KU Leuven
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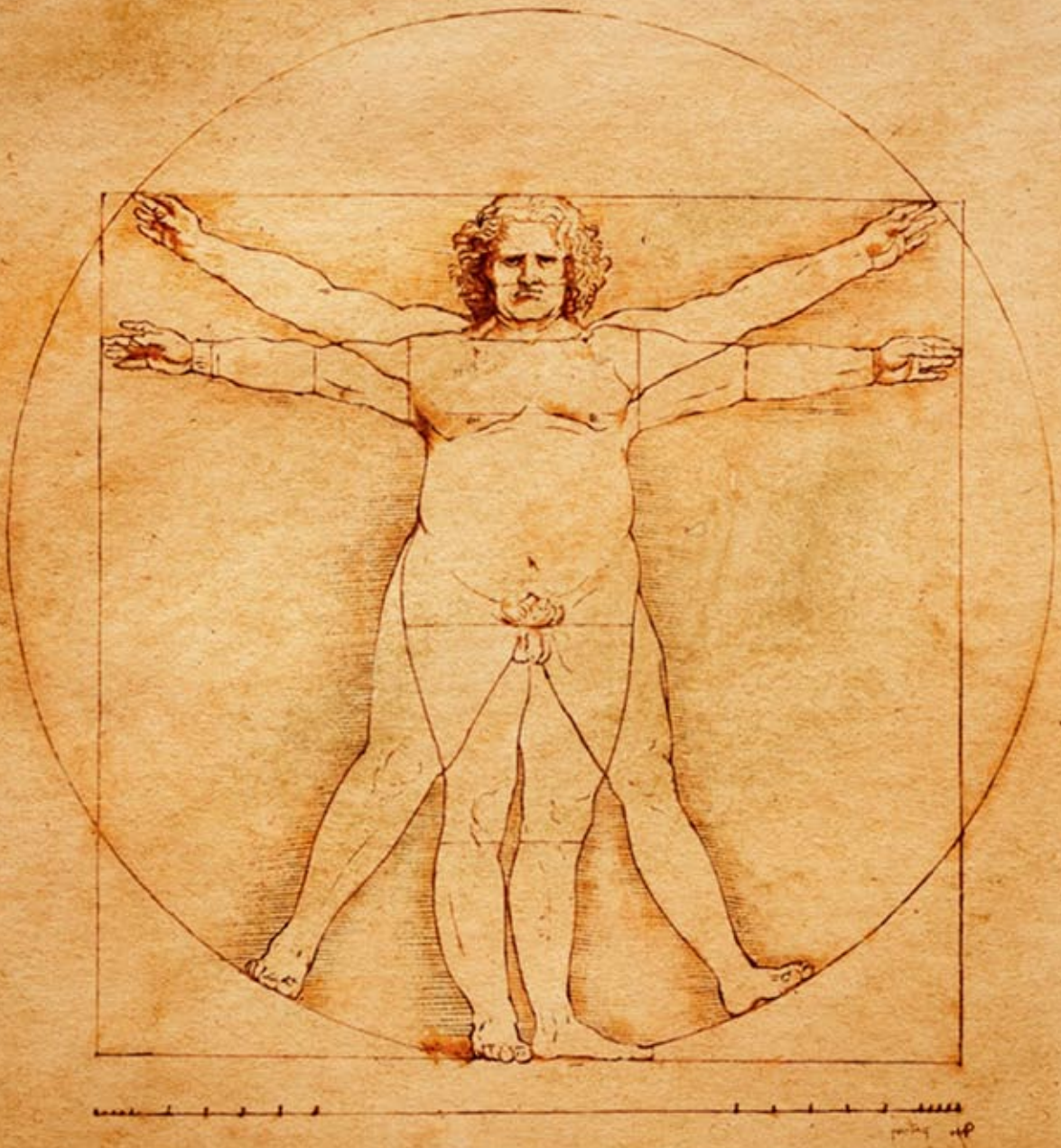

Role of Gelatinases in the Development of Adipose Tissue

Matthias Van Hul

Doctoral Thesis in Biomedical Sciences
Leuven, 2012

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ROLE OF GELATINASES IN DEVELOPMENT OF ADIPOSE TISSUE

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tissue
versus
MMP-2
MMP-9
MMPs
levels
Tolylsam
adipocyte
body
obesity
lower
differentiation
Figure
cells
gelatinase
inhibition
activity
inhibitor
protein
density
adipocytes
preadipocytes
fat
weight
tissues
expression
development
Table
SC
MMP
HFD
controls
whereas
significantly
weeks
reduced
GON
Tolylsam-treated
vitro
associated
addition
models
factors
g/day
day
high
affected
active
affect
Data
two
using
3T3-F442A
formation
cholesterol
littermates
study
specific
MMP-9+/+
energy
regulation
insulin
matrix
pads
total
inhibitors
cell
blood
may
experiments
wild-type
complex
induced
gelatinases
factor
A.U
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vivo
deficiency
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results
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proteins
several
plasma
domain
treatment
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revealed
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inhibitor-treated
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“Not all those who wander are lost.”

J.R.R. Tolkien

“An expert is a person who has made all the mistakes
which can be made, in a very narrow field.”

Niels Bohr

LIST OF ABBREVIATIONS **9**

INTRODUCTION **11**

1. Obesity **11**
1.1 The outbreak of an epidemic 11
1.2 Definition and diagnosis of obesity 12
1.3 Medical complications of obesity 13
1.4 The burden of excess weight 14
1.5 Causes of obesity 15
1.6 Treatment of obesity 15

2. Adipose tissue **17**
2.1 More than just storage 17
2.2 The adipocyte and its surroundings 18
2.3 Adipose tissue development 19
2.4 Adipogenesis 21
2.5 Murine models to study adipogenesis 23

3. Matrix Metalloproteinases (MMPs) **25**
3.1 A historical perspective 25
3.2 Classification and general structure 25
3.3 Regulation of MMP activity 27
3.4 Models to study MMPs 33
3.5 Function 34
3.6 Synthetic inhibitors of MMPs 37
3.7 MMPs and adipose tissue development 38
3.8 Gelatinases 39
3.9 Genes of gelatinases 40
3.10 MT1-MMP/TIMP-2 mediated MMP-2 activation 41

AIMS **45**

MATERIALS AND METHODS **47**

1. Animal models **47**
1.1 Mouse strains 47
1.2 Murine models 47
1.2.1 Nutritionally induced obesity in gelatinase deficient mice (model 1) 49
1.2.2 Nutritionally induced obesity in mice treated with a gelatinase inhibitor (model 2) 49
1.2.3 Treatment with a gelatinase inhibitor of mice with established obesity (model 3) 51
1.2.4 de novo adipogenesis (model 4) 52
1.3 Analysis 52
1.3.1 Histological analysis 52
1.3.2 Gelatinase determination 53
1.3.3 Analysis of mRNA expression 54
1.3.4 Metabolic parameters 55

2. Cell culture experiments **55**
2.1 *in vitro* differentiation 55
2.2 *In vitro* gene silencing 55
2.3 Analysis 57
2.3.1 Assessment of cell proliferation and viability 57
2.3.2 Differentiation assay 57
2.3.3 Gelatinase determination 57

2.3.4 Analysis of mRNA expression 57

3. Statistical analysis 58

RESULTS 61

1. Effect of MMP-9 deficiency on adipose tissue development 61
1.1 Body weight and adipose tissue composition of mice kept on standard fat diet 61
2.2 Body weight and adipose tissue composition of mice kept on high fat diet 64

2. Effect of MMP-2 deficiency on adipose tissue development 70
2.1 Body weight and adipose tissue composition of mice kept on standard fat diet 70
2.2 Body weight and adipose tissue composition of mice kept on high fat diet 72

3. Effect of gelatinase inhibition on adipose tissue development in wild-type mice 77

4. Effect of gelatinase inhibition on adipose tissue development in leptin deficient (*ob/ob*) mice 83

5. Effect of gelatinase inhibition on adipose tissue development in wild-type obese mice 87

6. Effect of gelatinase inhibition on adipose tissue development in MMP-2 deficient mice 94

7. Effect of gelatinase inhibition on *de novo* adipogenesis 97

8. *In vitro* experiments 99
8.1 Effect of gelatinase inhibition on 3T3-F442A preadipocyte differentiation 99
8.2 Effect of MMP-2 knock-down on 3T3-F442A preadipocytes differentiation 103

DISCUSSION 107
Background 107
Lessons from MMP knock-out mice 108
Gelatinase inhibition in murine models of obesity 109
Lessons from *in vitro* experiments 113
General conclusion 114
MMPs as therapeutic targets 114
Limitations and suggestions for future experiments 115

LIST OF PUBLICATIONS DISCUSSED IN THIS THESIS 117

REFERENCES 118

SUMMARY 143

SAMENVATTING 145

ACKNOWLEDGMENTS 147

CURRICULUM VITAE 151

LIST OF PUBLICATIONS 153

LIST OF ABBREVIATIONS

A.U.	Arbitrary units
ALT	Alanine aminotransferase
aP2	Adipocyte fatty acid-binding protein 2
APMA	p-aminophenylmercuric acid
AST	Aspartate aminotransferase
AUC	Area under the curve
BCA	Bicinchonic acid
BCS	Bovine calf serum
BMI	Body mass index
BSA	Bovine serum albumin
DAPI	4'-6-diamidino-2-phenylindole
DEX	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FFA	Free fatty acids
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GON	Gonadal
GPDH	Glycerolphosphate dehydrogenase
HDL	High density lipoprotein
HFD	High fat diet
IBMX	3-isobutyl-1-methylxanthine
kDa	kiloDalton
KO	Knock-out
LDL	Low density lipoprotein
MEF	mouse embryonic fibroblasts
MMP	Matrix metalloproteinase

MMPI	Matrix metalloproteinase inhibitor
MRI	Magnetic resonance imaging
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptor
Pref	Preadipocyte factor
RT-PCR	Reverse transcriptase polymerase chain reaction
SC	Subcutaneous
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFD	Standard fat diet
SVF	Stromal-vascular fraction
T3	Triiodo-L-thyronine
TIMP	Tissue inhibitor of MMP
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WHR	Waist-to-hip-ratio
WT	Wild-type

INTRODUCTION

1. Obesity

1.1 The outbreak of an epidemic

Ever since his emergence, man has been challenged by harsh environmental conditions, especially when it came to gathering food supplies. Periods of relative abundance were alternated by periods of food scarcity. During these periods of famine, many would perish and only those individuals that had accumulated enough energy would eventually survive. It is therefore not surprising that the human body, driven by a ruthless process of natural selection, has developed complex ways to overcome these difficult times by efficiently storing excess energy whenever available: calories that have not been used for metabolism or physical activity are stored as fat. This allows compact storage of energy, which can rapidly be summoned during more difficult times.

Technological and industrial progress in the modern western world has since put an end to this constant struggle for survival by securing constant and abundant food production. In addition to being copious, food is now more palatable, cheap and easily accessible. At the same time, the ever-increasing automation in industry, changing modes of transportation, and increasing urbanization rendered hard labor almost redundant. These changes in dietary and physical activity patterns exposed the human species to a whole new lifestyle to which it is poorly adapted. Ironically, yesterday's survivors have become the victims of their own success, as their bodies constantly continue to feel the need to retain as much energy as possible and accumulate fat ceaselessly. In fact, the complex mechanisms that once protected us from wasting energy have proven so efficient, that we now face a new problem. As it turned out, the human body does not have a natural defense against excessive energy storage. This, in combination with the observation that the adipose tissue is the only organ in the human body with seemingly unlimited growth potential, has left us to deal with the ever-growing problem of obesity and all its adverse effects on health.

Today, with nearly 500 million people considered obese and more than 1.6 billion struggling with overweight (1), obesity has become the most common nutritional disorder worldwide and its prevalence is still rapidly increasing (2)

(Figure 1). Once considered a high-income country problem, overweight and obesity are now also on the rise in low- and middle-income countries, particularly in urban settings (3). In 2010, around 43 million children under five were overweight, of which 35 million are living in developing countries. The worldwide prevalence of childhood overweight and obesity increased from 4% in 1990 to 7% in 2010. This trend is expected to reach 9%, or ≈60 million, in 2020 (4). This rampant advance has prompted the World Health Organization (WHO) to declare obesity a worldwide epidemic and to urge governments to undertake actions. Unfortunately, obesity prevention and research is still in its infancy, as the condition has too long been regarded as a mere cosmetic issue and complications remained underestimated.

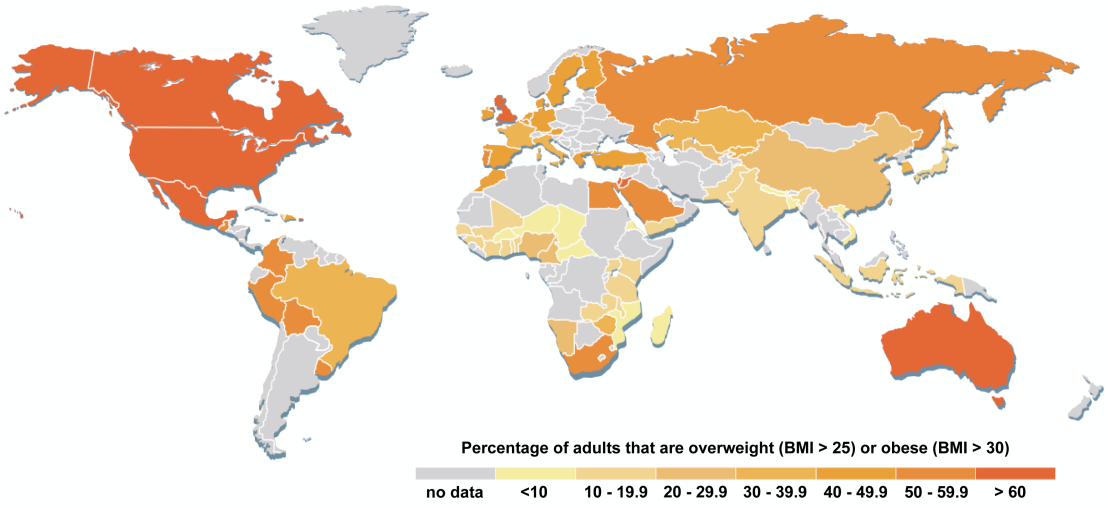


Figure 1. Global prevalence of adults that are overweight or obese. It is important to note that the data presented are not directly comparable since they vary in terms of sampling procedures, age ranges and the year(s) of data collection. Figure adapted from (5).

1.2 Definition and diagnosis of obesity

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. Regional adiposity can be accurately quantified using imaging techniques such as magnetic resonance imaging (MRI) and computed tomography (CT). However, these techniques are impractical for routine measurements, as they are expensive and complex. For practical purposes, some simple anthropometric measurements were designed. The most commonly used measurement of total adiposity is the body mass index (BMI), calculated by

dividing weight by the square of the height (kg/m^2)(6). BMI values between 18.5 and 25 are considered normal, whereas lower values indicate underweight and higher values denote overweight. The term 'obesity' is used for values above 30 and values above 40 are referred to as 'morbid obese' or 'extreme obese'. BMI provides the most useful population-level measure of overweight and obesity, as it is the same for both sexes and for all ages of adults. However, it should be considered a rough guide because it does not actually measure the percentage of body fat and therefore may not correspond to the same degree of fatness in different individuals. Other relatively simple methods for the diagnosis of obesity include the waist circumference (WC) and the waist-to-hip ratio (WHR) (7, 8). Of these three measurements, only the waist-to-hip ratio takes into account the differences in body structure.

1.3 Medical complications of obesity

In addition to the psychological burden of being obese, accumulation of adipose tissue has been linked in numerous epidemiological studies to several adverse health consequences and life-threatening conditions (9, 10), even overthrowing smoking as the leading preventable cause of death worldwide (11).

Raised BMI is a major risk factor for diseases such as:

- cardiovascular diseases (mainly heart disease and stroke);
- non-insulin-dependent diabetes mellitus (type 2 diabetes);
- musculoskeletal disorders (especially osteoarthritis);
- hypertension;
- atherosclerosis;
- obstructive sleep apnea;
- certain types of cancers (endometrial, breast, and colon).

Although it has been shown that the risk for these non-communicable diseases increases with the increase in BMI, the molecular changes in obesity that promote these disorders are not completely understood.

Insulin resistance appears to be the link between overweight/obesity and the adverse metabolic syndromes related to excess adiposity (12-15). Indeed, for many years it has been apparent that overweight/obese individuals tend to be insulin resistant and become more insulin sensitive with weight loss. In normal

circumstances, insulin stimulates glucose uptake into skeletal muscle, inhibits hepatic gluconeogenesis, and decreases adipose-tissue lipolysis and hepatic production of very-low-density lipoproteins (VLDL). Insulin signaling in the brain decreases appetite and prevents glucose production by the liver through neuronal signals from the hypothalamus. Insulin resistance, in contrast, leads to the release of free fatty acids from adipose tissue, increased hepatic production of very-low-density lipoproteins and decreased high-density lipoproteins. Increased production of non-esterified ("free" or unsaturated) fatty acids, inflammatory cytokines, and adipokines and mitochondrial dysfunction contribute to impaired insulin signaling, decreased skeletal muscle glucose uptake, increased hepatic gluconeogenesis, and β cell dysfunction, leading to hyperglycemia and ultimately to diabetes type 2. In addition, insulin resistance leads to the development of hypertension by impairing vasodilation induced by nitric oxide. Although not entirely understood, several mechanisms have been proposed to explain how increased adipose tissue mass affects whole body insulin resistance and cardiovascular risk. One theory is that increased adipose derived inflammatory cytokines and hormones induce a chronic inflammatory state that not only increases cardiovascular risk, but also antagonizes insulin signaling and mitochondrial function and thereby impairs glucose hemostasis (13, 16). Another theory suggests that release of free fatty acids and their subsequent accumulation in non-adipose tissues leads to the buildup of bioactive lipids that inhibit insulin signaling and metabolism (17-20).

1.4 The burden of excess weight

Since 1980 worldwide obesity has more than doubled. If this tendency is not addressed, WHO predicts that by 2015 numbers will increase to 2.3 billion overweight and 700 million obese adults. These are alarming prospects, given the high mortality associated with excess body weight. In European countries obesity is responsible for 10 to 13 % of the annual death toll.

In addition to its medical complications, obesity has a significant economic impact that is felt on all levels of society from individuals, to corporations, to governments. Medical costs involve both direct and indirect costs (21, 22). In European countries, obesity accounts for 2-8 % of the total health care. For

Belgium, medical costs linked to overweight and obesity are estimated at 1.2 billion € per year and additional costs due to high levels of absenteeism are believed to exceed 2 billion € annually.

1.5 Causes of obesity

The fundamental cause of obesity and overweight is an energy imbalance between calories consumed and calories expended. Although the major culprit of the increased prevalence of obesity appears to be 'obesogenic' (environmental changes that promote excessive caloric intake and discourage physical activity), the importance of genetic predisposition can not be neglected. Indeed, obesity often runs in families, which is one indicator that obesity has a genetic component. Twin, adoption and family studies have shown that susceptibility to obesity is partly determined by genetic factors and that there is a synergistic relationship between genes and environment: the magnitude of a subject's response to changes in lifestyle or environmental conditions depends on its genetic predisposition.

With few exceptions, obesity is a complex multifactorial disease (23). This complicates the identification of genes and mutations causing it and, as for other complex human diseases, possible candidate genes do not address genetic causes in the population as a whole. Still, they provide useful insights into the pathways involved in body weight regulation. Currently, positive associations with obesity phenotypes have been reported for more than 120 genes (24). Thus, the traditional view that obesity is self-inflicted and merely an issue of willpower and lifestyle is loosing scientific support, but remains still deeply rooted in common belief.

1.6 Treatment of obesity

Although regular physical activity and judicious dieting can result in weight loss, this requires fair amounts of effort and personal discipline. The slow progression of weight loss, caused by the human body's reluctance to squander energy, is a major drawback for many, and too often attempts are ceased prematurely. To make matters worse, those who do succeed are generally unable to maintain their weight loss in the long term. Many studies have indicated that a rebound in

weight will occur in up to 90% of them, as the adopted preventive lifestyle is easily abandoned once the desired results have been obtained. Indeed, there are considerable environmental and societal forces that make it difficult for most people to maintain healthy habits.



Figure 2. Remedies for corpulence have been around for ages but were long sold merely as cosmetics. This advertisement for "Allan's Anti-Fat" ran in the July 5, 1878 *Daily Star* (Marion, Ohio) and claims to "act upon the food in the stomach, preventing its being converted into fat."

Therapeutic agents that accelerate and/or help maintain weight loss may therefore represent a useful tool in the long-term treatment of obesity (Figure 2). In the search for targets for anti-obesity agents, most attention has been given to centrally acting neuropeptides that regulate food intake (appetite or satiety) or to peripheral mechanisms that reduce nutrient absorption from the gut. Although these approaches are the most likely to be intensively pursued in the future, they have met with only moderate success and serious safety concerns, which tend to discourage their use. In Europe, only one drug has been approved for long-term use: Orlistat (with brand names Xenical and Alli) reduces fat absorption in the bowels by inhibiting pancreatic lipase. Two others, Sibutramine (with brand names Reductil, Meridia and Sibutrex), that inhibits deactivation of several neurotransmitters in the brain to decrease appetite, and

Rimonabant (Acomplia), that acts through blockage of the endocannabinoid system, had been approved but were officially withdrawn by the EMEA (European Medicines Agency) in 2009 and 2010 respectively, due to safety issues (25, 26). This has left a vast gap in the current pharmacological treatment options and an unmet medical need for more effective and safe pharmacotherapy for obesity. Therefore, new strategies such as pharmacologically increasing metabolic rate and thermogenesis (27), inhibition of preadipocyte proliferation/differentiation (28), inducing apoptosis of adipocytes (29), converting white to brown adipose tissue (30), are currently being explored. Although it is unlikely that a single agent will suffice for adequate treatment, a combination of therapies and long-term treatment may eventually result in sustained success (31).

2. Adipose tissue

2.1 More than just storage

Fat is stored in the form of triglycerides and contained within specialized cells, the adipocytes. These cells are found throughout the whole body, but accumulate in certain regions to form adipose tissue. Mammals have two different types of adipose tissue: white and brown adipose tissue, with white adipose tissue being more abundant. Brown adipose tissue, which derives its color from rich vascularization and densely packed mitochondria, is important for regulating body temperature. In contrast to the energy storing white fat depots, it is a site of energy dissipation. Mitochondria from brown adipocytes have a unique carrier called uncoupling protein (UCP1) that transfers protons without subsequent production of ATP (32). As a consequence, the lipid in brown adipose tissue releases energy directly as heat, instead of serving as a energy substrate. Morphologically, brown adipocytes are multilocular and contain less overall lipid than their white counterparts, which are unilocular. In humans, brown adipose tissue surrounds the heart and great vessels in infancy but tends to disappear over time until only scattered cells can be found within white fat pads (33).

Besides adipocytes, adipose tissue also contains fibroblastic connective tissue cells, macrophages, leukocytes and preadipocytes. It is highly vascularized and innervated.

The traditional view of the adipose tissue was that of a passive reservoir for excess energy that also helps to insulate and cushion the body. Previous data, however, suggest that (white) adipose tissue should be regarded as a dynamic organ with pleiotropic functions, as it is an important source of hormones and cytokines (34-36). These cytokines are referred to as adipokines and include interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor alpha (TNF-α), visfatin, retinol binding protein-4 (RBP4) and chemerin. Adipose derived hormones include leptin, adiponectin and resistin. Furthermore, adipose tissue is also a major site for metabolism of sex steroids and glucocorticoids (37).

In addition to being the source of many bioactive peptides, that act at both the local (autocrine/paracrine) and systemic (endocrine) level, the adipose tissue also expresses numerous receptors that enable it to respond to a wide range of afferent signals from traditional hormonal systems as well as the central nervous system. Through this interactive network, the adipose tissue is integrally involved in the coordination of a variety of biological processes as diverse as homeostasis, blood pressure, immune response, neuroendocrine function, angiogenesis and energy balance (38, 39). It is therefore not surprising that both excess and deficiency of adipose tissue have severe adverse systemic consequences.

2.2 The adipocyte and its surroundings

Although various cell types contain esterified lipids, adipocytes are distinguished by their unique morphology allowing storage of large quantities of lipids. Approximately 60 to 85% of the weight of white adipose tissue is lipid that is stored in a unilocular droplet. The rest is made up of cytoplasm, mitochondria, nucleus and other organelles that are pushed against the plasma membrane, giving the cell a signet-ring shape.

About 90 to 99% of the intracellular fat is triglyceride, but small amounts of free fatty acids, diglyceride, cholesterol, phospholipid and minute quantities of cholesterol ester and monoglyceride are also present. With up to 90 % of the total lipid mixture, myristic, plamitic, palmitoleic, stearic, oleic, and linoleic acid

are the most abundant fatty acids present. Relative amounts may vary, depending on diet composition.

Besides adipocytes, the adipose tissue contains preadipocytes, fibroblasts, vascular smooth muscles cells, endothelial cells, neurons, resident monocytes/macrophages, and lymphocytes (40-43) . Collectively, these cells are referred to as the stromal-vascular fraction (SVF). Like adipocytes, the SVF produces several adipokines, yet it differs in the type and amount of molecules produced. For example, leptin and adiponectin are primarily secreted by adipocytes (44), whereas SVF cells contribute to most of the release of inflammatory mediators and interleukins such as tumor necrosis factor-α or interleukin-6 (45). In addition, the SVF of different adipose tissue depots exhibits unique adipokine expression and secretion profiles, thereby contributing to the major differences between subcutaneous and visceral fat depots (46).

Structural support and anchorage for the adipocytes and the SVF is provided by the extracellular matrix (ECM). Components of the ECM are produced intracellularly by resident cells, and secreted into the extracellular space via exocytosis. These include structural proteins (collagens and elastins) and various classes of adhesion proteins, such as fibronectin, laminin, elastins and proteoglycans. The ECM not only ensures tissue architecture, but also has a pivotal role in the biological functions of the adipose organ: it forms a reservoir of sequestrated bioactive molecules, acts as a scaffold for cell migration, communicates to cells via integrins, …

Adipocytes are surrounded by a distinctive pericellular basement membrane composed of mostly collagen IV and laminin, whereas heparan sulfate proteoglycan and fibronectin are almost completely missing. Its composition can vary quantitatively and qualitatively between different fat depots, and the ECM drastically changes in consistency during adipocyte differentiation and adipose tissue development (47).

2.3 Adipose tissue development

The “adipose organ” is highly plastic and can respond rapidly to changes in nutrient intake. It is the only organ in the body that can markedly change in mass during adult life (48), with an almost unlimited growth potential. Two distinct,

yet intertwined, processes are distinguished (49, 50). During 1) adipocyte hypertrophy, existing adipocytes will increase in size, as they accumulate excess triglycerides. This often precedes 2) adipocyte hyperplasia, also referred to as adipogenesis, which is the recruitment of new adipocytes from precursor cells in the adipose tissue and involves the proliferation, migration, clustering and differentiation of these preadipocytes.

Similar to neoplastic tissues, expansion of the adipose tissue relies on angiogenesis – the formation of blood vessels from pre-existing vessels - for the supply of nutrients and oxygen, for the transport of lipids and signaling molecules and for the elimination of waste products (51-53). Each adipocyte needs to be in close proximity to at least one blood capillary.

In addition, extensive proteolytic remodeling of the extracellular matrix (ECM) occurs simultaneously in the expanding adipose tissue. Two types of proteolytic systems have been implicated in the degradation of ECM and basement membrane components: the plasminogen/plasmin (fibrinolytic) and matrix metalloproteinase (MMP) systems. In addition, the MMPs can regulate cell behavior by interfering with the perception of its surrounding (cfr infra).

In human adults, changes in fat mass can mainly be attributed to changes in fat cell volume. The number of fat cells is set during childhood and adolescence and is believed to stay constant in adulthood in both lean and obese individuals. In early onset obesity, neither adipocyte death nor generation rate is markedly altered, suggesting a tight regulation of fat cell number in this condition during adulthood. Even after marked weight loss, the adipocyte number remains more or less the same. This is probably one of the reasons why obese individuals have difficulties maintaining weight loss, as re-uptake of available triglycerides will rapidly occur in 'starving' adipocytes.

While the extent of hyperplasia in adults is still under debate, it is difficult to explain extreme corpulence by hypertrophy alone. It is possible that prolonged exposure to excessive caloric surpluses forces the body to adjust its programmed 'body weight set-point' and allow hyperplasia to occur (54).

Nevertheless, since adipose tissue is a highly dynamic organ, with a constant need to rejuvenate its cell population, adipogenesis from preadipocytes is

constantly taking place in order to replace old adipocytes. Adipocytes have a turnover rate of approximately 10% per year, with half of the adipocytes replaced every 8 years (55).

2.4 Adipogenesis

Adipocytes derive from multipotent mesenchymal stem cells (MSC), which can also differentiate into myocytes, chondrocytes and osteocytes. Most of our knowledge about adipogenesis comes from *in vitro* studies of precursor cells (cfr infra), and there are little data on *in vivo* depot-specific aspects of differentiation. However, although the full developmental program remains unknown, it is generally accepted that adipogenesis occurs in two phases. A determination phase involves the commitment of a pluripotent stem cell to the adipocyte lineage and results in the conversion of the stem cell to a preadipocyte. This cell still morphologically resembles its precursor but has lost the ability to differentiate into any cell type other than the adipocyte. In a second phase, referred to as terminal differentiation, the preadipocyte undergoes morphological changes, gradually acquires the machinery that is necessary for lipid transport and synthesis, insulin sensitivity and the secretion of adipocyte-specific proteins and finally develops into a mature adipocyte. The molecular mechanisms regulating the terminal differentiation are more extensively characterized than the determination because most studies focusing on adipogenesis have used cell lines (such as 3T3-L1, 3T3-F442A, C3H10T1/2, cfr infra) already directed towards adipocyte differentiation. These studies have indicated that several transcription factors, cofactors and signaling intermediates from numerous pathways coordinate adipocyte maturation in a very complex way (56). Growth and differentiation of preadipocytes is controlled by communication between individual cells or between cells and the extracellular environment. Various hormones, cytokines and growth factors that affect adipocyte differentiation in a positive or negative manner have been identified and act via specific receptors to transduce external growth and differentiation signals through a cascade of intracellular events (57). Among extracellular signaling factors and pathways regulating adipogenesis are: insulin, glucocorticoids, retinoic acid (RA), growth hormone (GH), insulin-like growth

factor (IGF-1), EGF, Transforming growth factor (TGF-ß), interleukin-1 (IL-1), interferon-γ, Tumor necrosis factor (TNF-α), transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs), Wnt proteins, sonic hedgehog (SHH), Notch signaling pathway, DLK1/PREF1 signaling, insulin, insulin growth factor 1 (IGF1), fibroblast growth factors (FGFs), MAPK pathway, … Transcriptional factors include, but are not restricted to, peroxisome proliferator-activated receptor γ (PPARγ), several members of the CCAAT-enhancer-binding protein (C/EBP) family, transcription factor homologous to CCAAT-enhancer binding protein (CHOP), Krüppel-like factors (KLFs), KROX20, early B-cell factor (EBF), SREB1c, EPAS1, STAT5a, cyclic AMP response element-binding protein (CREB), the clock gene BMAL1. Transcriptional cofactors include histone acetyltransferases (HATs), CREB-binding protein (CBP), p300, BRG1, BRM, TRAP220 (also called PPAR-binding protein), PPAR-interacting protein (PRIP), cyclin D3-cyclin-dependent kinases (CDK6 and CDK4), transcriptional co-factor with PDZ-binding motif (TAZ).

In rat and mouse, committed preadipocytes start differentiating into mature adipocytes right after birth (58), whereas in humans adipose tissue development already begins during late embryonic development, though a majority of the differentiation occurs only shortly after birth (59). All mammals retain the ability to differentiate preadipocytes in response to their body's energy storage demands throughout their life spans.

Mature lipid-filled adipocytes are terminally differentiated cells and are therefore generally considered incapable of division. However, this assumption has been questioned, as several studies have reported that adipocyte dedifferentiation may occur after either acute injury or in a malignant context (60, 61). These findings were supported by *in vitro* observations. For example, treatment of differentiated adipocyte cells with TNF-α (62) or MMP-11 (63) leads to lipid depletion and reversal of adipocyte phenotype. This suggests that adipocytes exhibit a plasticity depending on the microenvironment, though more research on the subject in needed.

2.5 Murine models to study adipogenesis

In vitro, adipogenesis has been studied using several cellular models that involve almost all stages of development. Embryonic stem cells, derived from the inner cell mass of mouse blastocysts, can differentiate into mature adipocytes, when given a combination of retinoic acid and pro-adipogenic hormones. Similarly, mouse embryonic fibroblasts (MEFs), which are isolated after disaggregation of embryos at embryonic day E12–14, commit to the adipocyte lineage when treated with a cocktail of defined hormonal inducers. However, the most widely used and best-characterized *in vitro* models for studying the conversion of preadipocytes into adipocytes are the 3T3-L1 and 3T3-F442A adipogenic cell lines (57). These cells are derived from disaggregated Swiss 3T3 mouse embryos and are immortalized through clonal isolation (64). They have undergone determination and are therefore restricted to the adipocyte lineage, but are believed to differ in the developmental stage at which cells were arrested when derived. Once confluent, they will spontaneously differentiate into mature adipocytes and accumulate triglycerides. Differentiation can be maximized by the addition of adipogenic stimuli, including glucocorticoids, cyclic AMP agonists - usually a cocktail of insulin, triiodothyronine (T3), dexamethasone (DEX) and isobutyl-methylxanthine (IBMX) (65) - and insulin (66-68). Conversion to mature adipocytes takes about 12 days and can be evaluated by staining with Oil Red O, that binds to accumulated triglycerides in the cytoplasm (69).

Although these *in vitro* models share many similarities with primary adipocytes — including triglyceride storage, insulin sensitivity and expression of adipocyte-specific genes — there are some important differences. For example, differentiation induced *in vitro* is usually incomplete and results in multilocular adipocytes whereby triglycerides are stored in many droplets, whereas white adipocytes *in vivo* characteristically contain a single large lipid droplet (70). Also, differentiated 3T3-L1 and 3T3-F442A cells express only marginal levels of leptin as compared to primary adipocytes (71).

In vivo study of preadipocyte differentiation is less common as it suffers several major drawbacks. First, primary cultures are often heterogeneous as it is difficult to isolate preadipocytes from other fibroblast-like cells. Second, as preadipocyte differentiation is a continuous process, it is nearly impossible to obtain

preadipocytes at a similar developmental stage. Third, large amounts of fat tissue are required because of the relatively low percentage of preadipocytes in adipose tissue. Finally, primary cultures have a limited life span, compelling the need to harvest new cells repeatedly and narrowing the time window for experimental procedures.

To combine the benefits of *in vitro* cell lines with the stringency of an *in vivo* environment, it is possible to transplant adipogenic cells lines, like 3T3-F442A, subcutaneously into immunodeficient or allogeneic mice. Transplanted 3T3-F442A cells are able to differentiate into unilocular adipocytes that express leptin at levels comparable to those in normal tissues (71). If the cells have been labeled or transplanted in an area normally depleted of endogenous adipose tissue, they can be distinguished and isolated for further analysis.

3. Matrix Metalloproteinases (MMPs)

3.1 A historical perspective

In 1962 Jerome Gross and Charles Lapière described a diffusible enzyme originating from resorbing tadpole tails that was able to degrade gels made of native fibrillar collagen (72). Eight years later, the zymogen of this 'collagenase' was isolated and characterized (73). This initial observation became the starting point of a new, slowly but steadily growing research field.

As research continued, more enzymes were discovered and collagenase was renamed collagenase-1 and finally MMP-1, and became the first member of the matrix metalloproteinase (MMP) family, so called because of their dependence on metal ions for catalytic activity and their potency to degrade structural proteins of the extracellular matrix (74). To date, at least 26 MMPs are identified in vertebrates, 24 of which are present in humans. This 'system' of proteolytic enzymes is capable of degrading all components of the extracellular matrix, including interstitial and basement membrane collagens, proteoglycans, fibronectin and laminin, as well as several non-ECM proteins, such as adhesion molecules, cytokines, protease inhibitors and other (pro-)MMPs. MMPs have the ability to regulate cell behavior in several ways and are thereby involved in a large variety of physiological and pathological processes (75).

3.2 Classification and general structure

The most commonly used categorization of MMPs is based partly on structural characteristics and partly on functionality and substrate specificity. This results in the following subgroups:

- Collagenases (MMP-1, -8, -13)
- Gelatinases (MMP-2, -9)
- Stromelysins (MMP-3, -10, -11)
- Membrane type MMPs (MMP-14, -15, -16, -17, -24, -25)
- Other MMPs

Although the individual MMPs are products of different genes dispersed throughout the genome, they share significant sequence homology and have a

similar well-conserved domain structure (76) (Figure 3). In general, a MMP consists of:

- A signal peptide: typically, this sequence is 17-20 amino acids in length and contains a high proportion of hydrophobic residues. It directs the protein to the lumen of the endoplasmic reticulum (ER) for secretion and is removed after enzymatic digestion by an endopeptidase.
- An autoinhibitory pro-domain: this domain is about 80 amino acids long and plays an important role in the regulation of MMP activity. It contains a highly conserved cysteine residue that interacts with the zinc ion in the catalytic domain and prevents binding and cleavage of the substrate (77). This so-called 'cysteine switch' maintains the enzyme latency until it is removed or disrupted (78).
- A catalytic domain: this domain is approximately 160-170 amino acids in length and is organized as an oblate sphere. The active site is a groove that runs across the catalytic domain and includes a Zn^{2+} binding motif of three histidine residues (His-Glu-xxx-Gly-His-xxx-xxx-Gly-xxx-xxx-His), which coordinates a zinc atom and a water molecule at the active site.

  The 3-dimensional architecture of the catalytic domain is highly conserved and the active site contains six recognition pockets: S1-S3 and S1'-S3'. The most prominent subsite within the MMP catalytic domain is the S1'-specificity pocket, a binding site for substrate or inhibitor molecules. The S1' pocket differs considerably in size and shape among the various MMPs.

  The gelatinases (MMP-2 and MMP-9) incorporate in their catalytic domain three fibronectin type II modules inserted immediately before the zinc-binding motif (79).
- A hinge domain: The catalytic domain and the hemopexin domain are connected by a flexible hinge or linker region that varies in length and composition among the various MMPs. It often contains several proline residues and influences substrate specificity (80).
- A hemopexin domain: this domain of about 200 amino acids is found in every MMP with the exception of MMP-7, -23 and -26 (81, 82). It influences the binding of TIMPs (tissue inhibitors of metalloproteinases)

and certain substrates, and affects membrane activation and proteolytic activities (83, 84).

- A transmembrane domain: 6 MMPs have been identified that have an additional C-terminal extension of about 80-110 amino acids that forms a single-pass transmembrane domain that anchors the protein to the cell membrane. At the C-terminal end, they either have a short cytoplasmatic tail (MMP-14, -15, -16 and -24) or a hydrophobic region that acts as a glycosylphosphatidyl inositol (GPI) anchoring signal (MMP-17 and MMP-25)(85, 86).

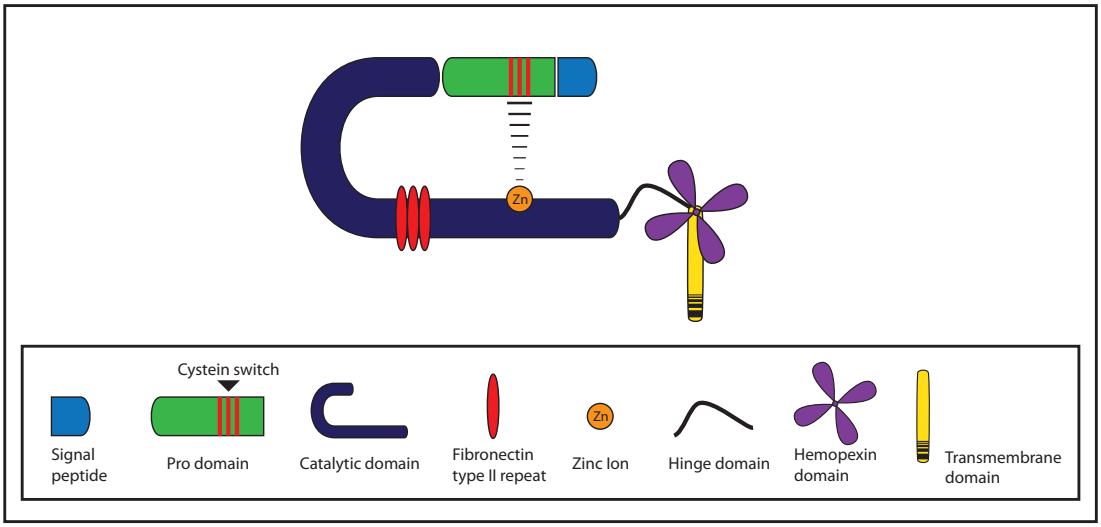


Figure 3. General structure of MMPs.

3.3 Regulation of MMP activity

Considering the degenerative potential of MMPs, it is not surprising that they are tightly regulated in order to maintain tissue integrity and function. This occurs at multiple levels, including transcription, modulation of mRNA half-life, protein synthesis, secretion, subcellular or extracellular localization, zymogen activation, inhibition of proteolytic activity and protease degradation (Figure 4). In normal conditions most MMPs are present at very low levels in the tissue, if at all. Their production, secretion and activation are rapidly induced when active tissue remodeling is needed. Each MMP has its complex and highly individualized pattern of expression.

- Transcriptional regulation

  MMP gene expression is regulated by numerous stimulatory and suppressive factors, acting on multiple signaling pathways. These include several cytokines and growth factor such as interleukins, interferons, epidermal growth factor (EGF), keratinocyte growth factor (KGF), nerve growth factor (NGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), tumor necrosis factor α (TNF-α), Transforming growth factor ß (TGF-ß) and Basigin (87).

- Post-transcriptional regulation

  Although MMP gene expression is primarily regulated at transcriptional level, there is also evidence about modulation of mRNA stability in response to growth factors and cytokines (88-91) and several studies demonstrated microRNA mediated RNA interference as a novel evolutionarily conserved mechanism for the regulation of gene expression at the post-transcription level (92-98). In addition, alternative splicing of some MMP transcripts can occur and affect activity and/or localization (99, 100).

- Secretion

  Most MMPs are constitutively secreted after they are synthetized. However some cells (such as inflammatory cells) are able to store MMPs in specific granules, which can be released promptly following cell stimulation (101).

- Activation

  With the exception of furin-processed MMPs (MMP-11, MMP-19, MMP-23, MMP-25, MMP-28 and the MT-MMPs), that contain a furin-susceptible insert between their propetide and catalytic domains (102), all MMPs are secreted as inactive proenzymes. Activation of these zymogens is one of the critical regulatory steps of matrix degradation. The proMMPs are kept inactive by the formation of an intramolecular complex between the cysteine residue in its propeptide domain and the essential zinc atom in the catalytic domain, thereby blocking access to the active site.

Activation is achieved by dissociation of the cysteine residue from the complex, generally by proteolytic cleavage of the N-terminal pro-domain. This mechanism is referred to as the "cysteine-switch" (78).

- Inactivation

  Further regulation of MMPs occurs by interaction with two types of endogenous inhibitors: the specific (the tissue inhibitors of metalloproteinases, TIMPs) and the non-specific inhibitors (such as α2-macroglobulin, an abundant plasma protein that represents the major inhibitor of MMPs in tissue fluids (103).

  So far, four mammalian TIMP family members have been identified (TIMP-1 to -4) and together they are able to reversibly inhibit all MMPs, although they differ in their ability to inhibit specific MMPs (104). They act by binding to the activated form of MMPs, but the gelatinases (MMP-2 and MMP-9) are able to form complexes with the non-inhibitory C-terminal end of TIMP-2 en -1, respectively, while still in the latent form. It has been proposed that low amounts of TIMP-2 accelerate the activation of MMP-2, whereas high amounts block its activity (cfr infra).

- Compartimentalization

  Because many extracellular signaling events occur at or near the cell membrane, targeting relevant MMPs towards this region might be more effective than simply have the appropriate MMPs diffuse. Additionally, this may provide a way to restrict the proteolytic activity of some MMPs to the pericellular space, keeping the rest of the extracellular environment unaffected. Furthermore, because surface receptors give cells the capacity to sense the presence and location of specific substrates, localized delivery of specific MMPs would allow the cell to adequately interact with its environment.

  While this specific localization is rather obvious for MT-MMPs, specific cell-interactions also have been described for other MMPs. For example, MMP-1 and MMP-2 can bind to integrins (105, 106), MMP-9 to CD44 (107) and MMP-7 to surface proteoglycans (108).

MMP-2 also interacts with MT1-MMP and this complex formation is essential for its activation (109-111).

MMPs are also bound to the cell-surface by their own substrates. Latent proMMP-9, for example, binds with high affinity to type IV collagen α2 chains, the major structural component of basement membranes. This concentrates proMMP-9 in close vicinity of the cell and in direct contact with its target (112, 113).

- Catabolism

  Proteolytic processing of MMPs provides another efficient way of regulating their activity. While some cleavages completely inactivate MMPs, others can alter their inherent characteristics. Removal of the hemopexin domain, for example, can 1) generate truncated enzymes that lose their ability to cleave some substrates but retain their ability to cleave others, 2) affect the substrate specificity, 3) diminish the affinity for inhibitors or 4) impair the ability of certain MMPs to localize to the pericellular space.

- Clearance

  MMPs can bind several proteins that can in turn be captured by scavenger receptors, endocytosed and permanently cleared. Examples are α2-macroglobulin, which has a bait region recognized by nearly all MMPs (103) and Thrombospondin 2 (TSP-2), which binds MMP-2 and is endocytosed by the low-density lipoprotein receptor-related protein (LRP)(114, 115).

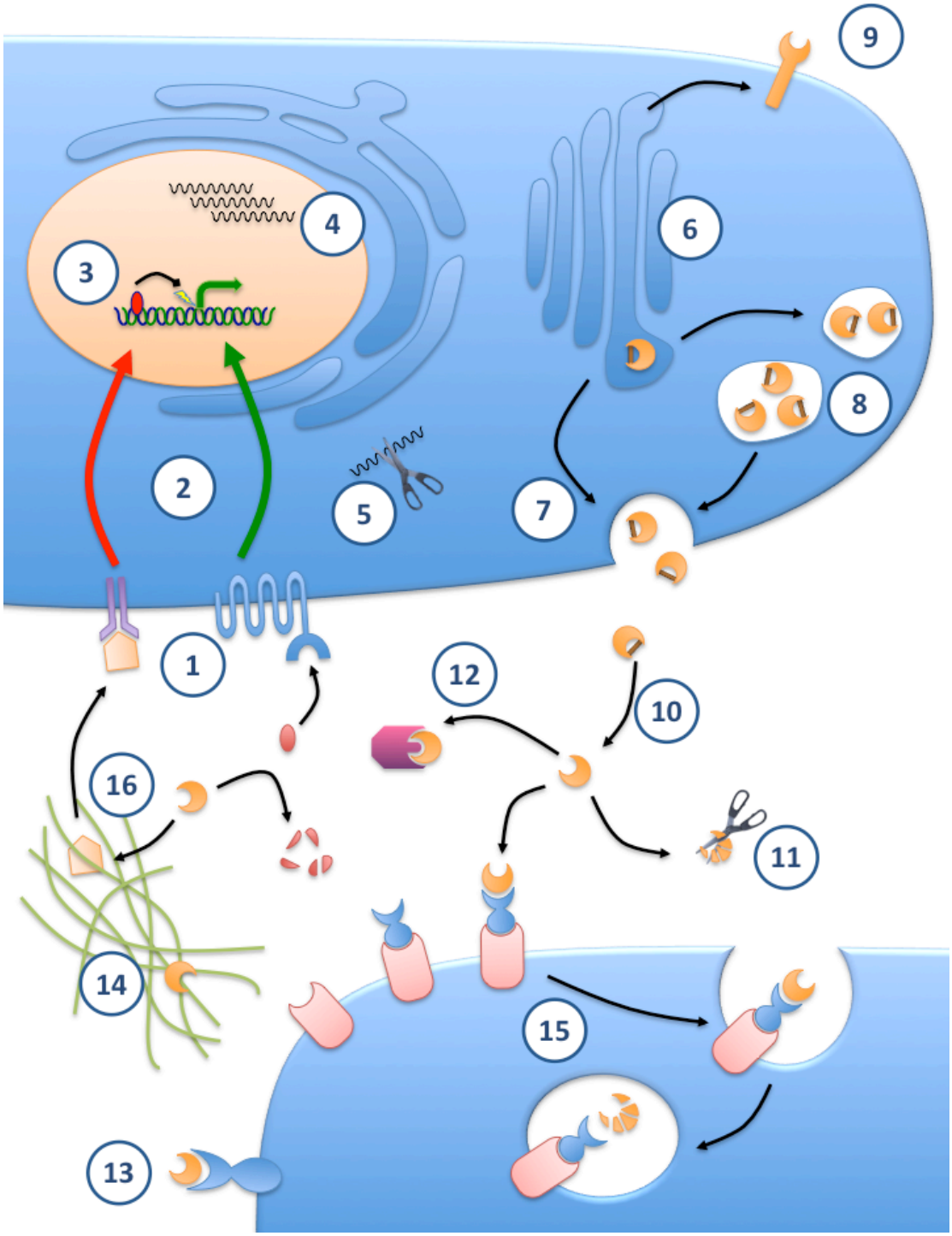


Figure 4. General representation of MMP regulation. MMP regulatory mechanisms include stimulating and inhibiting signaling (1), intracellular signal transduction (2), transcriptional activation and repression (3), post-transcriptional mRNA processing (4), mRNA degradation (5), intracellular activation of furin susceptible MMPs (6), constitutive secretion (7), regulated secretion (8), cell surface expression (9), proteolytic activation (10), proteolytic processing and/or inactivation (11), protein inhibition (12), cell surface localization (13), ECM localization (14), endocytosis and intracellular degradation (15), and release or degradation of signaling molecules (16). Figure based on (75).

Legend to Figure 4


Signaling receptors


Scavenger receptor


Pro-MMP


Active MMP


Degraded MMP


MMP Inhibitor


Membrane-bound MMP


MMP-binding surface receptor


Signaling molecules


MMP-binding protein


Extracellular matrix


RNAse/protease

3.4 Models to study MMPs

One of the most commonly used approaches to study protein function *in vivo* is the use of genetically engineered animals, with either gain-of-function (transgenic) or loss-of-function (knock-out) models. This strategy provides powerful tools to identify essential functions of individual proteins in a variety of physiological and pathological conditions.

Surprisingly, the majority of MMP knock-out mice generated so far show no major physiological alterations (Table 1). Although it is possible that MMPs are not important until after embryonic development, it is more likely that enzymatic redundancy, enzymatic compensation and/or adaptive development can account for the lack of severe phenotypes. Indeed, these animal models are constitutive and therefore loss of one MMP could be enzymatically compensated for by related MMPs. Therefore, most of the so far described knock-out models only show significant defects in regulating tissue response to environmental challenges, such as wounding, infection and inflammation.

MMP/TIMP gene	Mutant mouse phenotype
MMP-1	No overt phenotype (C. Lopez-Otin, unpublished)
MMP-2	Reduced body size (116), reduced neovascularization (117), decreased mammary branching (118), reduced lung saccular development (119), reduced tumor progression (120), reduced bone density (121)
MMP-3	Altered structure of neuromuscular junctions (122), reduced purse stringing during wound healing (123), altered mammary branching (118), enhanced adipose tissue development (124)
MMP-7	Innate intestinal immunity defects (125), decreased re-epithelialization after lung injury (126)
MMP-8	Increased skin tumors (127), resistance to tumor necrosis factor (TNF)-induced lethal hepatitis (128)
MMP-9	Defective endochondral ossification (129), defective neuronal remyelination after nerve injury (130), delayed healing of bone fractures (131), impaired vascular remodeling (132), delayed growth plate vascularization (129), delayed myelinization (133)
MMP-10	Increased pulmonary inflammation and mortality (134)

MMP-11	Delayed mammary tumorgenesis (135), adipocyte hypertrophy (136)
MMP-12	Diminished recovery from spinal cord crush (137), increased angiogenesis due to decreased angiostatin (138), delayed myelinization (133)
MMP-13	Bone remodeling defects (139, 140), reduced hepatic fibrosis (141), increased collagen accumulation in atherosclerotic plaques (142)
MMP-14	Skeletal remodeling defects (143-146), defective angiogenesis (147), inhibition of tooth eruption and root elongation (148), defects in lung and submandibular gland (147, 149)
MMP-19	Induced obesity (150)
MMP-20	Defects in tooth enamel (151)
MMP-23	No phenotype reported
MMP-24	Abnormal response to sciatic nerve injury (152)
MMP-28	Increased inflammatory response in lung (153)
TIMP-1	Accelerated endometrial gland formation (154), impaired learning and memory (155), accelerated hepatocyte cell-cycle progression (156), increased resistance to Pseudomonas infection (157), resistance to nutritionally induced obesity (158)
TIMP-2	Motor defects (159)
TIMP-3	Accelerated apoptosis in mammary glands (160), impaired bronchiole branching (161), enhanced metastatic dissemination (162)
TIMP-4	No phenotype reported (163)

Table 1. Murine MMP- and TIMP-null mutant phenotypes. Adapted from (164, 165).

3.5 Function

For a long time, MMP function was thought to be limited to the breakdown of extracellular matrix components to allow tissue remodeling and growth. However, matrix degradation has biological consequences beyond the mere creation of space for cells to migrate and proliferate. For example, cleavage of ECM proteins can produce specific fragments with independent biological activity or result in the release of ECM-bound biological agents, such as growth factors. Furthermore, MMP substrates are not restricted to structural matrix

proteins but also include various circulating, cell surface and pericellular proteins, such as peptide growth factors, chemotactic molecules, tyrosine kinase receptors, chemokines, cytokines, clotting factors, cell-adhesion molecules, and other MMPs. Taken together, MMPs are able to alter cell-matrix and cell-cell interactions; to release, activate or inactivate autocrine or paracrine signaling molecules; and to activate or inactivate cell receptors (Figure 5).

As a consequence, MMPs are implicated in a wide variety of physiological and pathological processes. Indeed, MMPs contribute to morphogenesis, angiogenesis, tissue repair, menstruation, … and several members of the MMP family have been linked with diseases such as periodontitis, rheumatoid arthritis, chronic heart failure, chronic inflammation, macular degeneration and tumor cell invasion. Their active participation in these pathological conditions has been the basis for the substantial effort to generate synthetic inhibitors for therapeutic use (166).

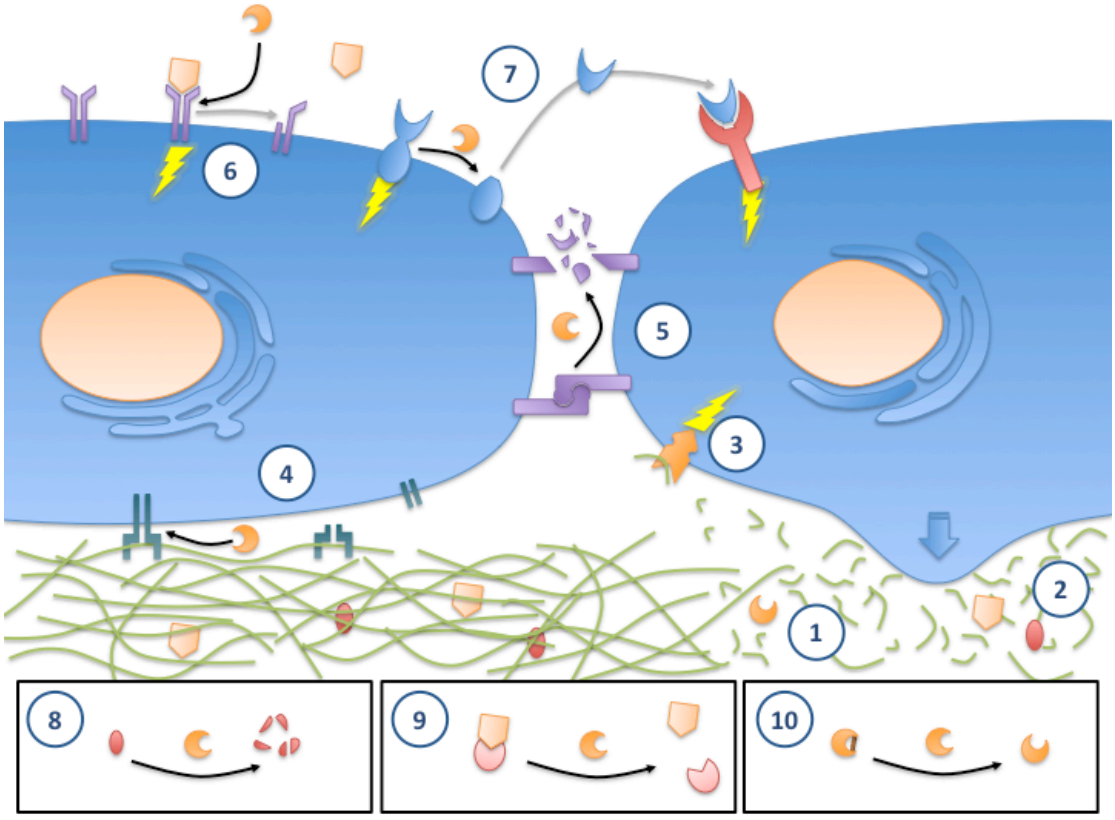
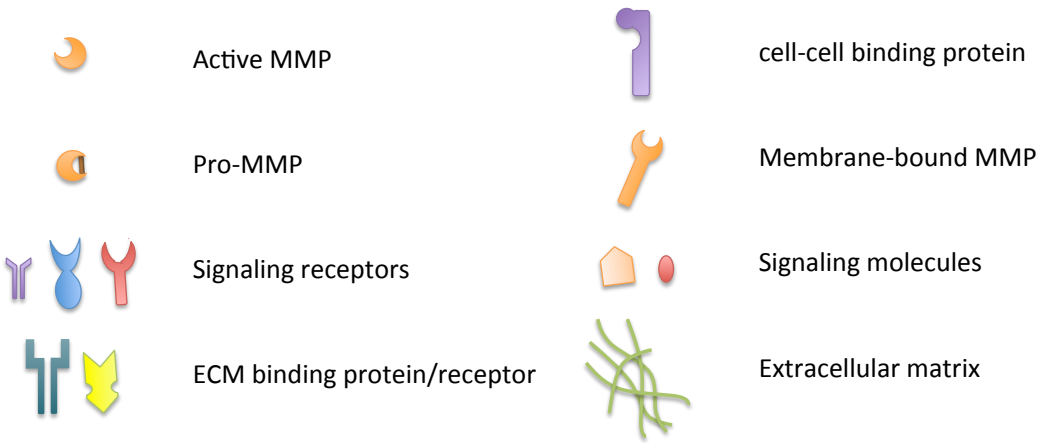


Figure 5. Potential mechanisms by which MMPs regulate cell behavior include degradation of ECM permitting cell migration/growth/proliferation (1), release of signaling molecules from the ECM (2), generation of bioactive ECM fragments (3), alteration of cell-matrix (4) and cell-cell interactions (5), activation/inactivation of cell receptors (6), shedding of bioactive fragments from cell-surface proteins (7), inactivation (8) and activation of signaling molecules (9), feedback via autoactivation (10).

Legend to Figure 5



3.6 Synthetic inhibitors of MMPs

Considering the wide variety of pathological states they are associated with, MMPs are regarded as viable drug targets with high therapeutic potential (167). It is therefore not surprising that great efforts are being made to develop synthetic compounds able to potently block their *in vivo* activity. In the last three decades over 60 synthetic matrix metalloproteinase inhibitors (MMPIs) have been pursued as clinical candidates, targeting indications such as cancer, arthritis, cardiovascular diseases and many others (168-170). However, despite promising results with *in vitro* and *in vivo* disease models, clinical development of all MMPIs (except doxycycline, Periostat, for periodontal disease) has been discontinued due to safety reasons and/or lack of efficacy. The disappointing results can be attributed to (I) poor selectivity of MMPIs, (II) poor understanding of targeted MMP function in pathology and during the different stages of disease progression. It has become clear from the advance in our understanding of MMP functions, that different MMPs may have opposite function depending on temporal and/or spatial parameters. For example, while some MMPs promote tumor progression, others provide protective effects (171-174). Similarly, opposite roles for different MMPs have been observed in arthritis (175) and in plaque stability during atherosclerosis (176). As a result, the strategy of broadly blocking MMPs to counter disease progression has failed; MMP targets and MMP anti-targets should be more precisely defined for each specific condition.

In conclusion, the disappointing results observed in clinical trials with currently available MMPIs underline the absolute need for compounds that are able to fully discriminate between different members of the MMP family. To achieve this, it may be necessary to switch to a different approach of targeting the MMPs. Indeed, most current MMP inhibitors are designed to target a highly conserved zinc group in the active site of MMPs. Though effective, these inhibitors generally display poor specificity. New inhibitors that do not incorporate a zinc-binding group in their chemical structure might provide more specific inhibition (166). To achieve this, contemporary MMPI design focuses on targeting the subdomains in the catalytic domain. The prospect of more specific MMPIs being developed in the near future, still justifies MMPs as potential therapeutic targets and drives basic research on MMPs (177).

3.7 MMPs and adipose tissue development

The first indications that MMPs play a role in adipocyte differentiation came from *in vitro* observations: in 1997 Brown et al reported that conditioned medium of rat adipocytes contained MMP-2 like gelatinolytic activity and it was suggested that this might play a role in their organization in multi-cellular clusters (178). Later, in 2001, Lijnen et al documented the presence of MMP-2 in adipose tissue of mice and observed elevated MMP-2 expression in adipose tissues of mice with nutritionally induced obesity as well as in genetically obese db/db mice (179). In that same year, Bouloumié et al provided evidence that human adipose tissue releases MMP-2 and -9 and that this secretion is modulated during adipocyte differentiation (180). Moreover, they suggested MMP activities to be directly involved in the regulation of adipocyte differentiation, since their inhibition with the broad-spectrum inhibitor Batimastat resulted in a blockade of adipocyte differentiation. In 2002, Maquoi et al measured mRNA levels of 16 MMPs and 4 tissue inhibitors of MMPs (TIMPs) in adipose tissue isolated from mice maintained for 15 weeks on a standard or high-fat diet (181). With the exception of MMP-8, all MMP and TIMP transcripts were detected in both gonadal and subcutaneous fat depots. In obese mice, the expression of MMP-3, -11, -12, -13, and -14 and TIMP-1 mRNAs was upregulated, whereas that of MMP-7, -9, -16, and -24 and TIMP-4 was downregulated. Additionally, *in vitro* conversion of adipogenic 3T3-F442A cells into mature adipocytes was associated with substantial modulations of MMP and TIMP expression. This *in vitro* adipogenesis was reduced in the presence of the synthetic MMP inhibitor CT1746. Later that year, Croissandeau et al confirmed the differential expression of MMP-2 and -9 during adipogenesis and reported that treatment of 3T3-L1 preadipocytes with the broad-spectrum MMP inhibitor Ilomastat or the more restricted MMP-2 Inhibitor I prevented their differentiation into adipocytes in a dose-dependent manner (182). In 2003, Chavey et al performed experiments similar to those of Maquoi et al in two genetic models of obesity (ob/ob and db/db mice) and in a diet-induced model of obesity (AKR mice). They found that mRNA levels for MMP-2, MMP-3, MMP-12, MMP-14, MMP-19, and TIMP-1 were strongly induced in obese adipose tissues compared with lean tissues, whereas MMP-7 and TIMP-3 mRNAs were

markedly decreased in obesity (183). They also reported that the synthetic MMP inhibitor Batimastat decreases adipose conversion of 3T3-L1 and primary rat preadipocytes, confirming previous observations.

In the years that followed, efforts were made to narrow down the list of crucial MMPs involved in adipose tissue development by subjecting different MMP- and TIMP-deficient mice to a high fat diet (HFD) and evaluating their fat mass development. This revealed enhanced adipose tissue growth in mice lacking MMP-3 (124), MMP-11 (136) or MMP-19 (150). These mice had higher body weight, increased adiposity and adipocyte hypertrophy compared with control littermates. In contrast, MMP-10 deficient mice did not differ markedly from their wild-type counterparts (184). TIMP-1 deficient mice on a HFD gained less weight as compared to controls and developed less adipose tissue (158). Interestingly, long-term systemic overexpression of TIMP-1 by adenoviral gene transfer did not significantly affect body weight or fat mass in mice kept on HFD, even though overall MMP activity was reduced in both plasma and adipose tissue (185).

Taken together, these data indicate that adipose tissue development is associated with profound changes in the MMP/TIMP balance, supporting their important role during this process. MMP-3, MMP-11 and MMP-19 appear to be involved in a negative regulatory process that controls homeostasis *in vivo*, whereas MMP-10 seems less important.

3.8 Gelatinases

The gelatinase subfamily of MMPs consists of gelatinase A (MMP-2, 72 kDa gelatinase) and gelatinase B (MMP-9, 92 kDa gelatinase). They are distinguished from other MMPs by three head-to-tail repeats inserted within their catalytic domain that resemble the type II repeats of the gelatin-binding region of fibronectin. These repeats are encoded by three extra exons and enable the gelatinases to bind and degrade denatured collagens, making them the main enzymes able to degrade type IV collagen and gelatin, the product of collagen degradation after lysis by collagenases (79). As type IV collagen is an integral part of basement membranes and disruption of the basement membrane is a key

step in cell proliferation, gelatinases are believed to play a crucial role in tissue expansion and re-organization.

Physiologically only a few cell types express MMP-9 including trophoblasts, osteoclasts, leucocytes, dendritic cells and adipocytes, and, in that respect, MMP-9 differs from MMP-2, which is expressed by a wide variety of cell types in normal conditions (75).

MMP-2 and MMP-9 share similar substrate specificities (Table 2), yet with subtle differences. For example, MMP-9 is a much less effective gelatinase than MMP-2, and their gelatin breakdown patterns are different (186). Moreover, the gelatinases differ in regulation mechanisms, expression profiles, localization patterns, … As a result, the gelatinases, although strongly related, exert different physiological functions.

Gelatinases are of particular interest in obesity research as they are secreted by (pre)adipocytes and are modulated during *in vitro* adipocyte differentiation.

3.9 Genes of gelatinases

The human *MMP-2* and *MMP-9* genes are located on chromosome $16q^{13}$ and near chromosome $20q^{11.2}$-$q^{13.1}$, respectively. Their structures have been completely determined and resemble each other (187, 188): both genes consist of 13 exons and 12 introns and the corresponding introns have identical locations with respect to the exons. However, the *MMP-9* gene is only about 7.7 kb while the gene for MMP-2 is about 27 kb in size. Also, the *MMP-9* gene distinguishes itself from other *MMP* genes by the presence of a 48-residue sequence in exon 9.

Despite having closely related gene structures, MMP-2 and MMP-9 differ quite distinctly in the regulation of their gene expression. Sequencing of the promoter regions revealed several differences that have impact on their regulation. For example the MMP-9 promoter contains a TATA like region at positions -29 to -25 and a 12-O-tetradecanoyl-phorbol-13-acetate (TPA) response element-like sequence at positions -79 to -73, which is similar to the promoter regions of other MMPs such as collagenases and stromelysins. In contrast, the *MMP-2* gene promoter has no TATA motif-like sequence, lacks the TPA response element and shares characteristics of housekeeping gene promoters. This may explain why, unlike most other MMPs, MMP-2 activity shows little regulation at

transcriptional level, and most of the regulation occurs at the post-transcriptional level and during enzyme activation and inactivation (88, 189, 190).

Genetic polymorphisms in the promoters of both MMP-2 and MMP-9 that influence gene transcription and expression have been described, and may be implicated in susceptibility to various diseases. For example, the -1562 C>T polymorphism in the MMP-9 promoter leads to differential transcription and is associated with increased susceptibility to neoplastic and vascular diseases.

Since these functionally related *MMP* genes are located on different chromosomes and differ greatly in composition of their regulatory regions, it is likely that they diverged from a common ancestor and evolved in parallel during different courses of selection throughout evolution. This resulted in gene products that share a high homology but differ in respect to their expression profile, substrate specificity and biological function.

3.10 MT1-MMP/TIMP-2 mediated MMP-2 activation

Unlike most other MMPs, MMP-2 is constitutively expressed and has little regulation at transcriptional level under normal conditions. In addition, proMMP-2 is refractory to activation by serine proteinases. Instead, MMP-2 activity is controlled through 1) some degree of post-transcriptional mRNA stabilization, 2) protein inactivation and clearance, and 3) a unique multistep mechanism of enzyme activation in which MMP-2, TIMP-2 and a MT-MMP cooperate closely (191, 192). Three MT-MMPs (MT1-MMP, MT2-MMP and MT3-MMP) have been shown to activate proMMP-2, although detailed activation studies were conducted primarily with MT1-MMP. In the proposed model, proMMP-2 is tethered by TIMP-2 via its non-inhibitory C-terminal end. TIMP-2 then binds to the active site of MT1-MMP using its inhibitory N-terminal end, acting as a bridge, and thereby localizing the complexed proMMP-2 to the cell surface. The prodomain of the recruited proMMP-2 is thus presented to an adjacent TIMP-2–free (uninhibited) MT1-MMP. The two MT1-MMP proteins interact with each other through their hemopexin domain, forming a tetrameric proMMP-2 activation complex. This leads to the cleavage of the MMP-2 propeptide between Asn^{37} and Leu^{38}, generating an activated 64 kDa

intermediate that is further processed to the fully activated 62 kDa form by an intermolecular autocleavage via a membrane-bound MMP-2 (Figure 6). This MT1-MMP/TIMP-2 mediated MMP-2 activation is only possible if the TIMP-2 concentration is low, with sufficient TIMP-2 to generate the trimolecular complex but not enough to saturate all the MT1-MMPs required for proteolysis of the propeptide. TIMP-2 can therefore be regarded as a dual regulator of ECM remodeling: at low concentrations, TIMP-2 will promote MMP-2 activation; at high levels it will bind to and inhibit all MT1-MMP activity, preventing proMMP-2 activation.

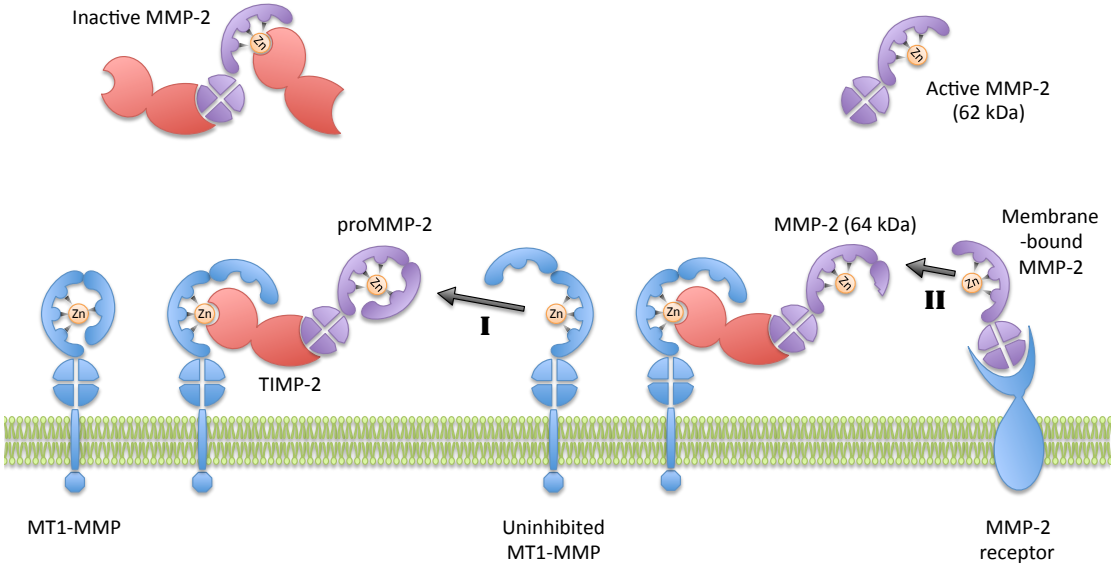


Figure 6. Cell surface activation of proMMP-2. TIMP-2 binds MT1-MMP via its inhibiting N-terminal domain and proMMP-2 via its non-inhibiting C-terminal domain. proMMP-2 is first cleaved by an adjacent uninhibited MT1-MMP molecule (I) followed by a second cleavage by a membrane-bound active MMP-2 (II) to produce a fully activated MMP-2 (62 kDa). Active MMP-2 can be inhibited by TIMP-2 via its N-terminal domain. Based on (75).

Gelatinase	Matrix substrates	Bioactive substrates		
	Degradation	Degradation/inactivation	activation	Release/shedding
MMP-2	Denatured collagens (gelatins), collagens I, IV, V, VII, X, XI, fibronectin, laminin, aggrecan, elastin, large tenascin C, vitronectin, decorin, neurocan, brevican	Ym1, S100A8, S100A9 (193) SDF-1 (194) Fractalkine (195) Osteopontin (195) galectin-1 (195) galectin-3 (196) CCL11 (197) IGFB (198)	IL-1ß (199, 200) TGF-ß (107) TNF- α (201) Endothelin-1 (202) Pro-MMP-1 Pro-MMP-9 (203) Pro-MMP-13 (204)	MCP-3 (205) FGFR-1 (206) FGF-2 (207)
MMP-9	Denatured collagens (gelatins), collagens I, IV, V, VII, X, XI, XIV, aggrecan, fibronectin, laminin, elastin, entactin, vitronectin	SDF-1 (194) CTAP-III (208) GRO- α (208) PF-4 (208) Ym1 (193) galectin-3 (196) plasminogen (209) α1-proteinase inhibitor (210) IL-2Ra (211)	IL-1ß (199, 200) TGF-ß (107) IL-8 (208) TNF- α (201) Pro-MMP-2 Pro-MMP-13	CD18 from macrophages (212) CCL11, CCL7, CCL17 (213) VEGF (214)

Table 2. Targets of the gelatinases. MCP-3 (CCL7): Chemo-attractant for monocytes and regulator of macrophage function; SDF-1 (CXCL12): Stromal derived Factor -1. MMP cleavage of SDF-1alpha results in loss of binding to its cognate receptor CXCR-4. This is reflected in a loss of chemoattractant activity for CD34(+) hematopoietic progenitor stem cells and lymphocytes; Fractalkine (CX3CL1, neurotactin): The soluble form potently chemoattracts T cells and monocytes, while the cell-bound chemokine promotes adhesion of leukocytes to activated endothelial cells.

AIMS

Dramatic tissue remodeling occurs within the expanding adipose tissue. However, whereas mechanisms leading to adipocyte hypertrophy and hyperplasia have been extensively studied, data concerning the regulation of the extracellular matrix (ECM) remodeling during fat mass increment are still scarce. In these studies we concentrated on two related, yet distinct, members of the MMP family: MMP-2 and MMP-9. Both are know to be produced and secreted by adipocytes and are modulated during adipogenesis.

Our main objectives were:

- To evaluate the effect of either MMP-2 or MMP-9 deficiency on adipose tissue formation in a murine model of obesity.
- To evaluate whether modulation of gelatinase activity has the potential to affect adipose tissue development *in vivo*
- To asses the effects of gelatinase inhibition and knock-down on adipogenesis

MATERIALS AND METHODS

1. Animal models

1.1 Mouse strains

MMP-2 deficient (MMP-2$^{-/-}$, genetic background C57Bl/6J) and MMP-9 deficient (MMP-9$^{-/-}$, genetic background 129sv:CDI or C57Bl/6J) mice and corresponding wild-type littermates (MMP-2$^{+/+}$ and MMP-9$^{+/+}$) were obtained from heterozygous breeding pairs as described elsewhere (116, 129). Genotyping was performed using the following primers: for MMP-2$^{+/+}$ mice: 5'-CAACGATGGAGGCACGAGTG-3' and 5'-GCCGGGGAACTTGATGATGG-3'; for MMP-2$^{-/-}$ mice: 5'-TGCAAAGCGCATGCTCCAGA-3' and 5'-TGTATGTGATCTGGTTCTTG-3'; for MMP-9$^{+/+}$ mice: 5'-GCATACTTGTACCGCTATGG-3' and 5'-TAACCGGAGGTGCAAACTGG-3'; and for MMP-9$^{-/-}$ mice: 5'-GCATACTTGTACCGCTATGG-3' and 5'-GACCACCAAGCGAAACAT-3'. After a denaturation step at 95°C for 5 min, isolated DNA was subjected to polymerase chain reaction (PCR) consisting of denaturation at 94°C for 1 min, followed by 1 min of annealing at 62°C and 1 min of elongation at 72°C for 35 cycles.

For experiments with the gelatinase inhibitor, C57Bl/6N mice were bred in the KULeuven animal facilities. Genetically obese ob/ob mice (genetic background 100% C57Bl/6) and athymic Balb/c NUDE mice were purchased from Charles River Laboratories (Les Oncins, France).

All animal experiments were approved by the local ethical committee (KULeuven, P06022 and P03112) and were performed in accordance with the guiding principles of the American Physiological Society and the International Society on Thrombosis and Haemostasis (215).

1.2 Murine models

Four different *in vivo* murine models were used in this thesis (Figure 7).

For all models, weight and food intake were measured at weekly intervals during the experimental period. Feeding efficiency was calculated as weight gain normalized to energy intake (in mg/kJ). Body temperature was measured using a rectal probe (TR-100, Fine Science Tools, Foster City, CA). To evaluate energy expenditure, spontaneous physical activity was measured at the end of the experiment during 3 consecutive days after a one-day acclimatization period

(except model 4). For this purpose, mice were placed in individual cages equipped with a turning wheel linked to a computer to record full turns at night (19:00-07:00). Data were averaged per mouse and are given as means ± SEM for the number of animals studied. At the end of the experimental period and after overnight fasting, mice were anesthetized by intraperitoneal injection of 60 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL). Blood was collected via the retro-orbital sinus on trisodium citrate (final concentration of 0.01 M) and plasma was stored at -80°C. Intra-abdominal (gonadal, GON) and inguinal subcutaneous (SC) fat pads were removed and weighed; portions were snap-frozen in liquid nitrogen for histological and zymographic analysis, RNA or protein extraction. Other organs including kidneys, lungs, spleen, pancreas, liver and heart were also weighed.

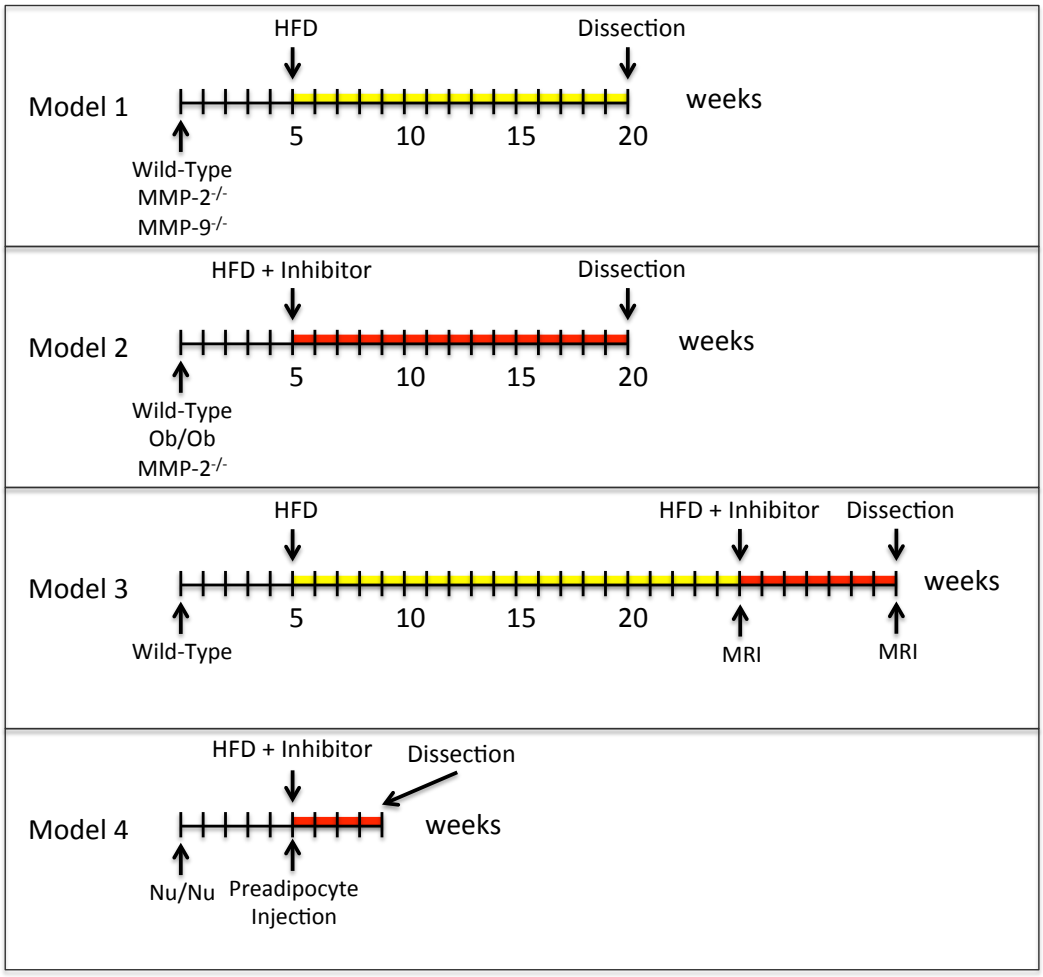


Figure 7. *In vivo* murine models to study adipose tissue development. HFD: high fat diet; MRI, Magnetic Resonance Imaging

1.2.1 Nutritionally induced obesity in gelatinase deficient mice (model 1)

Five weeks old male mice (wild-type, MMP-9$^{-/-}$ or MMP-2$^{-/-}$) were kept in microisolation cages on a 12h day/night cycle and fed at libitum with a high fat diet (HFD, Harlan Teklad TD 88137, Zeist, The Netherlands; 42% kcal as fat, 20.1 kJ/g) for 15 weeks.

For comparison, mice of each genotype were kept on standard fat diet (SFD; KM-04-k12, Muracon, Carfil, Oud-Turnhout, Belgium; 13% kcal as fat, 10.9 kJ/g).

1.2.2 Nutritionally induced obesity in mice treated with a gelatinase inhibitor (model 2)

Five weeks old male wild-type mice (C57Bl/6 genetic background) were fed the HFD for 15 weeks with (n = 10) or without (n = 10) addition of 100 mg/kg per day of Tolylsam ((R)-3-methyl-2-[4-(3-p-tolyl-[1,2,4]oxadiazol-5-yl)benzene sulfonylamino]-butyric acid, molecular weight = 412.5, Figure 8), an inhibitor of MMP-2, -9 and -12 (Table 3) (a kind gift of Shionogi & Co, Ltd, Osaka, Japan)(216).

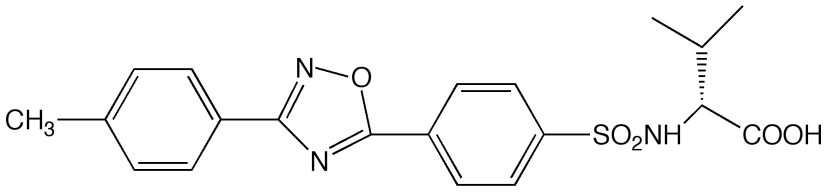


Figure 8. Chemical structure of Tolylsam.

MMP	1	**2**	3	8	**9**	**12**	13
Tolylsam	>10	**0.005**	5.558	0.397	**0.049**	**0.034**	0.201

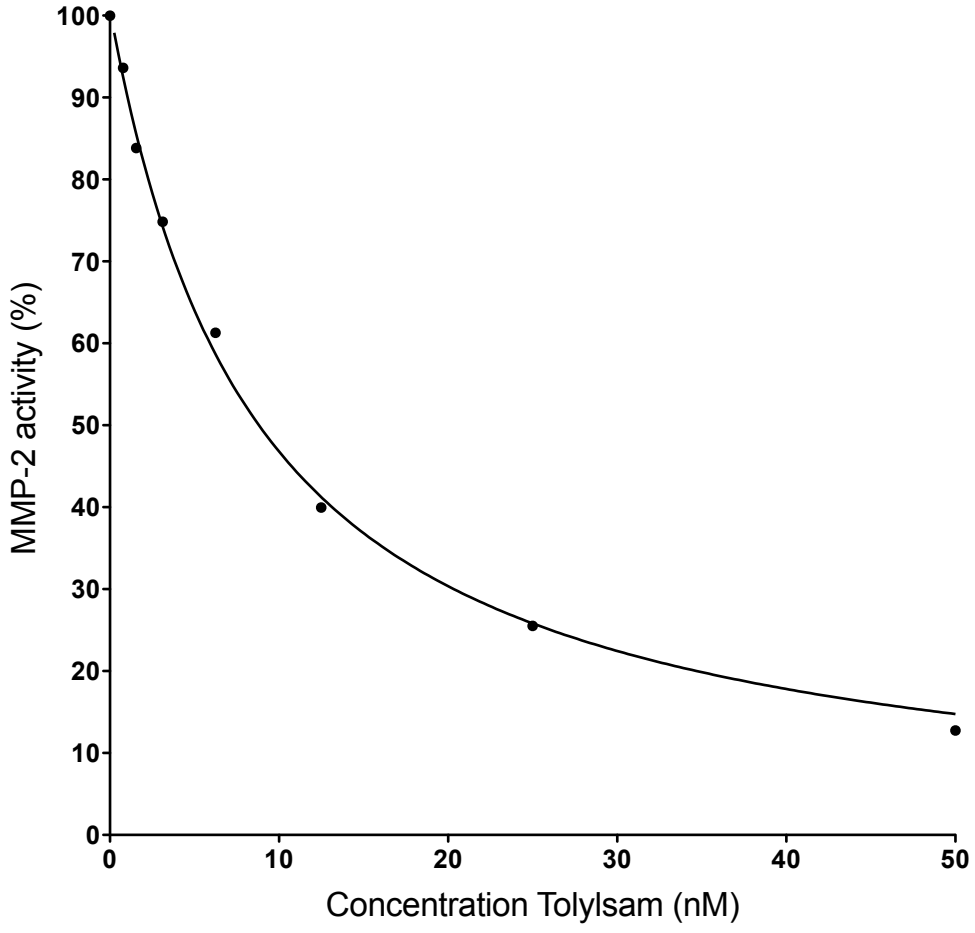
Table 3. IC50 values of Tolylsam for human MMPs (μM)

In vitro inhibitory potential of Tolylsam was confirmed by measuring the Ki value against murine MMP-2. Briefly, recombinant murine MMP-2 (Abcam, Cambridge UK; final concentration 0.3 nM) was preincubated with Tolylsam (final concentration: 0-50 nM) for 5 min to construct a calibration curve. MMP-2 fluorogenic substrate M-2350 (Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-

NH_2; Bachem, Bubendorf, Switzerland) was then added to a final concentration of 10 μM, and initial rates of substrate hydrolysis were determined by monitoring the increase in fluorescence (λ_{ex} = 328 nm, λ_{em} = 393 nm). The K_i value, computed form non-linear regression analysis using the Morrison equation (Eq. 1) for tight binding inhibitors (217), and was found to be ~ 8.5 nM. All measurements were performed at 37°C in TNCB buffer (50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05 % w/v Brij-35, pH 7.5).

$$v_i = v_0 \cdot \left[1 - \frac{([E] + [I] + K_i) - \sqrt{([E] + [I] + K_i)^2 - 4 \cdot [E] \cdot [I]}}{2 \cdot [E]}\right]$$

Equation 1: Morrison equation where [E] and [I] are the enzyme and Tolylsam concentrations, respectively.



In addition, Tolylsam completely blocked gelatinase activity in adipose tissue extracts on zymography, when added to the developing buffer.

1.2.3 Treatment with a gelatinase inhibitor of mice with established obesity (model 3)

Five weeks old male wild-type mice (C57Bl/6 genetic background) were kept in microisolation cages on a 12h day/night cycle and fed at libitum with HFD for 20 weeks. Then, 5 mice were given the HFD supplemented with 100 mg/kg per day of Tolylsam for an additional period of 7 weeks; 5 control mice were continued on HFD alone. Weight and food intake of the mice were measured at weekly intervals. Body, abdomen and fat pad volumes were measured at the start (i.e. week 25) and the end (i.e. week 32) of the experiments using non-invasive Magnetic Resonance Imaging (MRI) (collaboration with MoSAIC, KUleuven). Therefore, mice were anesthetized using isofluorane (2.5% for induction and 1.5% for maintenance during scanning) in O_2 and positioned on an animal bed. Temperature and respiration were monitored and maintained at 37°C and > 60/min, respectively. Fat-suppressed and non-suppressed images were acquired using a Bruker Biospin 9.4 Biospec Tesla small animal scanner (Bruker Biospin, Ettlingen, Germany) equipped with an actively shielded gradient set of 600 mT/m using a respiration triggered spin echo sequence (RARE) with 55 continuous slices of 0.6 mm thickness in interlaced mode (other parameters were: repetition time (TR) = 6000 ms, echo time (TE) = 15.9 ms, field of view (FOV) = 5.0 × 10.0 cm, a matrix of 256 × 400, two dummy scans and two averages). The fat-suppressed scan was repeated with the same receiver gain setting as for the non-suppressed dataset (fat suppression bandwidth = 1400 Hz). Both datasets were imported into in-house developed software for further processing. For radiofrequency (RF) irradiation and detection a 7.2 cm quadrature resonator (Bruker Biospin, Ettlingen) was used. The segmentation algorithm will automatically segment the body and fatty tissue, allowing calculation of the fat percentage per body volume. Another algorithm segments the abdominal (ABD) and SC fat depots by delineation of the visceral cavity, allowing automatic calculation of the SC (indicated in the images with a red color) and ABD fat (indicated in blue) volumes.

1.2.4 de novo adipogenesis (model 4)

To induce *de novo* fat pad formation (218-220), $10x10^6$ 3T3-F442A preadipocytes, grown to near confluency and resuspended in 200 µl phosphate buffered saline (PBS), were injected subcutaneously in the back of 6 week old male athymic Balb/c NUDE mice. Mice were kept in microisolation cages on a 12h day/night cycle and fed a HFD for 4 weeks supplemented with or without 100 mg/kg per day of Tolylsam. At the end of the experiment, *de novo* formed fat pads were removed and weighed; paraffin sections (10 µm) were prepared for histology.

1.3 Analysis

1.3.1 Histological analysis

Fat tissues were fixated overnight in Shandon Zinc Formal-Fixx (Anatomical Pathology International, Chester, UK) for 20 h, washed 3 times (10 min each) in PBS, transferred to 70% ethanol at 4°C and paraffin-embedded; 10-µm sections were processed onto Superfrost plus slides (Menzel-Glaser, Braunschweig, Germany). The mean adipocyte size was determined by computer-assisted image analysis (KS300, Zeiss, Jena, Germany) of SC and GON fat sections stained with hematoxylin and eosin under standard conditions. The adipocyte density was calculated by dividing the number of adipocytes by the total area of the analyzed section (ten areas divided over four sections were analyzed per animal and the data were subsequently averaged). The vasculature of the SC and GON fat was visualized using biotinylated *Bandeiraea (Griffonia) simplicifolia* BSI lectin (Sigma-Aldrich, Bornem, Belgium) (221), followed by signal amplification with the Tyramide Signal Amplification Cyanine system (Perkin Elmer, Boston, MA). The average blood vessel size was calculated by dividing the total stained area by the number of vessels and blood vessel density is expressed as the number of vessels per measured surface area (9-12 sections were analyzed per animal and results were then averaged). Blood vessel density was normalized to the adipocyte number (222).

Macrophage infiltration in adipose tissues was quantified after Mac3 staining, and expressed as percent of section area. Collagen was stained with Sirius red and quantified as percentage stained area per total tissue section area. Quality of

collagen fibers was estimated by Sirius red polarization microscopy, allowing to quantify thick, tightly packed collagen fibers (orange-red) and thin, loosely assembled fibers (yellow-green) (223).

1.3.2 Gelatinase determination

Extracts were prepared by lysing adipose tissues in 10 mM Phosphate buffer, pH 7.2, containing 150 mM NaCl, 1% Triton X-100, 0.5 % Na deoxycholate and 0.2 % Na azide. Total protein concentration was determined using the bicinchoninic acid method (BCA™ Protein Assay Kit assay, Pierce, Rockford, IL). Samples were analyzed by zymography on gelatin-containing gels as described (224, 225). 20μg of protein was applied to non-reduced SDS-polyacrylamide gel electrophoresis using 10% gels containing 0.1% gelatin. Gels were renaturated by exchanging SDS with Triton X-100 (2.5%) and then incubated for 24 hours at 37°C in developing buffer (50 mM Tris-HCl, pH 7.5, containing 7 mM $CaCl_2$, 0.2 M NaCl, and 0.02% Brij-35). Gels were subsequently stained with Coomassie (0.5% Coomassie R250, 30% MeOH, 10% acetic acid) for 3 hours, followed by destaining (50% MeOH and 10% acetic acid). To activate proMMPs, protein extracts were incubated with 0.5 mM p-aminophenylmercuric acid (APMA) for 16 hours at 37°C prior to loading on gels. Gels were scanned (Epson Perfection V700 PHOTO scanner, Nagano, Japan) and bands quantified with NIH imageJ 1.30 software (226).

Alternatively, 20μg total protein was diluted in assay buffer (50 mM Tris-HCl, pH 7.4, containing 0.1 % Brij-35, 10 mM $CaCl_2$ and 10 μM $ZnCl_2$) containing 5 μM quenched fluorescently labeled substrate (OmniMMP Fluorogenic substrate, Biomol, Plymouth Meeting, PA) (227). For background correction, equivalent samples were measured in the presence of EDTA (50 mM Tris-HCl, pH 7.4, containing 0.1 % Brij-35, 50 mM EDTA). MMP activity was monitored for 12 hours with a Spectra Gemini fluorometer (Molecular Devices Corp., Sunnyvale, CA) and analyzed with SOFTmax pro 4.0 (Molecular Devices Corp., Sunnyvale, CA).

1.3.3 Analysis of mRNA expression

Adipose tissues were homogenized using lysing matrix tubes (Qbiogene, Carlsbad, CA) in a Hybaid ribolyser (Thermo, Wallham, MA). Isolation of total DNA-free RNA was performed using the RNeasy Mini kit from Qiagen (Basel, Switzerland). In short, the lysates were transferred in a QIAshredder for homogenisation. An equal volume of 70% ethanol was added to the samples to allow binding of the RNA on the membrane of the RNeasy column. To avoid contamination of genomic DNA, a DNAse treatment was performed. RNAse-free water was used to elute the RNA. RNA concentrations were determined with the RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR) or using a nanodrop spectrometer (ThermoFisher Scientific, Waltham, MA). Samples were aliquoted and stored at -80°C.

Reverse Transcription reactions was performed to amplify the isolated RNA. This was done from 50 ng RNA of each sample at 48°C during 60 min with MultiscribeTM Reverse Transcriptase (Applied Biosystems by life technologies, Carlsbad, CA).

Expression of mRNA of target genes and an endogenous control gene (GAPDH or ß-actin) was measured by quantitative real-time PCR using primers and 6-carboxy-fluorescein (FAM) labeled probes or gene assays (Applied Biosystems, Table 4) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Transcript levels were determined in duplicate and normalized to the housekeeping gene (GAPDH or ß-actin). Data were obtained as cycle threshold (Ct) values, and expressed as copy number of target mRNA relative to 10^5 copies of GAPDH or ß-actin.

gene	Expression assay product number
GAPDH	Mm99999915_g1
ß-actin	Mm00607939_s1
MMP-9	Mm01240562_g1
MMP-2	Mm00439506_m1

Table 4. Expression assay product numbers of target genes and endogenous control genes (Applied Biosystems).

1.3.4 Metabolic parameters

Blood glucose concentrations were measured using Glucocard strips (Menarini Diagnostics, Firenze, Italy). Levels of triglycerides, total cholesterol, HDL cholesterol and LDL cholesterol in plasma were determined using standard laboratory assays. Insulin levels were measured with a specific ELISA (Mercodia, Uppsala, Sweden). Adiponectin, leptin, and resistin levels were determined using commercially available ELISA's (R&D Systems, Wiesbaden, Germany).

Triglycerides in faeces were measured with the Triglycerides FS kit (DiaSys Diagnostic Systems GmbH & Co. KG, Holzheim, Germany).

2. Cell culture experiments

2.1 *in vitro* differentiation

Murine 3T3-F442A preadipocytes were grown in basal medium (Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% bovine calf serum iron supplemented (BCS; Hyclone Laboratories Inc, Logan, UT) and 1% PenStrep (Invitrogen; 10,000 units/ml penicillin and 10 mg/ml streptomycin)) in an atmosphere of 95% humified air-5% CO_2 at 37°C. Cells were passaged when preconfluent. To induce differentiation, cells were seeded at a density of 10×10^3 cells cm^{-2} and grown to confluency (referred to as 'day 0'). After two days, differentiation was induced by replacing basal medium with induction medium (DMEM supplemented with 10% BCS, 17 nM insulin, 2 nM triiodothyronine (T3), 100 nM dexamethasone (DEX) and 100 µM methylisobutylxanthine (IBMX)) for 48 hours, after which, cultures were maintained in differentiation medium (DMEM with 10% BCS, 17 nM insulin and 2 nM T3). Alternatively, post-confluent cells were incubated in medium containing varying concentrations of synthetic MMP-inhibitor (Tolylsam), diluted in dimethyl sulfoxide (DMSO), or containing only DMSO (final concentration 1/1000). Medium, RNA and protein were collected at different time points during the experiment.

2.2 *In vitro* gene silencing

To obtain long term, stable gene silencing of MMP-2 in 3T3-F442A preadipocytes, the 'MISSION shRNA lentiviral transduction particles' system

(Sigma-Aldrich, St. Louis, MO) was used. MISSION shRNA lentiviral particles are transduction-ready viral particles for gene silencing in mammalian cells. For MMP-2 (NM_008610), five different clones were provided (TRCN0000031224-31225-31226-31227-31228) (Table 5), cloned into the pLKO.1 vector. MISSION non-target shRNA control transduction particles (SHC002V) cloned into the same vector were used as negative control. Transduction was further enhanced with the 'ExpressMag®' transduction system (Sigma-Aldrich). Briefly, for each lentiviral construct, triplicates of 60 x 10^3 3T3-F442A preadipocytes were seeded in a 6-well plate in basal medium. The next day, a mixture of 10 µl lentiviral particles and 30 µl ExpressMag® beads were added to the cells in fresh basal medium. After another day, the medium was replaced by fresh basal medium containing a lethal dose of 2 µg/ml puromycin. Medium with puromycin was refreshed every two days until puromycin-resistant colonies of 3T3-F442A cells appeared. The puromycin-resistant clones were further expanded and assayed for knock-down of the MMP-2 target gene. Cells transduced with the negative control (SHC002V) were subjected to the same selection procedure. Preadipocytes with or without MMP-2 gene silencing were differentiated as described above.

TRC Number	**Sequence**
TRCN0000031224	CCGG GC TGA AAG ATA CCC TCA AGA ACT CGA GTT CTT GAG GGT ATC TTT CAG CTT TTTG
TRCN0000031225	CCGG CC GGG ATA AGA AAT ATG GAT TCT CGA GAA TCC ATA TTT CTT ATC CCG GTT TTTG
TRCN0000031226	CCGG GCT GTG TTC TTC GCA GGG AAT CTC GAG ATT CCC TGC GAA GAA CAC AGC TTT TTG
TRCN0000031227	CCGG GCA ATA CCT GAA TAC TTT CTA CTC GAG TAG AAA GTA TTC AGG TAT TGC TTT TTG
TRCN0000031228	CCGG CGG TGG TCAT AGCT ACT TCT TCT CGAG AAG AAG TAG CTA TGA CCA CCG TTT TTG

Table 5. Sequence of the shRNA hairpins used for stable knock-down of MMP-2. Target sequences are underlined.

2.3 Analysis

2.3.1 Assessment of cell proliferation and viability

Cell proliferation was measured on preconfluent cells using the WST-1 colorimetric assay (Roche), according to the manufacturer's information.

Cellular viability was assessed by Trypan Blue dye exclusion assay. At experimental day 12, cell were trypsinized and stained with 0.4% Trypan blue. The number of blue cells was counted and expressed as percentages.

2.3.2 Differentiation assay

At experimental day 12, the extent of differentiation was assessed by quantification of Oil Red O uptake by lipid-containing cells (69). Therefore, cells were washed with PBS, fixed in 1.5% glutaraldehyde in PBS for 5 min, stained for 3 hours with a 0.2% Oil Red O solution (Sigma-Aldrich), washed and maintained in tissue culture water for microscopic visualization. Then, cells were dried overnight and the Oil Red O dye was extracted by adding isopropanol for 5 min. Total lipid accumulation was quantified by measuring absorbance of the solution at 510 nm on an EL808 plate reader, using KC4 data analysis software (Bio-tek Instruments Inc, VT, USA).

2.3.3 Gelatinase determination

Conditioned medium was collected and frozen at -80°C. Cell extracts were prepared by washing the cells with PBS followed by trypsinisation for 5 min at 37°C. Cells were collected, centrifuged and washed with PBS. After centrifugation, the pellet was dissolved in extraction buffer (10 mM Phosphate buffer, pH 7.2, containing 150 mM NaCl, 1% Triton X-100, 0.5 % Na deoxycholate and 0.2 % Na azide) and kept on ice for 10 min. After a final centriguation step, the supernatans was collected and frozen at -80°C.

Zymography was performed with either protein extracts or conditioned medium, as described above.

2.3.4 Analysis of mRNA expression

Cell extracts were prepared by washing the cells with PBS followed by lysing, using RLT buffer from the RNAeasy kit (Qiagen, Basel, Switserland). Total DNA-

free RNA extraction, reverse transcription reactions and real-time PCR were performed as described above. mRNA levels of adipogenic markers and genes of interest (Table 6) were normalized to that of endogenous GAPDH or ß-actin mRNA.

gene	Primers/probe sets and expression assay product number for genes of interest
ß-actin	Mm01205647_g1
GAPDH	Mm99999915_g1
MMP-2	Mm00439506_m1
MMP-9	Mm00607939_s1
MMP-14	Mm01318969_g1
TIMP-1	Mm00441818_m1
TIMP-2	Mm00441825_m1
adiponectin	Mm00456425_m1
VEGF-A	Mm00437304_m1
Pref1	fw: aaccatggcagtgcatctg rev: agcattcgtactggcctttc probe: aaatagacgttcgggcttgcacctc
PPAR-γ	fw: ctgtcggtttcagaagtgcct rev: atctccgccaacagcttctc probe: cccaaacctgatggcattgtgagaca
aP2	fw: ccttcaaactgggcgtgg rev: cgttttctctttattgtggtcgact probe: atgctcttcaccttcctgtcgtctgcg

Table 6. Primers and probes or expression assay product numbers of target genes and endogenous control genes (Applied Biosystems).

3. Statistical analysis

GraphPad Prism 4.03 software was used to determine statistical differences and statistical significant correlations (GraphPad, La Jolla, CA). Data are represented as means ± SEM. Differences between experimental groups were analyzed with the non-parametric Mann-Whitney test. When comparing three groups, the non-parametric Kruskal-Wallis test was used. If a statistical difference was detected ($p < 0.05$), the difference between the individual groups was determined using

the Dunn´s multiple comparison test. Comparison of weight and weight gain progress curves was performed by two-way repeated measures ANOVA. Statistical significance was set at $p < 0.05$.

RESULTS

1. Effect of MMP-9 deficiency on adipose tissue development

Van Hul M, Piccard H, Lijnen HR. Gelatinase B (MMP-9) deficiency does not affect murine adipose tissue development. Thrombosis and haemostasis. 2010. 104:165-71.

1.1 Body weight and adipose tissue composition of mice kept on standard fat diet

At the age of five weeks, the MMP-$9^{-/-}$ mice (50% 129sv : 50% CDI) were slightly leaner than their wild-type counterparts (MMP-$9^{+/+}$) (Table 7, p = 0.2), but at the end of the 15-weeks SFD feeding period, this difference disappeared. Body length was 9.4 ± 0.1 cm for both groups at age 18 to 22 weeks. Food intake was comparable in both groups (4.5 ± 0.06 g/day for MMP-$9^{-/-}$ and 4.3 ± 0.10 g/day for MMP-$9^{+/+}$ mice, p = 0.2), but the feeding efficiency of MMP-$9^{-/-}$ mice (2.4 ± 0.2 mg/kJ) was higher than that of wild-type mice (1.8 ± 0.3 mg/kJ, p = 0.05).

All organs, including subcutaneous (SC) and gonadal (GON) fat depots, were of similar weight, except for the spleen, which was smaller in the MMP-$9^{-/-}$ mice (Table 7).

	MMP-$9^{+/+}$ (n = 7)	MMP-$9^{-/-}$ (n = 7)
Body weight start (g)	21 ± 1.2	19 ± 1.0
Body weight end (g)	34 ± 1.8	33 ± 0.7
SC fat (g)	0.4 ± 0.1	0.5 ± 0.1
GON fat (g)	0.6 ± 0.2	0.7 ± 0.1
Lungs (mg)	168± 5	167 ± 11
Spleen (mg)	127 ± 13	84 ± 7*
Heart (mg)	177 ± 5	170 ± 4
Pancreas (mg)	186 ± 15	216 ± 11
Liver (mg)	1153 ± 135	963 ± 55
Kidneys (mg)	452 ± 12	437 ± 21

Table 7. Effect of MMP-9 deficiency on adipose tissue and organ weights of mice (50% 129sv : 50% CDI) kept on SFD for 15 weeks. Data are means ± SEM of n experiments in each group. * p < 0.05 versus MMP-$9^{+/+}$ mice.

Histological analysis of adipose tissue sections did not reveal any differences in size and density of adipocytes or blood vessels between MMP-$9^{-/-}$ and MMP-$9^{+/+}$ mice (Table 8). Metabolic parameters, including plasma levels of glucose, triglycerides, insulin, leptin and cholesterol, were comparable for both genotypes (Table 9).

	MMP-$9^{+/+}$ (n = 7)	MMP-$9^{-/-}$ (n = 7)
Adipocyte size (μm^2)		
SC fat	1770 ± 299	1810 ± 162
GON fat	3680 ± 696	3300 ± 272
Adipocyte density ($x10^{-6}/\mu m^2$)		
SC fat	664 ± 92	597 ± 51
GON fat	329 ± 51	323 ± 27
Blood vessel size (μm^2)		
SC fat	54 ± 2.4	51 ± 2.3
GON fat	60 ± 2.3	60 ± 3.3
Blood vessel density ($x10^{-6}/\mu m^2$)		
SC fat	418 ± 52	370 ± 47
GON fat	234 ± 44	239 ± 24
Normalized blood vessel density		
SC fat	0.6 ± 0.1	0.6 ± 0.1
GON fat	0.7 ± 0.1	0.8 ± 0.1

Table 8. Effect of MMP-9 deficiency on adipocyte and blood vessel size and density in adipose tissues of mice (50% 129sv : 50% CDI) kept on SFD for 15 weeks. Data are means ± SEM of n experiments in each group. No significant differences were observed versus MMP-$9^{+/+}$ mice.

MMP-9 antigen levels in plasma of MMP-$9^{+/+}$ mice were 46 ± 7 ng/ml, and were undetectable in plasma of MMP-$9^{-/-}$ mice. MMP-2 antigen levels were comparable in plasma of MMP-$9^{+/+}$ and MMP-$9^{-/-}$ mice (140 ± 13 versus 153 ± 17 ng/ml, p = 0.6).

Likewise, MMP-9 antigen was not detected in either SC or GON adipose tissue extracts of MMP-$9^{-/-}$ mice. For MMP-$9^{+/+}$ mice, MMP-9 levels were 0.52 ± 0.19 ng/µg total protein for SC and 0.37 ± 0.06 ng/µg total protein for GON adipose tissue. MMP-2 antigen levels in adipose tissues were not different for MMP-$9^{+/+}$

and MMP-9$^{-/-}$ mice (for SC: 1.60 ± 0.10 versus 1.44 ± 0.12 ng/µg total protein, p = 0.3; for GON: 0.68 ± 0.07 versus 0.66 ± 0.06 ng/µg total protein, p = 1.0).

	MMP-9$^{+/+}$ (n = 7)	MMP-9$^{-/-}$ (n = 7)
Glucose (mg/dl)	99 ± 15	95 ± 10
Insulin (ng/ml)	0.7 ± 0.1	0.6 ± 0.1
Leptin (ng/ml)	12 ± 2.0	10 ± 2.5
Triglycerides (mg/dl)	21 ± 2	24 ± 2
Total cholesterol (mg/dl)	26 ± 3	27 ± 3
HDL cholesterol (mg/dl)	24 ± 2	25 ± 3
LDL cholesterol (mg/dl)	ND	ND

Table 9. Effect of MMP-9 deficiency on plasma metabolic parameters of mice (50% 129sv : 50% CDI) kept on SFD for 15 weeks. Data are means ± SEM of n experiments in each group. ND: not detected. No significant differences were observed versus MMP-9$^{+/+}$ mice.

2.2 Body weight and adipose tissue composition of mice kept on high fat diet

Five weeks old male MMP-9$^{-/-}$ mice (50% 129sv : 50% CDI) had lower body weight at the start (p = 0.2) but gained somewhat more weight on the HFD, resulting in comparable body weights as the MMP-9$^{+/+}$ littermates at the age of 20 weeks (Figure 9 and Table 10).

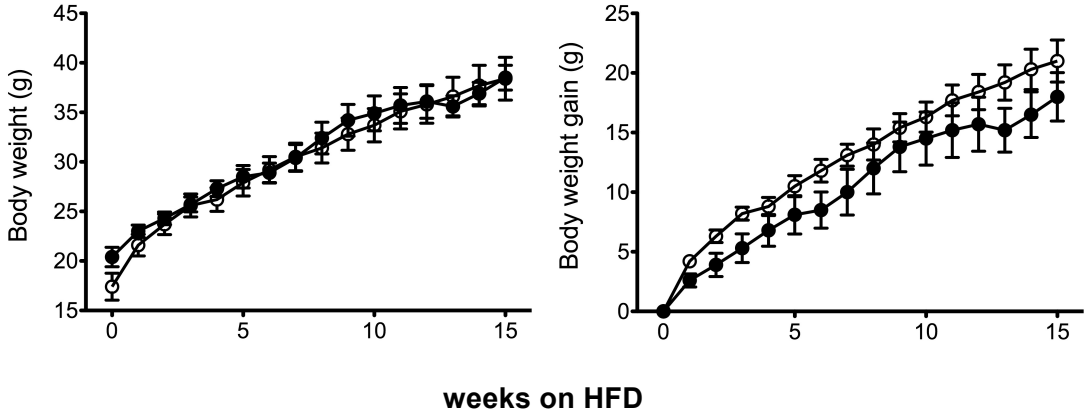


Figure 9. Body weight and weight gain of MMP-9$^{-/-}$ (open circles) and MMP-9$^{+/+}$ mice (black circles) kept on HFD for 15 weeks. Data are means ± SEM of 7 and 11 experiments for MMP-9$^{+/+}$ and MMP-9$^{-/-}$ mice respectively.

	MMP-9$^{+/+}$ (n = 7)	MMP-9$^{-/-}$ (n = 11)
Body weight start (g)	20 ± 1.0	17 ± 1.4
Body weight end (g)	39 ± 1.3	38 ± 2.2
SC fat (g)	1.1 ± 0.1	1.1 ± 0.2
GON fat (g)	1.4 ± 0.2	1.4 ± 0.2
Lungs (mg)	165 ± 6	149 ± 5
Spleen (mg)	142 ± 17	107 ± 3
Heart (mg)	164 ± 7	169 ± 3
Pancreas (mg)	244 ± 28	227 ± 14
Liver (mg)	1340 ± 160	1530 ± 215
Kidneys (mg)	428 ± 14	441 ± 20

Table 10. Effect of MMP-9 deficiency on adipose tissue and organ weights of mice (50% 129sv : 50% CDI) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. No significant differences were observed versus MMP-9$^{+/+}$ mice.

Food intake was comparable for MMP-9$^{-/-}$ and MMP-9$^{+/+}$ mice (3.2 ± 0.04 g/day and 3.3 ± 0.08 g/day) and feeding efficiency was somewhat higher for MMP-9$^{-/-}$ mice (3.3 ± 0.4 mg/kJ versus 2.7 ± 0.5 mg/kJ, p = 0.3).

The weight of isolated SC and GON adipose tissues and that of other organs did not differ between the two groups (Table 10), and histological analysis revealed comparable adipocyte size, adipocyte density, blood vessel size and blood vessel density (Table 11). Metabolic parameters were also comparable for both genotypes (Table 12).

	MMP-9$^{+/+}$ (n = 7)	MMP-9$^{-/-}$ (n = 11)
Adipocyte size (μm^2)		
SC fat	3590 ± 394	3730 ± 415
GON fat	4820 ± 442	5100 ± 435
Adipocyte density ($x10^{-6}/\mu m^2$)		
SC fat	308 ± 30	315 ± 40
GON fat	217 ± 19	214 ± 19
Blood vessel size (μm^2)		
SC fat	41 ± 1.7	47 ± 3.7
GON fat	46 ± 2.6	49 ± 3.5
Blood vessel density ($x10^{-6}/\mu m^2$)		
SC fat	297 ± 48	307 ± 44
GON fat	247 ± 59	198 ± 29
Normalized blood vessel density		
SC fat	0.9 ± 0.1	1.0 ± 0.1
GON fat	1.0 ± 0.2	1.0 ± 0.1

Table 11. Effect of MMP-9 deficiency on adipocyte and blood vessel size and density in adipose tissues of mice (50% 129sv : 50% CDI) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. No significant differences were observed versus MMP-9$^{+/+}$ mice.

	MMP-9$^{+/+}$ (n = 7)	MMP-9$^{-/-}$ (n = 11)
Glucose (mg/dl)	105 ± 15	111 ± 11
Insulin (ng/ml)	1.1 ± 0.1	1.2 ± 0.1
Leptin (ng/ml)	17 ± 3.3	18 ± 3.1
Triglycerides (mg/dl)	52 ± 8	40 ± 11
Total cholesterol (mg/dl)	119 ± 13	112 ± 34
HDL cholesterol (mg/dl)	109 ± 12	97 ± 26
LDL cholesterol (mg/dl)	15 ± 3	15 ± 8

Table 12. Effect of MMP-9 deficiency on plasma metabolic parameters of mice (50% 129sv : 50% CDI) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. ND: not detected. No significant differences were observed versus MMP-9$^{+/+}$ mice.

Because obesity phenotypes depend on the genetic background, we replicated our study on HFD in a pure C57Bl/6 background, which is believed to be a more obesity-prone strain (228). After 15 weeks on HFD, no differences were observed between the MMP-9$^{-/-}$ mice (n = 7) and the control littermates (n = 6) in terms of body weight, fat mass or size/density of adipocytes or blood vessels (Table 13). Again, metabolic parameters were not affected by MMP-9 deficiency (data not shown).

	MMP-9$^{+/+}$ (n = 6)	MMP-9$^{-/-}$ (n = 7)
Body weight start (g)	18 ± 0.4	17 ± 0.6
Body weight end (g)	34 ± 2.1	33 ± 1.8
SC fat (g)	0.7 ± 0.1	0.7 ± 0.1
GON fat (g)	1.4 ± 0.3	1.5 ± 0.2
Adipocyte size (μm^2)		
SC fat	2230 ± 213	2260 ± 221
GON fat	3860 ± 427	3540 ± 392
Adipocyte density ($x10^{-6}/\mu m^2$)		
SC fat	487 ± 43	479 ± 47
GON fat	277 ± 31	310 ± 46
Blood vessel size (μm^2)		
SC fat	42 ± 1.2	45 ± 2.6
GON fat	43 ± 3.7	42 ± 3.4
Blood vessel density ($x10^{-6}/\mu m^2$)		
SC fat	689 ± 57	553 ± 85
GON fat	438 ± 47	420 ± 44
Normalized blood vessel density		
SC fat	1.4 ± 0.1	1.3 ± 0.1
GON fat	1.6 ± 0.2	1.4 ± 0.2

Table 13. Effect of MMP-9 deficiency on adipose tissues of C57Bl/6 mice kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. No significant differences were observed versus the corresponding MMP-9$^{+/+}$ mice.

Gelatinase expression and activity on HFD (50% 129sv : 50% CDI background)

RT-PCR analysis confirmed the absence of MMP-9 mRNA in both SC and GON adipose tissues of MMP-9$^{-/-}$ mice, whereas it was detected in tissues of wild-type littermates. mRNA levels of MMP-2 were comparable for MMP-9$^{+/+}$ and MMP-9$^{-/-}$ mice (normalized to GAPDH: 719 ± 52 versus 714 ± 102 A.U. for SC, p = 0.96 and 395 ± 40 versus 451 ± 40 A.U. for GON, p = 0.34; normalized to ß-actin: 556 ± 90 versus 626 ± 67 A.U. for SC, p = 0.54 and 413 ± 35 versus 458 ± 37 A.U. for GON, p = 0.39).

MMP-2 antigen levels were not different between MMP-9$^{-/-}$ and MMP-9$^{+/+}$ adipose tissues (for SC: 1.92 ± 0.16 ng/µg protein versus 1.69 ± 0.11 ng/µg protein, p = 0.25; for GON: 0.58 ± 0.06 ng/µg protein versus 0.70 ± 0.07 ng/µg protein, p = 0.19). MMP-9 antigen was not detected in adipose tissue of MMP-9$^{-/-}$ mice and was present at levels of 0.60 ± 0.06 ng/µg protein in SC and 0.38 ± 0.07 ng/µg protein in GON fat of MMP-9$^{+/+}$ mice.

Zymography on gelatin-containing gels revealed four gelatinolytic bands in SC and GON adipose tissues of MMP-9$^{+/+}$ mice, corresponding to proMMP-2 (72 or 68 kDa), active MMP-2 (58 kDa) and proMMP-9 (92 kDa). Active MMP-9 could not be detected. Extracts of adipose tissues of MMP-9$^{-/-}$ mice displayed similar patterns, with the exception of proMMP-9, which was absent (Figure 10 and Table 14). Treatment with APMA resulted in higher levels of 58 kDa and the appearance of 62 kDa activation forms of MMP-2 in SC and GON fat of both genotypes and in concomitantly reduced 72 kDa and 68 kDa forms (Figure 10).

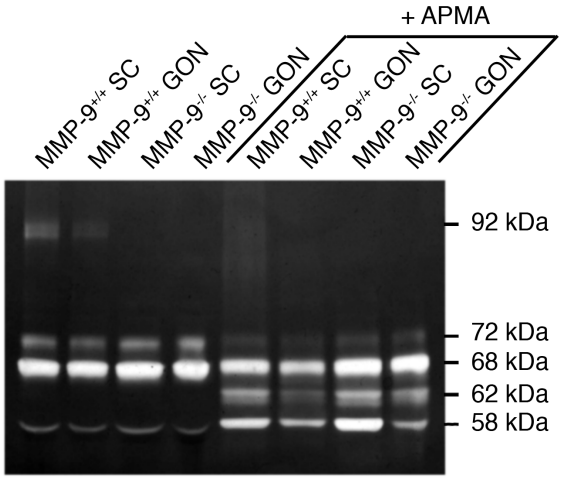


Figure 10. Zymographic analysis of SC and GON adipose tissues of MMP-9$^{-/-}$ and MMP-9$^{+/+}$ mice, before and after activation with APMA.

	MMP-9$^{+/+}$	MMP-9$^{-/-}$
SC adipose tissue		
92 kDa Pro-MMP-9	3.1 ± 0.8	ND
82 kDa Active MMP-9	ND	ND
72 kDa Pro-MMP-2	11 ± 2.6	11 ± 1.4
62 kDa Active MMP-2	ND	ND
68 kDa Pro-MMP-2	23 ± 6.3	22 ± 2.8
58 kDa Active MMP-2	3.8 ± 0.6	4.3 ± 0.4
GON adipose tissue		
92 kDa Pro-MMP-9	2.6 ± 0.1	ND
82 kDa Active MMP-9	ND	ND
72 kDa Pro-MMP-2	5.6 ± 0.8	7.4 ± 1.2
62 kDa Active MMP-2	ND	ND
68 kDa Pro-MMP-2	16 ± 1.9	17 ± 2.5
58 kDa Active MMP-2	4.1 ± 0.7	3.7 ± 0.7

Table 14. Gelatinolytic activity in adipose tissues of MMP-9$^{+/+}$ and MMP-9$^{-/-}$ mice (50% 129sv : 50% CDI) kept on HFD for 15 weeks. Data are means ± SEM and are expressed in arbitrary units. ND: not detected. No significant differences were observed versus the corresponding MMP-9$^{+/+}$ mice.

MMP-9 antigen levels in plasma of MMP-9$^{+/+}$ mice on HFD were 72 ± 9 ng/ml and were undetectable in plasma of MMP-9$^{-/-}$ mice. MMP-2 antigen levels were comparable in plasma of MMP-9$^{+/+}$ and MMP-9$^{-/-}$ mice (152 ± 16 versus 159 ± 11 ng/ml, p = 0.6). Thus, MMP-2 levels on HFD were similar to those on SFD for both genotypes, whereas MMP-9 levels in wild-type mice were higher on HFD than on SFD (p = 0.04).

2. Effect of MMP-2 deficiency on adipose tissue development

Van Hul M, Lijnen HR. A functional role of gelatinase A in the development of nutritionally induced obesity in mice. Journal of thrombosis and haemostasis. 2008. 6:1198-206.

2.1 Body weight and adipose tissue composition of mice kept on standard fat diet

At 5 weeks of age, the body weight and length of MMP-2$^{-/-}$ (KO) mice kept on SFD was lower than that of age and sex matched wild-type (WT) and heterozygous littermates (HE) (Figure 11, A-B).

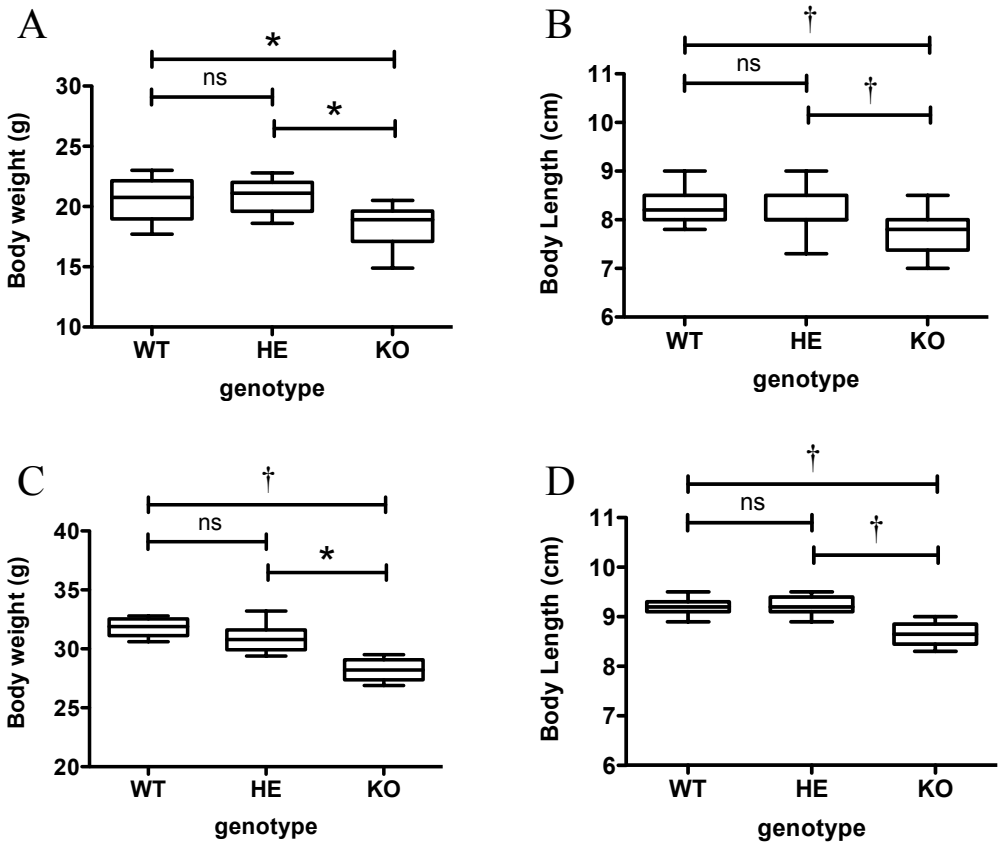


Figure 11. Body weight (A- B) and length (C-D) of MMP-2$^{+/+}$(WT), MMP-2$^{+/-}$ (HE) and MMP-2$^{-/-}$ (KO) mice at 5 and 20 weeks of age. Dunn's multiple comparison test revealed significantly lower body weight and length for MMP-2$^{-/-}$, but not MMP-2$^{+/-}$ mice, as compared to controls. * p <0.05, † p < 0.005.

At 20 weeks of age, the body weight and length of MMP-2$^{-/-}$ mice remained significantly lower than that of age and sex matched MMP-2$^{+/+}$ mice (Figure 11,

C-D), whereas food intake was comparable (4.6 ± 0.17 versus 4.5 ± 0.16 g/day, p = 0.88). Energy expenditure for MMP-$2^{+/+}$ and MMP-$2^{-/-}$ mice, as measured in cages with a computer-linked turning wheel, was comparable (11,890 ± 2600 versus 12,730 ± 1330 turning cycles/12h, p = 1.00). Also the rectal temperature was not different for MMP-$2^{+/+}$ or MMP-$2^{-/-}$ mice (38.6 ± 0.04 °C versus 38.7 ± 0.06 °C, p = 0.63).

2.2 Body weight and adipose tissue composition of mice kept on high fat diet

During HFD feeding, weight gain was significantly reduced for MMP-$2^{-/-}$ as compared to MMP-$2^{+/+}$ mice (Figure 12), resulting in significantly lower body weight at the end of the 15 weeks experimental period.

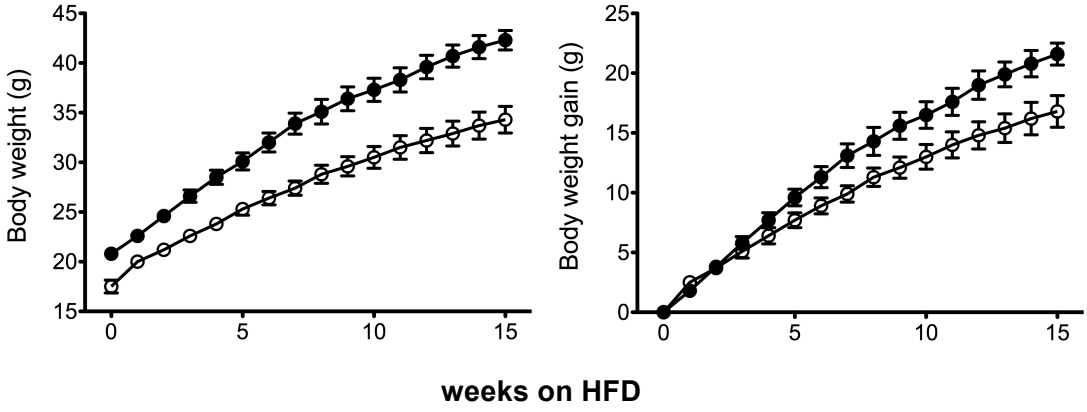


Figure 12. Body weight and weight gain of MMP-$2^{-/-}$ (white circles) and MMP-$2^{+/+}$ (black circles) mice kept on a high-fat diet (HFD) for 15 weeks. Data are means ± SEM. Analysis by two-way repeated-measures ANOVA revealed significantly ($p < 0.0001$) reduced body weight and weight gain for MMP-$2^{-/-}$ mice.

	MMP-$2^{+/+}$ (n = 10)	MMP-$2^{-/-}$ (n = 9)
Body weight start (g)	21 ± 0.3	18 ± 0.7‡
Body weight end (g)	42 ± 1.0	34 ± 1.3‡
SC fat (g)	1.6 ± 0.1	0.9 ± 0.1†
GON fat (g)	2.5 ± 0.1	1.5 ± 0.1‡
Lungs (mg)	166 ± 18	163 ± 15
Spleen (mg)	83 ± 5.3	77 ± 4.6
Heart (mg)	148 ± 5.4	136 ± 7.1*
Pancreas (mg)	262 ± 18	201 ± 14*
Liver (mg)	2650 ± 155	1390 ± 140‡
Kidneys (mg)	401 ± 20	375 ± 18

Table 15. Effect of MMP-2 deficiency on adipose tissue and organ weights of mice (C57Bl/6) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. * $p < 0.05$, † $p < 0.005$, ‡ $p < 0.0005$ versus MMP-$2^{+/+}$ mice.

Food intake (3.0 ± 0.02 g/day versus 3.1 ± 0.03 g/day for MMP-$2^{+/+}$ mice, p = 0.9) and feeding efficiency (weight gain normalized to caloric intake; 2.7 ± 0.3 mg/kJ versus 3.2 ± 0.3 mg/kJ, p = 0.11) was not different for MMP-$2^{-/-}$ mice.

The weight of the isolated SC and GON adipose tissues was also significantly lower for the MMP-$2^{-/-}$ mice. The weight of kidneys, lungs and spleen did not differ between the two genotypes, whereas heart, pancreas, and liver weights were lower in MMP-$2^{-/-}$ mice (Table 15).

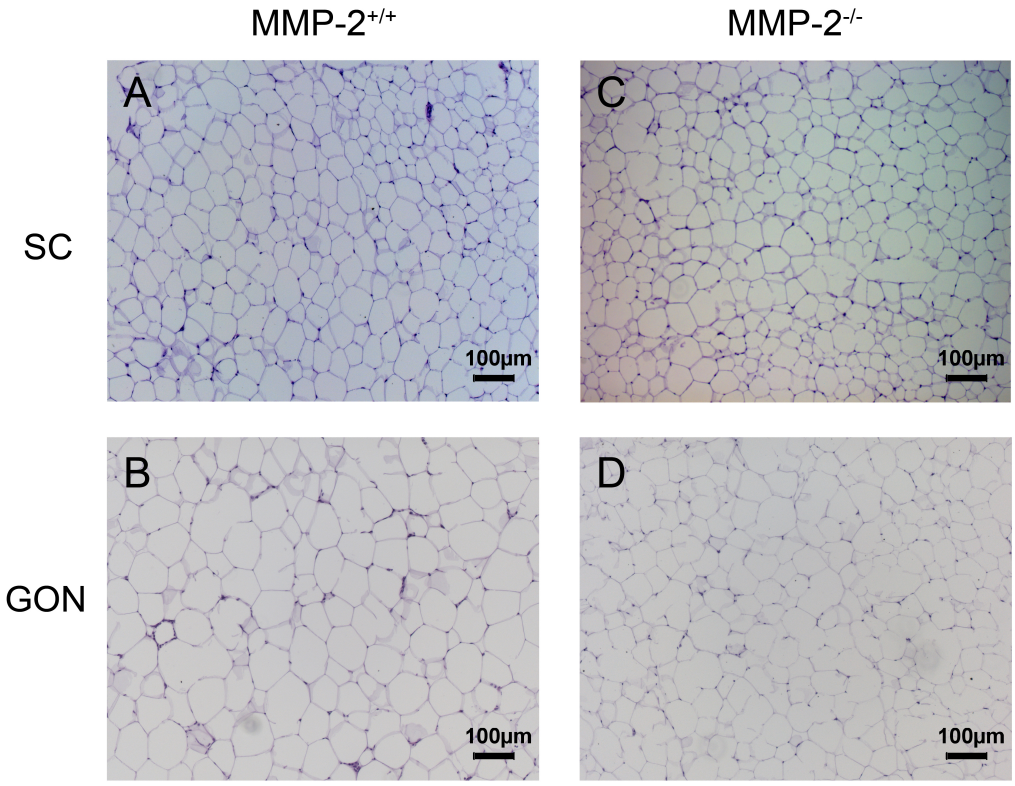


Figure 13. Representative haematoxylin-eosin stained paraffin sections of subcutaneous (A, C) and gonadal (B, D) adipose tissues of MMP-$2^{-/-}$ (C, D) and MMP-$2^{+/+}$ mice (A, B) after 15 weeks of HFD.

Histological analysis of haematoxylin-eosin stained paraffin sections revealed a smaller adipocyte size in both SC and GON adipose tissues of MMP-$2^{-/-}$ mice, corresponding to a higher adipocyte density (Table 16 and Figure 13).

Staining with an endothelial cell specific lectin showed no differences in blood vessel size or density, even after normalization for adipocyte density (Table 16).

Total collagen content, visualized by Sirius Red staining, was higher in SC (15.7 ± 0.67 % versus 13.4 ± 0.49 % in controls, p = 0.009) and in GON (14.3 ± 0.73 % versus 12.1 ± 0.50 % in controls, p = 0.01) adipose tissues of MMP-$2^{-/-}$ mice.

	MMP-2$^{+/+}$ (n = 10)	MMP-2$^{-/-}$ (n = 9)
Adipocyte size (μm^2)		
SC fat	4160 ± 170	3350 ± 270*
GON fat	6860 ± 310	5430 ± 520*
Adipocyte density ($x10^{-6}/\mu m^2$)		
SC fat	255 ± 17	338 ± 30*
GON fat	151 ± 7.9	203 ± 28*
Blood vessel size (μm^2)		
SC fat	44 ± 4.5	35 ± 3.1
GON fat	45 ± 3.6	43 ± 2.4
Blood vessel density ($x10^{-6}/\mu m^2$)		
SC fat	320 ± 56	312 ± 60
GON fat	227 ± 26	261 ± 56
Normalized blood vessel density		
SC fat	1.3 ± 0.2	1.0 ± 0.2
GON fat	1.4 ± 0.2	1.4 ± 0.3

Table 16. Effect of MMP-2 deficiency on adipocyte and blood vessel size and density in adipose tissues of mice (C57Bl/6) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. * $p < 0.05$ versus MMP-2$^{+/+}$ mice.

Obese MMP-2$^{-/-}$ mice displayed significantly lower levels of glucose, insulin and cholesterol, as compared to their wild-type littermates, whereas triglyceride levels were not affected (Table 17).

	MMP-2$^{+/+}$ (n = 10)	MMP-2$^{-/-}$ (n = 9)
Glucose (mg/dl)	187 ± 13	139 ± 16*
Insulin (ng/ml)	1.8 ± 0.3	0.9 ± 0.2*
Leptin (ng/ml)	18 ± 0.7	12 ± 1.0†
Triglycerides (mg/dl)	48 ± 3.3	56 ± 8.9
Total cholesterol (mg/dl)	176 ± 19	114 ± 11*
HDL cholesterol (mg/dl)	166 ± 14	117 ± 11*
LDL cholesterol (mg/dl)	35 ± 4.5	18 ± 1.9†

Table 17. Effect of MMP-2 deficiency on plasma metabolic parameters of mice (C57Bl/6) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. * $p < 0.05$, † $p < 0.0005$ versus MMP-2$^{+/+}$ mice.

Plasma levels of resistin were comparable in MMP-2$^{-/-}$ and MMP-2$^{+/+}$ mice (12 ± 0.5 ng/ml versus 11 ± 1.0 ng/ml, p = 0.24), whereas adiponectin levels were significantly higher (8.92 ± 0.64 µg/ml versus 6.77 ± 0.37 µg/ml, p = 0.008) and leptin levels significantly lower (12 ± 1.0 ng/ml versus 18 ± 0.80 ng/ml, p = 0.0008) in MMP-2$^{-/-}$ mice.

VEGF-A protein levels in adipose tissue extracts of MMP-2$^{+/+}$ and MMP-2$^{-/-}$ mice were comparable for SC (10 ± 0.9 versus 10 ± 0.6 pg/mg protein) and GON (13 ± 1.5 versus 16 ± 1.5 pg/mg protein) fat tissues.

TIMP-1 mRNA levels were comparable for MMP-2$^{+/+}$ and MMP-2$^{-/-}$ mice in both SC (13 ± 3.0 versus 10 ± 1.0 A.U.) and GON (76 ± 15 versus 50 ± 9 A.U.) adipose tissues. Also, TIMP-2 mRNA levels were similar for MMP-2$^{+/+}$ and MMP-2$^{-/-}$ mice in SC (2290 ± 150 versus 2720 ± 200 A.U.) and GON (3380 ± 320 versus 3060 ± 300 A.U.) fat.

On zymograms, four gelatinolytic bands were consistently observed in adipose tissues from wild-type mice, corresponding to proMMP-9 (92 kDa), proMMP-2 (72 or 68 kDa, differently glycosylated) and active MMP-2 (58 kDa). Active MMP-2 (62 kDa form) and active MMP-9 (82 kDa) were not consistently detected. Zymography confirmed the absence of MMP-2 in the MMP-2$^{-/-}$ SC and GON adipose tissues (Figure 14) and revealed an upregulation of MMP-9 in SC adipose tissues of MMP-2$^{-/-}$ mice (14 ± 1.4 versus 5.2 ± 1.2 A.U. for MMP-2$^{+/+}$, p = 0.03), but not in GON adipose tissues (5.2 ± 0.6 versus 5.5 ± 0.4 A.U.).

Activation with APMA for 16 hours resulted in reduced levels of the latent forms of MMP-2, with concomitant increase of active MMP-2 (58 kDa) and appearance of 62 kDa active MMP-2.

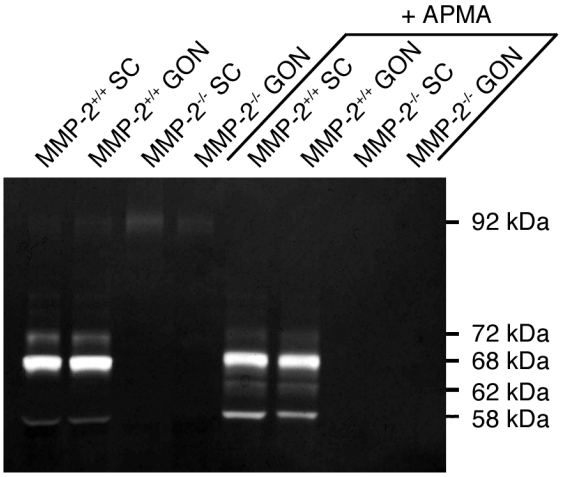


Figure 14. Zymographic analysis of SC and GON adipose tissues of MMP-2$^{-/-}$ andMMP-2$^{+/+}$ mice kept on HFD for 15 weeks, before and after activation with APMA.

In SC adipose tissue of MMP-2$^{-/-}$ mice also a significant upregulation of MMP-9 mRNA was observed (157 ± 16 A.U. versus 70 ± 6 A.U. in MMP-2$^{+/+}$ samples, $p < 0.0001$). This difference was not observed in the GON adipose tissues (31 ± 4 A.U. versus 32 ± 6 A.U.).

Zymography with plasma samples further confirmed the absence of MMP-2 in MMP-2$^{-/-}$ mice and revealed upregulation of MMP-9 (10 ± 1.0 versus 5.2 ± 0.5 A.U. in MMP-2$^{+/+}$ mice, $p = 0.0003$).

3. Effect of gelatinase inhibition on adipose tissue development in wild-type mice

Van Hul M, Lijnen HR. A functional role of gelatinase A in the development of nutritionally induced obesity in mice. Journal of thrombosis and haemostasis. 2008. 6:1198-206.

Wild-type mice fed the HFD supplemented with Tolylsam (100 mg/kg per day) had a lower body weight gain resulting in a lower body weight after 15 weeks as compared to the mice on HFD only (Figure 15).

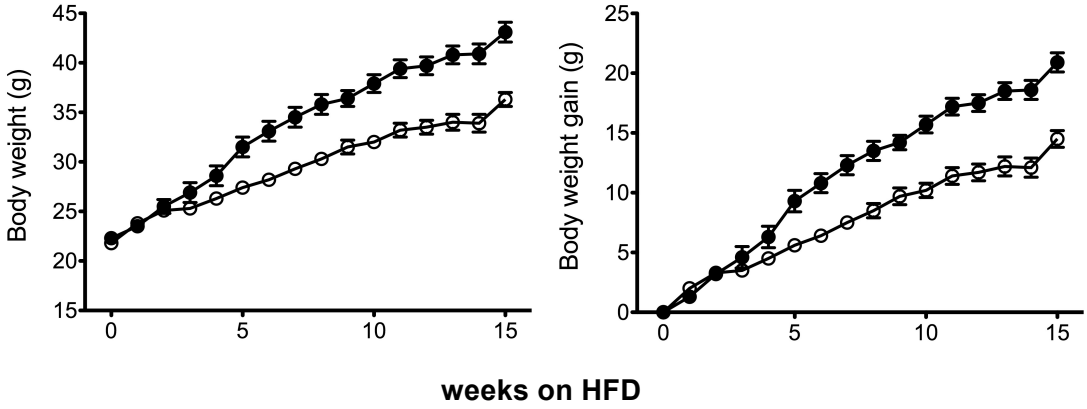


Figure 15. Body weight and weight gain of Tolylsam-treated (open circles) and placebo-treated wild-type mice (black circles) kept on HFD for 15 weeks. Data are means ± SEM. Analysis by two-way repeated-measures ANOVA revealed significantly ($p < 0.0001$) reduced body weight and weight gain.

Food intake of inhibitor-treated mice (2.2 ± 0.04 g/day) was higher than that of untreated mice (2.0 ± 0.05 g/day, $p = 0.003$), but the feeding efficiency was lower (2.9 ± 0.4 mg/kJ versus 4.9 ± 0.7 mg/kJ, $p = 0.02$).

The weight of SC and GON fat pads was significantly lower in the inhibitor-treated as compared to the placebo-treated mice (Table 18). Histological analysis revealed a smaller adipocyte size (Table 19) and smaller blood vessels in adipose tissues (Figure 16, Table 19). The blood vessel density was not affected in inhibitor-treated mice, but after normalization for adipocyte density, this was significantly lower for both SC and GON fat pads (Table 19). Total collagen

content was higher in SC (12.9 ± 0.45 % versus 10.8 ± 0.36 % in controls, p = 0.0005) but not in GON (8.9 ± 0.40 % versus 9.4 ± 0.41 % in controls, p = 0.4) adipose tissues of treatment.

	Controls (n = 10)	Inhibitor (n = 10)
Body weight start (g)	22 ± 0.3	22 ± 0.4
Body weight end (g)	43 ± 1.0	36 ± 0.7‡
SC fat (g)	1.6 ± 0.1	0.8 ± 0.1‡
GON fat (g)	2.1 ± 0.1	1.3 ± 0.1‡
Lungs (mg)	172 ± 17	189 ± 13
Spleen (mg)	106 ± 5.6	96 ± 3.3
Heart (mg)	149 ± 5.8	149 ± 3.8
Pancreas (mg)	255 ± 20	173 ± 10†
Liver (mg)	2620 ± 172	2470 ± 99
Kidneys (mg)	450 ± 16	414 ± 8.1

Table 18. Effect of gelatinase inhibition on adipose tissue and organ weights of mice (C57Bl/6) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. † p < 0.005, ‡ p < 0.0005 versus controls.

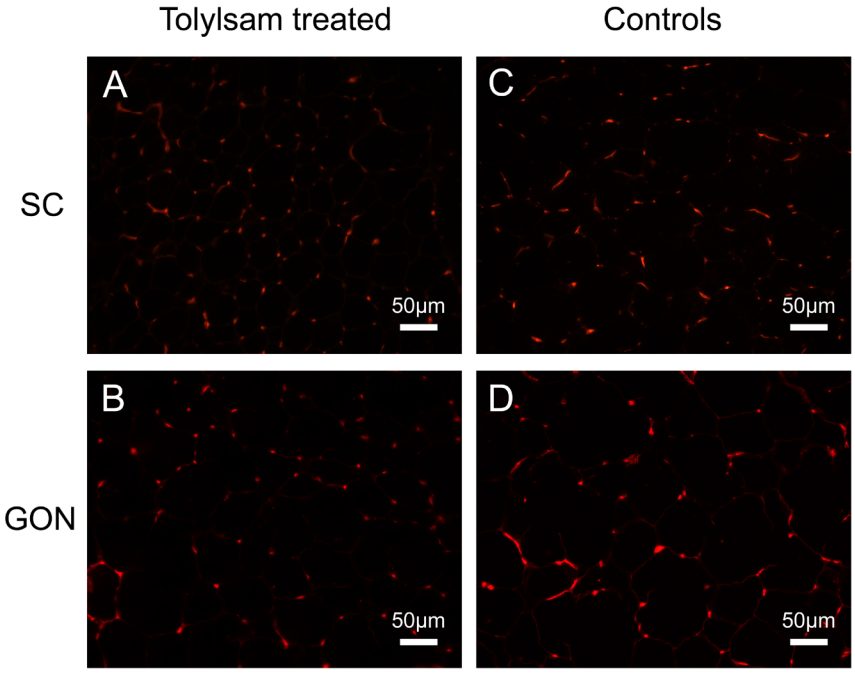


Figure 16. Lectin staining of SC (A, C) and GON (B, D) adipose tissues of Tolylsam-treated (A, B) and control mice (C, D) after 15 weeks of HFD.

	Controls (n = 10)	Inhibitor (n = 10)
Adipocyte size (μm^2)		
SC fat	4330 ± 142	3040 ± 169‡
GON fat	6800 ± 261	4960 ± 241‡
Adipocyte density ($x10^{-6}/\mu m^2$)		
SC fat	239 ± 8.1	355 ± 22†
GON fat	150 ± 5.1	208 ± 10†
Blood vessel size (μm^2)		
SC fat	41 ± 1.9	28 ± 1.7†
GON fat	45 ± 1.7	30 ± 2.7†
Blood vessel density ($x10^{-6}/\mu m^2$)		
SC fat	320 ± 14	330 ± 19
GON fat	236 ± 9	214 ± 13
Normalized blood vessel density		
SC fat	1.3 ± 0.1	0.9 ± 0.1†
GON fat	1.5 ± 0.1	1.0 ± 0.1‡

Table 19. Effect of gelatinase inhibition on adipocyte and blood vessel size and density in adipose tissues of mice (C57Bl/6) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. † $p < 0.005$, ‡ $p < 0.0005$ versus controls.

Metabolic parameters were not significantly affected by Tolylsam treatment, with the exception of reduced leptin, glucose and triglyceride levels (Table 20).

	Controls (n = 10)	Inhibitor (n = 10)
Glucose (mg/dl)	142 ± 14	101 ± 16*
Insulin (ng/ml)	1.8 ± 0.3	1.2 ± 0.3
Leptin (ng/ml)	17 ± 0.8	10 ± 1.1†
Triglycerides (mg/dl)	68 ± 5	47 ± 5*
Total cholesterol (mg/dl)	163 ± 13	181 ± 11
HDL cholesterol (mg/dl)	152 ± 5	169 ± 10
LDL cholesterol (mg/dl)	ND	ND

Table 20. Effect of gelatinase inhibition on plasma metabolic parameters of mice (C57Bl/6) kept on HFD for 15 weeks. Data are mean ± SEM of n experiments in each group.* $p < 0.05$, † $p < 0.005$ versus controls. ND, not detected.

Plasma levels of resistin (16 ± 1.2 ng/ml versus 14 ± 0.70 ng/ml, p = 0.13) and adiponectin (4.73 ± 1.6 µg/ml versus 2.5 ± 0.67 µg/ml, p = 0.48) were comparable in Tolylsam-treated and in control mice, whereas leptin levels were significantly reduced (10 ± 1.1 ng/ml versus 17 ± 0.70 ng/ml, p = 0.0003) in Tolylsam-treated mice.

VEGF-A levels in SC adipose tissues were not affected by Tolylsam treatment (11 ± 0.7 versus 10 ± 0.3 pg/mg protein for controls), but were enhanced in GON adipose tissues (22 ± 1.6 versus 13 ± 0.5 pg/mg protein for controls, p < 0.0001).

Treatment with Tolylsam did not affect TIMP-1 mRNA levels in SC (17 ± 2.9 versus 22 ± 3.2 A.U. for controls) or GON (60 ± 5.1 versus 74 ± 6.6 A.U. for controls) adipose tissues. Similarly, TIMP-2 mRNA levels were not affected in either SC (2240 ± 130 versus 2020 ± 100 A.U. for controls) or GON (2250 ± 140 versus 2770 ± 350 A.U. for controls) adipose tissues.

Gelatinase expression and activity

Overall MMP activity in adipose tissue extracts, monitored using a fluorescent broad-spectrum substrate, was only slightly decreased in the samples treated with inhibitor, as compared to placebo (p = 0.58).

In both SC and GON adipose tissues of inhibitor-treated mice mRNA levels of MMP-2 were significantly lower than in adipose tissues of controls (for SC: 553 ± 41 A.U. versus 1040 ± 53 A.U., p < 0.0001 and for GON: 282 ± 22 A.U. versus 554 ± 41 A.U., p = 0.0002), whereas mRNA levels of MMP-9 were higher in inhibitor-treated tissues (for SC: 77 ± 3.9 A.U. versus 44 ± 3.8 A.U., p = 0.0006 and for GON: 20 ± 2.1 A.U. versus 12 ± 0.9 A.U., p = 0.002).

Zymography of SC adipose tissue extracts revealed no difference in 92 kDa proMMP-9 and 68 kDa or 72 kDa proMMP-2 levels. 58 kDa active MMP-2 levels were, however, strongly reduced in the inhibitor-treated mice. Thus, 58 kDa active MMP-2 levels amounted to 11 ± 2.4 % of the total MMP-2 species (active plus latent) for inhibitor-treated SC adipose tissues, as compared to 26 ± 2.1 % for placebo-treated samples (p < 0.0005). Active MMP-9 could not consistently be detected (Table 21, Figure 17).

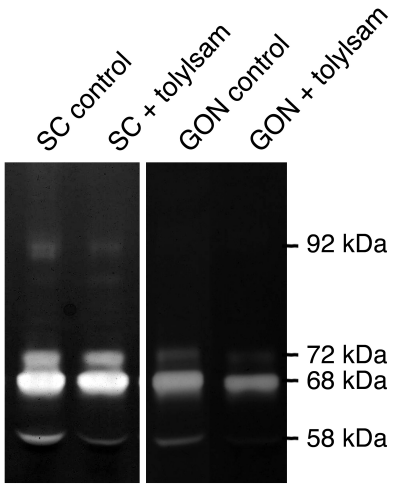


Figure 17. Zymographic analysis of SC and GON adipose tissues of Tolylsam-treated and placebo-treated mice after 15 weeks on HFD.

	Controls	Inhibitor
SC adipose tissue		
92 kDa Pro-MMP-9	3.5 ± 0.3	3.7 ± 0.2
82 kDa Active MMP-9	ND	ND
72 kDa Pro-MMP-2	17 ± 1.4	15 ± 0.9
62 kDa Active MMP-2	ND	ND
68 kDa Pro-MMP-2	27 ± 2.1	27 ± 1.9
58 kDa Active MMP-2	10 ± 1.4	3.5 ± 0.8*
GON adipose tissue		
92 kDa Pro-MMP-9	ND	ND
82 kDa Active MMP-9	ND	ND
72 kDa Pro-MMP-2	15 ± 2.2	6 ± 0.7*
62 kDa Active MMP-2	ND	ND
68 kDa Pro-MMP-2	29 ± 1.3	15 ± 1.0*
58 kDa Active MMP-2	12 ± 1.8	0.9 ± 0.4*

Table 21. Effect of Tolylsam on gelatinolytic activity in adipose tissues of mice kept on HFD for 15 weeks. ND: not detected. Data are means ± SEM and are expressed in arbitrary units. * $p < 0.0005$ versus placebo-treated mice.

In extracts from GON fat tissue a significant decrease of 68 kDa and 72 kDa proMMP-2 levels, as well as of 58 kDa active MMP-2 levels was observed. The 58 kDa active MMP-2 levels corresponded to 6 ± 3.4 % of the total MMP-2 species

for inhibitor-treated GON adipose tissues, as compared to 28 ± 3.3 % for placebo-treated samples ($p < 0.0005$). Pro-MMP-9 (92 kDa) or active MMP-9 (82 kDa) could not consistently be detected.

4. Effect of gelatinase inhibition on adipose tissue development in leptin deficient (*ob/ob*) mice

Van Hul M, Lijnen HR. Matrix metalloproteinase inhibition impairs murine adipose tissue development independently of leptin. Endocrine journal. 2011. 58:101-7.

Leptin deficient (ob/ob) mice fed the HFD supplemented with Tolylsam showed lower body weight gain than control mice, resulting in significantly reduced body weight after 15 weeks (52 ± 0.8 g versus 59 ± 1.0 g, $p < 0.0001$, Figure 18 and Table 22).

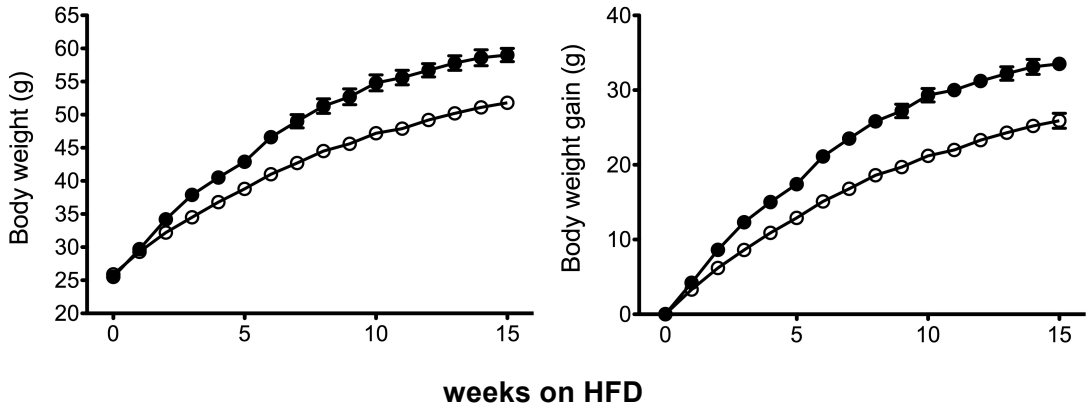


Figure 18. Body weight and weight gain of Tolylsam-treated (open circles) and placebo-treated ob/ob mice (black circles) kept on a high-fat diet (HFD) for 15 weeks. Data are means ± SEM. Analysis by two-way repeated-measures ANOVA revealed significantly ($p < 0.0001$) reduced body weight and weight gain.

Food intake was comparable (4.0 ± 0.08 g/day versus 3.9 ± 0.08 g/day for controls, $p = 0.6$), indicating a lower feeding efficiency (weight gain normalized to caloric intake; 3.1 ± 0.34 mg/kJ versus 4.2 ± 0.57 mg/kJ for controls, $p = 0.09$) for treated mice. Physical activity was very low in both groups (222 ± 41 turns/12h versus 250 ± 71 turns/12h, $p = 0.5$), and body temperature, measured at week 0, 7, and 15, did not indicate differences in thermogenesis between the two groups (35.8 ± 0.12 °C versus 35.8 ± 0.11 °C for controls).

	Controls (n = 10)	Inhibitor (n = 8)
Body weight start (g)	26 ± 0.6	26 ± 0.6
Body weight end (g)	59 ± 1.0	52 ± 0.8†
Weight gain (g)	33 ± 0.7	26 ± 1.0†
SC fat (g)	3.4 ± 0.2	2.9 ± 0.1*
GON fat (g)	3.7 ± 0.1	3.4 ± 0.1
Lungs (mg)	151 ± 5.3	166 ± 12
Spleen (mg)	87 ± 3.3	80 ± 4.3
Heart (mg)	164 ± 4.5	148 ± 4.8
Pancreas (mg)	182 ± 14	175 ± 13
Liver (mg)	4810 ± 268	5150 ± 174
Kidneys (mg)	436 ± 15	395 ± 18

Table 22. Effect of gelatinase inhibition on adipose tissue and organ weights of ob/ob mice (C57Bl/6) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. * $p < 0.05$, † $p < 0.0005$ versus controls.

The fat depots were somewhat smaller in the Tolylsam-treated mice, although this was only significant for the SC fat pads. The weight of other organs was comparable for both groups (Table 22). A trend towards smaller adipocytes was noted in the inhibitor-treated mice, but was not significant. A similar observation was made for blood vessel size, whereas the blood vessel density was significantly lower in Tolylsam-treated SC and GON adipose tissues. The normalized blood vessel density, i.e. adjusted for adipocyte density, thus was lower in the Tolylsam-treated mice (Table 23).

Tolylsam treatment did not affect total collagen content in SC (13.5 ± 1.1 % versus 12.7 ± 0.5 % in controls) or in GON (8.8 ± 0.9 % versus 9.8 ± 0.9 % in controls) adipose tissues. Collagen structural disorganization (monitored as ratio thick/thin fibers) in Tolylsam-treated and control adipose tissues was also similar (SC: 0.48 ± 0.07 versus 0.39 ± 0.03 and GON: 0.51 ± 0.11 versus 0.57 ± 0.12).

	Controls (n = 10)	Inhibitor (n = 8)
Adipocyte size (μm^2)		
SC fat	5880 ± 351	5120 ± 372
GON fat	8440 ± 533	7790 ± 354
Adipocyte density ($x10^{-6}/\mu m^2$)		
SC fat	176 ± 13	205 ± 15
GON fat	123 ± 10	132 ± 7
Blood vessel size (μm^2)		
SC fat	43 ± 4.7	39 ± 2.7
GON fat	44 ± 2.4	38 ± 2.2
Blood vessel density ($x10^{-6}/\mu m^2$)		
SC fat	413 ± 47	324 ± 19*
GON fat	291 ± 20	218 ± 13†
Normalized blood vessel density		
SC fat	2.4 ± 0.3	1.7 ± 0.1*
GON fat	2.4 ± 0.2	1.7 ± 0.2

Table 23. Effect of gelatinase inhibition on adipocyte and blood vessel size and density in adipose tissues of ob/ob mice (C57Bl/6) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. * $p < 0.05$, † $p < 0.005$ versus controls.

Plasma levels of glucose, insulin, total cholesterol and LDL cholesterol did not markedly differ, but triglyceride and HDL cholesterol concentrations were lower in the Tolylsam-treated mice (Table 24).

	Controls (n = 10)	Inhibitor (n = 8)
Glucose (mg/dl)	130 ± 16	113 ± 10
Insulin (ng/ml)	3.7 ± 0.6	4.8 ± 0.7
Leptin (ng/ml)	ND	ND
Triglycerides (mg/dl)	60 ± 6.7	36 ± 6.0*
Total cholesterol (mg/dl)	231 ± 18	198 ± 12
HDL cholesterol (mg/dl)	150 ± 9.3	120 ± 6.0*
LDL cholesterol (mg/dl)	54 ± 6.3	51 ± 6.6

Table 24; Effect of gelatinase inhibition on plasma metabolic parameters of ob/ob mice (C57Bl/6) kept on HFD for 15 weeks. ND: not detected. Data are means ± SEM of n experiments in each group.* $p < 0.05$ versus controls.

mRNA levels of MMP-2 were significantly higher upon Tolylsam treatment of the SC (732 ± 105 A.U. versus 402 ± 50 A.U., p = 0.01), but not the GON adipose tissue (430 ± 46 A.U. versus 490 ± 34 A.U., p = 0.3). mRNA levels of MMP-9 were also induced after treatment with Tolylsam in SC (52 ± 4.2 A.U. versus 35 ± 6.0 A.U., p = 0.04) but not in GON (19 ± 3.1 A.U. versus 15 ± 4.5 A.U., p = 0.5) fat tissues. On zymograms, four gelatinolytic bands were consistently observed in adipose tissue extracts, corresponding to proMMP-9 (92 kDa), proMMP-2 (72 or 68 kDa, differently glycosylated) and active MMP-2 (58 kDa). Active MMP-2 (62 kDa form) and active MMP-9 (82 kDa) were not consistently detected. Zymography (Figure 19) revealed no significant difference in proMMP-9 levels (4.0 ± 0.2 versus 6.4 ± 1.3 A.U., p = 0.1 for SC and 1.8 ± 0.1 versus 1.9 ± 0.2 A.U., p = 0.8 for GON). Total MMP-2 levels were also not different (39.4 ± 0.2 versus 36.5 ± 0.2 A.U., p = 0.3 for SC and 15.7 ± 0.2 versus 16.9 ± 0.1A.U., p = 0.5 for GON). Distribution of different molecular forms of MMP-2 (in percentage) shows similar amounts of 72 kDa proMMP-2 (20 ± 2.2 versus 19 ± 1.1 %, p = 0.7 for SC and 12 ± 1.4 versus 9.4 ± 0.7 %, p = 0.1 for GON) and 68 kDa proMMP-2 (75 ± 1.8 versus 71 ± 1.2 %, p = 0.1 for SC and 79 ± 1.9 versus 77 ± 1.4 %, p = 0.5 for GON). Active MMP-2 levels (58 kDa) were, however, reduced in both SC (4.5 ± 0.4 versus 9.3 ± 1.1 %, p = 0.01) and GON (8.6 ± 1.0 versus 13 ± 1.5 %, p = 0.03) inhibitor-treated adipose tissues.

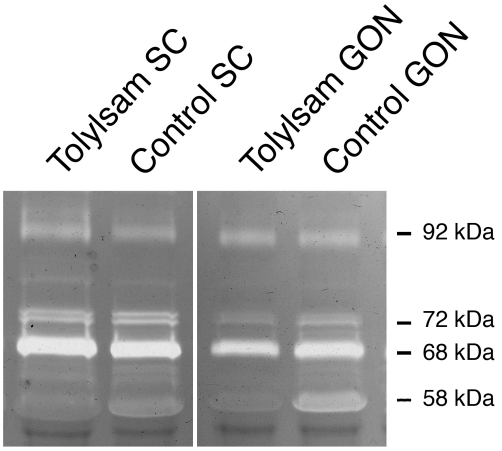


Figure 19. Zymographic analysis of SC and GON adipose tissues of Tolylsam-treated and non-treated ob/ob mice after 15 weeks of HFD.

5. Effect of gelatinase inhibition on adipose tissue development in wild-type obese mice

Van Hul M, Lijnen HR. Effect of weight loss on gelatinase levels in obese mice. Clinical and experimental pharmacology & physiology. 2011. 38:647-9.

Van Hul M, Lupu F, Dresselaers T, Lijnen HR. Matrix metalloproteinase inhibition affects adipose tissue mass in obese mice (submitted for publication)

Wild-type mice kept on HFD for 20 weeks were divided into two groups of similar weight (n = 5 each). One group was further kept on HFD, while the second group was fed a HFD supplemented with Tolylsam (100 mg/kg/day). MRI scanning at the start of the experimental period (after 1 week) did not reveal significant differences between control and Tolylsam-treated mice (total body fat 16 ± 0.70 cm^3 versus 15 ± 0.49 cm^3; SC fat 10 ± 0.46 cm^3 versus 9.8 ± 0.15 cm^3 and GON fat 5.9 ± 0.24 cm^3 versus 5.5 ± 0.34 cm^3). However, as a result of the MRI procedure, that required overnight fasting, mice in both groups showed a slight weight loss (Figure 20 A). Whereas between weeks 2 and 7 of the experiment, the control mice gained weight again (44 ± 0.90 g to 49 ± 0.89 g, $p < 0.0001$), the mice treated with Tolylsam did not (43 ± 1.3 g versus 42 ± 1.9 g, $p = 0.29$), resulting in a significantly higher body weight of the controls at the end of the experiment (Table 25). Food intake was comparable for both groups (3.9 ± 0.09 versus 3.7 ± 0.20 g/day for controls; $p = 0.4$).

Physical activity (4566 ± 1344 versus 4704 ± 985 turns/12h for controls; $p = 0.9$) and body temperature (35.9 ± 0.2 °C versus 35.8 ± 0.2 °C for controls; $p = 0.5$) were not affected by Tolylsam treatment. The mass of isolated SC and GON fat depots was significantly lower in the Tolylsam-treated mice, whereas spleen and kidneys appeared enlarged (Table 25). With inhibitor treatment, SC fat contributes to total body weight for 3.4 ± 0.2% versus 5.1 ± 0.1% for controls ($p = 0.008$), with corresponding values of 4.1 ± 0.2% versus 4.7 ± 0.1% ($p = 0.016$) for GON fat. MRI scanning confirmed significantly lower volumes of total body fat, SC and GON fat depots after Tolylsam treatment as compared to controls (Figure 20 B, Table 26).

	Controls (n = 5)	Inhibitor (n = 5)
Body weight start (g)	45 ± 1.0	46 ± 1.6
Body weight end (g)	49 ± 0.89	42 ± 1.9*
Weight gain (g)	3.9 ± 0.4	-4.2 ± 0.6†
SC fat (g)	2.4 ± 0.07	1.4 ± 0.15*
GON fat (g)	2.2 ± 0.05	1.6 ± 0.12*
Lungs (mg)	196 ± 15	227 ± 16
Spleen (mg)	117 ± 2.6	168 ± 12*
Heart (mg)	157 ± 3.1	167 ± 6.7
Pancreas (mg)	180 ± 21	205 ± 13
Liver (g)	3.7 ± 0.18	3.5 ± 0.20
Kidneys (mg)	386 ± 8.8	444 ± 21*

Table 25. Effect of gelatinase inhibition on adipose tissue and organ weights of obese mice kept on HFD for 20 weeks, followed by an additional 7 weeks on HFD, with or without Tolylsam. Data are means ± SEM of 5 experiments in each group. * $p < 0.05$, † $p < 0.0005$ versus controls.

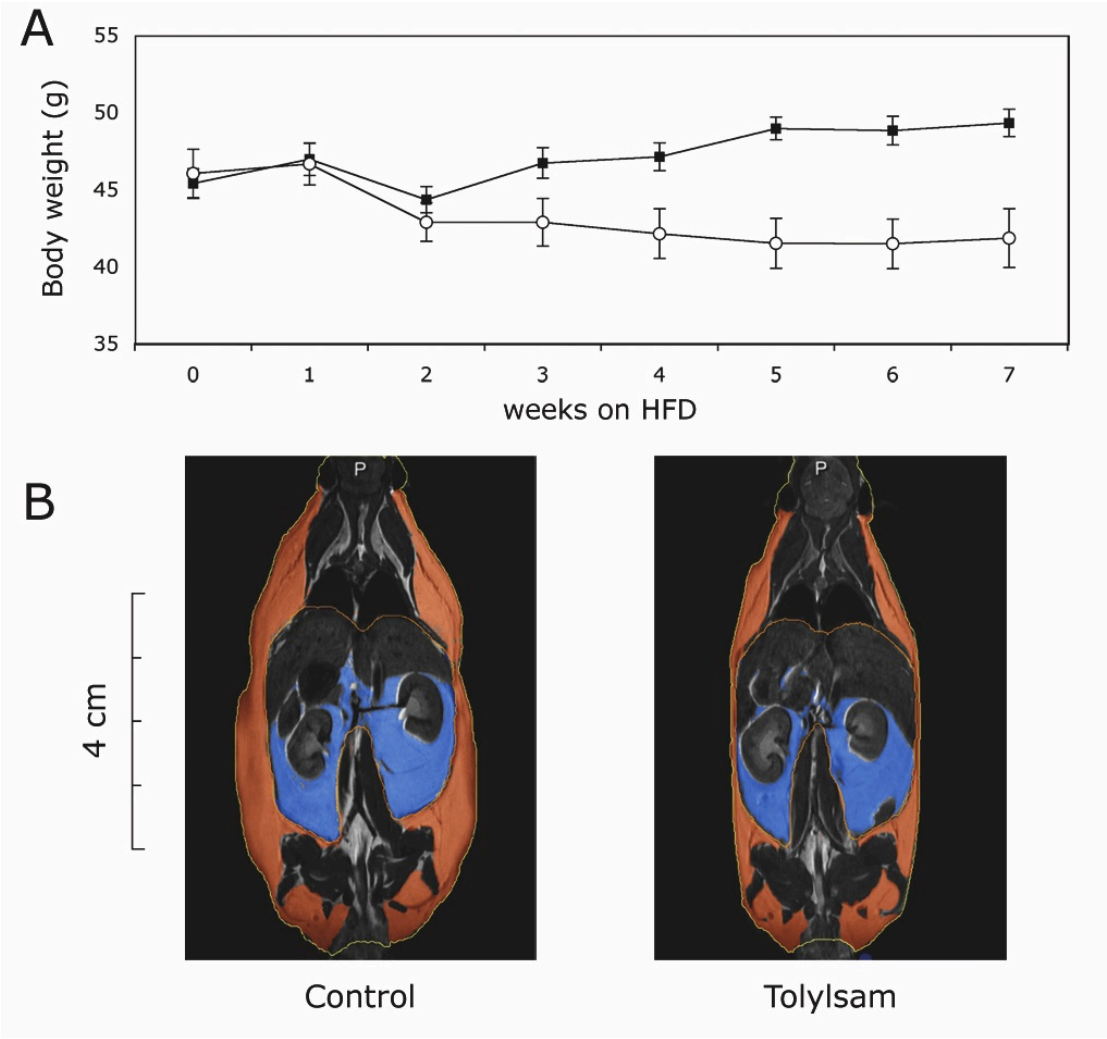


Figure 20. Effect of Tolylsam treatment on body weight of obese C57Bl/6 mice kept on a high fat diet for 7 weeks (panel A). Data are means ± SEM of 5 experiments each with (open circles) or without (black circles) addition of Tolylsam. Analysis by two-way repeated-measures ANOVA confirmed significantly lower body weight ($p < 0.0001$) upon Tolylsam treatment. Panel B shows representative MRI analysis of control and Tolylsam-treated mice at the end of the experiments; subcutaneous and abdominal fat are colored red and blue, respectively; the yellow and orange lines delineate total body area and abdominal cavity, respectively.

	Controls (n = 5)	Inhibitor (n = 5)
Total body (cm^3)	41 ± 0.57	34 ± 1.3*
Total fat (cm^3)	18 ± 0.66	12 ± 0.79†
SC fat (cm^3)	12 ± 0.40	7.6 ± 0.53†
GON fat (cm^3)	6.0 ± 0.26	4.1 ± 0.34*

Table 26. Quantification of MRI analysis of control and Tolylsam-treated mice at the end of the experiments. Data are means ± SEM of 5 experiments each. * $p < 0.005$ and † $p < 0.0005$ versus controls.

Quantitative analysis of isolated SC and GON fat depots revealed significant adipocyte hypotrophy, associated with higher adipocyte density following Tolylsam treatment (Table 27). Blood vessel size was not significantly different between both groups and blood vessel density was also similar after normalization to adipocyte density.

	Controls (n = 5)	Inhibitor (n = 5)
Adipocyte size (μm^2)		
SC fat	5160 ± 161	3710 ± 248†
GON fat	6530 ± 186	4910 ± 94‡
Adipocyte density ($x10^{-6}/\mu m^2$)		
SC fat	199 ± 7.4	283 ± 20*
GON fat	155 ± 4.8	205 ± 3.7‡
Blood vessel size (μm^2)		
SC fat	51 ± 2.4	54 ± 4.7
GON fat	50 ± 3.8	52 ± 3.9
Blood vessel density ($x10^{-6}/\mu m^2$)		
SC fat	242 ± 19	334 ± 27*
GON fat	175 ± 9.0	237 ± 13†
Normalized blood vessel density		
SC fat	1.2 ± 0.10	1.2 ± 0.08
GON fat	1.1 ± 0.05	1.2 ± 0.05

Table 27. Adipocyte and blood vessel size and density in adipose tissues of obese mice kept on HFD for 20 weeks, followed by 7 weeks on HFD with or without Tolylsam. Data are means ± SEM of 5 experiments in each group. * $p < 0.05$, † $p < 0.005$, ‡ $p < 0.0005$ versus controls.

Sirius-red staining revealed that Tolylsam treatment resulted in preservation of collagen as compared to controls: total collagen content in treated SC adipose tissues of 7.7 ± 0.7 % versus 4.4 ± 0.51 % ($p = 0.005$), with corresponding values for GON adipose tissues of 3.4 ± 0.4 % versus 2.3 ± 0.3 % ($p = 0.07$). Collagen structural disorganization, monitored as ratio thick/thin fibers, was less prominent in Tolylsam-treated SC (0.42 ± 0.07 versus 0.22 ± 0.02; $p = 0.05$) and GON (0.43 ± 0.05 versus 0.26 ± 0.06; $p = 0.06$) adipose tissue. In addition, quantitative analysis of electron micrographs (Figure 21) confirmed significantly more collagen-rich areas in SC (0.3 ± 0.04 versus 0.006 ± 0.001μm^2; $p < 0.0001$), but not in GON (0.17 ± 0.03 versus 0.14 ± 0.03 μm^2; $p = 0.6$) adipose tissues of

inhibitor-treated mice. Tolylsam treatment did not significantly affect macrophage accumulation in SC (2.86 ± 1.05 % versus 0.99 ± 0.41 % for controls; p = 0.22) or GON (1.35 ± 0.59 % versus 0.76 ± 0.27 %; p = 0.42) adipose tissues.

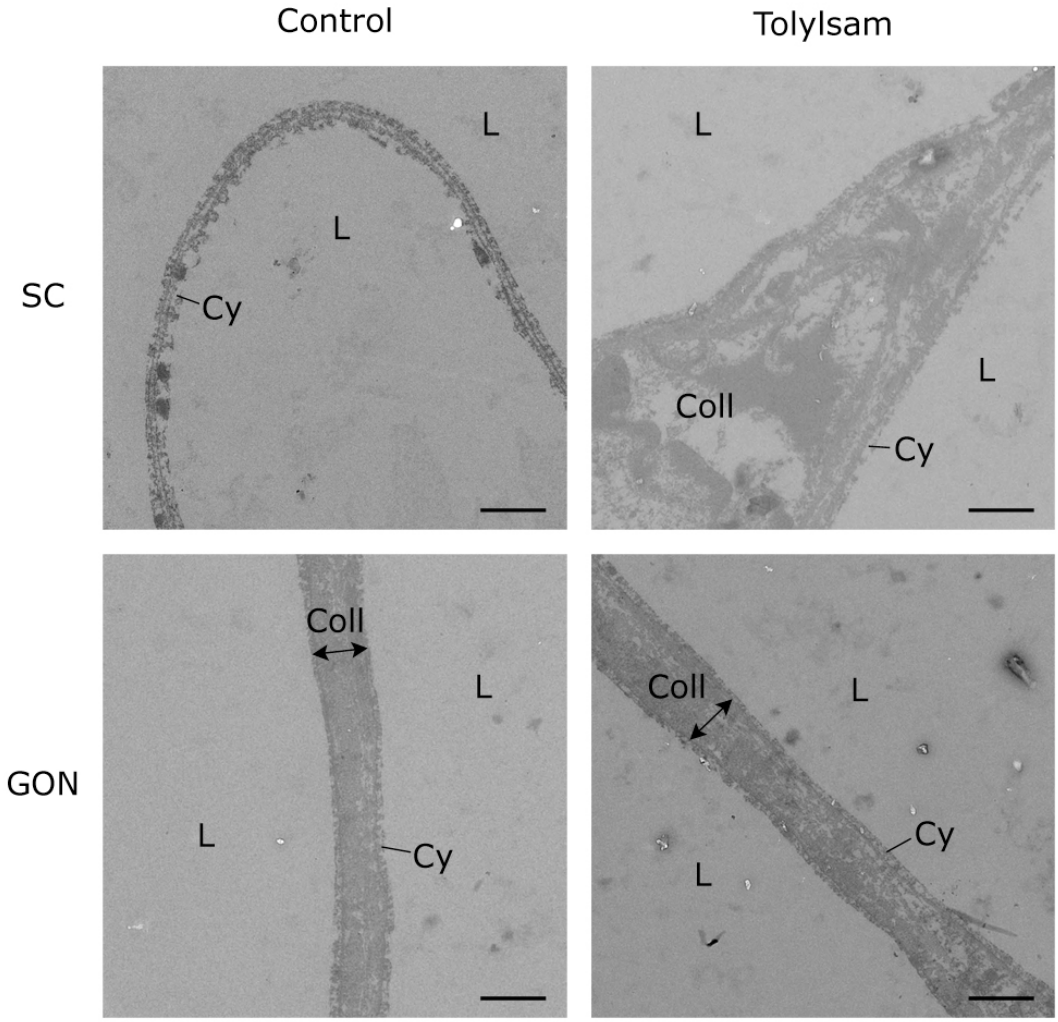


Figure 21. Electron microscopy analysis of subcutaneous (SC) and gonadal (GON) adipose tissue of obese mice kept on high fat diet with Tolylsam or controls without. Representative micrographs show adipocytes containing a large lipid droplet (L) and a thin rim of cytoplasm (Cy). Collagen (Coll) is accumulated in the intracellular space between adipocytes for the Tolylsam-treated tissues. The scale bar corresponds to 2 μm.

Analysis of plasma metabolic parameters at the end of the experiments did not reveal significant differences between Tolylsam-treated and control mice in levels of glucose, insulin or HDL cholesterol, whereas levels of triglycerides, total cholesterol and LDL cholesterol were significantly reduced by Tolylsam

treatment (Table 28). Triglyceride levels in faeces were similar for both groups (6.1 ± 1.7 versus 5.0 ± 2.3 mg/g faeces, p = 0.7).

	Controls (n = 5)	Inhibitor (n = 5)
Glucose (mg/dl)	134 ± 27	121 ± 28
Insulin (ng/ml)	1.8 ± 0.18	2.2 ± 0.44
Leptin (ng/ml)	22 ± 2.3	8.0 ± 1.8†
Triglycerides (mg/dl)	96 ± 12	42 ± 4.4*
Total cholesterol (mg/dl)	372 ± 12	194 ± 13‡
HDL cholesterol (mg/dl)	181 ± 25	134 ± 11
LDL cholesterol (mg/dl)	172 ± 22	52 ± 5.1†

Table 28. Metabolic parameters of obese mice (C57Bl/6) kept on HFD for 20 weeks, followed by 7 weeks on HFD with or without Tolylsam. Data are means ± SEM of 5 experiments in each group.* p < 0.05 versus controls.

Plasma leptin levels were significantly lower in inhibitor-treated versus control mice (Table 28) and correlated positively with body weight (r = 0.79; p = 0.009), and weight of SC (r = 0.73; p = 0.02) and GON (r = 0.85; p = 0.003) fat, for both groups combined. Leptin mRNA expression was also somewhat reduced in Tolylsam-treated as compared to control adipose tissues: 7232 ± 848 A.U. versus 11376 ± 1793 A.U. (p = 0.08) for SC fat, and 6518 ± 1026 A.U. versus 8196 ± 1491 A.U. (p = 0.38) for GON fat.

Gene expression analysis furthermore revealed upregulation of MMP-2 mRNA in SC (p = 0.016) but not in GON (p = 0.56) adipose tissues treated with Tolylsam, whereas MMP-9 mRNA was significantly upregulated in both SC (p = 0.046) and GON (p = 0.013) inhibitor-treated adipose tissues (Figure 22). TIMP-1 and TIMP-2 mRNA expression in SC or GON adipose tissues were not significantly affected by Tolylsam treatment (data not shown). In agreement with these data, MMP-2 antigen levels in extracts of SC adipose tissues were enhanced upon Tolylsam treatment (p = 0.004), but not in extracts of GON adipose tissues (p = 0.56), and MMP-9 antigen levels following Tolylsam treatment were enhanced in extracts of both SC (p = 0.002) and GON (p = 0.076) adipose tissues. In contrast, Tolylsam

did not affect plasma antigen levels of MMP-2 (105 ± 2.7 ng/ml versus 114 ± 4.2 ng/ml; p = 0.12) or of MMP-9 (67 ± 8.1 ng/ml versus 54 ± 5.0 ng/ml; p = 0.21).

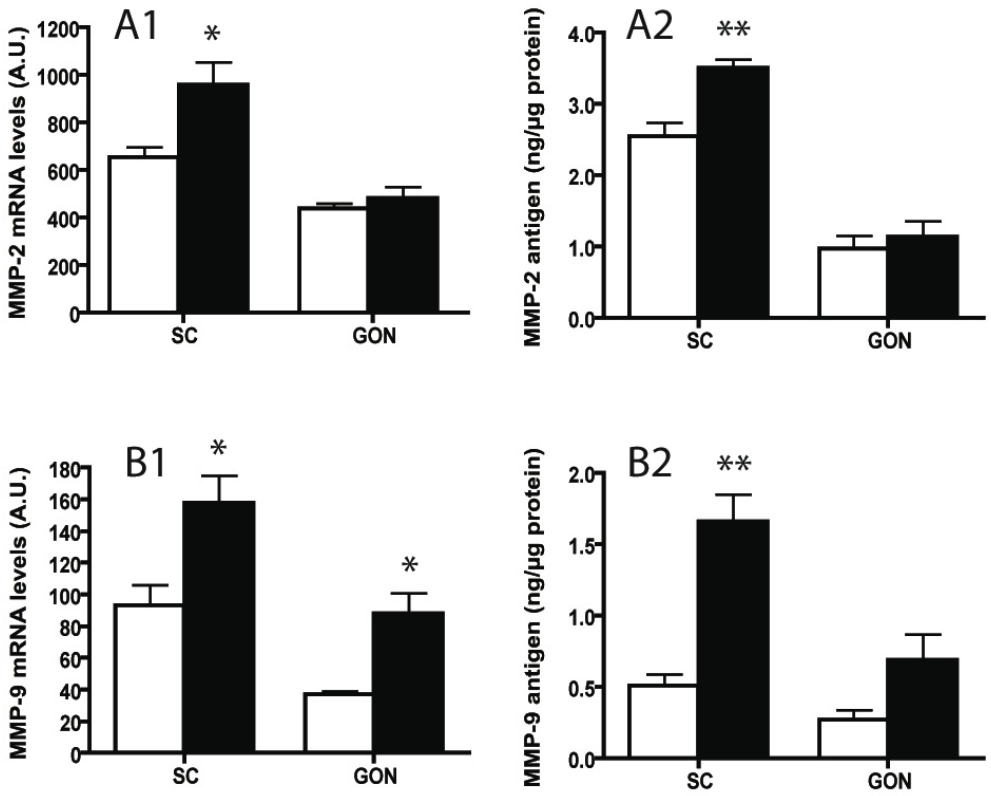


Figure 22. MMP-2 (panels A) and MMP-9 (panels B) mRNA (panels 1) and protein (panels 2) levels in extracts of control (open bars) or Tolylsam-treated (black bars) SC and GON adipose tissues. Data are means ± SEM of 5 determinations. *p < 0.05 and ** p < 0.005 versus controls.

6. Effect of gelatinase inhibition on adipose tissue development in MMP-2 deficient mice

Manuscript in preparation

MMP-2$^{-/-}$ mice fed the HFD supplemented with Tolylsam showed lower body weight gain than control MMP-2$^{-/-}$ mice, resulting in significantly reduced body weight after 15 weeks (Figure 23 and Table 29). Food intake (3.5 ± 0.12 versus 3.5 ± 0.08 g/day for controls; p = 0.5) and body temperature (37.3 ± 0.30 versus 37.2 ± 0.24 C° for controls; p = 0.8) was comparable for both groups.

Weight of SC and GON fat depots was significantly lower between treatment groups. All other organs, with the exception of a smaller spleen and heart, had a comparable weight, as compared to control MMP-2$^{-/-}$ mice (Table 29).

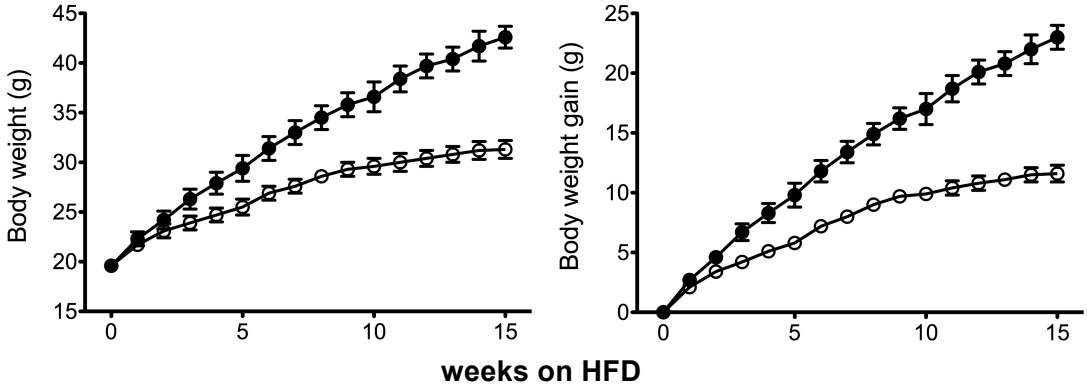


Figure 23. Body weight and weight gain of Tolylsam-treated (open circles) and placebo-treated (black circles) MMP-2$^{-/-}$ kept on HFD for 15 weeks. Data are means ± SEM. Analysis by two-way repeated-measures ANOVA revealed significantly ($p < 0.0001$) reduced body weight and weight gain.

Histological analysis revealed smaller mean adipocyte and blood vessel size in the fat depots of inhibitor-treated mice. The density of adipocytes was higher after treatment with Tolylsam and blood vessel density, normalized for adipocyte density, was significantly lower (Table 30). Total collagen content was higher in SC (19.5 ± 1.4 % versus 14.3 ± 0.51 % in controls, p = 0.006) and in GON (15.0 ± 0.87 % versus 12.5 ± 0.49 % in controls, p = 0.03) adipose tissues of Tolylsam-treated mice.

	Controls (n = 10)	Inhibitor (n = 9)
Body weight start (g)	20 ± 0.5	20 ± 0.5
Body weight end (g)	43 ± 1.1	31 ± 0.9†
SC fat (mg)	1730 ± 66	642 ± 54†
GON fat (mg)	2055 ± 99	795 ± 59†
Lungs (mg)	212 ± 14	189 ± 14
Spleen (mg)	114 ± 5.9	94 ± 4.6*
Heart (mg)	146 ± 5.1	130 ± 3.3*
Pancreas (mg)	335 ± 6.1	329 ± 12
Liver (mg)	2740 ± 217	2577 ± 70
Kidneys (mg)	433 ± 12	408 ± 6.7

Table 29. Effect of Tolylsam on adipose tissue and organ weights of MMP-2$^{-/-}$ mice kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. * p < 0.05, † p < 0.0005 versus MMP-2$^{-/-}$ controls.

	Controls (n = 10)	Inhibitor (n = 9)
Adipocyte size (μm^2)		
SC fat	2460 ± 105	1470 ± 180‡
GON fat	3290 ± 207	1920 ± 168‡
Adipocyte density ($x10^{-6}/\mu m^2$)		
SC fat	296 ± 12	503 ± 51†
GON fat	244 ± 22	370 ± 26†
Blood vessel size (μm^2)		
SC fat	42 ± 2.2	31 ± 1.3†
GON fat	44 ± 1.5	37 ± 1.9*
Blood vessel density ($x10^{-6}/\mu m^2$)		
SC fat	267 ± 15	386 ± 44*
GON fat	202 ± 5.2	230 ± 17
Normalized blood vessel density		
SC fat	0.9 ± 0.1	0.7 ± 0.1*
GON fat	0.9 ± 0.1	0.6 ± 0.1*

Table 30. Effect of Tolylsam on adipocyte and blood vessel size and density in adipose tissues of MMP-2$^{-/-}$ mice (C57Bl/6) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group. * p < 0.05, † p < 0.005, ‡ p < 0.0005 versus MMP-2$^{-/-}$ controls.

Follow-up of metabolic parameters showed significantly reduced levels of circulating leptin and triglycerides, whereas LDL cholesterol levels were higher. Glucose, total cholesterol, HDL cholesterol and insulin levels were not significantly affected (Table 31).

	Controls (n = 10)	Inhibitor (n = 9)
Glucose (mg/dl)	190 ± 15	162 ± 16
Insulin (ng/ml)	3.3 ± 0.7	1.9 ± 0.5
Leptin (ng/ml)	15 ± 0.9	2.7 ± 0.7†
Triglycerides (mg/dl)	51 ± 5.4	21 ± 3.4†
Total cholesterol (mg/dl)	220 ± 6.8	228 ± 10.6
HDL cholesterol (mg/dl)	190 ± 4.1	195 ± 10.9
LDL cholesterol (mg/dl)	19 ± 3.8	29 ± 1.6*

Table 31. Effect of Tolylsam on plasma metabolic parameters of MMP-2$^{-/-}$ mice (C57Bl/6) kept on HFD for 15 weeks. Data are means ± SEM of n experiments in each group.* $p < 0.05$, † $p < 0.005$ versus MMP-2$^{-/-}$ controls.

7. Effect of gelatinase inhibition on *de novo* adipogenesis

Manuscript in preparation

Injection of 3T3-F442A preadipocytes in the back of NUDE mice resulted in the formation of *de novo* fat pads after 4 weeks of HFD feeding in both the Tolylsam-treated and the control mice. Food intake and total body weight gain were comparable in both groups (Table 32).

Weight of the *de novo* fat pads was significantly lower in treated mice, as compared to controls. Haematoxylin/eosin staining confirmed the presence of adipocytes in the newly formed fat pads of both experimental groups (Figure 24 A,B). Quantitative image analysis revealed somewhat smaller adipocytes in Tolylsam-treated mice, although this was not significant, whereas adipocyte density was not affected. Upon Tolylsam treatment, blood vessels in *de novo* adipose tissue were smaller, but the density was comparable, also after normalization to the adipocyte density (Table 32).

	Controls (n = 10)	Inhibitor (n = 10)
Body weight start (g)	18 ± 0.5	18 ± 0.6
Body weight end (g)	23 ± 0.4	22 ± 0.4
Food intake (g/day)	3.7 ± 0.2	3.8 ± 0.1
SC fat (mg)	106 ± 16	99 ± 13
GON fat (mg)	142 ± 24	118 ± 16
De novo fat (mg)	21.4 ± 1.9	16.2 ± 0.8*
Adipocyte size (μm^2)	1081 ± 97	811 ± 61
Adipocyte density ($x10^{-6}/\mu m^2$)	435 ± 18	432 ± 9.1
Blood vessel size (μm^2)	54 ± 1.1	48 ± 0.8†
Blood vessel density ($x10^{-6}/\mu m^2$)	814 ± 58	733 ± 43
Normalized blood vessel density	1.8 ± 0.1	1.7 ± 0.1

Table 32. Effect of Tolylsam on body weight and fat depots in NUDE mice injected with preadipocytes and kept on high fat diet for 4 weeks. Data are means ± SEM of n experiments in each group. *p < 0.01 and † p < 0.0001 versus controls.

Histological analysis of Sirius Red stained sections of *de novo* adipose tissue revealed higher total collagen content in fat pads of treated mice, as compared to controls (23 ± 2.4 % versus 14 ± 2.1 %; p = 0.004) (Figure 24 C-D).

Quantification of ECM breakdown by analysis under polarized light, showed that the ratio of thick/thin fibers was significantly higher after Tolylsam treatment (0.26 ± 0.03 versus 0.14 ± 0.03; p = 0.003), compatible with less structural disorganization (Figure 24 E,F).

Analysis of metabolic parameters did not reveal significant effects of Tolylsam treatment on triglyceride levels (62 ± 9.7 versus 106 ± 24 mg/dl; p = 0.14), whereas total cholesterol (186 ± 15 versus 125 ± 6.0 mg/dl; p = 0.001) and HDL cholesterol (162 ± 14 versus 120 ± 6.9 mg/dl; p = 0.02) were enhanced.

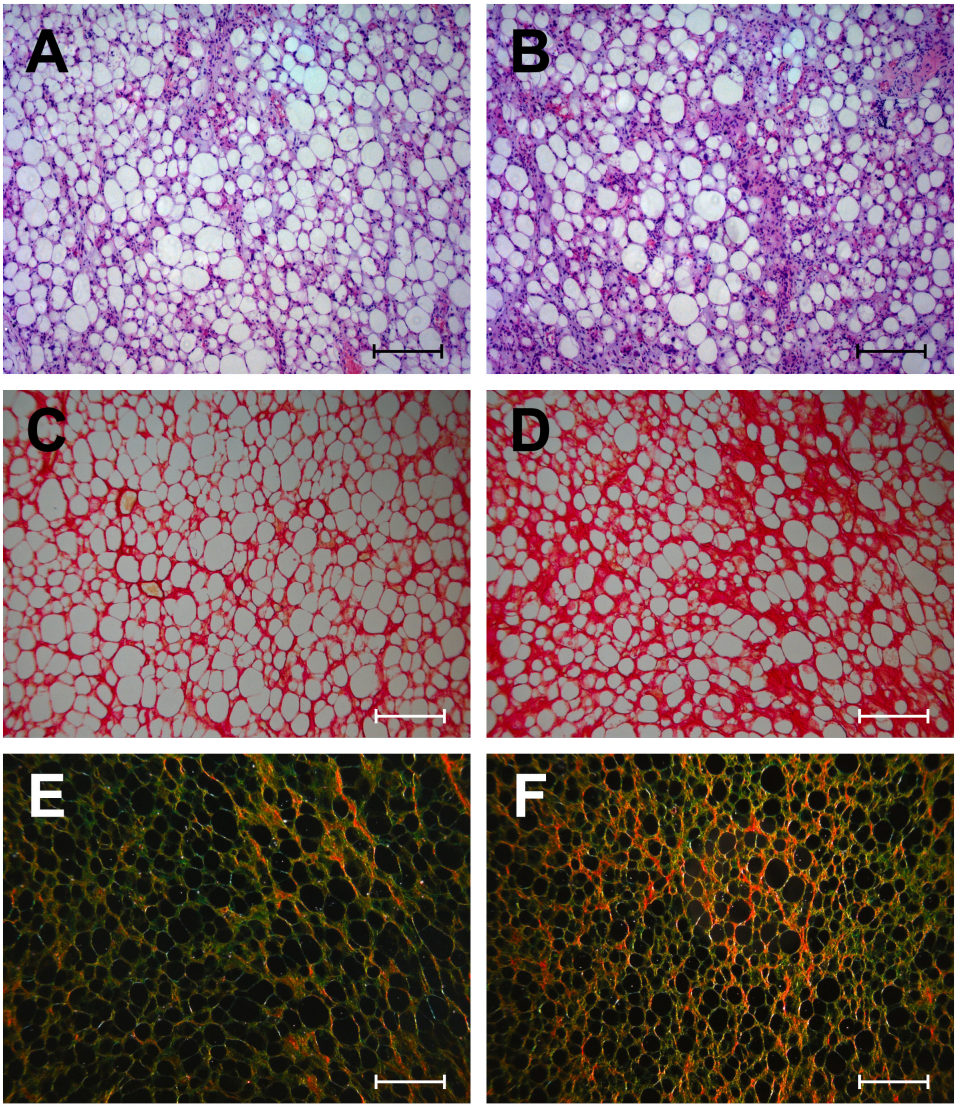


Figure 24. Haematoxylin/eosin (A, B) and Sirius Red staining under normal (C, D) or polarized light (E, F) of *de novo* formed fat pads of NUDE mice, kept on HFD with (right) or without (left) addition of Tolylsam. Intact collagen is colored red and loosely organized collagen fibers are colored green and yellow (E, F). Scale bars represent 200 µm.

8. *In vitro* experiments

8.1 Effect of gelatinase inhibition on 3T3-F442A preadipocyte differentiation

Manuscript in preparation

3T3-F442A preadipocytes were used as a model to study adipocyte differentiation *in vitro*. Two days after reaching confluence (designated as day 0) basal medium was replaced with an induction medium for 48 hours. Thereafter, differentiation medium was added for another 9 days. Throughout the differentiation process, medium was complemented with different concentrations (10, 25, 50 or 100 µM) of the synthetic MMP inhibitor Tolylsam, diluted in DMSO (final concentration 1/1000 for all groups). Media with or without addition of DMSO were used as negative controls.

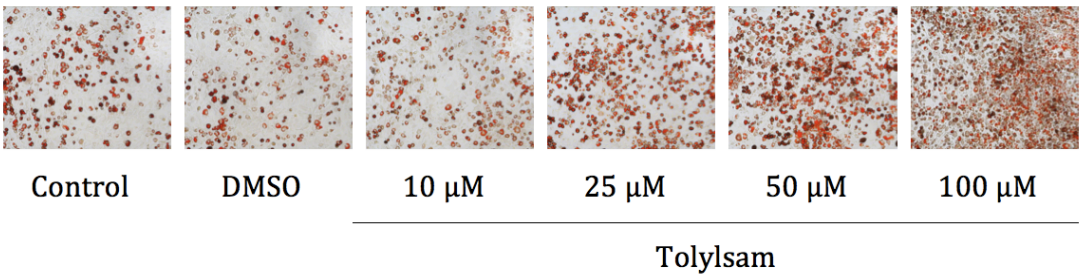


Figure 25. Oil Red O staining of 3T3-F442A cells after 12 days of differentiation. Control cells were compared to DMSO treated cells (final concentration 1/1000) and cells treated with four different doses of Tolylsam (10, 25, 50 or 100 µM).

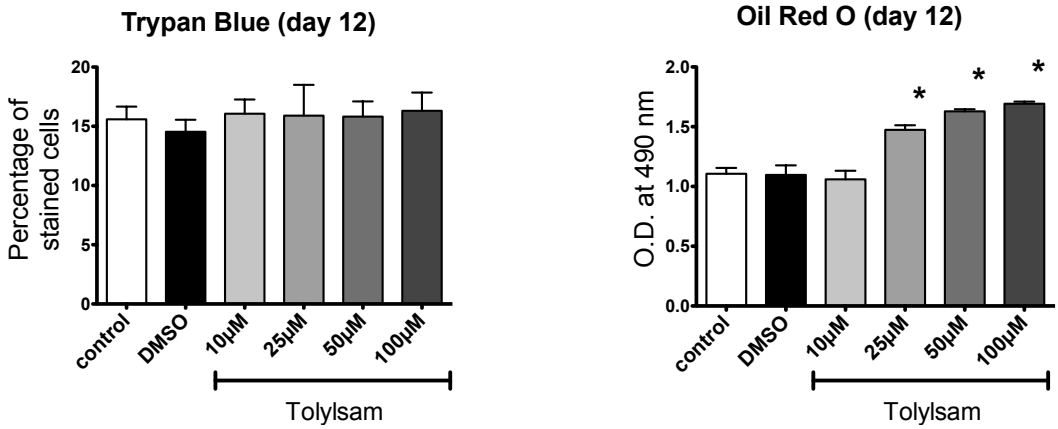


Figure 26. Effect of different concentrations of Tolylsam on the extent of *in vitro* differentiation of 3T3-F442A preadipocytes into mature adipocytes. Quantification of Trypan Blue and Oil Red O staining at Day 12 of differentiation without or with the addition of 10, 25, 50 or 100 µM of Tolylsam. Data are means ± SEM of 4 (Trypan Blue) or 6 (Oil Red O) determinations, * $p < 0.05$ versus control.

Cells that have undergone differentiation in presence of vehicle (DMSO) were not distinct from the control cells. However, addition of Tolylsam to cultured preadipocytes enhanced differentiation in a dose dependent manner (Figure 26 and Figure 25).

Incubation of confluent cells with various concentrations of Tolylsam for 48 hours revealed no toxic side effects in the WST-1 cytotoxicity assay (data not shown). Additionally, cell viability was checked with Trypan Blue staining at experimental day 12. Dead cells stain blue, while live cells exclude the dye. Treatment with DMSO was comparable to the control, which both induced approximately 15 % cell death. Tolylsam-treated cells were, for every concentration, comparable to controls and DMSO with respect to cell death (Figure 26, all $p > 0.05$).

Adipocyte differentiation can be monitored by evaluating the expression pattern of several adipogenic markers such as adiponectin (adipoQ, secreted by adipose tissue), adipocyte protein-2 (aP2; carrier protein for fatty acids), peroxisome proliferator-activated receptor-γ (PPAR-γ; nuclear receptor involved in fatty acid metabolism and adipocyte differentiation) and preadipocyte factor-1 (Pref-1; downregulation of which is required for adipose conversion). To collect the necessary mRNA samples, the previous experiment was repeated using the lowest concentration of inhibitor that showed a significant difference as compared to the controls (25 μM). Cells treated with DMSO were used as a negative control and mRNA samples were collected every two days.

In agreement with previous observations, Oil Red O quantification at experimental day 12 indicated a significant increase of intracellular lipid accumulation after addition of Tolylsam, as compared to DMSO (not shown). Additionally, the mRNA levels of pro-adipogenic markers (PPAR-γ, aP2 and adiponectin) were enhanced by the end of the differentiation procedure and mRNA levels of Pref-1 was somewhat lower (Figure 27).

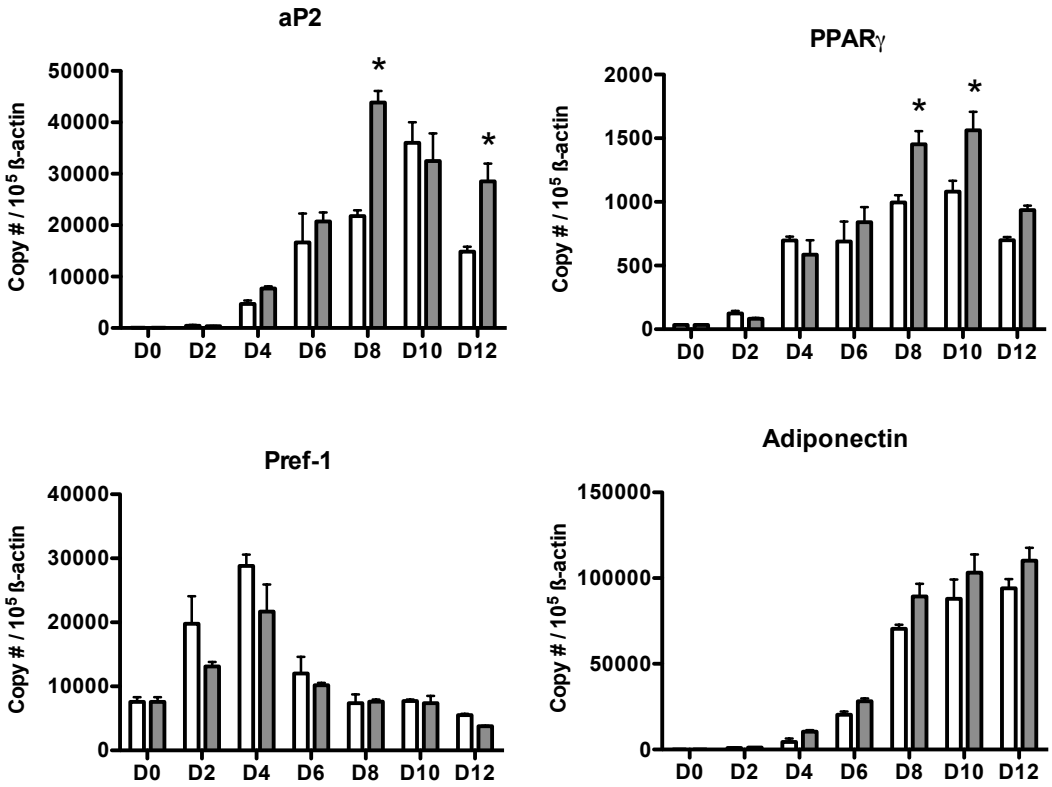


Figure 27. Time-course of the expression of adipogenic markers (preadipocyte factor (Pref-1), peroxisome proliferator-activated receptor (PPAR) γ, adiponectin and adipocyte fatty acid-binding protein 2 (aP2)) during differentiation of 3T3-F442A preadipocytes in the presence of 25 µM Tolylsam (grey bars) or dimethylsulphoxide vehicle (DMSO, white bars). Data are means ± SEM of 4 determinations, * $p < 0.05$ versus controls after Bonferroni correction for multiple testing.

Monitoring of the time course of the expression of MMPs and TIMPs revealed that expression of MMP-2, MMP-9, MMP-14, TIMP-2 was downregulated by Tolylsam at day 2 (and day 4 for MMP-2), but not at the end of the experiment. TIMP-1 mRNA levels were not affected. Expression of VEGF-A was somewhat, though not significantly, upregulated by Tolylsam (Figure 28).

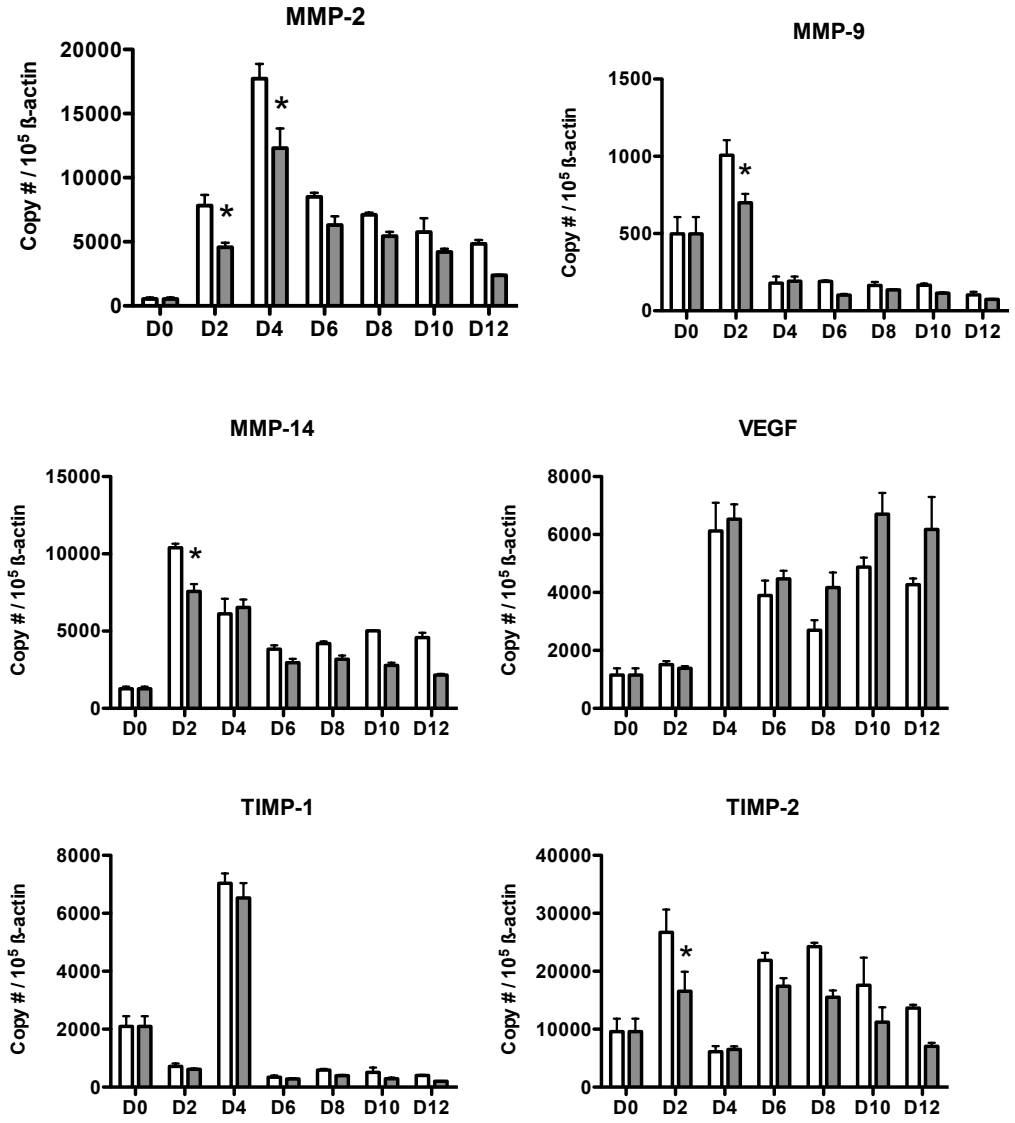


Figure 28. Time-course of the expression of MMP-2, MMP-9, MMP-14, VEGFa, TIMP-1 or TIMP-2 during differentiation of 3T3-F442A preadipocytes in the presence of 25 µM Tolylsam (grey bars) or dimethylsulphoxide vehicle (DMSO, white bars). Data are means ± SEM of 4 determinations, * $p < 0.05$ versus controls after Bonferroni correction for multiple testing.

8.2 Effect of MMP-2 knock-down on 3T3-F442A preadipocytes differentiation

Manuscript in preparation

Using Mission TRC shRNA lentiviral particles directed against MMP-2, stable knock-down of MMP-2 gene expression was achieved in 3T3-F442A preadipocytes with 5 different plasmids (constructs TRCN0000031224-8, hereafter indicated as C1-C5), amounting to 35% - 92% MMP-2 downregulation at experimental day 0 (two days after reaching confluence), as compared to the control plasmid SHC002V (scrambled shRNA, indicated as 2V). Knock-down was verified on gelatin zymograms by comparing conditioned medium of cells transfected with the different constructs with conditioned medium of control cells and unconditioned medium (Figure 29).

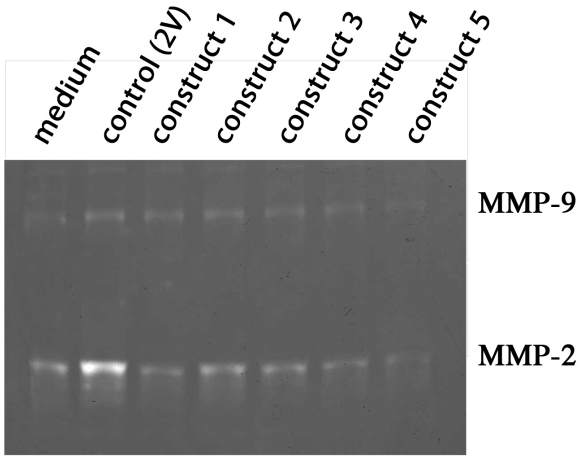


Figure 29. Zymography with unconditioned medium (lane 1), conditioned medium of negative control cells (lane 2) and conditioned medium of cells transfected with five different constructs against MMP-2 (lane 3 – 7).

For all constructs, MMP-2 gene silencing resulted in impaired differentiation of 3T3-F442A preadipocytes, as visualized by Oil Red O staining and analyzed by light microscopy. Quantitative analysis at experimental day 12 confirmed significantly lower intracytoplasmatic lipid content as compared to control (Figure 30).

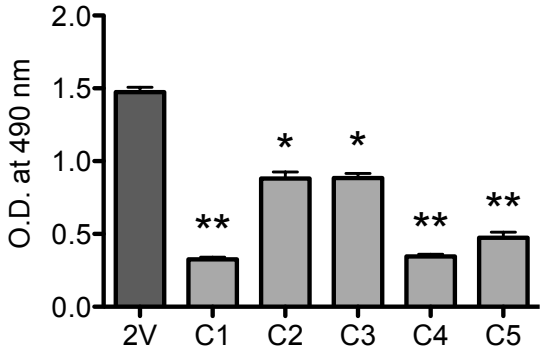


Figure 30. Quantification of Oil Red O staining of cells transfected with scrambled shRNA (2V) or cells transfected with different shRNA constructs against MMP-2 (C1-C5). * $p < 0.05$, ** $p < 0.005$, versus control (2V).

C5 had the most stable MMP-2 knock-down over the 12 day period and was chosen for further analysis. Monitoring of adipogenic markers during differentiation confirmed higher expression of Pref-1 and lower expression of aP2, PPAR-γ and adiponectin, compatible with a lower degree of differentiation upon MMP-2 gene silencing. MMP-9 expression was not affected by MMP-2 knock-down, whereas MMP-14 expression was elevated near the end of the differentiation (Figure 31).

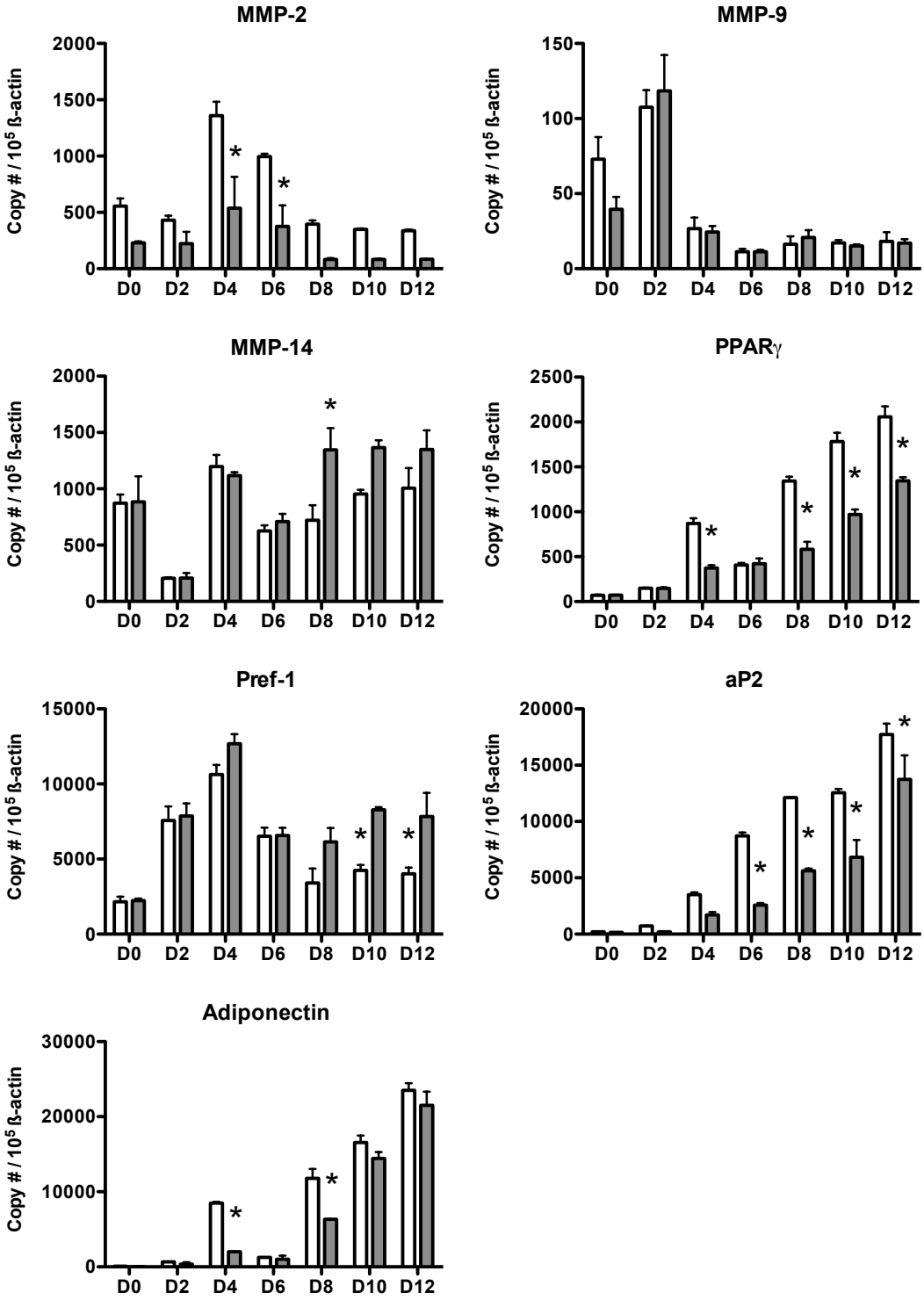


Figure 31. Time-course of the expression of MMP-2, MMP-9, MMP-14 and adipogenic markers (preadipocyte factor (Pref-1), peroxisome proliferator-activated receptor (PPAR) γ, adiponectin and adipocyte fatty acid-binding protein 2 (aP2)) during differentiation of 3T3-F442A cells transfected with shRNA against MMP-2 (grey bars) or scrambled shRNA (white bars). Data are means ± SEM of 4 determinations in 2 independent experiments, * $p < 0.05$ versus controls after Bonferroni correction for multiple testing.

DISCUSSION

Background

Over the last decades obesity and its associated adverse effects have become a worldwide issue, threatening the health of ever-growing numbers of individuals. Development of adipose tissue requires complex modifications of cells and their environments in which several growth factors, cytokines, hormones and proteinases are involved. Recent studies have revealed that the matrix metalloproteinase (MMP) family of proteinases performs a complex and sophisticated role herein by targeting both matrix and non-matrix substrates. Several reports have suggested the potential to modulate adipose tissue development by regulating MMP activity and/or expression. Thus, administration of the broad-spectrum MMP inhibitor Galardin to mice on high fat diet resulted in significantly reduced adipose tissue mass (229). Genetically obese *ob/ob* mice gained somewhat less weight when treated with the MMP inhibitor Bay 12-9566 (230), whereas the relatively gelatinase specific inhibitor Ro 28-2653 had moderate effects on adipose tissue development and cellularity (231). Although these studies support a functional role of the MMP system in adipogenesis and adipose tissue development, the role of individual MMPs remained to be identified, mainly because of overlapping substrate specificity of MMPs and the lack of specific synthetic inhibitors. Follow-up experiments in MMP deficient mice showed that deficiency of MMP-3 (stromelysin-1), MMP-11 (stromelysin-3) or MMP-19 in mice led to enhanced development of adipose tissue when fed a high fat diet (124, 136, 150). In contrast, MMP-14 (membrane type 1 MMP) deficiency led to impaired adipose tissue development (232), whereas deficiency of MMP-10 (stromelysin-2) had no apparent effect on adiposity (184). Furthermore, TIMP-1 deficient mice on a high fat diet gained less weight than wild-type littermates and developed less adipose tissue (158). In contrast, TIMP-2 deficient mice gained more weight than wild-type controls, both on standard chow and high fat diet (233). Thus, MMP activity may be associated with both stimulation and repression of adipose tissue growth. Therefore, the effect of individual MMP family members has to be evaluated separately.

In the present study we have focused on the contribution of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) to adipose tissue development using mice with specific gene inactivation or a more specific gelatinase inhibitor. Both gelatinases may represent particularly interesting targets, as they are secreted by adipocytes (180), their expression is modulated with obesity (181, 183, 234), and a functional role of gelatinases was suggested in the differentiation of preadipocytes into mature adipocytes (180, 235). Furthermore, in humans, MMP-9 plasma levels are enhanced in obese subjects (236-239), whereas for MMP-2 conflicting data have been reported with studies reporting no differences in circulating MMP-2 levels of obese subjects (240) and others observing a decrease of MMP-2 levels with obesity (238, 241).

Lessons from MMP knock-out mice

MMP-9$^{-/-}$ mice did not show gross anatomic abnormalities, and when kept on either SFD or HFD developed adipose tissue to the same extent as their MMP-9$^{+/+}$ littermates. They were somewhat smaller at the age of five weeks but, due to a somewhat higher feeding efficiency, they caught up with their wild-type littermates by the age of 7 weeks. MMP-9$^{-/-}$ mice were previously reported to have smaller long bones (tibia, femur) at birth as a consequence of delayed vascularization and ossification, resulting in a reduced body size. However, subsequent remodeling resolves this condition, resulting in normal bones by eight weeks of age (129). Our MMP-9$^{-/-}$ and MMP-9$^{+/+}$ mice had the same body length around the age of 20 weeks, confirming this finding. Additionally, MMP-9 deficiency had no effect on metabolic parameters, adipose tissue angiogenesis or adipocyte size and density. The elevated MMP-9 blood levels, reported in human studies, may reflect the proinflammatory state of the circulating mononuclear cells (236) and therefore be a consequence of obesity rather than a contributing factor. Indeed, C-reactive protein (CRP) levels, a mediator of systemic inflammation with the ability to induce MMP-9 upregulation in leukocytes (242), are increased in obese subjects and are positively correlated with BMI and MMP-9 levels (243).

MMP-2$^{-/-}$ mice were reported to be smaller at birth and to have slower growth rates (116). This was confirmed in our study, with adult mice being

approximately 10% shorter than their wild-type counterparts. When kept on HFD, MMP-2$^{-/-}$ mice have lower body weight and fat mass as compared to wild-type littermates, associated with marked adipocyte hypotrophy. This may reflect decreased triglyceride storage, since MMP-2 may affect the conversion of circulating plasmatic triglycerides to their storage form as intracellular lipid droplets, maybe by impairing synthesis and/or activity of the lipoprotein lipase. Circulating triglyceride levels in plasma of MMP-2$^{-/-}$ mice were somewhat, but not significantly, elevated as compared to MMP-2$^{+/+}$ littermates.

The weight of several other organs also remained lower in the MMP-2$^{-/-}$ mice. The significantly lower pancreas weight may be related to the observed lower circulating insulin levels in the MMP-2$^{-/-}$ mice. However, the lower plasma insulin levels could also be related to greater insulin sensitivity due to lower adiposity. Indeed, the glucose concentrations and HOMA-IR, are also lower for the MMP-2$^{-/-}$ mice. Analysis of other metabolic parameters furthermore revealed lower levels of cholesterol in MMP-2$^{-/-}$ mice on HFD, suggesting that the cholesterol metabolism is affected.

In conclusion, our data from the gelatinase deficient mice suggest a functional role of MMP-2, but not of MMP-9, in the development of adipose tissue. This is supported by the findings that MMP-2$^{-/-}$, but not MMP-9$^{-/-}$, mice kept on HFD have lower body weight and fat mass as compared to wild-type littermates, associated with marked adipocyte hypotrophy.

Gelatinase inhibition in murine models of obesity

In addition to the gelatinase deficient mouse models, we show the potential to reduce adipose tissue development by pharmacologic inhibition of gelatinases.

The competitive gelatinase inhibitor used in our study, Tolylsam, has been modified so that it is more specific for MMP-2, MMP-9 and MMP-12. IC50 values of this inhibitor, as measured in rats, are: 0.005 μM for MMP-2; 0.049 μM for MMP-9; 0.034 μM for MMP-12; 5.6 μM for MMP-3; 0.40 μM for MMP-8; and 0.20 μM for MMP-13. IC50 for other MMPs and other proteinases is >10 μM. Tolylsam was used effectively at a dose of 50 mg/kg per day in a cerebral aneurysm model in the rat (216). However, since the serum concentration of Tolylsam achieved

upon oral gavage is several fold lower in mice than in rats, we used a dose of 100 mg/kg per day.

Overall, treatment of mice with Tolylsam was well tolerated, did not affect food intake and did not lead to any notable adverse side effects.

Wild-type mice fed with high fat diet supplemented with this inhibitor, showed the same effect on adipose tissue as the MMP-2$^{-/-}$ mice. Treated mice also had reduced fat pads and smaller adipocytes as compared to untreated controls. This adipocyte hypotrophy may be associated with variations in adipokine levels. We have indeed observed that both MMP-2 deficiency and Tolylsam treatment were associated with significantly reduced levels of leptin and with enhanced levels of adiponectin. These variations probably correspond with the variations in body weight and fat mass. Previous studies have reported that body weight (obesity) is negatively correlated with adiponectin levels (244, 245). In contrast, secretion of leptin is positively correlated with body fat mass and adipocyte size (246). However, Tolylsam also reduces adipose tissue development in genetically obese mice (*ob/ob*), deficient in leptin, indicating that its effect is the result of an alternate mechanism.

An interesting observation was that mice treated with inhibitor from the age of five weeks on had smaller blood vessels and lower normalized blood vessel densities as compared to their controls (Table 33). Adipose tissue related angiogenesis, in contrast, was not different for MMP-2$^{-/-}$ mice and controls. Whereas the reduced adipose tissue formation in MMP-2$^{-/-}$ mice on HFD thus appears unrelated to altered angiogenesis, it cannot be excluded that in Tolylsam-treated mice this anti-angiogenic effect plays a role. Indeed, MMP-9, which is inhibited by Tolylsam releases matrix-bound VEGF and thereby induces angiogenesis (247). We observed increased levels of VEGF-A in GON, but not in SC adipose tissues of Tolylsam-treated mice, whereas in either SC or GON fat of MMP-2$^{+/+}$ and MMP-2$^{-/-}$ mice the levels were comparable. It is conceivable that MMP-2 deficiency as such does not affect angiogenesis in fat pads, whereas a combined inhibition of MMP-2, -9 and -12 does. Evidence supporting this, comes from studies using MMP-2/MMP-9 double knock-out mice. Like the single null mutants these mice do not show an overt phenotype. However, they display an amplified vascular defect after injury (248). This observation has been

independently confirmed by tumour invasion and vascularization studies (249). In contrast, administration of Tolylsam to mice with established obesity impaired adipose tissue growth, without affecting the pre-existing vasculature. Indeed, blood vessel density was higher in Tolylsam-treated fat tissues, but this difference disappeared after normalization to adipocyte density. This could imply that the effect of Tolylsam on adipose tissue is dual: it may slow down fat mass accumulation by inhibiting neo-vascularization of the adipose tissue, thereby depriving adipocytes of sufficient access to nutrition and circulating triglycerides, and by inhibiting adipocyte hyperplasia, either directly by affecting cell function or indirectly by reorganization of its surroundings. Indeed, administration of Tolylsam was associated with preservation of ECM collagen. This was also observed after injection of preadipocytes into the back of NUDE mice. In this model, which represents an established model of *in vivo* adipogenesis, we found that development of *de novo* fat pads was significantly impaired upon Tolylsam treatment. This was associated with a trend towards adipocyte hypotrophy. Blood vessel size was lower in Tolylsam-treated *de novo* adipose tissue, suggesting an anti-angiogenic effect in early phase development of blood vessels. Normalized blood vessel density was however not significantly different as also observed in the SC and GON adipose tissues of the obese mice. Similarly to the obese mice, Tolylsam-treated adipose *de novo* tissues had higher collagen levels at the end of the experiments. This is compatible with reduced MMP activity. It is conceivable that the higher content of structurally intact collagen in Tolylsam-treated fat hampers hypertrophic growth of adipocytes, which allows continued weight gain in the HFD control group of the diet study. A similar mechanism has previously been proposed to explain the effect of a broad-spectrum MMP inhibitor on adipose tissue development in a murine model of nutritionally induced obesity (229).

Thus, on the one hand, inhibition of gelatinases by Tolylsam may be associated with preservation of collagen, contributing to adipocyte hypotrophy, whereas on the other hand it may impair adipose tissue related angiogenesis (Table 33).

	Adipocytes		Blood vessels	
	size	density	size	density
MMP-9$^{-/-}$ versus MMP-9$^{+/+}$	≈	≈	≈	≈
MMP-2$^{-/-}$ versus MMP-2$^{+/+}$	↓	↑	≈	≈
MMP-12$^{-/-}$ versus MMP-12$^{+/+}$	≈	≈	≈	≈
Tolylsam-treated wild-type versus placebo-treated controls	↓	↑	↓	↓
Tolylsam-treated Ob/Ob mice versus placebo-treated controls	≈	≈	(↓)	↓
Tolylsam-treated obese mice versus placebo-treated controls	↓	↑	≈	≈
Tolylsam-treated MMP-2$^{-/-}$ mice versus placebo-treated MMP-2$^{-/-}$	↓	↑	↓	↓
De novo fat pads of Tolylsam-treated NuNu mice versus placebo-treated controls	(↓)	≈	↓	≈

Table 33. Effect of MMP-2, -9, -12 deficiency and Tolylsam treatment on adipocyte or blood vessel size and density in mice kept on HFD. Brackets indicate a borderline non-significance.

Inhibitor treatment of five weeks old wild-type mice resulted in a downregulation of MMP-2 expression in SC and GON adipose tissue, but an upregulation of MMP-9 expression. Overall, we did however obtain a marked inhibition of active MMP-2 levels, whereas active MMP-9 levels remained very low. Paradoxically, gelatinase A (MMP-2) and gelatinase B (MMP-9) mRNA expression in the adipose tissues were enhanced upon inhibitor treatment in ob/ob mice and diet-induced obese mice. Thus, it cannot be excluded that gelatinase inhibition in adipose tissues is partially offset by increased expression. Modulation of MMP expression associated with the use of synthetic inhibitors was previously reported for Batimastat (250), Ro 28-2653 (231) and galardin (229).

Given the fact that food intake, physical activity and body temperature were comparable in both treated and control mice in all experimental models,

Tolylsam does not seem to act through reduction of satiety or increased basal metabolism, although additional parameters, such as oxygen consumption, should be verified to provide a more detailed metabolic profile.

Lessons from *in vitro* experiments

Previous *in vitro* studies with proteinase inhibitors and neutralizing antibodies suggested that MMP-2 and MMP-9 play a key role in adipocyte differentiation (180). Our results from the *in vitro* knock-down experiments confirm that it is possible to reduce adipocyte differentiation by targeting the expression of MMP-2, implicating MMP-2 as a potential mediator of adipogenesis.

In contrast to the knock-down experiments, administration of Tolylsam to 3T3-F442A preadipocytes resulted in a dose-dependent enhancement of differentiation, as monitored by Oil Red O staining and expression of adipogenic markers. The apparent contradiction between both *in vitro* studies may be related to the fact that gene knock-down specifically eliminates the target gene, whereas pharmacologic inhibition may affect other MMPs.

Moreover, conflicting data exists on the effect of MMP inhibition using this *in vitro* differentiation model. Treatment of 3T3-F442A preadipocytes with 10 µM of the synthetic MMP inhibitors CT1746 or Batimastat (BB-94) reduced adipose conversion *in vitro* (180, 181). Similar observations were made in 3T3-L1 preadipocytes with 10 µM Batimastat or 25 µM Ilomastat (Galardin) (182, 183). In contrast, Alexander *et al. (251)* reported that 10 µM Ilomastat promotes differentiation, rather than inhibiting it. Not only the concentration of inhibitor, but also the time window wherein it was added to the cells and the concentration and origin of serum supplemented to the medium may be crucial. Therefore, it might be necessary to use 3D matrix systems as a more relevant model of differentiation in vitro (252).

It should also be kept in mind, that while *in vitro* experiments using preadipocyte cell lines provide necessary information at cellular level, it is more difficult to predict the consequences of MMP modulation in a complex biological system. Indeed, while the lack of confounding factors, such as vasculature, extracellular matrix, heterogeneity and 3D organization of cells, is reduced to a minimum *in*

vitro, this impedes extrapolation towards an *in vivo* setting. A detailed understanding of MMP activity *in vivo* therefore remains essential if we are to unravel how MMPs interact with their complex biological environment.

General conclusion

In conclusion, this study reveals divergent functions for gelatinases in the development of adipose tissue: MMP-2 appears to enhance adipose tissue development in mice on high fat diet, at least in part by contributing to adipocyte growth, whereas MMP-9 does not seem crucial. In addition, adipose tissue growth can be reduced by administration of Tolylsam, which is not only a gelatinase inhibitor but also impairs blood vessel formation. This insight might be useful in the search for pharmacological MMP inhibitors that may affect this process.

MMPs as therapeutic targets

While considerable evidence implicates MMPs as important players in several biologic processes, the actual mechanisms underlying their involvement remain mostly unresolved. This is partly due to the multiplicity of MMPs and their distinct but overlapping substrate specificity. Although such a multiplicity of MMPs is advantageous in case of loss of function of one family member, via redundant and compensatory mechanisms, it often confounds efforts to fully understand MMP function and limits the effectiveness of specific therapeutic approaches.

The MMP system is made even more complex by the extensive interactions between the different members that can both positively and negatively influence each other's regulation. Cell-type and tissue-specific regulation, inducible and constitutive expression, and discrepancies between *in vitro* and *in vivo* patterns of expression further contribute to the complexity of the mechanisms affected by MMPs.

Although targeting MMP-2 may lead to reduced fat mass accumulation in mice, inhibiting MMP-2 as a potential therapeutic strategy to treat obesity in humans should be considered with great care. Indeed, MMP-2 deficiency may be associated with serious side effects. While MMP-2 deficient mice have been

described as being overtly normal, inactivating MMP-2 mutations in humans are associated with severe osteolysis and arthritis.

In 2000, a consanguineous Saudi Arabian family with two affected siblings displaying a Nodulosis-Arthropathy-Osteolysis phenotype (NAO; OMIM 605156) was described (253, 254). Via a genome wide search, two family-specific homoallelic non-sense mutations were identified in the MMP-2 gene: R101H and Y244X (255).

In southern Italy, Zankl *et al.* reported a 21-year-old woman who suffered from a severe form of osteolysis that resembled the symptoms of Winchester syndrome (OMIM 277950), caused by a homozygous missense mutation (E404K) in the active site of MMP-2 (256). Similarly, Rouzier *et al.* described two Algerian sisters with a Winchester-like phenotype (257). In both patients, a homozygous deletion in exon 8 of the MMP2 gene was identified. More recently, two affected brothers were reported with a novel mutation (658+2T>C) (258).

How MMP-2 inactivation causes the features of Torg, NAO, and Winchester syndrome is unclear and it may seem contradictory that loss of proteinase activity promotes an osteolytic phenotype. However, MMP-2 also has many other substrates that may affect osteoblast maturation or osteoclast activity. TGFß1 represents such a substrate as it is involved in both processes (107).

It should be kept in mind that complications caused by total MMP-2 deficiency from birth on may not necessarily occur upon pharmacologic inhibition at a later stage when essential developmental processes have already occurred.

Limitations and suggestions for future experiments

The analysis of single gelatinase null mutants revealed surprisingly benign phenotypes with knock-out mice displaying no overt malformations. This lack of serious defects indicates proteolytic redundancy, enzymatic compensation, and/or adaptive development. Since substrate specificities of MMPs may overlap, it is conceivable that lack of MMP-9/MMP-2 is compensated for by another protease (259). For example, in MMP-$2^{-/-}$ mice we observed an upregulation of MMP-9 in SC adipose tissue. As for MMP-$9^{-/-}$ mice, we did not find an upregulation of MMP-2, the most likely candidate, but there are 17 other MMPs and some ADAM (a disintegrin and metalloproteinase) family members with

reported activity against gelatin (260). Deficiency of gelatinases may also affect some of the other proteinases that depend on MMP-2 or MMP-9 for their activation.

Therefore, pharmacologic inhibition represents an alternative model for studying MMP function *in vivo*. Unfortunately, despite intense efforts in the last 25 years, the design of selective inhibitors has proved to be extremely difficult since all MMP members share strikingly similar structures around and within their active sites [119]. The broad inhibitory spectrum of small-molecule MMP inhibitors add to the complexity of interpreting results in experimental set-ups and is the main reason why many clinical trials using these compounds ended with negative results.

Two interesting strategies should be considered for selective MMP targeting in future experiments. One approach is the generation of blocking peptides or monoclonal antibodies (mAb) that target exosite binding sites of MMPs to their macromolecular substrates. Another is the use of RNA interference technology to specifically bind specific MMP target mRNAs and interfere with protein translation and/or promote mRNA degradation. However, problems and difficulties with stability, efficacy of tissue targeting, and off-target effects are first to be addressed as they challenge their potential therapeutic application.

List of publications discussed in this thesis

1. Van Hul M, Piccard H, Lijnen HR. Gelatinase B (MMP-9) deficiency does not affect murine adipose tissue development.
Thromb Haemost. 2010 Jul;104(1):165-71. Epub 2010 Apr 29. PubMed PMID: 20431850. (section 1)

2. Van Hul M, Lijnen HR. A functional role of gelatinase A in the development of nutritionally induced obesity in mice.
J Thromb Haemost. 2008 Jul;6(7):1198-206. Epub 2008 Jul 1. PubMed PMID: 18433461. (sections 2 and 3)

3. Van Hul M, Lijnen HR. Matrix metalloproteinase inhibition impairs murine adipose tissue development independently of leptin.
Endocr J. 2011;58(2):101-7. Epub 2011 Jan 13. PubMed PMID: 21242647. (section 4)

4. Van Hul M, Lupu F, Dresselaers T, Lijnen HR. Matrix metalloproteinase inhibition affects adipose tissue mass in obese mice (submitted for publication) (section 5)

5. Van Hul M, Lijnen HR. Effect of weight loss on gelatinase levels in obese mice.
Clin Exp Pharmacol Physiol. 2011 Sep;38(9):647-9. doi: 10.1111/j.1440-1681.2011.05552.x. PubMed PMID: 21671984. (section 5)

6. Van Hul M, Bauters D, Lijnen HR. Effect of gelatinase inhibition on in vitro and in vivo adipogenesis (manuscript in preparation) (section 7 and 8)

REFERENCES

1. **Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, Paciorek CJ, Singh GM, Gutierrez HR, Lu Y, Bahalim AN, Farzadfar F, Riley LM, Ezzati M**. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. Lancet. 2011. 377:557-67.

2. **Kim S, Popkin BM**. Commentary: understanding the epidemiology of overweight and obesity--a real global public health concern. International journal of epidemiology. 2006. 35:60-7; discussion 81-2.

3. **Popkin BM**. The nutrition transition and its health implications in lower-income countries. Public health nutrition. 1998. 1:5-21.

4. **de Onis M, Blossner M, Borghi E**. Global prevalence and trends of overweight and obesity among preschool children. The American journal of clinical nutrition. 2010. 92:1257-64.

5. **Popkin BM**. The world is fat. Scientific American. 2007. 297:88-95.

6. **Eknoyan G**. Adolphe Quetelet (1796-1874)--the average man and indices of obesity. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2008. 23:47-51.

7. **Gill T, Chittleborough C, Taylor A, Ruffin R, Wilson D, Phillips P**. Body mass index, waist hip ratio, and waist circumference: which measure to classify obesity? Sozial- und Praventivmedizin. 2003. 48:191-200.

8. **Huxley R, Mendis S, Zheleznyakov E, Reddy S, Chan J**. Body mass index, waist circumference and waist:hip ratio as predictors of cardiovascular risk--a review of the literature. European journal of clinical nutrition. 2010. 64:16-22.

9. **Must A, Spadano J, Coakley EH, Field AE, Colditz G, Dietz WH**. The disease burden associated with overweight and obesity. JAMA : the journal of the American Medical Association. 1999. 282:1523-9.

10. **Lewis CE, McTigue KM, Burke LE, Poirier P, Eckel RH, Howard BV, Allison DB, Kumanyika S, Pi-Sunyer FX**. Mortality, health outcomes, and body mass index in the overweight range: a science advisory from the American Heart Association. Circulation. 2009. 119:3263-71.

11. **Jia H, Lubetkin EI**. Trends in quality-adjusted life-years lost contributed by smoking and obesity. American journal of preventive medicine. 2010. 38:138-44.

12. **Boden G**. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes. 1997. 46:3-10.

13. **Hotamisligil GS**. Molecular mechanisms of insulin resistance and the role of the adipocyte. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2000. 24 Suppl 4:S23-7.

14. **Kahn SE, Hull RL, Utzschneider KM**. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 2006. 444:840-6.

15. **Shoelson SE, Lee J, Goldfine AB**. Inflammation and insulin resistance. The Journal of clinical investigation. 2006. 116:1793-801.

16. **Hotamisligil GS**. Inflammation and metabolic disorders. Nature. 2006. 444:860-7.

17. **Kraegen EW, Cooney GJ, Ye JM, Thompson AL, Furler SM**. The role of lipids in the pathogenesis of muscle insulin resistance and beta cell failure in type II diabetes and obesity. Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association. 2001. 109 Suppl 2:S189-201.

18. **Lelliott C, Vidal-Puig AJ**. Lipotoxicity, an imbalance between lipogenesis de novo and fatty acid oxidation. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2004. 28 Suppl 4:S22-8.

19. **Summers SA**. Ceramides in insulin resistance and lipotoxicity. Progress in lipid research. 2006. 45:42-72.

20. **Unger RH**. Minireview: weapons of lean body mass destruction: the role of ectopic lipids in the metabolic syndrome. Endocrinology. 2003. 144:5159-65.

21. **Wolf AM, Colditz GA**. Current estimates of the economic cost of obesity in the United States. Obesity research. 1998. 6:97-106.

22. **Wolf AM**. What is the economic case for treating obesity? Obesity research. 1998. 6 Suppl 1:2S-7S.

23. **Farooqi IS, O'Rahilly S**. Monogenic obesity in humans. Annual review of medicine. 2005. 56:443-58.

24. **Perusse L, Rankinen T, Zuberi A, Chagnon YC, Weisnagel SJ, Argyropoulos G, Walts B, Snyder EE, Bouchard C**. The human obesity gene map: the 2004 update. Obesity research. 2005. 13:381-490.

25. **Boekholdt SM, Peters RJ**. Rimonabant: obituary for a wonder drug. Lancet. 2010. 376:489-90.

26. **James WP, Caterson ID, Coutinho W, Finer N, Van Gaal LF, Maggioni AP, Torp-Pedersen C, Sharma AM, Shepherd GM, Rode RA, Renz CL**. Effect of sibutramine on cardiovascular outcomes in overweight and obese subjects. The New England journal of medicine. 2010. 363:905-17.

27. **Clapham JC, Arch JR, Chapman H, Haynes A, Lister C, Moore GB, Piercy V, Carter SA, Lehner I, Smith SA, Beeley LJ, Godden RJ, Herrity N, Skehel M, Changani KK, Hockings PD, Reid DG, Squires SM, Hatcher J, Trail B, Latcham J, Rastan S, Harper AJ, Cadenas S, Buckingham JA, Brand MD, Abuin A**. Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. Nature. 2000. 406:415-8.

28. **Hensler M, Bardova K, Jilkova ZM, Wahli W, Meztger D, Chambon P, Kopecky J, Flachs P**. The inhibition of fat cell proliferation by n-3 fatty acids in dietary obese mice. Lipids in health and disease. 2011. 10:128.

29. **Zhang Y, Huang C**. Targeting adipocyte apoptosis: A novel strategy for obesity therapy. Biochemical and biophysical research communications. 2011.

30. **Tiraby C, Langin D**. Conversion from white to brown adipocytes: a strategy for the control of fat mass? Trends in endocrinology and metabolism: TEM. 2003. 14:439-41.

31. **Aronne LJ, Powell AG, Apovian CM**. Emerging pharmacotherapy for obesity. Expert opinion on emerging drugs. 2011. 16:587-96.

32. **Klingenberg M, Huang SG**. Structure and function of the uncoupling protein from brown adipose tissue. Biochimica et biophysica acta. 1999. 1415:271-96.

33. **Virtanen KA, Nuutila P**. Brown adipose tissue in humans. Current opinion in lipidology. 2011. 22:49-54.

34. **Lau DC, Dhillon B, Yan H, Szmitko PE, Verma S**. Adipokines: molecular links between obesity and atheroslcerosis. American journal of physiology Heart and circulatory physiology. 2005. 288:H2031-41.

35. **Fischer-Posovszky P, Wabitsch M, Hochberg Z**. Endocrinology of adipose tissue - an update. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme. 2007. 39:314-21.

36. **Maeda K, Okubo K, Shimomura I, Mizuno K, Matsuzawa Y, Matsubara K**. Analysis of an expression profile of genes in the human adipose tissue. Gene. 1997. 190:227-35.

37. **Siiteri PK**. Adipose tissue as a source of hormones. The American journal of clinical nutrition. 1987. 45:277-82.

38. **Kim S, Moustaid-Moussa N**. Secretory, endocrine and autocrine/paracrine function of the adipocyte. The Journal of nutrition. 2000. 130:3110S-5S.

39. **Morrison RF, Farmer SR**. Hormonal signaling and transcriptional control of adipocyte differentiation. The Journal of nutrition. 2000. 130:3116S-21S.

40. **Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr.** Obesity is associated with macrophage accumulation in adipose tissue. The Journal of clinical investigation. 2003. 112:1796-808.

41. **Frayn KN, Karpe F, Fielding BA, Macdonald IA, Coppack SW**. Integrative physiology of human adipose tissue. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2003. 27:875-88.

42. **Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H**. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. The Journal of clinical investigation. 2003. 112:1821-30.

43. **Caspar-Bauguil S, Cousin B, Galinier A, Segafredo C, Nibbelink M, Andre M, Casteilla L, Penicaud L**. Adipose tissues as an ancestral immune organ: site-specific change in obesity. FEBS letters. 2005. 579:3487-92.

44. **Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW**. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology. 2004. 145:2273-82.

45. **Zeyda M, Farmer D, Todoric J, Aszmann O, Speiser M, Gyori G, Zlabinger GJ, Stulnig TM**. Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. International journal of obesity. 2007. 31:1420-8.

46. **Peinado JR, Jimenez-Gomez Y, Pulido MR, Ortega-Bellido M, Diaz-Lopez C, Padillo FJ, Lopez-Miranda J, Vazquez-Martinez R, Malagon MM**. The stromal-vascular fraction of adipose tissue contributes to major differences between subcutaneous and visceral fat depots. Proteomics. 2010. 10:3356-66.

47. **Divoux A, Clement K**. Architecture and the extracellular matrix: the still unappreciated components of the adipose tissue. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2011. 12:e494-503.

48. **Hausman DB, DiGirolamo M, Bartness TJ, Hausman GJ, Martin RJ**. The biology of white adipocyte proliferation. Obesity reviews : an official

journal of the International Association for the Study of Obesity. 2001. 2:239-54.

49. **Gregoire FM**. Adipocyte differentiation: from fibroblast to endocrine cell. Experimental biology and medicine. 2001. 226:997-1002.

50. **Smas CM, Sul HS**. Control of adipocyte differentiation. The Biochemical journal. 1995. 309 (Pt 3):697-710.

51. **Hausman GJ, Richardson RL**. Adipose tissue angiogenesis. Journal of animal science. 2004. 82:925-34.

52. **Bouloumie A, Lolmede K, Sengenes C, Galitzky J, Lafontan M**. Angiogenesis in adipose tissue. Annales d'endocrinologie. 2002. 63:91-5.

53. **Silverman KJ, Lund DP, Zetter BR, Lainey LL, Shahood JA, Freiman DG, Folkman J, Barger AC**. Angiogenic activity of adipose tissue. Biochemical and biophysical research communications. 1988. 153:347-52.

54. **Farias MM, Cuevas AM, Rodriguez F**. Set-point theory and obesity. Metabolic syndrome and related disorders. 2011. 9:85-9.

55. **Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Naslund E, Britton T, Concha H, Hassan M, Ryden M, Frisen J, Arner P**. Dynamics of fat cell turnover in humans. Nature. 2008. 453:783-7.

56. **Rosen ED, MacDougald OA**. Adipocyte differentiation from the inside out. Nature reviews Molecular cell biology. 2006. 7:885-96.

57. **Gregoire FM, Smas CM, Sul HS**. Understanding adipocyte differentiation. Physiological reviews. 1998. 78:783-809.

58. **Ailhaud G, Grimaldi P, Negrel R**. Cellular and molecular aspects of adipose tissue development. Annual review of nutrition. 1992. 12:207-33.

59. **Burdi AR, Poissonnet CM, Garn SM, Lavelle M, Sabet MD, Bridges P**. Adipose tissue growth patterns during human gestation: a histometric comparison of buccal and gluteal fat depots. International journal of obesity. 1985. 9:247-56.

60. **Andrade ZA, de-Oliveira-Filho J, Fernandes AL**. Interrelationship between adipocytes and fibroblasts during acute damage to the subcutaneous adipose tissue of rats: an ultrastructural study. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al]. 1998. 31:659-64.

61. **Meng L, Zhou J, Sasano H, Suzuki T, Zeitoun KM, Bulun SE**. Tumor necrosis factor alpha and interleukin 11 secreted by malignant breast

epithelial cells inhibit adipocyte differentiation by selectively down-regulating CCAAT/enhancer binding protein alpha and peroxisome proliferator-activated receptor gamma: mechanism of desmoplastic reaction. Cancer research. 2001. 61:2250-5.

62. **Souza SC, Palmer HJ, Kang YH, Yamamoto MT, Muliro KV, Paulson KE, Greenberg AS**. TNF-alpha induction of lipolysis is mediated through activation of the extracellular signal related kinase pathway in 3T3-L1 adipocytes. Journal of cellular biochemistry. 2003. 89:1077-86.

63. **Andarawewa KL, Motrescu ER, Chenard MP, Gansmuller A, Stoll I, Tomasetto C, Rio MC**. Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front. Cancer research. 2005. 65:10862-71.

64. **Green H, Kehinde O**. Sublines of mouse 3T3 cells that accumulate lipid. Cell. 1974. 1:113-6.

65. **Student AK, Hsu RY, Lane MD**. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. The Journal of biological chemistry. 1980. 255:4745-50.

66. **Chang TH, Polakis SE**. Differentiation of 3T3-L1 fibroblasts to adipocytes. Effect of insulin and indomethacin on the levels of insulin receptors. The Journal of biological chemistry. 1978. 253:4693-6.

67. **Costa M, Manen CA, Russell DH**. In vivo activation of cAMP-dependent protein kinase by aminophylline and 1-methyl, 3-isobutylxanthine. Biochemical and biophysical research communications. 1975. 65:75-81.

68. **Elks ML, Manganiello VC, Vaughan M**. Hormone-sensitive particulate cAMP phosphodiesterase activity in 3T3-L1 adipocytes. Regulation of responsiveness by dexamethasone. The Journal of biological chemistry. 1983. 258:8582-7.

69. **Ramirez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W**. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. Histochemistry. 1992. 97:493-7.

70. **Green H, Meuth M**. An established pre-adipose cell line and its differentiation in culture. Cell. 1974. 3:127-33.

71. **Mandrup S, Loftus TM, MacDougald OA, Kuhajda FP, Lane MD**. Obese gene expression at in vivo levels by fat pads derived from s.c. implanted 3T3-F442A preadipocytes. Proceedings of the National Academy of Sciences of the United States of America. 1997. 94:4300-5.

72. **Gross J, Lapiere CM**. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proceedings of the National Academy of Sciences of the United States of America. 1962. 48:1014-22.

73. **Harper E, Gross J**. Separation of collagenase and peptidase activities of tadpole tissues in culture. Biochimica et biophysica acta. 1970. 198:286-92.

74. **Birkedal-Hansen H**. From tadpole collagenase to a family of matrix metalloproteinases. Journal of oral pathology. 1988. 17:445-51.

75. **Sternlicht MD, Werb Z**. How matrix metalloproteinases regulate cell behavior. Annual review of cell and developmental biology. 2001. 17:463-516.

76. **Bode W, Fernandez-Catalan C, Tschesche H, Grams F, Nagase H, Maskos K**. Structural properties of matrix metalloproteinases. Cellular and molecular life sciences : CMLS. 1999. 55:639-52.

77. **Springman EB, Angleton EL, Birkedal-Hansen H, Van Wart HE**. Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proceedings of the National Academy of Sciences of the United States of America. 1990. 87:364-8.

78. **Van Wart HE, Birkedal-Hansen H**. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proceedings of the National Academy of Sciences of the United States of America. 1990. 87:5578-82.

79. **Trexler M, Briknarova K, Gehrmann M, Llinas M, Patthy L**. Peptide ligands for the fibronectin type II modules of matrix metalloproteinase 2 (MMP-2). The Journal of biological chemistry. 2003. 278:12241-6.

80. **Knauper V, Docherty AJ, Smith B, Tschesche H, Murphy G**. Analysis of the contribution of the hinge region of human neutrophil collagenase (HNC, MMP-8) to stability and collagenolytic activity by alanine scanning mutagenesis. FEBS letters. 1997. 405:60-4.

81. **Gururajan R, Grenet J, Lahti JM, Kidd VJ**. Isolation and characterization of two novel metalloproteinase genes linked to the Cdc2L locus on human chromosome 1p36.3. Genomics. 1998. 52:101-6.

82. **Park HI, Ni J, Gerkema FE, Liu D, Belozerov VE, Sang QX**. Identification and characterization of human endometase (Matrix metalloproteinase-26) from endometrial tumor. The Journal of biological chemistry. 2000. 275:20540-4.

83. **Murphy G, Allan JA, Willenbrock F, Cockett MI, O'Connell JP, Docherty AJ**. The role of the C-terminal domain in collagenase and stromelysin specificity. The Journal of biological chemistry. 1992. 267:9612-8.

84. **Sanchez-Lopez R, Alexander CM, Behrendtsen O, Breathnach R, Werb Z**. Role of zinc-binding- and hemopexin domain-encoded sequences in the substrate specificity of collagenase and stromelysin-2 as revealed by

chimeric proteins. The Journal of biological chemistry. 1993. 268:7238-47.

85. **Itoh Y, Kajita M, Kinoh H, Mori H, Okada A, Seiki M**. Membrane type 4 matrix metalloproteinase (MT4-MMP, MMP-17) is a glycosylphosphatidylinositol-anchored proteinase. The Journal of biological chemistry. 1999. 274:34260-6.

86. **Kojima S, Itoh Y, Matsumoto S, Masuho Y, Seiki M**. Membrane-type 6 matrix metalloproteinase (MT6-MMP, MMP-25) is the second glycosyl-phosphatidyl inositol (GPI)-anchored MMP. FEBS letters. 2000. 480:142-6.

87. **Fini ME, Cook JR, Mohan R**. Proteolytic mechanisms in corneal ulceration and repair. Archives of dermatological research. 1998. 290 Suppl:S12-23.

88. **Overall CM, Wrana JL, Sodek J**. Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. The Journal of biological chemistry. 1991. 266:14064-71.

89. **Delany AM, Brinckerhoff CE**. Post-transcriptional regulation of collagenase and stromelysin gene expression by epidermal growth factor and dexamethasone in cultured human fibroblasts. Journal of cellular biochemistry. 1992. 50:400-10.

90. **Delany AM, Jeffrey JJ, Rydziel S, Canalis E**. Cortisol increases interstitial collagenase expression in osteoblasts by post-transcriptional mechanisms. The Journal of biological chemistry. 1995. 270:26607-12.

91. **Vincenti MP**. The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes. Transcriptional and posttranscriptional regulation, signal transduction and cell-type-specific expression. Methods in molecular biology. 2001. 151:121-48.

92. **Akhtar N, Rasheed Z, Ramamurthy S, Anbazhagan AN, Voss FR, Haqqi TM**. MicroRNA-27b regulates the expression of matrix metalloproteinase 13 in human osteoarthritis chondrocytes. Arthritis and rheumatism. 2010. 62:1361-71.

93. **Chen KC, Wang YS, Hu CY, Chang WC, Liao YC, Dai CY, Juo SH**. OxLDL up-regulates microRNA-29b, leading to epigenetic modifications of MMP-2/MMP-9 genes: a novel mechanism for cardiovascular diseases. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2011. 25:1718-28.

94. **Gabriely G, Wurdinger T, Kesari S, Esau CC, Burchard J, Linsley PS, Krichevsky AM**. MicroRNA 21 promotes glioma invasion by targeting

matrix metalloproteinase regulators. Molecular and cellular biology. 2008. 28:5369-80.

95. **Nakasa T, Miyaki S, Okubo A, Hashimoto M, Nishida K, Ochi M, Asahara H**. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. Arthritis and rheumatism. 2008. 58:1284-92.

96. **Roy S, Khanna S, Hussain SR, Biswas S, Azad A, Rink C, Gnyawali S, Shilo S, Nuovo GJ, Sen CK**. MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovascular research. 2009. 82:21-9.

97. **Stanczyk J, Pedrioli DM, Brentano F, Sanchez-Pernaute O, Kolling C, Gay RE, Detmar M, Gay S, Kyburz D**. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. Arthritis and rheumatism. 2008. 58:1001-9.

98. **Sun L, Yan W, Wang Y, Sun G, Luo H, Zhang J, Wang X, You Y, Yang Z, Liu N**. MicroRNA-10b induces glioma cell invasion by modulating MMP-14 and uPAR expression via HOXD10. Brain research. 2011. 1389:9-18.

99. **Luo D, Mari B, Stoll I, Anglard P**. Alternative splicing and promoter usage generates an intracellular stromelysin 3 isoform directly translated as an active matrix metalloproteinase. The Journal of biological chemistry. 2002. 277:25527-36.

100. **Matsumoto S, Katoh M, Saito S, Watanabe T, Masuho Y**. Identification of soluble type of membrane-type matrix metalloproteinase-3 formed by alternatively spliced mRNA. Biochimica et biophysica acta. 1997. 1354:159-70.

101. **Hasty KA, Pourmotabbed TF, Goldberg GI, Thompson JP, Spinella DG, Stevens RM, Mainardi CL**. Human neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. The Journal of biological chemistry. 1990. 265:11421-4.

102. **Pei D, Weiss SJ**. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature. 1995. 375:244-7.

103. **Sottrup-Jensen L, Birkedal-Hansen H**. Human fibroblast collagenase-alpha-macroglobulin interactions. Localization of cleavage sites in the bait regions of five mammalian alpha-macroglobulins. The Journal of biological chemistry. 1989. 264:393-401.

104. **Woessner JF, Nagase H**. Matrix metalloproteinases and TIMPs. Oxford: Oxford University Press; 2000.

105. **Stricker TP, Dumin JA, Dickeson SK, Chung L, Nagase H, Parks WC, Santoro SA**. Structural analysis of the alpha(2) integrin I

domain/procollagenase-1 (matrix metalloproteinase-1) interaction. The Journal of biological chemistry. 2001. 276:29375-81.

106. **Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresh DA**. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell. 1996. 85:683-93.

107. **Yu Q, Stamenkovic I**. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes & development. 2000. 14:163-76.

108. **Yu WH, Woessner JF, Jr.** Heparan sulfate proteoglycans as extracellular docking molecules for matrilysin (matrix metalloproteinase 7). The Journal of biological chemistry. 2000. 275:4183-91.

109. **Caterina JJ, Yamada S, Caterina NC, Longenecker G, Holmback K, Shi J, Yermovsky AE, Engler JA, Birkedal-Hansen H**. Inactivating mutation of the mouse tissue inhibitor of metalloproteinases-2(Timp-2) gene alters proMMP-2 activation. The Journal of biological chemistry. 2000. 275:26416-22.

110. **Toth M, Bernardo MM, Gervasi DC, Soloway PD, Wang Z, Bigg HF, Overall CM, DeClerck YA, Tschesche H, Cher ML, Brown S, Mobashery S, Fridman R**. Tissue inhibitor of metalloproteinase (TIMP)-2 acts synergistically with synthetic matrix metalloproteinase (MMP) inhibitors but not with TIMP-4 to enhance the (Membrane type 1)-MMP-dependent activation of pro-MMP-2. The Journal of biological chemistry. 2000. 275:41415-23.

111. **Wang Z, Juttermann R, Soloway PD**. TIMP-2 is required for efficient activation of proMMP-2 in vivo. The Journal of biological chemistry. 2000. 275:26411-5.

112. **Olson MW, Toth M, Gervasi DC, Sado Y, Ninomiya Y, Fridman R**. High affinity binding of latent matrix metalloproteinase-9 to the alpha2(IV) chain of collagen IV. The Journal of biological chemistry. 1998. 273:10672-81.

113. **Toth M, Sado Y, Ninomiya Y, Fridman R**. Biosynthesis of alpha2(IV) and alpha1(IV) chains of collagen IV and interactions with matrix metalloproteinase-9. Journal of cellular physiology. 1999. 180:131-9.

114. **Yang Z, Kyriakides TR, Bornstein P**. Matricellular proteins as modulators of cell-matrix interactions: adhesive defect in thrombospondin 2-null fibroblasts is a consequence of increased levels of matrix metalloproteinase-2. Molecular biology of the cell. 2000. 11:3353-64.

115. **Yang Z, Strickland DK, Bornstein P**. Extracellular matrix metalloproteinase 2 levels are regulated by the low density lipoprotein-

related scavenger receptor and thrombospondin 2. The Journal of biological chemistry. 2001. 276:8403-8.

116. **Itoh T, Ikeda T, Gomi H, Nakao S, Suzuki T, Itohara S**. Unaltered secretion of beta-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2)-deficient mice. The Journal of biological chemistry. 1997. 272:22389-92.

117. **Kato T, Kure T, Chang JH, Gabison EE, Itoh T, Itohara S, Azar DT**. Diminished corneal angiogenesis in gelatinase A-deficient mice. FEBS letters. 2001. 508:187-90.

118. **Wiseman BS, Sternlicht MD, Lund LR, Alexander CM, Mott J, Bissell MJ, Soloway P, Itohara S, Werb Z**. Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. The Journal of cell biology. 2003. 162:1123-33.

119. **Kheradmand F, Rishi K, Werb Z**. Signaling through the EGF receptor controls lung morphogenesis in part by regulating MT1-MMP-mediated activation of gelatinase A/MMP2. Journal of cell science. 2002. 115:839-48.

120. **Itoh T, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S**. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer research. 1998. 58:1048-51.

121. **Inoue K, Mikuni-Takagaki Y, Oikawa K, Itoh T, Inada M, Noguchi T, Park JS, Onodera T, Krane SM, Noda M, Itohara S**. A crucial role for matrix metalloproteinase 2 in osteocytic canalicular formation and bone metabolism. The Journal of biological chemistry. 2006. 281:33814-24.

122. **VanSaun M, Herrera AA, Werle MJ**. Structural alterations at the neuromuscular junctions of matrix metalloproteinase 3 null mutant mice. Journal of neurocytology. 2003. 32:1129-42.

123. **Bullard KM, Lund L, Mudgett JS, Mellin TN, Hunt TK, Murphy B, Ronan J, Werb Z, Banda MJ**. Impaired wound contraction in stromelysin-1-deficient mice. Annals of surgery. 1999. 230:260-5.

124. **Maquoi E, Demeulemeester D, Voros G, Collen D, Lijnen HR**. Enhanced nutritionally induced adipose tissue development in mice with stromelysin-1 gene inactivation. Thrombosis and haemostasis. 2003. 89:696-704.

125. **Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, Lopez-Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, Parks WC**. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science. 1999. 286:113-7.

126. **Dunsmore SE, Saarialho-Kere UK, Roby JD, Wilson CL, Matrisian LM, Welgus HG, Parks WC**. Matrilysin expression and function in airway epithelium. The Journal of clinical investigation. 1998. 102:1321-31.

127. **Balbin M, Fueyo A, Tester AM, Pendas AM, Pitiot AS, Astudillo A, Overall CM, Shapiro SD, Lopez-Otin C**. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nature genetics. 2003. 35:252-7.

128. **Van Lint P, Wielockx B, Puimege L, Noel A, Lopez-Otin C, Libert C**. Resistance of collagenase-2 (matrix metalloproteinase-8)-deficient mice to TNF-induced lethal hepatitis. Journal of immunology. 2005. 175:7642-9.

129. **Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z**. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell. 1998. 93:411-22.

130. **Larsen PH, Wells JE, Stallcup WB, Opdenakker G, Yong VW**. Matrix metalloproteinase-9 facilitates remyelination in part by processing the inhibitory NG2 proteoglycan. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2003. 23:11127-35.

131. **Colnot C, Thompson Z, Miclau T, Werb Z, Helms JA**. Altered fracture repair in the absence of MMP9. Development. 2003. 130:4123-33.

132. **Galis ZS, Johnson C, Godin D, Magid R, Shipley JM, Senior RM, Ivan E**. Targeted disruption of the matrix metalloproteinase-9 gene impairs smooth muscle cell migration and geometrical arterial remodeling. Circulation research. 2002. 91:852-9.

133. **Larsen PH, DaSilva AG, Conant K, Yong VW**. Myelin formation during development of the CNS is delayed in matrix metalloproteinase-9 and -12 null mice. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006. 26:2207-14.

134. **Kassim SY, Gharib SA, Mecham BH, Birkland TP, Parks WC, McGuire JK**. Individual matrix metalloproteinases control distinct transcriptional responses in airway epithelial cells infected with Pseudomonas aeruginosa. Infection and immunity. 2007. 75:5640-50.

135. **Masson R, Lefebvre O, Noel A, Fahime ME, Chenard MP, Wendling C, Kebers F, LeMeur M, Dierich A, Foidart JM, Basset P, Rio MC**. In vivo evidence that the stromelysin-3 metalloproteinase contributes in a paracrine manner to epithelial cell malignancy. The Journal of cell biology. 1998. 140:1535-41.

136. **Lijnen HR, Van HB, Frederix L, Rio MC, Collen D**. Adipocyte hypertrophy in stromelysin-3 deficient mice with nutritionally induced obesity. Thrombosis and haemostasis. 2002. 87:530-5.

137. **Wells JE, Rice TK, Nuttall RK, Edwards DR, Zekki H, Rivest S, Yong VW**. An adverse role for matrix metalloproteinase 12 after spinal cord injury in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2003. 23:10107-15.

138. **Houghton AM, Grisolano JL, Baumann ML, Kobayashi DK, Hautamaki RD, Nehring LC, Cornelius LA, Shapiro SD**. Macrophage elastase (matrix metalloproteinase-12) suppresses growth of lung metastases. Cancer research. 2006. 66:6149-55.

139. **Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, Lopez-Otin C, Krane SM**. Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. Proceedings of the National Academy of Sciences of the United States of America. 2004. 101:17192-7.

140. **Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp-Kistner M, Angel P, Werb Z**. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. Development. 2004. 131:5883-95.

141. **Uchinami H, Seki E, Brenner DA, D'Armiento J**. Loss of MMP 13 attenuates murine hepatic injury and fibrosis during cholestasis. Hepatology. 2006. 44:420-9.

142. **Deguchi JO, Aikawa E, Libby P, Vachon JR, Inada M, Krane SM, Whittaker P, Aikawa M**. Matrix metalloproteinase-13/collagenase-3 deletion promotes collagen accumulation and organization in mouse atherosclerotic plaques. Circulation. 2005. 112:2708-15.

143. **Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, Pidoux I, Ward JM, Birkedal-Hansen H**. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell. 1999. 99:81-92.

144. **Holmbeck K, Bianco P, Chrysovergis K, Yamada S, Birkedal-Hansen H**. MT1-MMP-dependent, apoptotic remodeling of unmineralized cartilage: a critical process in skeletal growth. The Journal of cell biology. 2003. 163:661-71.

145. **Holmbeck K, Bianco P, Pidoux I, Inoue S, Billinghurst RC, Wu W, Chrysovergis K, Yamada S, Birkedal-Hansen H, Poole AR**. The metalloproteinase MT1-MMP is required for normal development and maintenance of osteocyte processes in bone. Journal of cell science. 2005. 118:147-56.

146. **Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K**. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix

metalloproteinase I. Proceedings of the National Academy of Sciences of the United States of America. 2000. 97:4052-7.

147. **Oblander SA, Zhou Z, Galvez BG, Starcher B, Shannon JM, Durbeej M, Arroyo AG, Tryggvason K, Apte SS**. Distinctive functions of membrane type 1 matrix-metalloprotease (MT1-MMP or MMP-14) in lung and submandibular gland development are independent of its role in pro-MMP-2 activation. Developmental biology. 2005. 277:255-69.

148. **Beertsen W, Holmbeck K, Niehof A, Bianco P, Chrysovergis K, Birkedal-Hansen H, Everts V**. On the role of MT1-MMP, a matrix metalloproteinase essential to collagen remodeling, in murine molar eruption and root growth. European journal of oral sciences. 2002. 110:445-51.

149. **Atkinson JJ, Holmbeck K, Yamada S, Birkedal-Hansen H, Parks WC, Senior RM**. Membrane-type 1 matrix metalloproteinase is required for normal alveolar development. Developmental dynamics : an official publication of the American Association of Anatomists. 2005. 232:1079-90.

150. **Pendas AM, Folgueras AR, Llano E, Caterina J, Frerard F, Rodriguez F, Astudillo A, Noel A, Birkedal-Hansen H, Lopez-Otin C**. Diet-induced obesity and reduced skin cancer susceptibility in matrix metalloproteinase 19-deficient mice. Molecular and cellular biology. 2004. 24:5304-13.

151. **Caterina JJ, Skobe Z, Shi J, Ding Y, Simmer JP, Birkedal-Hansen H, Bartlett JD**. Enamelysin (matrix metalloproteinase 20)-deficient mice display an amelogenesis imperfecta phenotype. The Journal of biological chemistry. 2002. 277:49598-604.

152. **Komori K, Nonaka T, Okada A, Kinoh H, Hayashita-Kinoh H, Yoshida N, Yana I, Seiki M**. Absence of mechanical allodynia and Abeta-fiber sprouting after sciatic nerve injury in mice lacking membrane-type 5 matrix metalloproteinase. FEBS letters. 2004. 557:125-8.

153. **Manicone AM, Birkland TP, Lin M, Betsuyaku T, van Rooijen N, Lohi J, Keski-Oja J, Wang Y, Skerrett SJ, Parks WC**. Epilysin (MMP-28) restrains early macrophage recruitment in Pseudomonas aeruginosa pneumonia. Journal of immunology. 2009. 182:3866-76.

154. **Zhou HE, Zhang X, Nothnick WB**. Disruption of the TIMP-1 gene product is associated with accelerated endometrial gland formation during early postnatal uterine development. Biology of reproduction. 2004. 71:534-9.

155. **Chaillan FA, Rivera S, Marchetti E, Jourquin J, Werb Z, Soloway PD, Khrestchatisky M, Roman FS**. Involvement of tissue inhibition of metalloproteinases-1 in learning and memory in mice. Behavioural brain research. 2006. 173:191-8.

156. **Mohammed FF, Pennington CJ, Kassiri Z, Rubin JS, Soloway PD, Ruther U, Edwards DR, Khokha R**. Metalloproteinase inhibitor TIMP-1 affects hepatocyte cell cycle via HGF activation in murine liver regeneration. Hepatology. 2005. 41:857-67.

157. **Lee MM, Yoon BJ, Osiewicz K, Preston M, Bundy B, van Heeckeren AM, Werb Z, Soloway PD**. Tissue inhibitor of metalloproteinase 1 regulates resistance to infection. Infection and immunity. 2005. 73:661-5.

158. **Lijnen HR, Demeulemeester D, Van Hoef B, Collen D, Maquoi E**. Deficiency of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) impairs nutritionally induced obesity in mice. Thrombosis and haemostasis. 2003. 89:249-55.

159. **Jaworski DM, Soloway P, Caterina J, Falls WA**. Tissue inhibitor of metalloproteinase-2(TIMP-2)-deficient mice display motor deficits. Journal of neurobiology. 2006. 66:82-94.

160. **Fata JE, Leco KJ, Voura EB, Yu HY, Waterhouse P, Murphy G, Moorehead RA, Khokha R**. Accelerated apoptosis in the Timp-3-deficient mammary gland. The Journal of clinical investigation. 2001. 108:831-41.

161. **Gill SE, Pape MC, Khokha R, Watson AJ, Leco KJ**. A null mutation for tissue inhibitor of metalloproteinases-3 (Timp-3) impairs murine bronchiole branching morphogenesis. Developmental biology. 2003. 261:313-23.

162. **Cruz-Munoz W, Sanchez OH, Di Grappa M, English JL, Hill RP, Khokha R**. Enhanced metastatic dissemination to multiple organs by melanoma and lymphoma cells in timp-3-/- mice. Oncogene. 2006. 25:6489-96.

163. **English JL, Kassiri Z, Koskivirta I, Atkinson SJ, Di Grappa M, Soloway PD, Nagase H, Vuorio E, Murphy G, Khokha R**. Individual Timp deficiencies differentially impact pro-MMP-2 activation. The Journal of biological chemistry. 2006. 281:10337-46.

164. **Page-McCaw A, Ewald AJ, Werb Z**. Matrix metalloproteinases and the regulation of tissue remodelling. Nature reviews Molecular cell biology. 2007. 8:221-33.

165. **Fanjul-Fernandez M, Folgueras AR, Cabrera S, Lopez-Otin C**. Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models. Biochimica et biophysica acta. 2010. 1803:3-19.

166. **Dorman G, Cseh S, Hajdu I, Barna L, Konya D, Kupai K, Kovacs L, Ferdinandy P**. Matrix metalloproteinase inhibitors: a critical appraisal of design principles and proposed therapeutic utility. Drugs. 2010. 70:949-64.

167. **Lia NG, Shib ZH, Tang YP, Duan JA**. Selective matrix metalloproteinase inhibitors for cancer. Current medicinal chemistry. 2009. 16:3805-27.

168. **Fingleton B**. Matrix metalloproteinases as valid clinical targets. Current pharmaceutical design. 2007. 13:333-46.

169. **Fingleton B**. MMPs as therapeutic targets--still a viable option? Seminars in cell & developmental biology. 2008. 19:61-8.

170. **Turk B**. Targeting proteases: successes, failures and future prospects. Nature reviews Drug discovery. 2006. 5:785-99.

171. **Lopez-Otin C, Matrisian LM**. Emerging roles of proteases in tumour suppression. Nature reviews Cancer. 2007. 7:800-8.

172. **Lopez-Otin C, Palavalli LH, Samuels Y**. Protective roles of matrix metalloproteinases: from mouse models to human cancer. Cell cycle. 2009. 8:3657-62.

173. **Coussens LM, Tinkle CL, Hanahan D, Werb Z**. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell. 2000. 103:481-90.

174. **Pozzi A, Moberg PE, Miles LA, Wagner S, Soloway P, Gardner HA**. Elevated matrix metalloprotease and angiostatin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization. Proceedings of the National Academy of Sciences of the United States of America. 2000. 97:2202-7.

175. **Mudgett JS, Hutchinson NI, Chartrain NA, Forsyth AJ, McDonnell J, Singer, II, Bayne EK, Flanagan J, Kawka D, Shen CF, Stevens K, Chen H, Trumbauer M, Visco DM**. Susceptibility of stromelysin 1-deficient mice to collagen-induced arthritis and cartilage destruction. Arthritis and rheumatism. 1998. 41:110-21.

176. **Johnson JL, George SJ, Newby AC, Jackson CL**. Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. Proceedings of the National Academy of Sciences of the United States of America. 2005. 102:15575-80.

177. **Dorman G, Kocsis-Szommer K, Spadoni C, Ferdinandy P**. MMP inhibitors in cardiac diseases: an update. Recent patents on cardiovascular drug discovery. 2007. 2:186-94.

178. **Brown LM, Fox HL, Hazen SA, LaNoue KF, Rannels SR, Lynch CJ**. Role of the matrixin MMP-2 in multicellular organization of adipocytes cultured in basement membrane components. The American journal of physiology. 1997. 272:C937-49.

179. **Lijnen HR, Maquoi E, Holvoet P, Mertens A, Lupu F, Morange P, Alessi MC, Juhan-Vague I**. Adipose tissue expression of gelatinases in mouse models of obesity. Thrombosis and haemostasis. 2001. 85:1111-6.

180. **Bouloumie A, Sengenes C, Portolan G, Galitzky J, Lafontan M**. Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. Diabetes. 2001. 50:2080-6.

181. **Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR**. Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. Diabetes. 2002. 51:1093-101.

182. **Croissandeau G, Chretien M, Mbikay M**. Involvement of matrix metalloproteinases in the adipose conversion of 3T3-L1 preadipocytes. The Biochemical journal. 2002. 364:739-46.

183. **Chavey C, Mari B, Monthouel MN, Bonnafous S, Anglard P, Van Obberghen E, Tartare-Deckert S**. Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. The Journal of biological chemistry. 2003. 278:11888-96.

184. **Lijnen HR, Van Hoef B, Rodriguez JA, Paramo JA**. Stromelysin-2 (MMP-10) deficiency does not affect adipose tissue formation in a mouse model of nutritionally induced obesity. Biochemical and biophysical research communications. 2009. 389:378-81.

185. **Demeulemeester D, Scroyen I, Voros G, Snoeys J, De Geest B, Collen D, Lijnen HR**. Overexpression of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) in mice does not affect adipogenesis or adipose tissue development. Thrombosis and haemostasis. 2006. 95:1019-24.

186. **Xia T, Akers K, Eisen AZ, Seltzer JL**. Comparison of cleavage site specificity of gelatinases A and B using collagenous peptides. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology. 1996. 1293:259-66.

187. **Huhtala P, Chow LT, Tryggvason K**. Structure of the human type IV collagenase gene. The Journal of biological chemistry. 1990. 265:11077-82.

188. **Huhtala P, Tuuttila A, Chow LT, Lohi J, Keski-Oja J, Tryggvason K**. Complete structure of the human gene for 92-kDa type IV collagenase. Divergent regulation of expression for the 92- and 72-kilodalton enzyme genes in HT-1080 cells. The Journal of biological chemistry. 1991. 266:16485-90.

189. **Brown PD, Levy AT, Margulies IM, Liotta LA, Stetler-Stevenson WG**. Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer research. 1990. 50:6184-91.

190. **Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H, Larjava H**. Transforming growth factor-beta 1 up-regulates type IV collagenase

expression in cultured human keratinocytes. The Journal of biological chemistry. 1991. 266:11436-41.

191. **Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI**. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. The Journal of biological chemistry. 1995. 270:5331-8.

192. **Hernandez-Barrantes S, Toth M, Bernardo MM, Yurkova M, Gervasi DC, Raz Y, Sang QA, Fridman R**. Binding of active (57 kDa) membrane type 1-matrix metalloproteinase (MT1-MMP) to tissue inhibitor of metalloproteinase (TIMP)-2 regulates MT1-MMP processing and pro-MMP-2 activation. The Journal of biological chemistry. 2000. 275:12080-9.

193. **Greenlee KJ, Corry DB, Engler DA, Matsunami RK, Tessier P, Cook RG, Werb Z, Kheradmand F**. Proteomic identification of in vivo substrates for matrix metalloproteinases 2 and 9 reveals a mechanism for resolution of inflammation. Journal of immunology. 2006. 177:7312-21.

194. **McQuibban GA, Butler GS, Gong JH, Bendall L, Power C, Clark-Lewis I, Overall CM**. Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. The Journal of biological chemistry. 2001. 276:43503-8.

195. **Dean RA, Overall CM**. Proteomics discovery of metalloproteinase substrates in the cellular context by iTRAQ labeling reveals a diverse MMP-2 substrate degradome. Molecular & cellular proteomics : MCP. 2007. 6:611-23.

196. **Ochieng J, Fridman R, Nangia-Makker P, Kleiner DE, Liotta LA, Stetler-Stevenson WG, Raz A**. Galectin-3 is a novel substrate for human matrix metalloproteinases-2 and -9. Biochemistry. 1994. 33:14109-14.

197. **Corry DB, Rishi K, Kanellis J, Kiss A, Song Lz LZ, Xu J, Feng L, Werb Z, Kheradmand F**. Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency. Nature immunology. 2002. 3:347-53.

198. **Fowlkes JL, Thrailkill KM, Serra DM, Suzuki K, Nagase H**. Matrix metalloproteinases as insulin-like growth factor binding protein-degrading proteinases. Progress in growth factor research. 1995. 6:255-63.

199. **Schonbeck U, Mach F, Libby P**. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. Journal of immunology. 1998. 161:3340-6.

200. **Ito A, Mukaiyama A, Itoh Y, Nagase H, Thogersen IB, Enghild JJ, Sasaguri Y, Mori Y**. Degradation of interleukin 1beta by matrix

metalloproteinases. The Journal of biological chemistry. 1996. 271:14657-60.

201. **Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL, et al.** Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature. 1994. 370:555-7.

202. **Fernandez-Patron C, Radomski MW, Davidge ST**. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. Circulation research. 1999. 85:906-11.

203. **Toth M, Chvyrkova I, Bernardo MM, Hernandez-Barrantes S, Fridman R**. Pro-MMP-9 activation by the MT1-MMP/MMP-2 axis and MMP-3: role of TIMP-2 and plasma membranes. Biochemical and biophysical research communications. 2003. 308:386-95.

204. **D'Angelo M, Yan Z, Nooreyazdan M, Pacifici M, Sarment DS, Billings PC, Leboy PS**. MMP-13 is induced during chondrocyte hypertrophy. Journal of cellular biochemistry. 2000. 77:678-93.

205. **McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM**. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. Blood. 2002. 100:1160-7.

206. **Levi E, Fridman R, Miao HQ, Ma YS, Yayon A, Vlodavsky I**. Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1. Proceedings of the National Academy of Sciences of the United States of America. 1996. 93:7069-74.

207. **Tholozan FM, Gribbon C, Li Z, Goldberg MW, Prescott AR, McKie N, Quinlan RA**. FGF-2 release from the lens capsule by MMP-2 maintains lens epithelial cell viability. Molecular biology of the cell. 2007. 18:4222-31.

208. **Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G**. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. Blood. 2000. 96:2673-81.

209. **Patterson BC, Sang QA**. Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). The Journal of biological chemistry. 1997. 272:28823-5.

210. **Liu Z, Zhou X, Shapiro SD, Shipley JM, Twining SS, Diaz LA, Senior RM, Werb Z**. The serpin alpha1-proteinase inhibitor is a critical substrate for gelatinase B/MMP-9 in vivo. Cell. 2000. 102:647-55.

211. **Sheu BC, Hsu SM, Ho HN, Lien HC, Huang SC, Lin RH**. A novel role of metalloproteinase in cancer-mediated immunosuppression. Cancer research. 2001. 61:237-42.

212. **Vaisar T, Kassim SY, Gomez IG, Green PS, Hargarten S, Gough PJ, Parks WC, Wilson CL, Raines EW, Heinecke JW**. MMP-9 sheds the beta2 integrin subunit (CD18) from macrophages. Molecular & cellular proteomics : MCP. 2009. 8:1044-60.

213. **Corry DB, Kiss A, Song LZ, Song L, Xu J, Lee SH, Werb Z, Kheradmand F**. Overlapping and independent contributions of MMP2 and MMP9 to lung allergic inflammatory cell egression through decreased CC chemokines. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2004. 18:995-7.

214. **Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D**. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nature cell biology. 2000. 2:737-44.

215. **Giles AR**. Guidelines for the use of animals in biomedical research. Thrombosis and haemostasis. 1987. 58:1078-84.

216. **Aoki T, Kataoka H, Morimoto M, Nozaki K, Hashimoto N**. Macrophage-derived matrix metalloproteinase-2 and -9 promote the progression of cerebral aneurysms in rats. Stroke; a journal of cerebral circulation. 2007. 38:162-9.

217. **Copeland RA**. Enzymes : a practical introduction to structure, mechanism, and data analysis. 2nd ed. New York ; London: Wiley; 2000.

218. **Mandrup S, Loftus TM, MacDougald OA, Kuhajda FP, Lane MD**. Obese gene expression at in vivo levels by fat pads derived from s.c. implanted 3T3-F442A preadipocytes. Proc Natl Acad Sci U S A. 1997. 94:4300-5.

219. **Neels JG, Thinnes T, Loskutoff DJ**. Angiogenesis in an in vivo model of adipose tissue development. FASEB J. 2004. 18:983-5.

220. **Lijnen HR, Christiaens V, Scroyen I, Voros G, Tjwa M, Carmeliet P, Collen D**. Impaired adipose tissue development in mice with inactivation of placental growth factor function. Diabetes. 2006. 55:2698-704.

221. **Laitinen L**. Griffonia simplicifolia lectins bind specifically to endothelial cells and some epithelial cells in mouse tissues. The Histochemical journal. 1987. 19:225-34.

222. **Cao R, Brakenhielm E, Wahlestedt C, Thyberg J, Cao Y**. Leptin induces vascular permeability and synergistically stimulates angiogenesis with FGF-2 and VEGF. Proceedings of the National Academy of Sciences of the United States of America. 2001. 98:6390-5.

223. **Whittaker P, Kloner RA, Boughner DR, Pickering JG**. Quantitative assessment of myocardial collagen with picrosirius red staining and circularly polarized light. Basic research in cardiology. 1994. 89:397-410.

224. **Alexander CM, Werb Z**. Targeted disruption of the tissue inhibitor of metalloproteinases gene increases the invasive behavior of primitive mesenchymal cells derived from embryonic stem cells in vitro. The Journal of cell biology. 1992. 118:727-39.

225. **Kleiner DE, Stetler-Stevenson WG**. Quantitative zymography: detection of picogram quantities of gelatinases. Analytical biochemistry. 1994. 218:325-9.

226. **Girish V, Vijayalakshmi A**. Affordable image analysis using NIH Image/ImageJ. Indian journal of cancer. 2004. 41:47.

227. **Fields GB**. Using fluorogenic peptide substrates to assay matrix metalloproteinases. Methods in molecular biology. 2001. 151:495-518.

228. **Bachmanov AA, Reed DR, Tordoff MG, Price RA, Beauchamp GK**. Nutrient preference and diet-induced adiposity in C57BL/6ByJ and 129P3/J mice. Physiology & behavior. 2001. 72:603-13.

229. **Lijnen HR, Maquoi E, Hansen LB, Van Hoef B, Frederix L, Collen D**. Matrix metalloproteinase inhibition impairs adipose tissue development in mice. Arteriosclerosis, thrombosis, and vascular biology. 2002. 22:374-9.

230. **Rupnick MA, Panigrahy D, Zhang CY, Dallabrida SM, Lowell BB, Langer R, Folkman MJ**. Adipose tissue mass can be regulated through the vasculature. Proceedings of the National Academy of Sciences of the United States of America. 2002. 99:10730-5.

231. **Demeulemeester D, Collen D, Lijnen HR**. Effect of matrix metalloproteinase inhibition on adipose tissue development. Biochemical and biophysical research communications. 2005. 329:105-10.

232. **Chun TH, Hotary KB, Sabeh F, Saltiel AR, Allen ED, Weiss SJ**. A pericellular collagenase directs the 3-dimensional development of white adipose tissue. Cell. 2006. 125:577-91.

233. **Stradecki HM, Jaworski DM**. Hyperphagia and leptin resistance in tissue inhibitor of metalloproteinase-2 deficient mice. Journal of neuroendocrinology. 2011. 23:269-81.

234. **Lijnen HR, Maquoi E, Demeulemeester D, Van Hoef B, Collen D**. Modulation of fibrinolytic and gelatinolytic activity during adipose tissue development in a mouse model of nutritionally induced obesity. Thrombosis and haemostasis. 2002. 88:345-53.

235. **Bourlier V, Zakaroff-Girard A, De Barros S, Pizzacalla C, de Saint Front VD, Lafontan M, Bouloumie A, Galitzky J**. Protease inhibitor treatments reveal specific involvement of matrix metalloproteinase-9 in human adipocyte differentiation. The Journal of pharmacology and experimental therapeutics. 2005. 312:1272-9.

236. **Ghanim H, Aljada A, Hofmeyer D, Syed T, Mohanty P, Dandona P**. Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation. 2004. 110:1564-71.

237. **Glowinska-Olszewska B, Urban M**. Elevated matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1 in obese children and adolescents. Metabolism: clinical and experimental. 2007. 56:799-805.

238. **Glowinska-Olszewska B, Urban M, Florys B**. [Selected matrix metalloproteinases (MMP-2, MMP-9) in obese children and adolescents]. Endokrynologia, diabetologia i choroby przemiany materii wieku rozwojowego : organ Polskiego Towarzystwa Endokrynologow Dzieciecych. 2006. 12:179-83.

239. **Patel C, Ghanim H, Ravishankar S, Sia CL, Viswanathan P, Mohanty P, Dandona P**. Prolonged reactive oxygen species generation and nuclear factor-kappaB activation after a high-fat, high-carbohydrate meal in the obese. The Journal of clinical endocrinology and metabolism. 2007. 92:4476-9.

240. **Belo VA, Souza-Costa DC, Lana CM, Caputo FL, Marcaccini AM, Gerlach RF, Bastos MG, Tanus-Santos JE**. Assessment of matrix metalloproteinase (MMP)-2, MMP-8, MMP-9, and their inhibitors, the tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in obese children and adolescents. Clinical biochemistry. 2009. 42:984-90.

241. **Kosmala W, Plaksej R, Przewlocka-Kosmala M, Kuliczkowska-Plaksej J, Bednarek-Tupikowska G, Mazurek W**. Matrix metalloproteinases 2 and 9 and their tissue inhibitors 1 and 2 in premenopausal obese women: relationship to cardiac function. International journal of obesity. 2008. 32:763-71.

242. **Bisoendial RJ, Birjmohun RS, Akdim F, van 't Veer C, Spek CA, Hartman D, de Groot ER, Bankaitis-Davis DM, Kastelein JJ, Stroes ES**. C-reactive protein elicits white blood cell activation in humans. Am J Med. 2009. 122:582 e1-9.

243. **Van Hul M, Piccard H, Lijnen HR**. Gelatinase B (MMP-9) deficiency does not affect murine adipose tissue development. Thrombosis and haemostasis. 2010. 104:165-71.

244. **Bullen JW, Jr., Bluher S, Kelesidis T, Mantzoros CS**. Regulation of adiponectin and its receptors in response to development of diet-induced

obesity in mice. American journal of physiology Endocrinology and metabolism. 2007. 292:E1079-86.

245. **Hu E, Liang P, Spiegelman BM**. AdipoQ is a novel adipose-specific gene dysregulated in obesity. The Journal of biological chemistry. 1996. 271:10697-703.

246. **Friedman JM, Halaas JL**. Leptin and the regulation of body weight in mammals. Nature. 1998. 395:763-70.

247. **Lee MH, Atkinson S, Murphy G**. Identification of the extracellular matrix (ECM) binding motifs of tissue inhibitor of metalloproteinases (TIMP)-3 and effective transfer to TIMP-1. The Journal of biological chemistry. 2007. 282:6887-98.

248. **Lambert V, Wielockx B, Munaut C, Galopin C, Jost M, Itoh T, Werb Z, Baker A, Libert C, Krell HW, Foidart JM, Noel A, Rakic JM**. MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2003. 17:2290-2.

249. **Masson V, de la Ballina LR, Munaut C, Wielockx B, Jost M, Maillard C, Blacher S, Bajou K, Itoh T, Itohara S, Werb Z, Libert C, Foidart JM, Noel A**. Contribution of host MMP-2 and MMP-9 to promote tumor vascularization and invasion of malignant keratinocytes. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2005. 19:234-6.

250. **Arlt M, Kopitz C, Pennington C, Watson KL, Krell HW, Bode W, Gansbacher B, Khokha R, Edwards DR, Kruger A**. Increase in gelatinase-specificity of matrix metalloproteinase inhibitors correlates with antimetastatic efficacy in a T-cell lymphoma model. Cancer research. 2002. 62:5543-50.

251. **Alexander CM, Selvarajan S, Mudgett J, Werb Z**. Stromelysin-1 regulates adipogenesis during mammary gland involution. The Journal of cell biology. 2001. 152:693-703.

252. **Justice BA, Badr NA, Felder RA**. 3D cell culture opens new dimensions in cell-based assays. Drug discovery today. 2009. 14:102-7.

253. **Al Aqeel A, Al Sewairi W, Edress B, Gorlin RJ, Desnick RJ, Martignetti JA**. Inherited multicentric osteolysis with arthritis: a variant resembling Torg syndrome in a Saudi family. American journal of medical genetics. 2000. 93:11-8.

254. **Al-Mayouf SM, Majeed M, Hugosson C, Bahabri S**. New form of idiopathic osteolysis: nodulosis, arthropathy and osteolysis (NAO) syndrome. American journal of medical genetics. 2000. 93:5-10.

255. **Martignetti JA, Aqeel AA, Sewairi WA, Boumah CE, Kambouris M, Mayouf SA, Sheth KV, Eid WA, Dowling O, Harris J, Glucksman MJ, Bahabri S, Meyer BF, Desnick RJ**. Mutation of the matrix metalloproteinase 2 gene (MMP2) causes a multicentric osteolysis and arthritis syndrome. Nature genetics. 2001. 28:261-5.

256. **Zankl A, Bonafe L, Calcaterra V, Di Rocco M, Superti-Furga A**. Winchester syndrome caused by a homozygous mutation affecting the active site of matrix metalloproteinase 2. Clinical genetics. 2005. 67:261-6.

257. **Rouzier C, Vanatka R, Bannwarth S, Philip N, Coussement A, Paquis-Flucklinger V, Lambert JC**. A novel homozygous MMP2 mutation in a family with Winchester syndrome. Clinical genetics. 2006. 69:271-6.

258. **Gok F, Crettol LM, Alanay Y, Hacihamdioglu B, Kocaoglu M, Bonafe L, Ozen S**. Clinical and radiographic findings in two brothers affected with a novel mutation in matrix metalloproteinase 2 gene. European journal of pediatrics. 2010. 169:363-7.

259. **Kolaczkowska E, Grzybek W, van Rooijen N, Piccard H, Plytycz B, Arnold B, Opdenakker G**. Neutrophil elastase activity compensates for a genetic lack of matrix metalloproteinase-9 (MMP-9) in leukocyte infiltration in a model of experimental peritonitis. J Leukoc Biol. 2009. 85:374-81.

260. **Visse R, Nagase H**. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 2003. 92:827-39.

SUMMARY

In 1999 the World Health Organization (WHO) declared obesity a worldwide epidemic. Today, it is estimated that around 500 million people worldwide are obese, and more than 1.6 billion are overweight. Diseases associated with excess body weight include among others: cardiovascular disease, hypertension, stroke, diabetes type II, atherosclerosis and certain forms of cancer. As a result, obesity recently became the number one cause of preventable death.

Obesity is defined as an excessively high amount of adipose tissue in relation to lean body mass. Development of adipose tissue is a complex process in which several growth factors, cytokines, hormones and proteinases contribute to adipogenesis (formation of adipocytes), angiogenesis (formation of blood vessels) and proteolytic remodeling of the extracellular matrix (ECM). The matrix metalloproteinases (MMPs) form a family of zinc-dependent endopeptidases that play an important role in these processes by degradation of ECM and basement membrane components, by altering cell interactions and/or by activation of latent growth factors. This has led to the hypothesis that modulation of the activity of certain MMPs may have an effect on adipose tissue development.

In the present study we have focused on the contribution of MMP-2 (gelatinase A) and MMP-9 (gelatinase B). It was previously shown that they are both expressed by adipocytes and modulated during development of adipose tissue. To evaluate their potential role in adipogenesis, we set up a range of *in vivo* and *in vitro* experiments using different murine models.

In vivo:

- MMP-2 and MMP-9 deficient mice were kept on a high fat diet (HFD) for 15 weeks and compared to wild-type littermates. This revealed no differences for the MMP-9-deficient mice. In contrast, MMP-2 deficient mice had a 20% reduction of body weight, associated with smaller fat pads en hypotrophy of adipocytes and preservation of collagen.
- Wild-type mice on a HFD treated with a relative gelatinase specific inhibitor (Tolylsam) for 15 weeks, developed less adipose tissue, resulting in lower body weights. This was associated with adipocyte

hypotrophy and reduced vascularization and higher levels of total collagen.

- Leptin deficient ob/ob mice (a genetically induced obesity model) were significantly leaner when treated with Tolylsam.
- Adipose tissue growth was impaired in wild obese type mice that were switched to a HFD supplemented with Tolylsam, whereas mice that were kept on the HFD only, continued to gain weight. Tolylsam treatment resulted in preservation of collagen.
- *De novo* fat pad formation in NUDE mice following injection of 3T3-F442A preadipocytes was impaired in mice treated with Tolylsam.
- MMP-2 deficient mice developed less adipose tissue when treated with Tolylsam. This was associated with adipocyte hypotrophy and reduced angiogenesis.

In vitro:

- Downregulation of MMP-2 expression in 3T3-F442A preadipocytes resulted in reduced differentiation into mature adipocytes.
- 3T3-F442A preadipocytes cultured in the presence of Tolylsam showed increased differentiation as compared to vehicle treated cells. This may be related to the relatively low substrate specificity of Tolylsam.

Taken together, these results reveal divergent functions for gelatinases in the development of adipose tissue: MMP-2 appears to enhance adipose tissue development in mice on high fat diet, at least in part by contributing to adipocyte growth, whereas MMP-9 does not seem crucial. In addition, adipose tissue development can be impaired by oral administration of the relatively gelatinase-specific inhibitor Tolylsam, which is not only a MMP inhibitor, preserving collagen in the ECM, but also impairs blood vessel formation.

SAMENVATTING

In 1999 werd zwaarlijvigheid (obesitas) door de Wereldgezondheidsorganisatie uitgeroepen tot wereldwijde epidemie. Naar schatting zijn er op dit ogenblik 500 miljoen mensen zwaarlijvig en kampen er meer dan 1.6 miljard met overgewicht. Verscheidene levensbedreigende complicaties werden in verband gebracht met een te hoog lichaamsgewicht, waaronder cardiovasculaire aandoeningen, hypertensie, beroerte, type II diabetes, atherosclerose en sommige vormen van kanker. Dit heeft ertoe geleid dat zwaarlijvigheid op het punt staat tabak te overtreffen als de belangrijkste vermijdbare doodsoorzaak in de westerse wereld.

Zwaarlijvigheid wordt gekenmerkt door een overmaat aan vetweefsel ten opzichte van spier- en botmassa. De ontwikkeling van vetweefsel is een complex proces waarbij onder invloed van verschillende groeifactoren, cytokines, proteinases en hormonen uitgesproken veranderingen optreden. Drie processen treden hierbij op: adipogenese (vorming van adipocyten), angiogenese (vorming van bloedvaten) en proteolytische hermodellering van extracellulaire matrix (ECM). Het matrix metalloproteïnase (MMP) systeem speelt een belangrijke rol in deze processen. MMP's zijn een familie van zinkafhankelijke endopeptidasen die ECM en basale membranen hydrolyseren en zo toelaten dat vetweefsel kan groeien en dat bloedvaten kunnen gevormd worden. Daarnaast kunnen MMP's groeifactoren proteolytisch activeren of vrijzetten uit de ECM en cel-cel interacties beïnvloeden. De gelatinases (gelatinase A of MMP-2 en gelatinase B of MMP-9) vormen een subgroep van deze MMP familie. Recent onderzoek heeft aangetoond dat zowel MMP-2 als MMP-9 geproduceerd worden door adipocyten en dat modulatie van gelatinolytische activiteit optreedt tijdens de ontwikkeling van vetweefsel. Deze studie beoogde daarom een beter inzicht te verwerven in de rol van deze gelatinases tijdens de ontwikkeling van vetweefsel en het ontstaan van obesitas. Hiertoe werden zowel *in vitro* als *in vivo* experimenten uitgevoerd, waarbij de muis als model gebruikt werd.

In vivo experimenten:

- MMP-2- en MMP-9-deficiënte muizen werden op een vetrijk dieet gehouden en vergeleken met wild-type muizen. Geen verschillen werden gevonden voor MMP-9 deficiënte muizen, maar MMP-2-deficiënte muizen werden gemiddeld 20% minder zwaar. Dit ging gepaard met verminderde vetopslag, hypotrofie van de adipocyten en behoud van de ECM.

- Wild-type muizen op vetrijk dieet en behandeld met Tolylsam, een relatief specifieke inhibitor tegen de gelatinases, ontwikkelden minder vetmassa in vergelijking met niet-behandelde muizen. Dit was geassocieerd met hypotrofie van de adipocyten, verminderde angiogenese en een hoger gehalte aan totaal collageen.
- Tolylsam was eveneens efficiënt in het reduceren van vetmassa bij leptine-deficiënte ob/ob muizen (een genetisch model voor obesitas).
- De ontwikkeling van vetweefsel werd afgeremd bij obese muizen op vetrijk dieet, wanneer ze behandeld werden met Tolylsam. Muizen die niet behandeld werden, bleven onverminderd vet accumuleren. Ook hier werd een behoud van ECM waargenomen.
- De *de novo* vorming van vetkussentjes bij NUDE muizen na injectie van 3T3-F442A preadipocyten was gereduceerd bij muizen behandeld met Tolylsam.
- MMP-2-deficiënte muizen ontwikkelden minder vetweefsel als ze Tolylsam toegediend kregen. Ze hadden tevens kleinere adipocyten en maakten minder en kleinere bloedvaten aan.

In vitro experimenten:

- Verminderde expressie van MMP-2 in 3T3-F442A preadipocyten resulteerde in verminderde differentiatie tot mature adipocyten.
- De differentiatie van 3T3-F442A preadipocyten was echter toegenomen door de aanwezigheid van Tolylsam in het medium. Dit is mogelijkerwijze te verklaren door de relatief beperkte substraat-specificiteit van Tolylsam.

Deze resultaten wijzen op een divergente functie voor de afzonderlijke gelatinases in de ontwikkeling van vetweefsel: MMP-2 lijkt de groei van het vetweefsel in muizen op een vetrijk dieet te doen toenemen, terwijl MMP-9 niet cruciaal lijkt. Bovendien kan de accumulatie van vetmassa worden verhinderd door het toedienen van Tolylsam, een inhibitor die relatief specifiek gericht is tegen de gelatinases, en zo collageen in de ECM beschermt en tevens inwerkt op de vorming van nieuwe bloedvaten.

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About six years ago, I decided to enrol in the Ph.D. program. This decision marked the beginning of a fascinating journey collecting experiences and encounters that ultimately would define me as a person. All this time, I could count on the support of so many people, to whom I would like to express my gratitude.

My first and foremost "thanks" goes to my promoter, Prof. H.R. Lijnen, for giving me the opportunity to work in his lab. I admit that, after hearing all kinds of horror stories about tyrannical promoters demanding long working hours and destroying your social life, I had mixt feelings of excitement and anxiety when I first joined the group… Fortunately, in my case, these stories turned out to be no more but urban legends. Sure, I had my share of weekend work, stress and deadlines, but Roger is a very enjoyable and accommodating person to work with. He gave me freedom to explore my research interests, at the same time providing the necessary guidance to prevent me from getting lost. I feel truly privileged to have been given the opportunity to wander freely through unknown scientific territory and yet to have been ensured of the support of an experienced scientific advisor. Although he had a lot of other duties as head of our department, he always managed to give me his full and dedicated attention. I really admire his efficiency, as well as his ability to put things into perspective and bring order within apparent chaos. As a future reference of what a boss should be, he will be hard to match.

Next, I would like to thank my current and past colleagues from the 'Lijnen' group for their expert technical assistance, valuable advice and constructive feedback: Berthe, Ilse, Bianca, Valerie, Annemie, Christine, Liesbeth, Sofie, Leentje and Inge. They have taught me a great number of techniques, while being insightful research partners themselves. I wish them all good luck in the further pursuit of their career and in their personal life.
To Dries, I hope that my contribution to the field will prove a solid foundation for his own doctoral studies.

Also, I should not forget my colleagues from the "Platelet lab". They involved me in their social activities so regularly that at times it was not entirely clear to which group I belonged. In particular, I would like to thank the members of "the gang". Laura, Karen, Jeroen, Serena, Soetkin, Jan, Christophe[2], Thomas, Alexandre, Yasha, Elke and Elise. I will miss our daily lunch breaks, which were the scene of lively and often hilarious discussions. I'm persuaded there is a pearl to be found amongst the heaps of ideas we set loose on the world. Our weekly 'journal clubs' on Wednesday were one of the highlights of my week and from now on I will have to adjust to the idea of no longer being up to date. I consider myself lucky to have been part of so many happy moments in your lives (a marriage, births, the acquisition of a house, a new love, ...) and I hope I was of some comfort during the sad times. I couldn't have wished for better colleagues and I look forward to a life long friendship.

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Outside the lab, an exhaustive list of people have contributed to my education or this thesis in one way or the other. I owe much gratitude to my family and friends for their endless support and understanding. More then once their rhetorical "How are you doing on your thesis?" was countered by my slightly sarcastic answer: "Just wrapping up!", a reflex which became an alternative way of greeting each other. I hope this thesis will finally shed some light on what I have been doing the last few years besides "torturing helpless animals", "playing with cells" and "curing obesity. Although I cannot mention everybody by name, I hope they know how appreciated their involvement has been.

Yet, more in particular, I would like to thank my parents for their unconditional support in every aspect of my life. Through their example they gave me the strength and confidence to always continue and press forward. I want to thank them for instilling in me the love of learning and the continuous desire for more knowledge.
It occurs to me that 'a child's gratitude' must be a contradictio in terminis, as we can only understand our parent's efforts and sacrifices when we have reached a certain maturity ourselves. Today, more than ever, I realize that none of this could have happened without them. This thesis is dedicated to them and I hope they see it for what it really is: a tribute to their good parenting!

My sisters, Noémi, Ariane and Rebecca, and my brother, Siméon, are acknowledged for their role in all those fond memories I have, for creating a warm domestic environment and for being there when needed. We may grow older and go our own way, but I look forward to the happy moments that are still to come. I wish them all the best in their future endeavours.
I also would like to mention the two latest arrivals in our family: Samuel and Céline. Though they are still too young to understand, I would nevertheless like to acknowledge them for the many laughters we shared and for reminding me of what is truly important in life.

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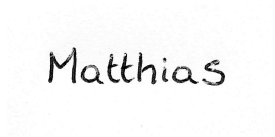
I must not forget my good friends Sarah, Jürgen, Stijn, Michi and "those from the scouts", who were always ready to help and who made sure I was continuously engaged and entertained along the way, providing me with much needed relaxation. Perhaps apologies are also in order, for all these times I was too busy/tired/stressed to join them on what would undoubtedly become another great adventure. If a man is to be judged by his friends, I have little to fear.

Finally, I thank my lucky stars for guiding me to the love of my life, Eva. Her endless patience, support and encouragement helped me through the, sometimes, tough moments as a PhD researcher. Her continuous efforts to come up with solutions, even when none seemed to be out there, graces her sympathetic character. She never stopped believing in me and it would be impossible to express in a few sentences how grateful I am for her love and caring. She truly is a princess among women and I am blessed to have her by my side. We have shared many happy years and I deeply hope many, many more will follow.



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Attended seminars regularly and followed different follow-up courses during PhD training including:
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LIST OF PUBLICATIONS

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PUBLICATIONS

- **Matrix metalloproteinase inhibition impairs in vivo adipogenesis and adipose tissue development**
  Van Hul M, Lupu F, Dresselaers T, Lijnen HR
  (submitted)

- **Caloric restriction improves coagulation and inflammation profile in obese mice**
  Lijnen HR, Van Hul M, Hemmeryckx B
  Thromb Res. 129:74-79 (2012)

- **Effect of gelatinase inhibition on adipogenesis and adipose tissue development**
  Van Hul M, Bauters D, Kindt N, Noppen B, Vanhove M, Lijnen HR
  Clin Exp Pharmacol Physiol. 39:49-56. (2012)

- **Role of Thrombospondin-2 in Murine Adipose Tissue Angiogenesis and Development**
  Van Hul M, Frederix L, Lijnen HR
  Obesity. [Epub ahead of print] (2011)

- **Effect of weight loss on gelatinase levels in obese mice.**
  Van Hul M, Lijnen HR
  Clin Exp Pharmacol Physiol. 38:647-649 (2011)

- **Matrix metalloproteinase inhibition impairs murine adipose tissue development independently of leptin**
  Van Hul M, Lijnen HR
  Endocr J. 58: 101-107 (2011)

- **Gelatinase B (MMP-9) deficiency does not affect murine adipose tissue development**
  Van Hul M, Piccard H, Lijnen HR
  Thromb Haemost. 104: 165-171 (2010)

- **A functional role of gelatinase A in the development of nutritionally induced obesity in mice**
  Van Hul M, Lijnen HR
  J Thromb Haemost. 6: 1198-206 (2008)

- **MFN2 mutation distribution and genotype/phenotype correlation in Charcot-Marie-Tooth type 2.**
  Verhoeven K, Claeys KG, Züchner S, Schröder JM, Weis J, Ceuterick C, Jordanova A, Nelis E, De Vriendt E, Van Hul M, Seeman P, Mazanec R, Saifi GM, Szigeti K, Mancias P, Butler IJ, Kochanski A, Ryniewicz B, De Bleecker J, Van den Bergh P, Verellen C, Van Coster R, Goemans N, Auer-Grumbach M, Robberecht W, Milic Rasic V, Nevo Y, Tournev I, Guergueltcheva V, Roelens F, Vieregge P, Vinci P, Moreno MT, Christen HJ, Shy ME, Lupski JR, Vance JM, De Jonghe P, Timmerman V.
  Brain. 129: 2093-2102 (2006)

INTERNATIONAL CONGRESSES

- Leducq International Network Against Thrombosis (LINAT) 2006
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  - "Deficiency of thrombospondin-2 does not affect murine adipose tissue angiogenesis or development" (oral presentation)
  - "Modulation of adipose tissue mass by matrix metalloproteinase inhibition" (poster)
- International Society on Thrombosis and Haemostasis (ISTH) 2011
  - Kyoto, Japan
  - "Caloric restriction of obese mice improves adiposity, inflammation and metabolic profile" (poster)
  - "Modulation of gelatinases during weight loss in obese mice" (poster)