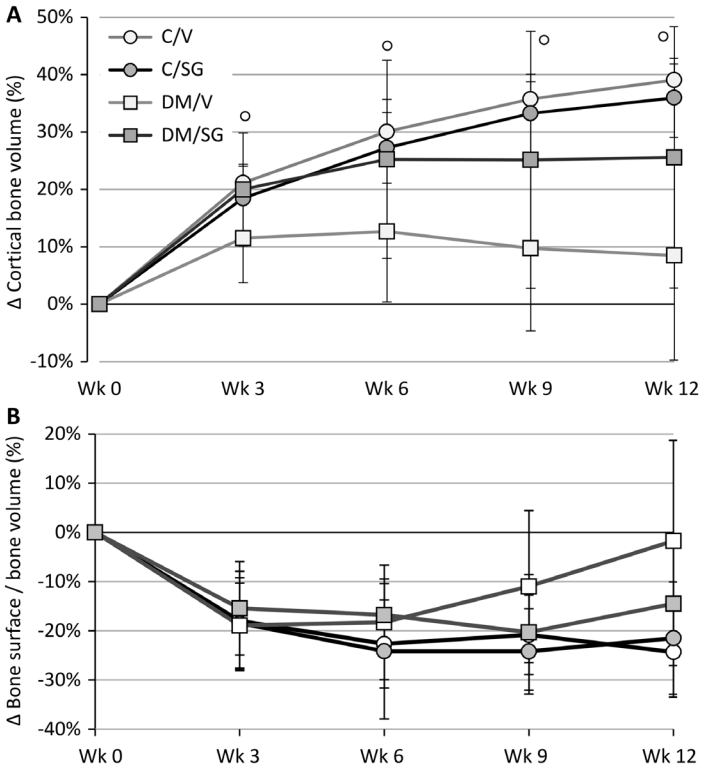

Figure 3.33. *(A) Cortical bone volume and (B) ratio of cortical bone surface over bone volume measured by micro-CT in the metaphysis. ° p < 0.05 in DM/V compared to C/V.*

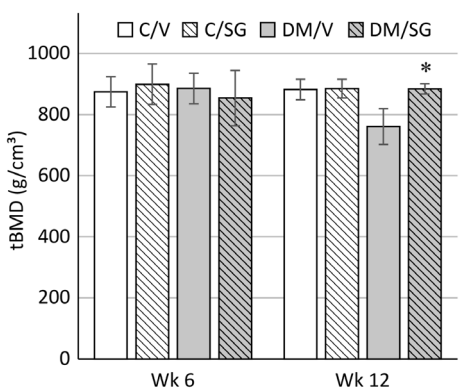

Figure 3.34. *tBMD measured with micro-CT in femoral necks in (A) week 6 and (B) week 12.* p < 0.05 versus DM/V.*

Table 3.10. *Mechanical properties of the right femoral bone as measured with three point bending and calculated using MathLab. Cortical transversal surface was calculated using micro-CT and a custom MathLab algorithm.*

		C/V	C/SG	DM/V	DM/SG
Stiffness (N/mm)	wk6	154.8 ± 19.0	148.4 ± 17.0	135.8 ± 11.3	150.0 ± 15.2
	wk12	173.4 ± 16.46	175.8 ± 15.3	140.4 ± 26.3	165.0 ± 25.9
Ultimate load (N)	wk6	87.76 ± 9.50	82.78 ± 8.01	74.86 ± 5.00†	84.52 ± 11.78
	wk12	88.03 ± 11.96	90.96 ± 9.77	66.60 ± 11.06†	81.96 ± 16.12*
Cortical transversal surface (mm^2)	wk6	7.13 ± 0.58	7.06 ± 0.57	6.42 ± 0.41	6.85 ± 0.40
	wk12	7.22 ± 0.23	7.23 ± 0.50	6.41 ± 0.40†	7.01 ± 0.58
Ultimate load / cortical transversal surface (N/mm^2)	wk6	12.31 ± 0.97	12.02 ± 1.38	11.59 ± 0.63	12.66 ± 1.22
	wk12	11.74 ± 1.36	12.58 ± 1.13	10.65 ± 1.26†	12.04 ± 1.29*
Ultimate displacement (mm)	wk6	0.65 ± 0.07	0.75 ± 0.27	0.63 ± 0.09	0.66 ± 0.13
	wk12	0.57 ± 0.12	0.55 ± 0.03	0.56 ± 0.11	0.55 ± 0.13
Energy to failure (mJ)	wk6	2.86 ± 0.73	2.79 ± 1.10	2.36 ± 0.84	2.93 ± 1.33
	wk12	2.25 ± 1.32	2.00 ± 0.29	1.60 ± 0.45	2.09 ± 1.14
Young's modulus (MPa/%)	wk6	402.3 ± 44.6	391.0 ± 53.9	400.2 ± 51.3	376.0 ± 100.2
	wk12	411.9 ± 55.7	424.9 ± 36.0	401.1 ± 63.8	417.4 ± 39.7
Ultimate stress (MPa)	wk6	279.0 ± 21.3	277.2 ± 25.4	262.3 ± 29.2	261.4 ± 66.0
	wk12	512.1 ± 38.2	529.6 ± 23.6	481.4 ± 74.1	513.9 ± 34.3
Ultimate strain (%)	wk6	8.0 ± 0.9	9.4 ± 4.2	7.5 ± 1.2	8.4 ± 1.9
	wk12	7.1 ± 1.7	6.9 ± 0.6	6.7 ± 1.3	6.9 ± 1.7
Toughness (J/mm^3)	wk6	9.02 ± 2.03	9.30 ± 3.43	8.21 ± 2.73	8.95 ± 4.49
	wk12	6.69 ± 3.58	6.01 ± 0.45	5.54 ± 1.62	6.35 ± 2.98

** $p < 0.05$ compared to untreated diabetic animals (DM/V).*
† $p < 0.05$ compared to untreated control animals (C/V).

Bone histomorphometric analysis of trabecular bone

Static bone histomorphometric analysis did not yield any significant results. Bone area (figure 3.36A), mineralized area (3.36B), osteoid perimeter (3.36C), eroded perimeter (3.36D), osteoclast perimeter (3.36E) and trabecular parameters (3.36F-H) were found equal in all groups. Bone area and trabecular parameters, however, revealed interesting trends supporting a protective effect of SG treatment on diabetic induced bone loss.

The same can be concluded for dynamic bone histomorphometric analysis. The medians of the MAR, AjAr, BFR, MLT and OMT further support a trend towards an improvement of bone metabolism in SG-treated diabetic rats in comparison to their untreated counterparts, but none of these measurements led to significant differences (figure 3.37, table 3.11).

In conclusion it can be summarized that, although most of the measured histomorphometric parameters did not reveal any significant differences, the combined evaluation of some of the static and dynamic bone parameters allows us to suggest a confirmation of the significant effect of sitagliptin on

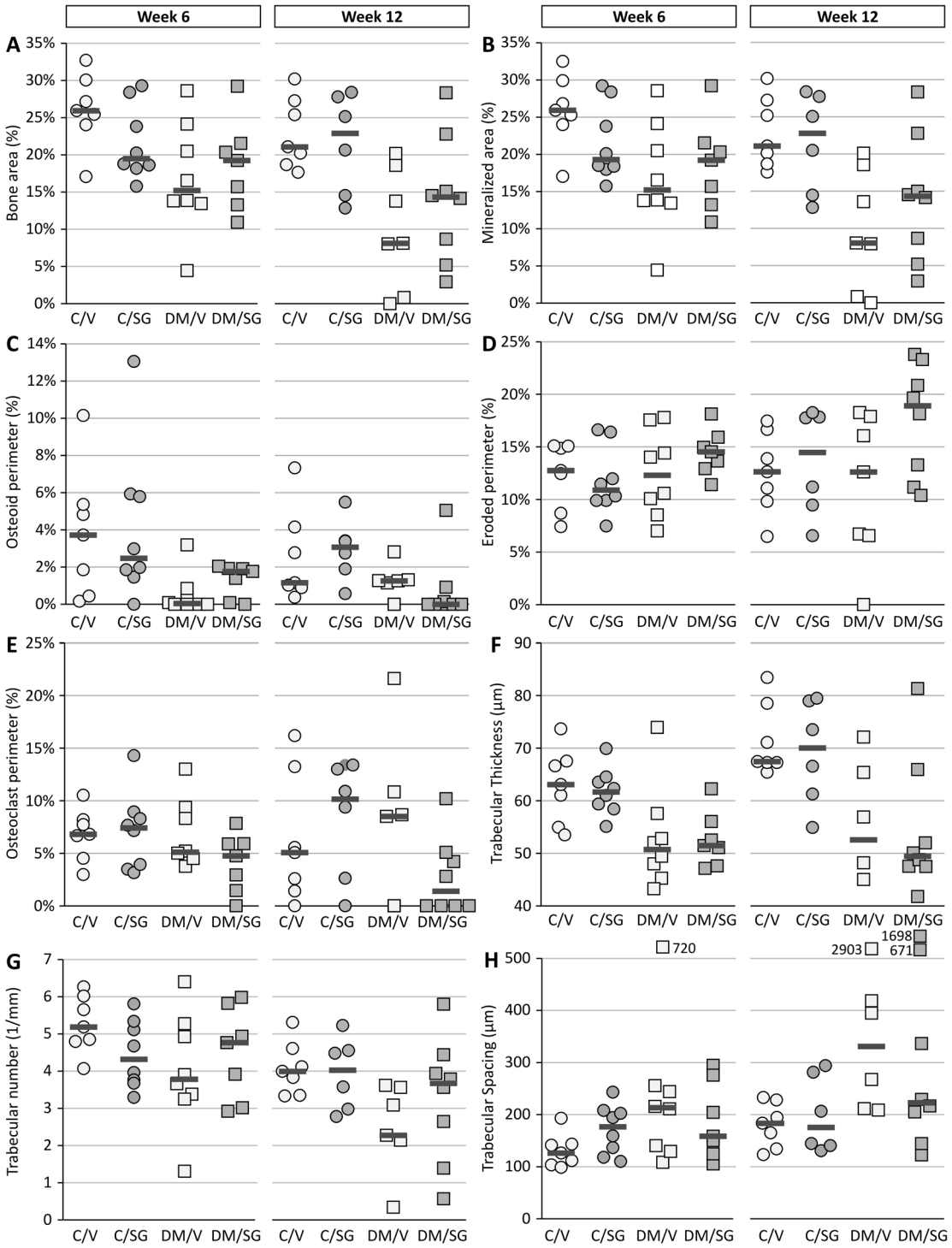

Figure 3.36. *(A) Bone area, (B) mineralized area, (C) osteoid perimeter, (D) eroded perimeter, (E) ratio of osteoclast perimeter to eroded perimeter, (F) trabecular thickness, (G) trabecular number and (H) trabecular spacing at 6 weeks (left) and 12 weeks (right) as determined by bone histomorphometry. No significant differences were observed.*

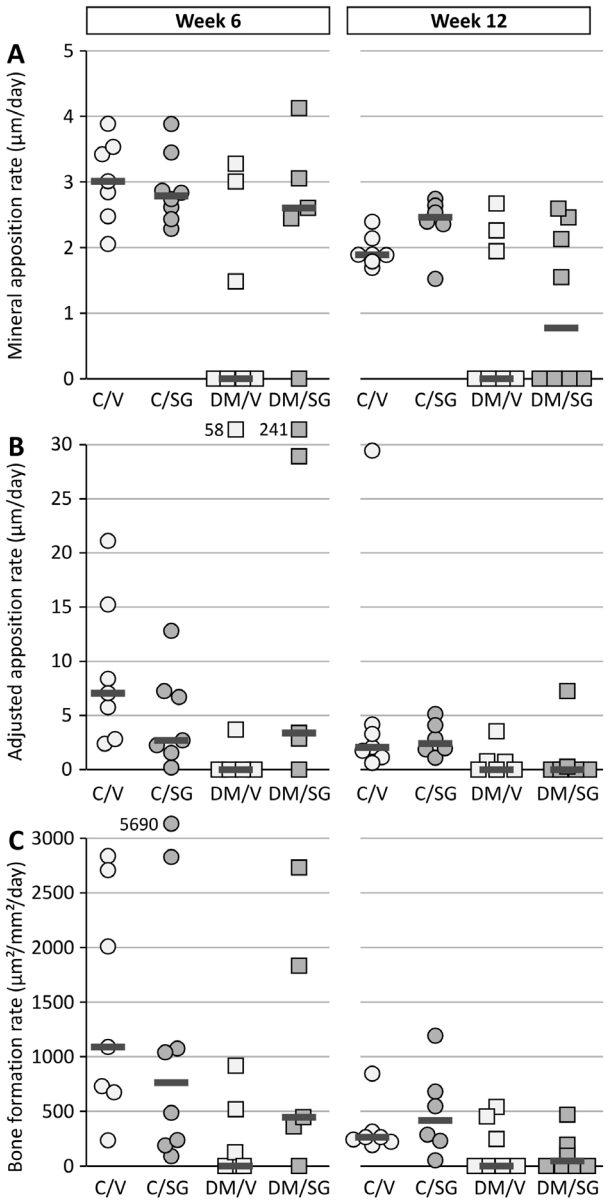

Figure 3.37. *(A) MAR, (B) AjAR and (C) BFR determined by bone histomorphometry. No significant differences were observed.*

diabetic bone pathology observed by micro-CT and three-point bending. This becomes also clear on the representative histological sections presented in figure 3.38. The absence of significant differences, however, can at least in part be ascribed to the high biological variability inherent to the bone histomorphometric analysis.

Table 3.11. *Dynamic bone histomorphometric parameters.*

		C/V	C/SG	DM/V	DM/SG
dlPm (%)	wk6	6.1 ± 3.8	6.3 ± 3.8	0.9 ± 1.4	5.1 ± 4.7
	wk12	2.5 ± 1.5	3.2 ± 2.3	1.8 ± 1.6	1.0 ± 0.7
OMT (days)	wk6	1.2 ± 0.5	1.3 ± 0.7	20.2 ± 18.4	8.0 ± 7.1
	wk12	2.1 ± 0.6	1.7 ± 1.1	2857 ± 2671	2500.3 ± 2072.3
MLT (days)	wk6	0.7 ± 0.6	5.2 ± 3.7	23.5 ± 17.9	7.5 ± 6.4
	wk12	2.4 ± 2.1	2.0 ± 1.6	21.6 ± 16.8	25.5 ± 15.2

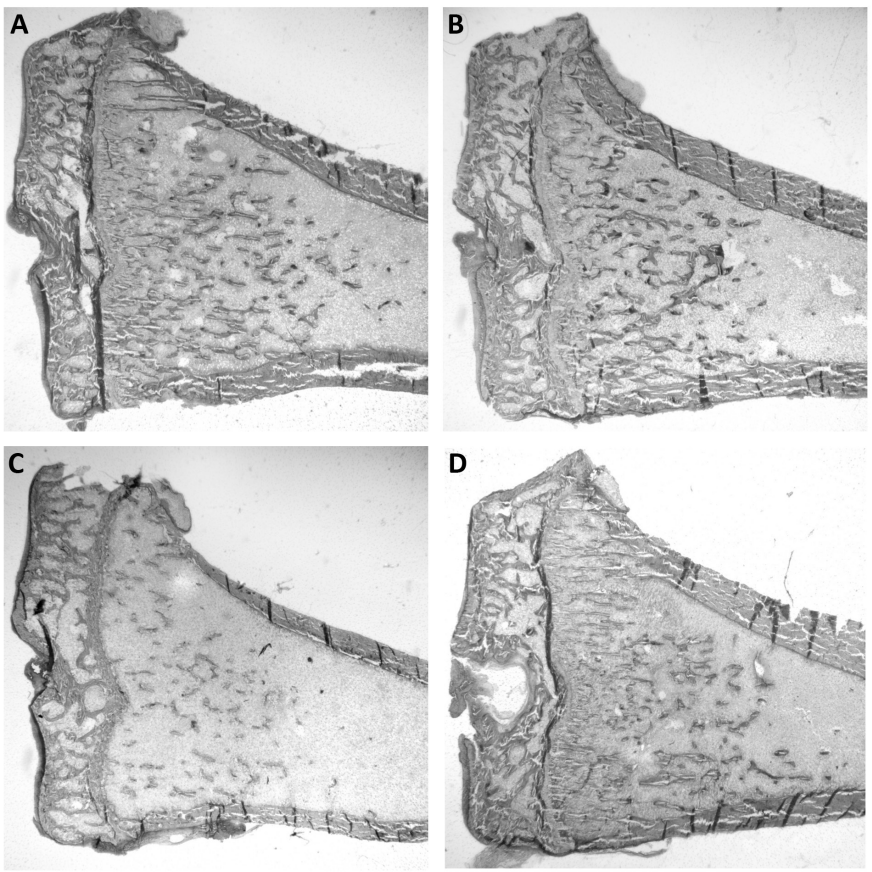

Figure 3.38. *Representative histological, Goldner stained metaphyseal bone sections of (A) C/V (B) C/SG (C) DM/V (D) DM/SG. Note the decreased trabecular number and reduced cortical thickness in the untreated diabetic rats (C).*

Growth plate thickness measurement reveals significant reductions of hypertrophic and proliferative zones in untreated, but not in sitagliptin-treated diabetic animals

At week 6, untreated diabetic animals displayed a reduced proliferative zone compared to control animals, whereas their SG-treated counterparts did not. In week 12, the same phenomenon was observed for the hypertrophic zone (figure 3.39).

3.6.3.6. Biochemical analysis of bone metabolism indicates reduced bone resorption through sitagliptin treatment

Serum calcium was found to be increased in diabetic animals treated with sitagliptin in comparison to untreated diabetic animals in week 6 as well as week 12, where it was found to be similar to controls (figure 3.40A). The serum phosphate was variable in all groups and remained unaffected through induction of diabetes or sitagliptin treatment (figure 3.40B).

Induction of diabetes resulted in a significantly increased excretion of calcium and a nonsignificant increase in phosphate in the urine compared with

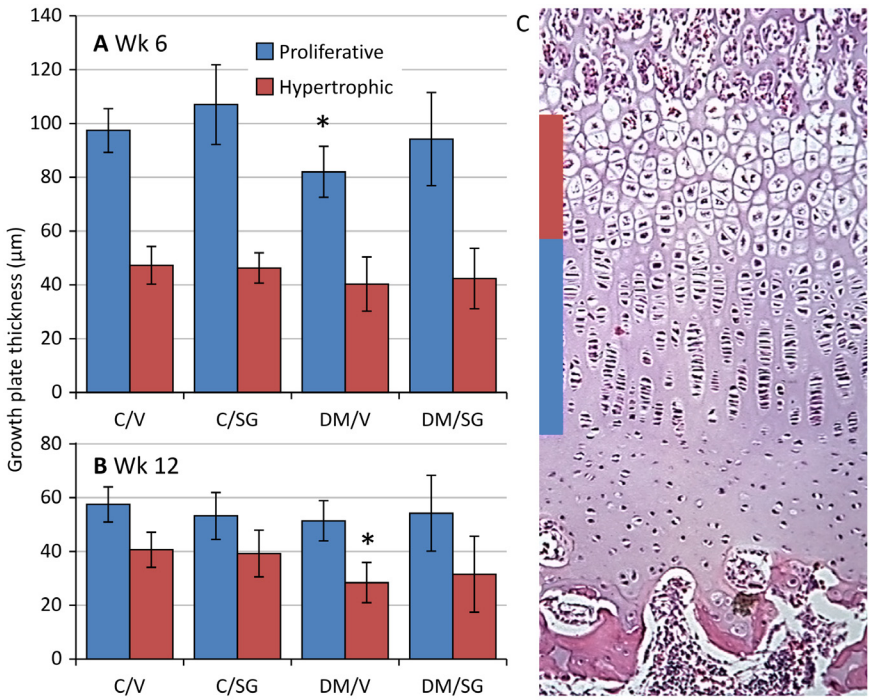

Figure 3.39. *Thickness of the growth plate at 6 weeks (A), 12 weeks (B), and an image of the growth plate (C), blue: proliferative zone, red: hypertrophic zone. * $p < 0.05$ vs C/V.*

control animals, both in week 6 and week 12. Sitagliptin treatment, however, did not affect the urinary excretion of these ions (figure 3.41).

To provide further support for the observed differences between sitagliptin-treated and untreated diabetic animals, bone metabolic biomarkers were determined. Serum osteocalcin levels, as a measure of osteoblast activity, showed a decrease in all diabetic animals in weeks 6 and 12 compared to baseline without any effect of SG treatment (figure 3.42A). Serum CTX-1 level, a measurement of osteoclast activity, increased drastically in all diabetic animals at week 6 compared to baseline. Interestingly, at week 12 CTX-1 levels in sitagliptin-treated diabetic animals were no longer different from to control levels and significantly lower than that of vehicle treated diabetic animals (fig-

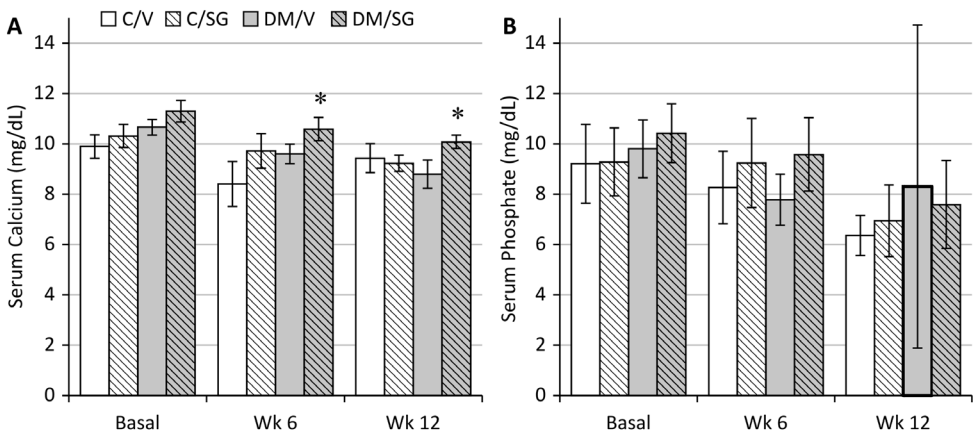

Figure 3.40. *Calcium (A) and phosphate (B) levels measured in the serum. * p < 0.05 versus DM/V.*

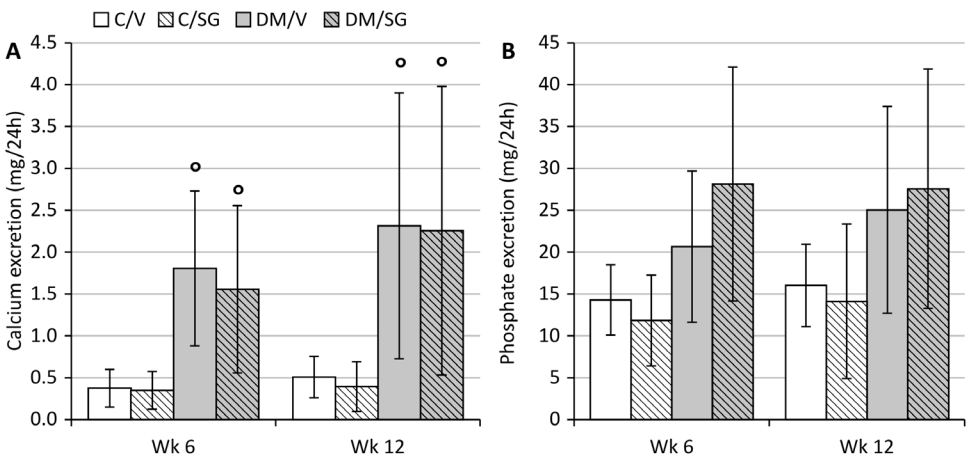

Figure 3.41. *Calcium (A) and phosphate (B) excretion measured in the urine in mg per 24 hours. ° p < 0.05 versus C/V and C/SG.*

ure 3.42B). No differences were observed when PTH and pentosidine levels of sitagliptin-treated and untreated diabetic animals were compared (figure 3.42C and D).

Analysis of serum MMP2 and MMP9, markers of bone turnover that are generally determinant of active bone osteoblasts as well as osteoclasts, revealed a significant decrease of the serum MMP2 concentration in the SG-treated diabetic group compared to the untreated diabetic group at week 6 (figure 3.43A). At week 12, diabetic animals showed increased MMP9 levels compared to controls, whereas the SG-treated diabetic animals did not display increased levels (figure 3.43B). However, an increased MMP concentration was already observed in diabetic animals compared to controls at the start of the experiment. No differences were observed comparing the prolidase activities of different animal groups (data not shown). Dipeptides Ala-Pro and Pro-Pro were found to be increased in the serum of diabetic animals at week 12,

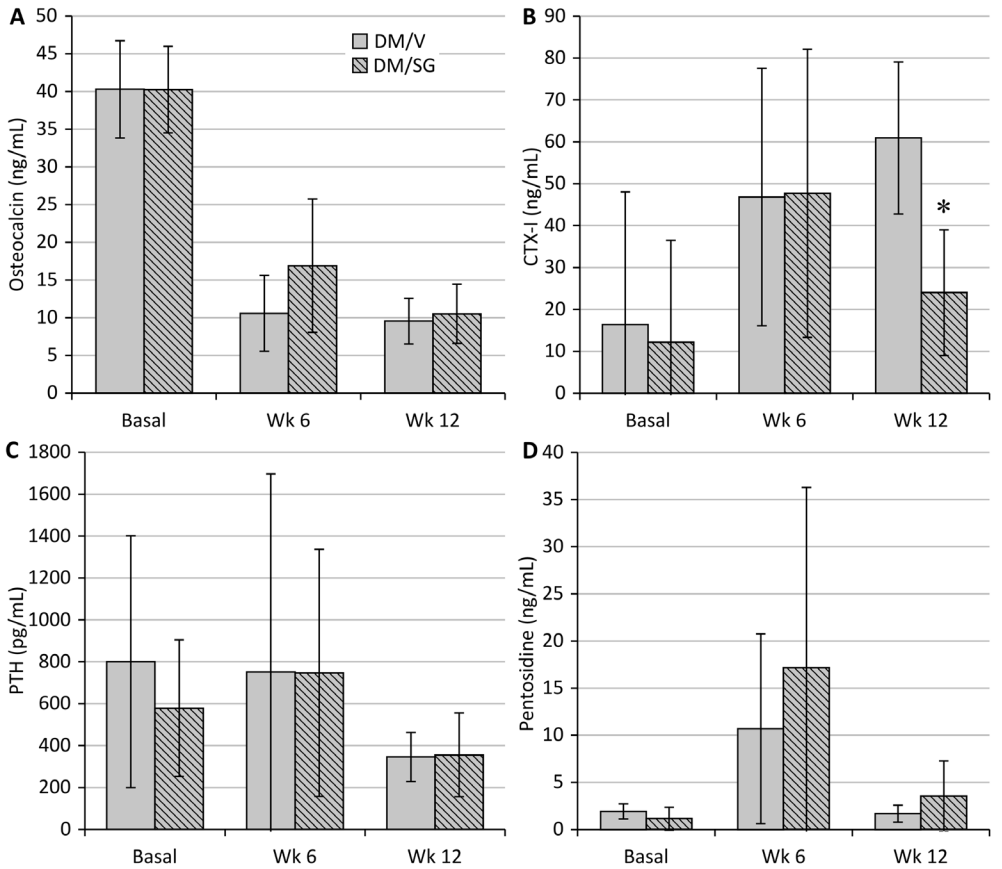

Figure 3.42. *(A) Serum osteocalcin, (B) CTX-1, (C) PTH and (D) pentosidine levels.*
** p < 0.05 vs DM/V.*

but this increase was not affected by SG treatment (data not shown). These results were published by the Laboratory of Medicinal Biochemistry of the University of Antwerp[384].

3.6.3.7. NAD/STZ-induced diabetes nor SG-treatment affect calcium deposition in the aorta

The abdominal aorta was isolated during sacrifice to assess the calcium content in the tunica media by Von Kossa staining. Although the apparent increase in Ca^{2+}-content in diabetic animals seems normalized through SG-

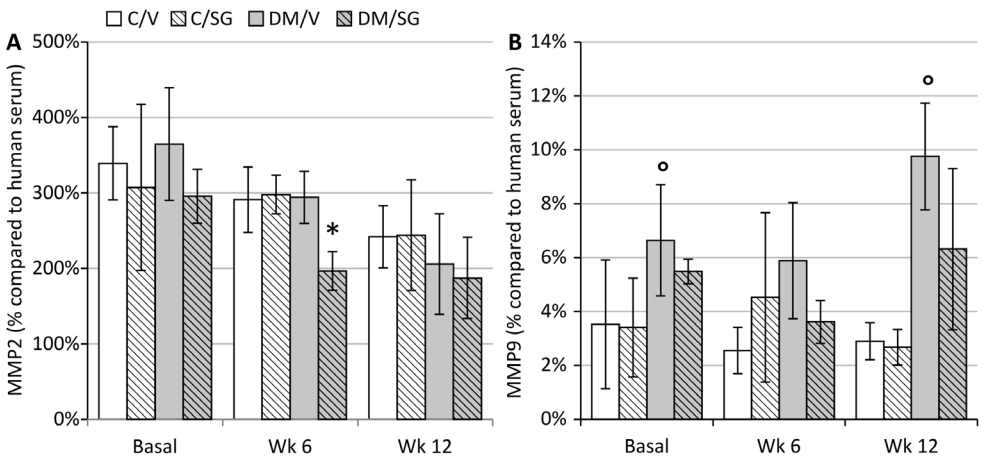

Figure 3.43. *(A) MMP2 and (B) MMP9 concentrations in the serum as measured by gelatin zymography. * p < 0.05 vs DM/V, ° p < 0.05 vs C/V.*

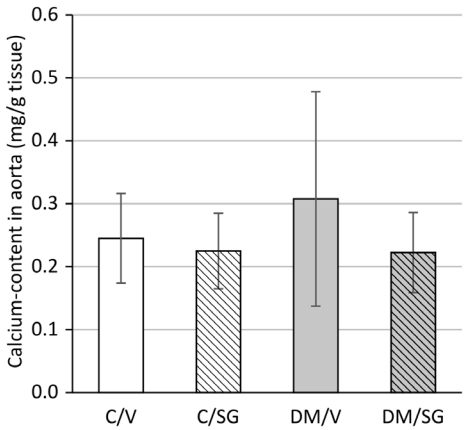

Figure 3.44. *Calcium-content as measured by Von Kossa staining in the abdominal aorta.*

treatment, this observation was not significant (figure 3.44).

3.6.4. Discussion

This study investigates the effect of an efficient inhibition of DPP4 activity on the development of diabetic bone pathology and, for the first time, clearly shows a beneficial effect of DPP4 inhibitor treatment on bone mass as well as bone strength in diabetic animals, independent of their blood glucose levels. The results of this experiment have been subject of a recent publication in the American Journal of Physiology – Endocrinology & Metabolism[385].

Treatment with nicotinamide and streptozotocin results in partial destruction of pancreatic β-cells, causing a hypoinsulemic diabetic state in all injected animals, which evolved quickly to blood glucose levels over 200 mg/dL. The observed substantial animal-to-animal variation is probably due to the difference in absorption of nicotinamide after intraperitoneal injection and thus the difference in cytotoxic effect on the pancreatic β-cells. Pancreatic β-islets are also known to recover in diabetic rats treated with DPP4 inhibitors: β-pancreatic islets were up to 40% reduced in a strain of spontaneously diabetic rats, after which they recovered up to 80% of their original mass through vildagliptin treatment[386]. A similar proliferative action was confirmed by the use of sitagliptin in STZ-induced diabetic mice[387]. The response of rodents as well as humans to the proliferating effect of incretin-based therapies on β-pancreatic cells was recently found to decline with age. The proliferative capacity of β-cells of the young rats used in this experiment is probably far higher compared to that of older human subjects[388]. Proliferation of β-cells due to inhibitor treatment, and the associated increase of insulin expression could also play a role in the conservation of bone volume through insulin signaling. Morphological assessment of H&E-stained pancreatic sections however did not reveal any differences in the appearance of β-pancreatic islets in sitagliptin treated as well as untreated diabetic animals at sacrifice, but they could have changed throughout the course of the experiment. Although variations of insulin levels could be caused by alteration of β-cell proliferation through sitagliptin treatment, ultimately affecting bone microstructure, these changes are probably negligible as they were not reflected in different blood glucose levels between animal groups.

Animals with high glucose and thus probably lower insulin levels clearly lost more body weight and exhibited growth stagnation, which can result in a reduction of BMD[389,390]. Sitagliptin treatment, as it did not influence blood glucose, did not result in an alteration of STZ-induced growth retardation. As expected from clinical studies, treatment with DPP4 inhibitors is either weight neutral[391] or results in weight loss[392] in obese type 2 diabetic subjects. This

effect was reported not to be directly associated to glycemic management. A similar metabolic control could be the mechanism effectuating the observed trend of an increased body weight in the lean STZ-induced diabetic model following SG treatment.

Although it had previously been shown that DPP4 activity is increased in obese and hyperglycemic subjects, a correlation between blood glucose levels and DPP4 activity was not observed in this study, using non-obese diabetic animals. The inhibition of DPP4 resulted in a significant reduction of the drinking volume as well as a reduction in polyuria, independent of glucose levels in both the diabetic and control animals. This is in direct contrast with the increased diuresis reported in healthy GLP-1-infused animals, involving a down-regulation of NHE3 activity in the renal proximal tubule (resulting in natriuresis through a reduction of sodium reabsorption)[393], which was also reported to occur in alogliptin treated animals through a yet unknown but NHE3-independent mechanism[394]. These mechanisms are possibly altered in the NAD/STZ-diabetic rat model, in which sitagliptin treatment significantly reduces the diabetic diuresis. The variation of the diabetes-induced diuresis and thirst ultimately determined their water intake and thus their sitagliptin dosage.

Although dosage was undoubtedly higher in diabetic groups than in controls, the inflicted DPP4 inhibition was similar in both sitagliptin-treated groups. As previously mentioned, clinical studies raised concern about an increased risk of pancreatitis[395], but no increases of serum amylase and lipase were detected in animals treated with the same dosage of sitagliptin in a separate experiment (data not shown) nor in the previous experiment involving ovariectomy.

As shown in our study, treatment with sitagliptin results in a significant attenuation of trabecular bone loss and a marked improvement of trabecular bone structure, which can be attributed to the conservation of trabecular number and trabecular spacing, without affecting trabecular thickness. Sitagliptin treatment in diabetic animals also resulted in a continued cortical bone growth (bone volume) when compared to untreated diabetic animals. A similar trend, but limited to the trabecular bone, was observed in the sitagliptin-treated control animals.

Furthermore, a significant increase was observed in the ultimate load to fracture the femora of sitagliptin-treated versus untreated diabetic animals, also when normalizing the ultimate load over the transversal cortical surface in the diaphysis, indicating an increase of bone density and strength in these animals.

Bone histomorphometry results to a certain extent mirrored measurements made by micro-CT. Also, the ratio of osteoclast to eroded perimeter

observed during histomorphometric analysis at week 12 was in line with the reduced serum concentrations of CTX-I in SG-treated diabetic animals. Serum osteocalcin levels remained unaffected and no differences were observed in the relatively low number of osteoblasts nor the amount of osteoid, suggesting that the SG-induced effect on the bone was not achieved by influencing bone forming cells but by reducing the number of bone resorbing cells. The bone phenotype of the controls seems to remain unaffected by sitagliptin treatment. No arguments could be found for the involvement of the AGE-RAGE axis or interactions at the level of the parathyroid gland in the SG-induced effect, shown by serum levels of pentosidine and PTH, respectively. Levels of active serum MMP9, responsible for the degradation of collagen, were found to be increased in diabetic animals compared to controls. MMP9 is also known to be responsible for the recruitment of osteoclasts to the bone[239,396,397]. Interestingly, SG was found to reduce the rise of MMP9 in the serum of diabetic animals.

The calcium and phosphate excretion measured over 24 hours was significantly increased in all diabetic animals, which is due to chronic metabolic acidosis and to be expected in a rat model based on STZ. The increased calciuria and phosphaturia were not affected by the sitagliptin treatment. No correlations could be found between DPP4-activity, glucose levels and calcium and phosphate excretion in the diabetic animals. Although osteoporosis and high calcium excretion in T2DM patients is strongly associated with artery calcification[398], the abdominal aorta of STZ-induced diabetic animals did not show a significantly increased calcium content.

Previous studies regarding the effect of DPP4 inhibitors on bone metabolism have been inconclusive. In an experimental study published in 2010, the effect of sitagliptin treatment was assessed on bone microstructure and integrity in male HFD-fed diabetic mice and ovariectomized mice[214]. Sitagliptin treatment effectuated an increase of vertebral BMD, trabecular architecture and bone mineral content, but this effect was associated with a significant reduction of the blood glucose levels, as they were using a type 2 diabetic model with only moderate hyperglycemia. Sitagliptin treatment of HFD-diabetic animals increased vertebral BMD and trabecular bone volume through trabecular thickness, trabecular number and a significantly reduced trabecular spacing, and also induced a shift towards increased mineralization, which also effectuated a decrease of the Young's modulus. The genetic inactivation of DPP4, however, did not result in a specific bone phenotype[214,369]. Another recent experimental study concluded that experimental inhibitor MK-0626 did not affect bone quality in diabetic MKR mice[399], which might be due to the effect of the mutation in the IGF-1 receptor in this model, which was shown to reduce proliferation of bone cells as well as osteoblast activity [303], and also

indirectly influence other incretins[400].

Small clinical studies have been inconclusive. In postmenopausal type 2 diabetic women, a 12 week treatment with sitagliptin was associated with a decrease of bone resorption markers[377]. In a study in which T2DM patients were treated with vildagliptin daily (100 mg) for a year, vildagliptin was found not to affect bone markers s-CTX, ALP, calcium and phosphate in serum[401]. A recent cohort study also did not observe any effect of DPP4 inhibitor use on fracture risk in T2DM patients[402]. Low doses of saxagliptin were associated with a significantly decreased metaphyseal trabecular bone volume, osteocytic density and osteoblastic number in healthy animals. In vitro in osteoblast-like cells, saxagliptin was found to inhibit insulin and IGF-1 induced phosphorylation and decrease the production of Coll1 and expression of Runx2 and osteocalcin[403]. The saxagliptin manufacturer first reported an increased fracture rate[403], but this was not confirmed by a recent clinical trial[404] and thus removed from the medication guide.

But although conclusions of specific experimental as well as clinical studies have been divergent, meta-analyses of clinical bone trials regarding fracture risk in diabetic patients have shown a trend towards a reduced fracture risk in patients treated with DPP4 inhibitors[405,406]. Then again, these trials were short-term, making it difficult to draw long-term conclusions regarding actual fracture outcome. One can safely say that current literature does not allow for definite conclusions.

Our study indicates a beneficial effect of sitagliptin treatment, possibly linked to a reduction of bone resorption. The here observed decrease in bone resorption is in line with the results of the recent publication indicating a decrease of bone resorption markers in postmenopausal diabetic women after a 12 week treatment with sitagliptin[377]. An effect of DPP4 inhibition on bone resorption was also proposed in the previously mentioned study observing a slightly increased BMD in sitagliptin treated mice[214]. Postprandially, bone resorption is inhibited either through GLP-2 alone or also through GLP-1 and GIP[196,254,258,262]. It should be noted that food intake is determining for the release of these substrates. A reason to assume GLP-2 is involved, is that the administration of GLP-2 in lean as well as obese mice results in a reduced food intake[407], which is also observed in our animals treated with sitagliptin. It would be interesting for further research to investigate the effect of DPP4 inhibition on osteoclasts in vitro. DPP4 inhibition could possibly affect osteoclast activity or development. A recent in vitro study reports an impairment of the development of human functional osteoclasts by the blockade of DPP4 using a monoclonal antibody, an effect associated with the inhibition of the p38-MAPK-phosphorylation pathway[234], a pathway which is among others influenced by GLP-1 but not GLP-2[408,409].

Whether the observed effects are due to a general improvement of bone metabolism through modulation of inflammatory processes, or that the effect can be attributed to variations of circulating DPP4 substrate concentrations, or to variations of substrate receptor expression in diabetic animals compared to healthy animals remains to be elucidated. The decreased bone resorption in sitagliptin treated diabetic animals might be a result of a prolonged half-life of DPP4 substrates associated with a beneficial effect on bone metabolism. In previously mentioned experiments, the effect of GLP-1[196], GLP-2[272] and GIP[252] on bone resorption has clearly been shown. Also the effects of neuropeptides and immunomodulators like VIP[282,316], PACAP[315], NPY[410], PYY[297], SP[282] and IGF-1[164], which also influence the activity of osteoclasts directly, should be taken into account. Besides having a direct effect on the activity of osteoclasts, the latter substrates may also exert an effect on bone metabolism through feeding and energy regulation[289,372]. Thus the observed effect on bone resorbing cells is most probably a well-orchestrated, sophisticated effect of different DPP4 substrates on these cells. The affinity of DPP4 for these substrates but also the organs in which they are synthesized as well as their exposure to DPP4 in their journey towards the bone and the affinity of these substrates to the present receptors have to be taken into account in the evaluation of the effect of these substrates.

As some substrates of DPP4 can circulate in varying concentrations according to hyperglycemia or other conditions, depending on their own expression levels as well as modulation by DPP4, determining the active concentration of these substrates is important to find out which DPP4 substrates are crucial mediators of observed effects after DPP4 inhibition. Some substrates, like NPY and PYY, are able to counteract effects of other substrates, which could explain the controversial results found in experimental as well as clinical studies. STZ treatment was also shown to result in an increase of NPY levels, possibly indirectly, induced by changes in expression of the Y1 and Y2 receptor[411]. It is worth mentioning that serum levels of GIP, GLP-1 and other DPP4 substrates as well as the expression of their receptors were found to be altered through hyperglycemia in experimental as well as clinical studies[230,260,412,413]. A two-fold down-regulation of the incretin receptors GIP and GLP-1 occurs in pancreatic islets during hyperglycemia[260]. Possibly, the same redistribution of GIP and GLP-1 receptors occurs in bone cells. Nevertheless, in a clinical study involving type 2 diabetic Japanese patients, the intact GLP-1 levels were shown to be unaffected through hyperglycemia[229].

Clearly, further research is needed to assign the observed improvement of bone quality to the activity of specific DPP4 substrates. An important problem in the currently available assays is the precise distinction between active and non-active (truncated or modulated) forms of DPP4 substrates. As some

substrates activities are truly dependent on the two truncated N-terminal peptides, the analytical development of these assays has proven to be a challenge. For a few DPP4 substrates like GIP and GLP-1, precise assays seem to have been developed in the meantime.

This investigation of the effect of DPP4 inhibition through sitagliptin on the pathophysiological development of diabetic osteoporosis clearly shows a beneficial effect of sitagliptin treatment on cortical and trabecular bone mass, microstructure and bone strength in diabetic animals, independent of their blood glucose levels. The results of this study strongly suggest a protective effect of DPP4 inhibition on the bone in diabetic animals, which might be the reason for a reduced fracture risk in the diabetic population treated with DPP4 inhibitors[405]. As diabetic patients have an increased risk of fractures combined with an increased tendency to fall due to peripheral neuropathy, diabetic retinopathy and abnormal fluctuations in glycemia and blood pressure resulting in light-headedness, these results may prove an additional clinical potential of DPP4 inhibitors in maintaining the strength of bone compromised by impaired glucose intolerance.

3.7. References

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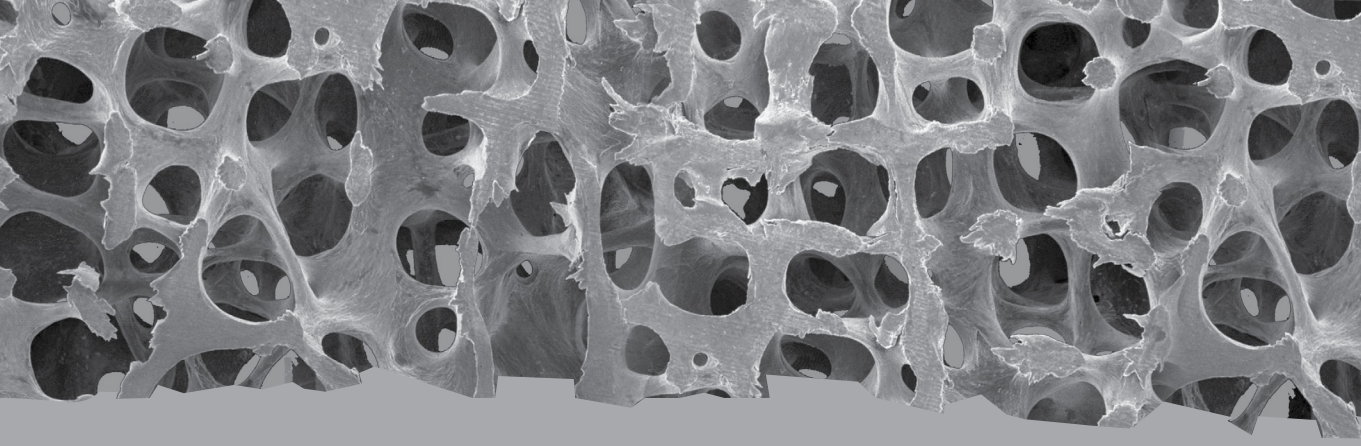

Chapter 4
General Discussion

4

Chapter 4: Contents

Conclusion and perspectives

In this thesis, a beneficial effect of DPP4 inhibition has been shown on kidney function, in a model of unilateral ischemia reperfusion, as well as on bone metabolism, in a model of diabetic osteoporosis.

DPP4 was first studied in the 70s and has been extensively characterized in different tissues throughout the last decades[1-6]. Together with its substrates, its structural properties and pharmacological characteristics were extensively studied, mainly resulting in the discovery of more and more DPP4 substrates as well as interactions with other proteins, biochemical aspects and the expansion towards clinical applications[7-11]. DPP4 was found to have an important regulatory role systemically in the circulation in its soluble form after cleavage from its membrane-bound hydrophobic anchor. In its anchored form, it has been found on endothelial and epithelial cells in a wide array of tissues[12-15], as well as in circulating immune cells[16]. DPP4-dependent regulatory systems are complex and variable, involving direct interactions with other proteins in lipid rafts[17], truncation and modulation of substrate proteins, and modulation of inflammatory processes[18]. The regulatory activity of DPP4 is dependent on its surroundings, since the expression of substrates as well as the occurrence of immune processes is determined by spatial as well as temporal factors. Some DPP4 substrates are expressed in specific organs and enter the circulation, where they have a specific half-life dependent on local activity of DPP4, other proteases, and other interactions. Other substrates do not enter the circulation and act locally, e.g. neuropeptides at the endings of nerve fibers. Furthermore, the activity of DPP4 substrates is mostly dependent on the binding on one or more specific receptors, which are not ubiquitously expressed but meticulously regulated to expression in specific regions. The specific expression of substrates and their receptors was also found to be variable throughout human development as well as throughout the development of various pathologies. In this perspective, it is important to understand that these regulatory interactions can vary extensively from strictly localized (for example punctual chemotactic and inflammatory activity of SDF-1α), to more general activity of specific incretins that have a wide array of consequences throughout the circulation, influencing feeding, glucose and energy metabolism, intestinal growth, and less straightforward complex secondary effects (e.g. GLP-1). Different examples further emphasize the complexity of the processes regulated by DPP4. The portal concentrations of DPP4 incretin substrates, for example, were shown to be more affected by DPP4 inhibition than the peripheral concentrations[19]. Another illustration is the intravenous administration of GLP-1, bypassing important hepato-portal sensors for GLP-1[20].

To conclude, the exact effects of the activity of any specific DPP4 substrate

within an organism has never been entirely characterized, but evidence has shown that it is complex and precisely regulated, and can play crucial roles that work in milliseconds up to hours, that are in effect throughout the entire lifespan or in a specific window of time throughout human development or a specific pathology[21,22].

Pharmacological characterization of DPPs has led to the development of specific inhibitors of their activity. Inhibition of DPP4 activity was found to result mainly in the increase of the incretin effect, mediated by DPP4 substrates GIP and GLP-1. Considering the clinical value of the incretin effect for diabetic patients, the inhibition of DPP4 activity soon led to the clinical application to improve the glucose tolerance in these patients. Clinical tests and meta-analysis confirmed the successful treatment of T2DM patients, alleviating common symptoms and resulting in an improved glucose tolerability. Although the treatment has been internationally approved and prescribed, the long-term effect of DPP4 inhibition on the patient as a whole is yet to be elucidated. Also, the physicochemical properties as well as the metabolism and elimination pathways of individual DPP4 inhibitors could modulate the effects of DPP4 inhibition. Although efficacy with regard to the glucose metabolism is consistent in large populations across ethnic groups, studies have unveiled rare but worrisome side-effects[23,24]. Moreover, specific studies have shown that treated T2DM patients, although showing improved glucose tolerance, appear to have normal GLP-1 activities[25]. A complete profile of DPP4 substrate activity in a wide array of patients displaying various (diabetes-related) pathologies in a clinical study should be interesting, but has never been established. In that regard, the measurement of active DPP4 substrates and their effects on intracellular signaling pathways following receptor binding has been proven to be a challenge due to the lack of appropriate methods[26].

In the kidney, DPP4 was found to be present mainly on proximal tubular cells but also on glomerular cells. Furthermore, DPP4 was found to exert catalytic activity in the tubular lumen. Serum soluble substrates of DPP4 also freely pass the glomerular barrier to bind their receptors on the luminal surface of tubular cells. Many substrates have been identified in the kidney, of which some are very well characterized and some are completely unknown[11]. Specific receptors of commonly known DPP4 substrates have been observed in specific kidney regions, which are summarized in figure 10 of the second chapter. These effects include an increase of natriuresis and diuresis (effectuated by GLP-1 and NPY, through NHE3 and occlusion of the vas efferens, respectively), an anti-apoptotic effect (effectuated by GLP-1, GLP-2 and NPY, mostly in proximal tubular cells), an anti-inflammatory effect (GLP-1, VIP and NPY), and effects on oxidative stress, angiogenesis and chemotaxis of progenitor cells (SDF-1α, GLP-1, VIP and NPY). Furthermore, DPP4 itself was found to

be involved in lymphocyte activation and leukocyte infiltration in the ischemic kidney, effects found to be influenced by DPP4 inhibition.

The study described in this thesis confirmed the hypothesis of a protective effect of DPP4 inhibitor treatment on the kidney in ischemic AKI, observing a significant, dose-dependent protective effect of vildagliptin on kidney function after experimental unilateral IRI. This remarkable improvement of kidney function was shown to be functional as well as morphological, and was associated with a reduction of apoptosis and necrosis, as well as a reduction of oxidative stress and inflammation. Furthermore, an increased infiltration of macrophages was reported in vildagliptin-treated animals, and also T-cell infiltration was affected. No change in diuresis or in renal hemodynamics was observed, and blood glucose levels were also shown to be unaffected by DPP4 inhibition.

The related publication in the American Journal of Physiology – Renal Physiology is the first to assess the effect of a single dose of DPP4 inhibitor in the setting of renal IRI in nondiabetic rats. Similar results were reported in type 2 diabetic rats[27]. Anti-apoptotic and anti-inflammatory effects could possibly be related to an increase of active GLP-1 or VIP levels. However, the question to which substrates exactly the observed effects can be attributed remains to be answered. In order to reveal the underlying mechanisms of the renoprotective effect of DPP4 inhibitors, the active concentration of substrates and the resulting substrate receptor activation needs to be discovered. This could be achieved through research focusing on the downstream activation pathways, or experimental studies evaluating the effect of individual substrates through exogenous administration or using receptor knockout animals. The usage of increased doses of DPP4 inhibitors, as well as administration of DPP4 inhibitors in other models (e.g. CKD) might yield to meaningful differential results. Interactions of DPP4 with other molecules like integrins should also be considered[28]. The results obtained can be of utmost value in the prevention of ischemic injury in the transplanted kidney. Furthermore, as kidney injury is a common complication in diabetic patients, treatment with DPP4 inhibitors could affect the occurrence of such injury, an effect which has not been evaluated in clinical meta-analyses but appears promising[29]. These results further support setting up studies regarding the potential use of DPP4 inhibitors in pathologies other than type 2 diabetes. The complete mechanism of the renoprotective effect resulting from DPP4 inhibition, which is without a doubt a complex phenomenon, remains however to be elucidated.

In the bone, DPP4 was found to be expressed on the surface of osteoblasts as well as osteoclasts. The role of some DPP4 substrates in the bone has been well established, as summarized in figure 10 of the previous chapter. Many of the extensively studied DPP4 substrates were found to bind to receptors

on the surface of osteoblasts (GIPR, GLP-1R, Y1, VPAC1, VPAC2, IGF-1R and nk-1R), osteoclasts (GIPR, GLP-1R, GLP-2R, CXCR4, VPAC1, PAC1 and nk-1R) and osteoblast progenitor cells (CXCR4). IGF-1 was also found to be produced by osteoblasts themselves, and NPY by osteoblasts as well as osteoclasts. NPY and PYY are also released by nerve fibers throughout the bone. NPY has been found to play an important role in hypothalamic control of the bone metabolism through a system that is closely interacting with the regulation of feeding, involving peptide hormones controlled by hypothalamic release and by adipocytes. Leptin, one of these peptide hormones, is closely associated with estradiol levels. These peptides and associated mechanisms were shown to be disrupted in menopause (leptin) as well as in diabetes (adiponectin), two situations associated with a systemic increase of DPP4 expression and with disruptions of the bone metabolism such as osteoporosis and associated elevated fracture risk. Inflammatory factors were also shown to influence osteoclastic activity.

In this thesis, the effect of DPP4 inhibition was evaluated in a model of postmenopausal osteoporosis and in STZ-induced diabetic osteoporosis. In general, trabecular bone loss was prevented as observed through micro-CT. Beneficial tendencies associated with DPP4 inhibitor treatment were observed in postmenopausal osteoporotic animals (but only raised significance very early after ovariectomy). Another recent study did not find any significant effects in DPP4 inhibitor treated osteoporotic animals either, but confirmed a tendency towards improvement of bone structure[30].

In the study conducted with STZ-induced diabetic animals, significantly less trabecular bone loss was observed in the diabetic group, with confirmed efficient DPP4 inhibition after treatment with sitagliptin through drinking water. Cortical bone was also found to be affected in sitagliptin-treated diabetic animals, as shown by a higher cortical bone volume in the diaphysis compared to untreated diabetic animals, resulting in increased bone strength. Results of bone histomorphometry pointed towards a decrease of osteoclastic activity in diabetic animals treated with sitagliptin. The results regarding the diabetic study were published in American Journal of Physiology – Endocrinology & Metabolism. Although no precise conclusions can be drawn regarding the involvement of specific DPP4 substrates, a tendency towards an increased bone mass and bone strength seems clear. In the meantime, clinical studies evaluating the effect of DPP4 inhibition on actual fracture risk have been indicating a positive effect associated with DPP4 inhibitor use in type 2 diabetic patients[31]. As the observed effect is associated with a decrease of bone resorption, incretin substrates as well as GLP-2 could be involved.

The results of the studies summarized in this thesis suggest a beneficial effect of DPP4 inhibition on kidney function in the experimental setting of re-

nal ischemic injury, as well as on bone metabolism in an experimental model of STZ-induced diabetes. Furthermore, it suggests DPP4 inhibition might also positively affect bone metabolism in other models. The additional beneficial effects of DPP4 inhibition which have been reported over the years give rise to the hypothesis that modulation of DPP4 activity could affect the development of many pathologies that are not necessarily related to diabetes. DPP4 inhibition has been shown to exert pleiotropic and generally beneficial effects in different organs. In the pancreas, the organ targeted by incretins to increase the release of insulin, prolonged half-life of active substrates GIP and GLP-1 were also shown to increase the proliferation and reduce apoptosis of insulin-producing pancreatic β-cells. A similar anti-apoptotic effect was observed in models of myocardial infarction, in normal[32,33], obese[33] as well as diabetic animals[34,35]. In general, DPP4 inhibition is known to exert an anti-inflammatory as well as anti-oxidative effect in T2DM[36,37]. Such effects were shown to be beneficial for atherosclerotic lesions and general endothelial function in the vessels of hypertensive as well as diabetic subjects[38-41]. An anti-inflammatory effect was also induced in topical DPP4 inhibition in experimental asthma[42]. Additional beneficial effects of DPP4 inhibition in lung injury were attributed to SDF-1α and the recruitment of progenitor cells[43]. Although some of these effects of DPP4 inhibition were confirmed in kidney injury in the presented study[44], others, like the contribution of SDF-1α in lung injury, have been found controversial in kidney repair[45,46]. In conclusion, further research is needed to grasp the underlying mechanisms of the pleiotropic effects of DPP4 inhibition in different organs. Also, caution should be exercised when treating patients with DPP4 inhibitors, as some side-effects have been observed in specific case-studies. Whether such complications are related to other not yet identified determinants related to age, ethnicity, other factors or additional pathologies, remains unclear. Many angles have to be considered when translating the potential therapeutic effect to the clinical setting. When evaluating the question whether DPP4 inhibition might exert a beneficial effect in any specific setting, regional and temporal variations of expressions of DPP4 and substrates have to be taken into account. Furthermore, the administration method is important, and pharmaceutical properties of DPP4 inhibitors result in different half-lives, binding affinities and thus transitory inhibitions of DPP4. DPP4 inhibitors are in permanent development, and, because of the complexity of DPP4 regulation, potential applications of these antidiabetic drugs are thoroughly tested and in practice. Future research will elucidate the intricate mechanisms of DPP4 inhibition more thoroughly, allowing for therapeutic applications beyond the incretin effect.

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Summary

DPP4 beyond glucose homeostasis: attenuation of acute kidney injury and diabetic bone loss through DPP4 inhibition

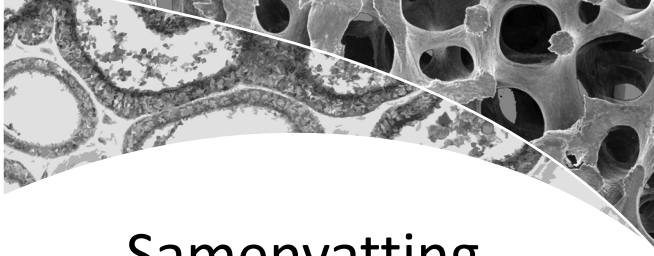
DPP4 is a N-terminal exopeptidase which plays an important role in protein regulation. It is ubiquitously expressed throughout the human body where it can be found anchored to the cellular membrane but also roaming free in the circulation. The substrates of DPP4, a growing list of peptides, are known to have important roles in the glucose metabolism (incretins) as well as in neurological and immunological mechanisms. As the inhibition of DPP4 results in an increased half-life of the incretins GIP and GLP-1, DPP4 inhibitors were developed as a treatment to improve glucose tolerance in type 2 diabetic patients, a condition associated with an increase of DPP4 activity.

Inhibition of DPP4 is known to exhibit protective effects on ischemic cardiomyopathy and lung injury, associated with anti-inflammatory, anti-oxidative and anti-apoptotic activities of specific DPP4 substrates. Acute ischemic injury of the kidney is a complication associated with high mortality in hospitalized patients. As DPP4 and its substrates are also expressed in the kidney, the potentially therapeutic effect of DPP4 inhibition in ischemically induced acute kidney injury was studied in rats in a model of 30-min unilateral renal ischemia, followed by contralateral nephrectomy. Saline or vildagliptin (1 mg/kg and 10 mg/kg) was administered intravenously 15 min before the surgery. Experimental animals were euthanized after 2, 12, and 48 hours of reperfusion. DPP4 inhibition resulted in a significant, dose-dependent lower serum creatinine compared to saline treated animals. Tubular morphology evaluated by PAS-PCNA revealed significantly reduced tubular necrosis at 12h in animals treated with 10 mg/kg vildagliptin. DPP4 inhibition did not affect regeneration but decreased apoptosis, as shown by a decreased Bax/Bcl-2 mRNA expression ratio and a decrease in apoptotic bodies on TUNEL-stained sections. Furthermore, vildagliptin treatment was shown to exert anti-inflammatory and anti-oxidative effects. Through a mechanism yet to be fully understood, vildagliptin treatment results in a functional protection of the kidney against ischemia reperfusion injury.

DPP4 incretin substrates not only increase secretion of insulin, but have also been found to affect the bone metabolism. DPP4 as well as many DPP4 substrate receptors are expressed on the surface of active bone cells, and some DPP4 substrates are directly released from active bone cells and sur-

rounding nerve fibers. The administration of DPP4 substrates have been found to positively affect bone metabolism. Menopause as well as diabetic hyperglycemia are conditions both associated with a decreased bone mineral density resulting in osteoporosis and an increased fracture rate, combined with an increased activity of DPP4. In a model of ovariectomy, the effect of DPP4 inhibition through gavage with sitagliptin was assessed on the pathophysiological development of osteoporosis. The inhibition of DPP4 activity in the serum was indeed shown to affect the bone metabolism, but results were only significant in control animals and in the early phase after ovariectomy. This could be due to the intensity of the effect of ovariectomy on the trabecular bone or on the complex interactions with DPP4 and metabolism. The effect of sitagliptin was also studied in a rat model of streptozotocin-induced diabetes. In this study, diabetic as well as control animals were treated with 2 g/L sitagliptin through drinking water, resulting in an efficient inhibition of DPP4 activity. Trabecular bone loss, the decrease in trabecular number, and the increase in trabecular spacing were found to be attenuated through sitagliptin treatment in STZ-diabetic rats, as shown by in vivo micro-CT. Micro-CT analysis further showed that sitagliptin prevented cortical bone growth stagnation in diabetic rats, resulting in stronger femora during three-point bending. Serum levels of the resorption marker CTX-1 were found to be significantly lower in sitagliptin-treated diabetic animals compared with untreated diabetic animals. Independent of glycemic management, sitagliptin treatment was shown to significantly attenuate bone loss and to increase bone strength in diabetic rats, probably through the reduction of bone resorption.

These results open perspectives for the application of DPP4 inhibitors in future scientific research, and show their potential for further therapeutic development. They are specifically relevant in diabetic patients, a population that is currently already being treated with DPP4 inhibitors, and which is at high risk for kidney and bone pathology. The results bundled in this thesis have been published as two articles to contribute to the search for unknown effects of DPP4 inhibition in the kidney and the bone, and to the acquisition of further insight in the pluripotent effects of DPP4 inhibition through its many substrates.



Samenvatting

DPP4 - meer dan glucose homeostase: vermindering van acute nierschade en diabetes-gerelateerd botverlies door DPP4 inhibitie

DPP4 is een N-terminaal exopeptidase dat een belangrijke rol speelt in eiwitregulatie. Het wordt overal in het menselijk lichaam tot expressie gebracht, zowel verankerd in de celmembraan als onder vrije vorm in de circulatie. De substraten van DPP4, een groeiende verzameling peptiden, spelen een belangrijke rol in het glucosemetabolisme (incretines), evenals in neurologische en immunologische mechanismen. Gezien de inhibitie van DPP4 het halfleven van de incretines GIP en GLP-1 verlengt, werden DPP4 inhibitoren ontwikkeld om de glucosetolerantie te verhogen in patiënten met diabetes type 2, een toestand die gepaard gaat met een stijging van de DPP4 activiteit.

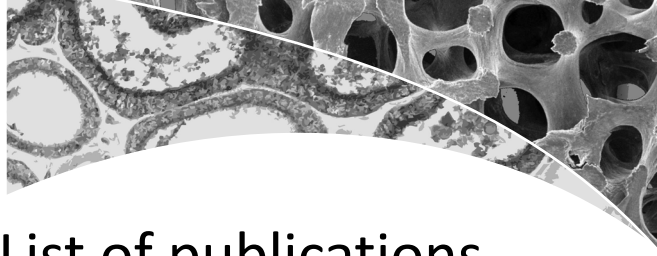
Het is geweten dat de inhibitie van DPP4 zorgt voor een beschermend effect in ischemische hartziekte en ischemische longschade, hetgeen geassocieerd werd met anti-inflammatoire, anti-oxidatieve en anti-apoptotische effecten van bepaalde DPP4 substraten. Acuut ischemisch nierfalen is een complicatie bij gehospitaliseerde patiënten die gepaard gaat met een hoge mortaliteit. Gezien DPP4 en zijn substraten ook worden teruggevonden in de nier, werd het potentieel therapeutisch effect van DPP4 inhibitie in ischemisch-geinduceerd acuut nierfalen bestudeerd in een unilateraal ischemisch ratmodel, waarbij een ischemische fase van 30 minuten werd gevolgd door contralaterale nefrectomie. Fysiologische zoutoplossing of vildagliptin (1 en 10 mg/kg) werden een kwartier voor de operatie intraveneus toegediend. Euthanasie van de proefdieren gebeurde na een periode van 2, 12 of 48 uur reperfusie. De inhibitie van DPP4 resulteerde in een significante dosisafhankelijke daling van het creatininegehalte in het serum. Tubulaire morfologie, geëvalueerd met behulp van PAS-PCNA kleuring, vertoonde een significant verminderde tubulaire necrose in dieren behandeld met 10 mg/kg vildagliptin na 12 uur reperfusie. DPP4 inhibitie had geen invloed op de regeneratie van proximale tubulaire cellen maar verminderde cellulaire apoptose, hetgeen aangetoond werd met een verminderde Bax/Bcl-2 mRNA expressieratio en een verminderd aantal apoptotische lichaampjes na TUNEL-kleuring. Ook werden anti-inflammatoire en anti-oxidatieve effecten waargenomen na vildagliptinbehandeling. De behandeling met vildagliptin zorgt dus voor een functionele bescherming van de nier tegen ischemie reperfusieschade door een tot nu nog niet volledig verklaard mechanisme.

Niet alleen zorgen DPP4 substraten voor de verhoogde secretie van insuline, ze spelen ook een belangrijke rol in het botmetabolisme. Zowel DPP4 als andere DPP4 substraten worden direct vrijgemaakt door actieve botcellen en zenuwvezels. Veel DPP4 substraten bleken na toediening een positieve invloed uit te oefenen op het botmetabolisme. Zowel menopauze als diabetische hyperglycemie zijn condities die geassocieerd zijn met een verminderde botdensiteit, osteoporose en een verhoogde incidentie van botbreuken, evenals een verhoogde activiteit van DPP4. In een postmenopauzaal ratmodel veroorzaakt door ovariëctomie werd het effect van DPP4 inhibitie door middel van gavage met sitagliptin nagegaan op de pathofysiologische ontwikkeling van osteoporose. Hoewel de activiteit van DPP4 zichtbaar een weerslag had op het botmetabolisme, werden slechts significante verschillen opgemerkt in controledieren en in de vroege fase na ovariëctomie, dit mogelijk omwille van het te uitgesproken effect van ovariëctomie op het trabeculair bot, en/of omwille van complexe interacties tussen DPP4 en het metabolisme. Verder werd het effect van sitagliptin ook nagegaan in een ratmodel van streptozotocin-geïnduceerde diabetes. In deze studie werden diabetische en controledieren behandeld met 2 g/L sitagliptin in het drinkwater, hetgeen resulteerde in een efficiënte DPP4 inhibitie. Trabeculair botverlies, evenals de daling van het trabeculair aantal en de stijging van de intertrabeculaire afstand, werden afgezwakt in diabetische dieren door de behandeling met sitagliptin, hetgeen aangetoond werd door middel van in vivo micro-CT. Verder werd door middel van deze techniek vastgesteld dat in sitagliptin-behandelde dieren de door diabetes geïnduceerde groeistagnatie van corticaal bot werd verhinderd, hetgeen resulteerde in sterker bot (gemeten thv de femur dmv driepuntsbuigingsanalyse). In het serum van sitagliptin-behandelde diabetische dieren werd eveneens een significante daling waargenomen van de resorptiemerker CTX-1 in vergelijking met de onbehandelde diabetische dieren. Er werd dus aangetoond dat de behandeling met sitagliptin resulteert in een verminderd botverlies en een verhoogde botsterkte, waarschijnlijk veroorzaakt door een verminderde botresorptie, en dit zonder de glycemie van de proefdieren te beïnvloeden.

Deze resultaten openen perspectieven voor de toepassing van DPP4 inhibitoren voor andere therapeutische doeleinden dan de behandeling van diabetes. Verder zijn de beschreven resultaten ook bijzonder relevant voor de diabetische populatie gezien deze momenteel reeds met DPP4 inhibitoren behandeld wordt en een verhoogd risico loopt op nier en botziekten. De resultaten besproken in deze thesis werden gepubliceerd in twee artikels teneinde bij te dragen tot de zoektocht naar onbekende effecten van DPP4 inhibitie in de nier en het bot, en verdere inzichten te bekomen in de pluripotente effecten van DPP4 inhibitie via zijn vele substraten.

Curriculum vitae

The author of this thesis was born on April the 17th 1983 in Ukkel, Belgium. After graduating secondary school in 2000, he started university studies to become a Bio-Engineer at the Vrije Universiteit Brussel (VUB). In September 2005, he received his masters degree in Bio-Engineering with a master thesis entitled 'Purification and characterization of lectins from the seeds of Dichrostachys cinerea'. The following years, he subscribed to scientific master-after-master programmes of the VUB, resulting in two additional master degrees, in sustainable development and human ecology and in environmental sciences, obtained in September 2007 with the master thesis entitled 'Epidemiological riddles concerning the consequences of in utero exposure to diethylstilbestrol (DES): absolute risk of clear cell adenocarcinoma and multigenerational inherited hypospadias'. After following a completely different course as a distributor and sales manager of accessories for woodwind instruments for the international market at a small Belgian firm called Woodwind Conceptions, and intrigued by a PhD position at the University of Antwerp, he applied and was accepted under the supervision of prof. Dr. P. D'Haese. At the start of his PhD there in 2009, he was still combining research with the online distribution of musical instruments after working hours. In 2010, he started followed additional studies at the faculty of medicine at the University of Antwerp with a credit contract. In March 2011, the author was awarded with the Young Nephrologist Award for Belgium (handed out by the Belgische Vereniging voor Nefrologie / ERA-EDTA) in ULB Erasme in Brussels after his oral presentation regarding DPP4 inhibition in the kidney. In June 2015, the draft of this PhD thesis was submitted and a bachelor degree in medicine was obtained. During the course of the PhD research, the category C laboratory animal science certificate was obtained, mandatory for setting up animal experiments.



List of publications

Glorie, L. L., Verhulst, A., Matheeussen, V., Baerts, L., Magielse, J., Hermans, N., D'Haese, P. C., De Meester, I., De Beuf, A. (2012). DPP4 inhibition improves functional outcome after renal ischemia-reperfusion injury. Am J Physiol Renal Physiol. 303(5):F681-688.

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Dankwoord

Deze thesis is het resultaat van een reeks experimenten, die de vrucht zijn van een geconcerteerde samenwerking van vele tientallen personen van verschillende departementen van de Universiteit Antwerpen en andere. Volgende mensen hebben een belangrijke rol gespeeld bij de voortgang en het afronden van dit werk.

In de eerste plaats wil ik graag mijn promotor Patrick D'Haese danken. Hoewel ik een achtergrond had als onderzoeker, kwam ik van een andere wereld en had ik geen ervaring in experimenteel biomedisch onderzoek. Eerlijkheid gebiedt me toe te geven dat ik voor mijn sollicitatiegesprek zaken moest opzoeken die voor de meeste masterstudenten in de biomedische wetenschappen voor de hand liggend zijn. Dus Patrick, dank voor je vertrouwen en steun tijdens al die jaren, in het bijzonder voor je opbouwende kritiek bij het verloop van mijn onderzoek, de tussentijdse evaluaties en je geduld bij het afronden van het schrijven van artikels en van dit eindwerk. Ik kijk op naar hoe u het hele labo zo efficiënt weet te leiden, en verschillende onderwerpen meester weet te blijven.

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De directe begeleiders van mijn onderzoek verdienen een prominente plaats in dit dankwoord. Annemie, ik wil jou, mijn eerste begeleider, danken voor de opleiding in celcultuur tijdens het prille begin van mijn thesis. Jammer genoeg heb ik die technieken nauwelijks gebruikt, maar tijdens jouw laatste momenten op het labo Pathofysiologie friste je mijn in vitro ervaringen op en wekte je mijn interesse voor het botonderzoek.

Annelies, ondertussen heb ook jij het labo verlaten, jij nam mijn begeleiding snel over, en je was diegene die me introduceerde tot het opereren van dieren. Dat was even wennen, en die lange dagen opereren met jou in de beginfase van mijn thesis en jouw laatste momenten op het labo Pathofysiologie waren een onvergetelijke ervaring, daar wil ik je van harte voor danken. Ook je begeleiding bij de daarop volgende analyses was onontbeerlijk.

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Naast het diensthoofd en mijn eerste begeleidsters verdienen mijn collega's van het Laboratorium Pathofysiologie het meeste lof. Geert, Rita, Hilde, jullie gezelschap en ondersteuning zijn voor mij van enorm belang geweest. Elke experimentele studie ging voor mij gepaard met organisatorische stress, en jullie wisten als geen ander die ludiek te kaderen, te relativeren en te verlichten. Jullie stonden altijd klaar, en jullie ervaring in assistentie maakte elke experimentele uitdaging comfortabel en efficiënt. Twee keer op een dag een verzameling dieren gaveren voor een grillige onderzoeker, met 12 uur tussen, je moet het maar willen doen. Simonne en Geert, menig maal hebben jullie mijn dag geconcentreerd staren in de microscoop kleur gegeven. Ludwig, jij stond ook altijd klaar voor de calciummetingen. De strak georganiseerde begeleiding, en de verwerking, de analyse en de organisatie van die stalen maakt jullie de ruggengraat van het labo. Telkens opnieuw gaven jullie al-

les probleemloos een plaats in de agenda en werd alles opgevolgd met een glimlach. Grote Geert, jouw kennis over bothistomorfometrie en informatica bleken vaak genoeg van onschatbare waarde bij analyses van stalen en interpretatie van resultaten. Maar ook de vele gesprekken met de lotgenoten waarmee ik het lokaal deelde waren van groot belang bij het op punt stellen van experimenten, delen van ervaringen en zeker niet onbelangrijk, het draaglijk maken van de ups en downs van wetenschappelijk onderzoek. Daarbij heb ik het over Tineke, Ellen, Stef en Benjamin, die ik me heb zien voorgaan in het doctoreren, evenals Nathalie, die me daar ongetwijfeld in opvolgt. Ik heb altijd opgekeken naar jullie concentratie en doorzettingsvermogen. Elkeen van ons heeft gedurende die jongvolwassen tijd op persoonlijk vlak ook een enorme groei doorgemaakt. Veel dank, ik heb met jullie op bureau en in het labo een fijne tijd beleefd.

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Het mooiste aan de professionele samenwerking met mijn collega's van het laboratorium Pathofysiologie was dat die zo'n sterk sociaal fundament had. Van elk van hen heb ik enorm veel leren kennen en ervaren, zowel binnen als buiten de grenzen van de universiteit. Wandelend op de campus, tijdens langdurig manueel werk, op terras in het buitenland bij een congres, op restaurant, bij het delen van muziek, filmavonden, diverse feestjes... Dank, collega's, ik zal jullie nooit vergeten.

De experimenten in verband met DPP4 en het botmetabolisme begonnen met analyses van proefdieren van een cardiovasculaire studie uitgevoerd in het Laboratorium Fysiologie, onder auspiciën van Prof. De Keulenaer. Ik wil hem, evenals Anne-Sophie, Kathleen en Marc van harte danken voor hun warme ontvangst en voor de stalen die mijn botonderzoek hebben ingeleid.

Bij het botonderzoek werd micro-CT gebruikt, waardoor ik in contact kwam met Prof. De Clerck. Haar ervaring was onontbeerlijk bij het op punt stellen van de in vivo-scans. Bij de daadwerkelijke uitvoering ervan stond Frank altijd klaar om te garanderen dat het scannen vlot verliep, en zijn gezelschap maakte de lange scandagen een hoop aangenamer. Prof. De Clerck, Frank, dank voor jullie expertise en bijstand, en voor de warme ontvangst.

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