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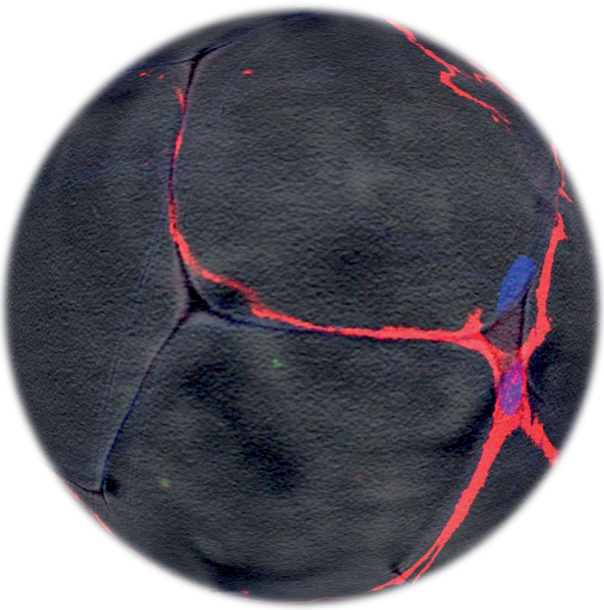
Characterisation of human adipose tissue - ceramide metabolism, depot differences and evaluation of dysfunctionality



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CHARACTERISATION OF HUMAN ADIPOSE TISSUE - CERAMIDE METABOLISM, DEPOT DIFFERENCES AND EVALUATION OF DYSFUNCTIONALITY

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To my family

ABSTRACT

Normal adipose tissue function is necessary for maintaining proper energy balance, as both excess and absence of this tissue lead to metabolic disturbances, such as insulin resistance. Adipose tissue can be dysfunctional in many ways, including disturbances in adipocyte size, lipolysis rate, adipokine secretion, inflammation, fibrosis or oxidative stress. Recently ceramides have been proposed as candidate molecules that mediate the development of adipocyte insulin resistance. The aim of this thesis is to evaluate different aspects of dysfunctionality in human adipose depots in relation to their potential contribution to insulin resistance and cardiovascular disease.

Paper I and II focus on the role of ceramides in human adipose tissue. We show that in obese women with high liver fat increased ceramide content in the subcutaneous adipose depot is most probably due to the increased sphingomyelin hydrolysis rather than *de novo* production. Moreover, sphingomyelinases, that are responsible for this reaction, are present in areas rich in apolipoprotein B, which may suggest that circulating lipoproteins may be a source of sphingomyelin for the local ceramide production within adipose tissue. Additionally, we show increased ceramide levels in the mediastinal as compared to the subcutaneous adipose tissue and show that ceramides in this depot are associated with inflammatory processes. In our unpublished data we demonstrate that ceramide induces inflammatory cytokine expression in both macrophages and adipocytes. Paper III investigates whether the mediastinal depot shows characteristics of brown adipose tissue. A comparison of several markers of brown and white fat between subcutaneous and mediastinal adipose tissue reveals that the mediastinal fat has higher expression levels of some brown (UCP1, PPARGC1A) and lower expression levels of white (SHOX9, HOXC8) markers. Gene ontology analysis indicates that mediastinal depot is enriched in genes related to mitochondrial function. In some sections of mediastinal fat positive UCP1 staining and presence of multilocular cells are observed. In Paper IV we investigate whether adipose tissue in patients with chronic kidney disease is dysfunctional. We report that subcutaneous adipose tissue in patients with kidney failure is characterized by higher numbers of phagocytic cells and smaller adipocytes, but shows no signs of fibrosis as compared to healthy subjects. Additionally, proteomic analysis shows differential expression patterns between the patients and controls. Among the proteins expressed at higher levels in the patients, alpha-1-microglobulin/bikunin precursor is the most significant and among those expressed at lower levels in the patients, the most significant is vimentin – a protein known to be involved in lipid droplet metabolism.

In summary, the work presented in this thesis demonstrates that adipose tissue ceramides can promote local inflammation, a process strongly linked to insulin resistance. Moreover, the mediastinal adipose depot shows some signs of brown fat, however the functional consequences remain to be evaluated. Finally, uremic adipose tissue shows adverse protein composition, which together with an increased number of phagocytic cells and smaller adipocyte size indicates that uremic adipose tissue is dysfunctional, which could lead to increased cardiovascular risk in chronic kidney disease patients.

LIST OF SCIENTIFIC PAPERS

I. Kolak M, **Gertow J**, Westerbacka J, Summers SA, Liska J, Franco-Cereceda A, Oresic M, Yki-Järvinen H, Eriksson P, Fisher RM. Expression of ceramide-metabolising enzymes in subcutaneous and intra-abdominal human adipose tissue. Lipids in Health and Disease, 2012, 11:115

II. **Gertow J**, Kjellqvist S, Ståhlman M, Cheung L, Gottfries J, Werngren O, Boren J, Franco-Cereceda A, Eriksson P, Fisher RM. Ceramides are associated with inflammatory processes in human mediastinal adipose tissue. Nutrition, Metabolism & Cardiovascular Diseases, 2014, 24: 124-131

III. Cheung L, **Gertow J**, Werngren O, Folkersen L, Petrovic N, Nedergaard J, Franco-Cereceda A, Eriksson P, Fisher RM. Human mediastinal adipose tissue displays certain characteristics of brown fat. Nutrition & Diabetes, 2013, 3(5), e66

IV. **Gertow J**, Ng CZ, Mamede Branca RM, Werngren O, Kjellqvist S, Bruchfeld A, MacLaughlin H, Eriksson P, Axelsson J and Fisher RM. Proteomic analysis reveals an altered protein composition of subcutaneous adipose tissue in patients with chronic kidney disease. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

AgRP	Agouti-related peptide
AMBP	Alpha-1-microglobulin/bikunin precursor
apoB	Apolipoprotein B
ASAH1	N-acylsphingosine amidohydrolase
ASAP	Advanced Study of Aortic Aneurysm
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CERK	Ceramide kinase
CERT	Ceramide transfer protein
CGT	Ceramide glycosyl transferase
CIDEA	Cell death activator CIDE-A
CKD	Chronic kidney disease
COBL	Cordon-bleu protein
CVD	Cardiovascular disease
DAB	3,3’-diaminobenzidine tetrachloride
DEGS1	Dihydroceramide desaturase
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FTO	Fat mass and obesity-associated protein
GFR	Glomerular filtration rate
GLUT4	Glucose transporter 4
GO	Gene ontology
HDL	High density lipoproteins
HLF	High liver fat
hMCSF	Human macrophage colony-stimulating factor
HOXC8	Homeobox protein Hox-C8
HPLC	High-performance liquid chromatography
HSL	Hormone-sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IHC	Immunohistochemistry
IL1B	Interleukin 1B
IL6	Interleukin 6
IR	Insulin resistance
IRS1	Insulin receptor substrate 1
IκK	IκB kinase

JNK	c-Jun N-terminal kinase
LASS1-6	Ceamide synthase 1-6
LDL	Low density lipoproteins
LPL	Lipoprotein lipase
MC4R	Melanocortin 4 receptor
MCP1/CCL2	Monocyte chemoattractant protein 1
mRNA	Messenger ribonucleic acid
NEFA	Non-esterified fatty acids
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLF	Normal liver fat
NPY	Neuropeptide Y
OPLS	Orthogonal projections to latent structures
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
PCSK1	Proprotein convertase 1
PKB	Protein kinase B
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PRDM16	PR domain containing 16
PVDF	Polyvinylidene difluoride
RMA	Robust multiple-array average
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGMS1-2	Sphingomyelin synthase
SHOX2	Short stature homeobox 2
SMPD1-4	Sphingomyelinase 1-4
SPHK1	Sphingosine kinase 1
SPT	Serine palmitoyl transferase
SPTLC1-2	Serine palmitoyltransferase, long chain base subunit 1-2
SVF	Stromal-vascular fraction
T2DM	Type 2 Diabetes Mellitus
TG	Triacylglycerols
TNF	Tumor necrosis factor α
UCP1	Uncoupling protein 1
UGCG	UDP-glucose ceramide glucosyltransferase
UPLC-QTOFMS	Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry
WAT	White adipose tissue
WHO	World Health Organisation
VLDL	Very low density lipoproteins
VSMC	Vascular smooth muscle cells
α-MSH	α-melanocyte-stimulating hormone

1 INTRODUCTION

1.1 Structure and function of human adipose tissue

Human adipose tissue is a loose connective tissue that in healthy, non-obese individuals constitutes about 20% (in men) or 25% (in women) body weight and is composed of several cell types. They can be classified into mature adipocytes and a so-called stromal-vascular fraction (SVF). SVF is a mixture of vascular (endothelial), inflammatory and progenitor cells, whose contribution to the total SVF may vary according to the depot or body mass (1). Blood vessels provide the tissue with necessary nutrients and enable gas exchange. Immune and inflammatory cells are represented mainly by macrophages, that remove apoptotic or necrotic cells or cell debris (2). Progenitor cells (or preadipocytes) upon appropiate stimulation (increased energy intake and insulin levels) undergo a major differentiation programme and become mature adipocytes (3). In this way adipose tissue can expand in order to store excess energy. Mature adipocytes constitute the major part of the tissue and it is here that the tissue's basic function – uptake, storage and release of fatty acids – takes place.

As mentioned above the main function of adipose tissue is the uptake of circulating non-esterified fatty acids (NEFA), both – albumin-bound as well as those that are released following hydrolysis of triacylglycerol (TG) in circulating lipoproteins by lipoprotein lipase (LPL), their esterification onto glycerol-3-phosphate and storage as triacylglycerols. Triacylglycerols can be hydrolysed back to NEFA and leave the adipocyte in the process called lipolysis. The sequential hydrolysis of triacylglycerols is catalysed by 3 enzymes - adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (4). The flux of NEFA to and from adipocytes is regulated by a number of hormones, such as insulin, leptin and adrenergic receptor agonists (adrenaline, noradrenaline). Apart from energy storage adipose tissue provides cushioning and insulation helping to maintain body temperature as well as helping to keep internal organs in place.

1.2 White adipose depots

The vast majority (about 85%) of adipose tissue in humans is located under the skin and therefore this depot is called subcutaneous adipose tissue. The rest forms visceral depots, such as mesenteric, omental, perirenal fat (in the abdominal cavity), or mediastinal, pericardial and epicardial fat (within the thoracic cavity). However lipid droplets can be

stored even inside muscle fibers (intramuscular fat) or in the liver (liver fat) (5). Adipose depots share many similarities, but some differences have also been consistently reported, in particular the high metabolic activity (expressed as the rate of stimulated lipolysis) observed in intra-abdominal adipocytes as compared to the subcutaneous cells (6-8), and reduced lipid turn-over of the lower-body (gluteofemoral) fat (9). The secretion of adipokines and cytokines also differs between adipose depots, with subcutaneous tissue secreting more leptin than the visceral depot (10), but the latter secreting more inflammatory molecules, such as IL8, MCP1, resistin and visfatin (11).

1.3 White and brown adipose tissue

The two major types of human fat that have been well recognized are white (WAT) and brown (BAT) adipose tissue. They both originate from the mesoderm, but from distinct progenitor cells and they are remarkably different in both structure and function (12, 13). WAT is composed of large adipocytes (50-150μm in diameter) containing one, large lipid droplet (unilocular cells) that fills almost the entire cell pushing other cell organelles to the periphery. The major role of white adipocytes is related to the regulation and storage of energy in the form of TG. BAT on the other hand is composed of adipocytes containing a number of smaller lipid droplets (multilocular cells) as well as many mitochondria (that give the tissue its brownish colour). BAT in humans is present mostly in infants, where it can comprise up to 5% body weight, but evidence exists also for the presence of active BAT in adults (14). BAT depots include mainly interscapular, supraclavicular, and suprarenal pads, but can also be present around the aorta and the heart (peri-aortic and pericardial) as well as around the kidney and trachea (14). One of the most unique features of brown adipocytes is the presence of uncoupling proteins, in particular uncoupling protein 1 (UCP1), on the inner mitochondrial membrane. UCP1 uncouples the respiratory chain from ATP production allowing dissipation of energy as heat. Thanks to this unique feature BAT enables non-shivering thermogenesis and generates heat, hence its relatively high amounts in newborns, hibernating animals, rodents and small mammals. Adult humans however present with much smaller quantities of brown fat.

In recent years a new type of cell has been identified in addition to the classical white and brown adipocytes, namely the beige (or brite = brown-in-white) adipocyte (15). These cells are present in both humans and rodents and are characterized by a gene expression pattern that is distinct from those of both white and brown adipocytes, in particular low basal UCP1 expression, which however can be potently stimulated by cyclic AMP. These beige

adipocytes are usually scattered among white adipocytes but they show comparable thermogenic potential to classical brown cells (15).

1.4 Adipose tissue as an endocrine organ

It was long believed that adipose tissue was a mere storage place for excess fat, however this view was drastically changed a few decades ago with discoveries of its secretory properties (16, 17) and today we have identified a whole set of proteins that are secreted from mature adipocytes (18, 19) as well as other cells present in the adipose tissue, such as preadipocytes and macrophages (11, 20). Now it is well established that the tissue participates in the regulation of body homeostasis by secreting a range of cytokines and hormones including leptin, adiponectin, resistin, interleukin-6 (IL-6), tumour necrosis factor α (TNF), monocyte chemoattractant protein-1 (MCP1/CCL2), naming just few of them.

Leptin is secreted almost exclusively (95%) by adipose tissue and the amount of the secreted hormone is proportional to the adipose mass. It acts centrally in the hypothalamus as well as on the periphery (21). The central effects of leptin include suppression of hunger and inducing the feeling of satiety by inhibiting neuropeptide Y (NPY) and agouti-related peptide (AgRP), and activating α-melanocyte-stimulating hormone (α-MSH) (22). The peripheral effects of leptin include a wide range of actions, such as regulation of energy expenditure, reproduction or immune cell modulation (23). Despite the initial excitement about leptin being a potential cure for obesity, obese individuals have higher circulating leptin levels (proportionally to the amount of body fat) and are resistant to it (24). Thus leptin fails to modulate feeding behavior and energy expenditure properly in obesity.

Adiponectin is another hormone specifically secreted by adipose tissue that affects metabolic processes, such as glucose levels and fatty acid oxidation (25). On the contrary to leptin, adiponectin levels rise during weight loss and higher circulating adiponectin has been associated with insulin sensitivity (26). Adiponectin has anti-oxidative and anti-inflammatory effects and is believed to prevent vascular pathologies, such as atherosclerosis (27).

In addition to leptin and adiponectin, adipose tissue produces and secretes several cytokines, some of which are released into the circulation, such as IL6, and some with more local, paracrine effects, like TNF and MCP1 (28, 29). These are pro-inflammatory cytokines that have been associated with inflammatory cell recruitment and impairment of insulin signaling (30, 31).

1.5 Adipose tissue dysfunction and its consequences

1.5.1 Obesity

One of the earliest symptoms of adipose dysfunctionality that may indicate metabolic complications is obesity. It develops when energy intake chronically exceeds the expenditure, however the underlying mechanisms may be related to the genetic background as well as environmental factors. There are several genetic conditions associated with obesity, such as leptin deficiency, polymorphisms in the melanocortin 4 receptor (MC4R) or fat mass and obesity-associated protein (FTO) genes (32-34); however they will not be discussed in detail in this section. Obesity is characterized by adipocyte hypertrophy (increase in size) and hyperplasia (increase in number) (35). According to the World Health Organization (WHO) overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health. The commonly used measure of obesity is the body mass index (BMI), where weight in kilograms is divided by the square of height in meters. BMI between 20 and 25 is considered normal 25-30 is overweight and above 30 indicates obesity. However, as useful as BMI may be to roughly estimate the body mass, it does not take into account body composition, which may play a major role in some individuals, for example in those with high muscle mass.

In the past centuries high body weight was considered a sign of wealth and fertility and the prevalence of obesity was higher in rich, well-developed countries. Accompanied by increasing availability of low-cost, energy-dense fast foods and beverages this trend has recently changed its direction and now it is many of the developing and low-income countries that struggle with an obesity pandemic: since 1980 worldwide obesity has more than doubled and in 2014 39% of adults were overweight and 13% were obese (WHO).

Obesity does not necessarily have to be related to metabolic complications (36), but in the majority of cases it is related to hypertension, insulin resistance and diabetes, cardiovascular disease, cancer and other comorbidities (37, 38).

1.5.2 Insulin resistance and diabetes

Insulin resistance is one of the conditions frequently (but not necessarily) associated with obesity and a central feature of Type 2 Diabetes Mellitus (T2DM). The definition of insulin resistance encompasses impaired insulin-stimulated glucose uptake, glucose intolerance and hyperinsulinemia (39) often accompanied by abnormal lipid and lipoprotein profiles. In other

words cells such as myocytes, adipocytes and hepatocytes that would normally respond to increased insulin levels with uptake of glucose, amino acids and fatty acids (for instance in a post-prandial state) no longer do so – they become resistant to insulin. As a result circulating glucose and NEFA levels remain elevated which further signals to pancreatic beta-cells to secrete insulin and promotes hepatic secretion of very low density-lipoproteins (VLDL). The reasons why cells fail to respond properly to insulin signaling are not clear, however several suggestions have been made. Even though genetic variants account for a small proportion of cases there is certain genetic predisposition for insulin resistance syndrome and several polymorphisms have been described within the insulin receptor substrate (IRS) family of proteins, PPARγ, PCSK1 and many others (40, 41). Other explanations include a decrease in the cellular amount and translocation of glucose transporters or a decrease in the number of insulin receptors, but most probably it is molecules that counteract insulin action and disrupt the insulin signaling cascade that are the most common culprits (42). The effects of insulin on glucose production, levels and uptake are antagonized by glucagon, adrenaline, cortisol, growth hormone and inflammatory cytokines, such as TNF, which has emerged as a powerful agent that can inhibit insulin receptor signaling by a modified form of IRS1 (43). Relatively new and exciting findings link adipose tissue ceramides to the development of insulin resistance and the metabolic syndrome and they will be discussed in more detail in section 1.5.5. Long-term elevation of glucose levels lead to glucotoxicity and results in the glycation and other modifications of proteins that stimulate oxidative stress and cytokine release from inflammatory cells triggering vascular dysfunction (44). Increased levels of NEFA cause lipotoxicity, where lipids accumulate in tissues not adapted for lipid storage, such as skeletal muscle, liver, kidney or pancreas where they further disrupt insulin signaling and lead to endoplasmic reticulum stress and the unfolded protein response (45). Currently the most effective treatment of insulin resistance and T2DM includes dietary intervention that reduces amounts of simple carbohydrates favoring fiber-rich products as well as several classes of drugs that increase insulin release and sensitivity (46-48). A first-line drug for T2DM is the biguanide metformin. It suppresses hepatic glucose production, enhances glucose uptake in the periphery and decreases absorption of glucose from the intestine. It also decreases insulin-induced suppression of fatty acid oxidation. A class of drugs called sulphonylureas acts on pancreatic beta cells, closing the ATP-sensitive potassium channels and promoting insulin release. However, while they help to lower glucose levels, they have no effect on lipid levels. Finally, the thiazolidinediones activate the PPARγ transcription factor and improve sensitivity to insulin as well as plasma lipids. Physical exercise and reducing body weight are also strongly recommended.

1.5.3 Cardiovascular disease (CVD)

CVD is globally the leading cause of death, representing 31% of all deaths in 2012 (WHO). The term “cardiovascular disease” includes disorders of the heart and blood vessels, the most common being angina, myocardial infarction, stroke, hypertension, peripheral artery disease, congenital heart disease and heart failure. A family history of heart disease increases the risk of CVD, with other major causes being smoking, physical inactivity, unhealthy diet and harmful use of alcohol, which means that many of the CVDs are preventable. The process lying behind some of the most common forms of CVD, such as myocardial infarction, stroke and angina, is atherosclerosis. Atherosclerosis is a thickening of the arterial wall as a result of inflammatory cells accumulation, migration and proliferation of smooth muscle cells and neointima formation, forming a fibrous cap (49). The mechanisms of atherosclerotic plaque accumulation are complex and multi-factorial, but it is believed that the major cause is related to the high levels and retention of cholesterol- and triacylglycerol-carrying LDL particles in the arterial wall where they are prone to oxidation and trigger inflammatory responses (monocyte recruitment and foam cells formation) in the intimal space (50). Atherosclerotic plaques may be stable, with little risk of rupture, or unstable, with high probability of rupture (51). Plaque rupture leads to thrombus formation in the vessel lumen which rapidly reduces or completely blocks the blood flow and causes death of the tissues supported by the affected artery. Most commonly this happens in the coronary artery and causes myocardial infarction or in the brain where it causes stroke. Several risk factors have been associated with atherosclerotic plaque development, including dyslipidemia, smoking, insulin resistance/diabetes, male sex, advanced age and obesity, in particular central (abdominal) obesity (52).

1.5.4 Chronic kidney disease (CKD) as a cardiovascular risk factor

The complexity of CVD, particularly its multifactorial background, often results in its simultaneous occurrence to other diseases, such as kidney disease. Chronic kidney disease is a complex disease where the number of deaths more than doubled between 1990 and 2013 (53) indicating its increasing prevalence. There are 3 major conditions underlying CKD that together are responsible for about 75% of all adult CKD cases – hypertension, diabetes and glomerulonephritis (54). CKD – as its name indicates – is a chronic condition and usually develops over a period of months or years. It is traditionally classified into 5 stages on the basis of estimated glomerular filtration rate (GFR), with stage 1 being the most modest, with almost normal or only mild decrease in GFR rate ($\geq$90 ml/min/1.73 m^2) and stage 5 being the

most advanced with GFR <15 ml/min/1.73 m^2, which indicates kidney failure and requires permanent renal replacement therapy in a form of dialysis or kidney transplantation. What is interesting about CKD is that patients with earlier stages of the disease are more likely to die of CVD than develop kidney failure (55, 56). CVD is responsible for about 50% of all-cause-mortality in patients who have received kidney transplant (55). CKD patients often present the traditional CVD risk factors, such as diabetes (insulin resistance in non-diabetic CKD patients), hypertension and dyslipidaemia (57-59). It is still a matter of debate as to why these patients have such a high CVD risk. However, since the involvement of adipose tissue dysfunction in CVD has been established, a new field for study is emerging. However, not many reports exist that explore possible links between dysfunctional adipose tissue and increased CVD risk in kidney disease (60-65). In support of this hypothesis are several key findings, including elevated circulating NEFA concentrations (65), altered circulating adipokines (62, 64) and increased systemic inflammation that correlates with fat mass (60) in patients with CKD. More interestingly, studies on isolated adipocytes showed that uremic serum increases basal lipolysis and decreases mRNA expression of perilipin – a lipid droplet-associated protein (63), which may lead to increased fatty acid release promoting ectopic fat deposition and the development of insulin resistance. Finally, the activity of adipose lipoprotein lipase was decreased in CKD patients leading to the formation of lipoproteins with a pro-atherogenic lipid composition (66). Altogether these data indicate that dysfunctional adipose tissue in CKD patients could be one of the factors increasing their risk of CVD development and that studies to verify this question are motivated.

1.5.5 Adipose tissue contribution to the development of IR and CVD

Dysfunctionality within adipose tissue encompasses a set of disturbances, such as dysregulated lipolysis or uptake of fatty acids, adipocyte hypertrophy, inflammation, adverse adipokine secretion or fibrosis (2, 19, 20, 67, 68). During energy oversupply adipocytes can expand so rapidly that new capillaries can barely keep up, leading to one of the earliest signs of adipose dysfunctionality – hypoxia. Additionally, adipocytes are surrounded by extracellular matrix, which in normal conditions provides flexibility to the tissue and enables adipocyte expansion. Abnormal collagen deposition is another hallmark of adipose dysfunctionality, restricting adipocyte growth and promoting an inflammatory phenotype (68). When adipose tissue becomes even more dysfunctional it impacts on both the local environment and exerts systemic effects. For example when the tissue's storage capacity is exceeded and it cannot accommodate more TG or when the lipolysis rate increases, the circulating NEFA levels rise. NEFA originating from perivascular fat can directly affect the

underlying vessel wall impairing endothelial cell function, accelerating lesion formation and inflammation (69). Circulating NEFA in turn may end up in tissues not suitable for fatty acid storage, such as liver, heart, skeletal muscle and pancreas. Ectopic fat deposition impairs insulin sensitivity, glucose metabolism and increases oxidative stress (70) and these symptoms are particularly pronounced in abdominal obesity, where excess of visceral fat, which is believed to have a higher lipolytic rate, is drained directly to the portal vein and exposes the liver to high concentrations of NEFA, adipokines and inflammatory cytokines. Additionally, this increased flux of NEFA to the liver favours the development of an atherogenic lipoprotein profile, in particular increased VLDL formation. A common hallmark of obesity is adipocyte hypertrophy, which is considered detrimental for adipose tissue function since the cell volume has been shown to positively correlate with insulin and TG and negatively with insulin sensitivity and HDL in both SAT as well as VAT (71). Moreover, even in non-obese subjects increased adipocyte size is often accompanied by tissue fibrosis which restricts its expansion capacity and secretion of proinflammatory cytokines which trigger recruitment of inflammatory cells in to adipose tissue and facilitate insulin resistance development (72).

The presence of inflammatory cells within the adipose tissue to a large extent relates to tissue macrophages, which exist in two activation states – a pro-inflammatory (M1 macrophages) and an anti-inflammatory (M2 macrophages) (73). M1 activity inhibits cell proliferation and causes tissue damage, while M2 activity promotes cell proliferation and tissue repair. These two activation states are possible thanks to the ability of a macrophage to (via two enzymes) metabolize arginine to produce nitric oxide (M1 activation) or ornithine (M2 activation). The former is further associated with molecules that attract other inflammatory cells, such as IL8 (neutrophils), MCP1 (monocytes), and with T cell polarization. Ornithine on the other hand is required for many repair processes, such extracellular matrix construction. The presence of tissue resident M2 macrophages is therefore a normal state, providing support and repair during cellular senescence or injury. The activation of the M1 phenotype however has been associated with obesity and insulin resistance (67). It is believed that expanding adipocytes that lose their storage capacity become necrotic and attract macrophages that form so-called crown-like structures around the necrotic cell and act as scavengers to remove cell debris and lipid droplets (74). NEFA which are released from hypertrophic/necrotic adipocytes can trigger pro-inflammatory responses in macrophages, contributing to increased systemic and local levels of inflammatory cytokines, such as TNF (75). Furthermore, activated M1 macrophages limit the plasticity of adipose tissue via secretion of matrix metalloproteinase 9

which degrades the extracellular matrix (76) and impairs adipocyte progenitor cell proliferation and differentiation (77).

Studies focusing on the ability of adipose tissue to initiate inflammatory processes became extensive after the discovery that adipose tissue can secrete a proinflammatory TNF and that TNF levels are increased in obesity (31, 78). In addition to the effects of TNF on insulin signaling that promote an insulin resistant phenotype (79), it also affects adipocyte metabolism, including increasing basal lipolysis and intracellular ceramide levels, inhibiting LPL expression and activity, and inducting apoptosis and adipose plasminogen activator-inhibitor-1 (PAI-1) synthesis (80). Circulating concentrations of other hormones and cytokines produced within adipose tissue, including leptin and IL6, that are elevated in obesity, correlate with measures of insulin sensitivity (81) mediating pro-inflammatory and chemoattractant processes. It has been shown that in response to a high-fat diet adipose tissue secretes MCP1 that promotes macrophage recruitment and inflammation (82), and in fact, adipose visceral progenitor cells were revealed to initiate the process of MCP1 secretion and macrophage infiltration. The direct effects of adipose tissue on the vascular wall have also been shown. In physiological conditions perivascular fat may have beneficial properties via reduction in vascular tone and anti-inflammatory and anti-proliferative effects of adiponectin, for instance (83). In pathological situations however, such as obesity, adipose tissue can secrete a whole set of adipokines that activate inflammatory cells (84) and exert proliferative and angiogenic effects on the vessel wall (85). It was demonstrated that adipocyte-conditioned media obtained from epicardial adipocytes from patients undergoing coronary artery bypass surgery combined with oleic acid induce a potent proliferation and migration of vascular smooth muscle cells (VSMC) and activate inflammatory responses (86). Adipokines, such as visfatin were shown to increase not only VSMC, but also endothelial cells proliferation (87) and resistin was shown to activate the endothelium by upregulation of adhesion molecules (88). (89). In addition to its potent chemoattractant properties in adipose tissue itself, MCP1 has been also shown to mediate neointimal hyperplasia and prominent adventitial inflammation and angiogenesis promoting atherosclerotic processes in carotid arteries (90).

Thus, adipose tissue, via all the mechanisms described above, on local as well as systemic levels contributes to the metabolic disturbances that may lead to cardiovascular diseases and insulin resistance.

1.5.6 Ceramide metabolism and role in IR

Ceramides are lipid molecules composed of a sphingoid base (sphingosine) linked to a fatty acid via an amide bond. The fatty acids in naturally occurring ceramides have variable length, usually between 14 and 26 carbons and are unsaturated or monounsaturated. Free ceramides are present in tissues in very small amounts and ceramides are usually part of complex lipids, such as glycosphingolipids or the major lipids of the lipid bilayer – phospholipids, where they are part of sphingomyelin. In greater amounts ceramides (together with other lipids, such as cholesterol, cholesteryl esters, TG and fatty acids) are present in *stratum corneum* of the skin and *vernix caseosa*, the waxy white substance coating the skin of newborn babies where they play a protective role against loss of water, form a physical barrier and probably also conserve heat (*vernix caseosa*). Ceramides are not only constituents of a lipid bilayer, but they are important signaling molecules and regulate several cellular processes, like proliferation, differentiation, apoptosis, migration or inflammation (91). Production of ceramides occurs via two major processes – *de novo* synthesis and the hydrolysis of sphingomyelin. De novo synthesis of ceramide takes place in the endoplasmic reticulum (ER). The first step of the synthesis is rate limiting and starts with palmitate and serine being condensed by serine palmitoyl transferase (SPT) to form 3-keto-dihydrosphingosine. The following chain of reactions transforms the intermediate products to form dihydroceramide and finally ceramide, which is then transported by the ceramide transfer protein (CERT) or vesicular trafficking to the Golgi apparatus where it can be further incorporated into sphingolipids or be transformed into ceramide-1-phosphate, for instance. The alternative pathway is the hydrolysis of sphingomyelin catalyzed by several sphingomyelinases that differ in their preferences for cofactors and pH (SMPD1, SMPD2, SMPD3 and SMPD4). Therefore the production of ceramide from sphingomyelin can take place in several cell compartments, such as the Golgi apparatus, the lysosome or the cell membrane.

Understanding ceramide synthesis makes one point clear – the rate of its production is largely driven by the availability of long-chain saturated fatty acids, in particular palmitate (C16:0) to which SPT is specific (±1 carbon) (92). Still it is possible for some extracellular stimuli to regulate the ceramide synthesis rate without the fatty acid dependence, for example it has been shown that stress signals - cytokines (TNF) and UV radiation – can directly upregulate SPT expression (92, 93). Increased ceramide levels have been consistently reported in skeletal muscle of insulin resistant or obese subjects and animals (94-96) and even modest elevation in ceramide content seems to be sufficient to antagonize insulin action (97). The proposed mechanisms by which ceramide promotes the insulin resistant phenotype are the

following. Ceramide may induce phosphorylation of inhibitory serine/threonine residues on insulin receptor substrate 1 (IRS1) via activation of several enzymes known for IRS1 inhibition, such as p38, c-Jun N-terminal kinase (JNK) and IκB kinase (IκK) (98). The stimulation of JNK and IκK by ceramide additionally leads to activation of transcription factors – c-Jun and NFκB (98), which promote an insulin resistant phenotype and mediate inflammatory processes. Moreover, ceramide inhibits phosphorylation of Akt/protein kinase B (PKB) – a central regulator of insulin action and its effects on metabolic processes, like glucose uptake (99) and inhibits GLUT4 translocation to the cell membrane (100). Much less extensive research has been performed on ceramides in human adipose tissue (101, 102) nevertheless the data that exists also indicates adverse associations between ceramide content in human fat and metabolic indices, such as liver fat content (101). Everything we have learned so far about the role of ceramide in mediating insulin resistance, especially in skeletal muscles, makes it an exciting candidate for a similar role in human adipose tissue. This is one of the scopes of this thesis and will be investigated and discussed further.

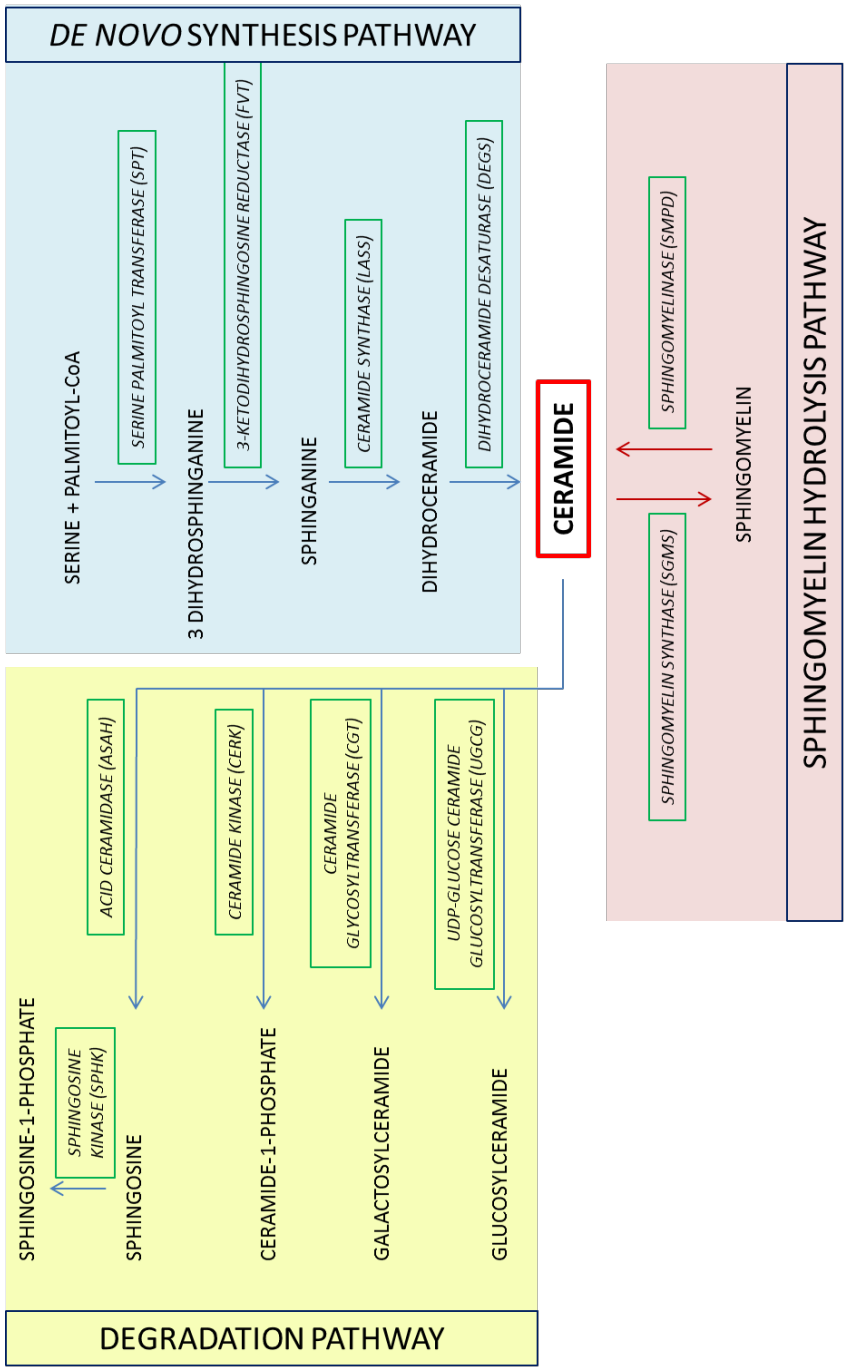


Figure 1. Schematic representation of ceramide synthesis and metabolism.

2 HYPOTHESIS AND AIMS

2.1 General Hypothesis

Adipose tissue plays a central role in the integration and regulation of metabolism, particularly via its capability of fatty acid handling and endocrine actions. However metabolic activity and properties related to lipid storage capacity, lipolysis rate and adipokine secretion vary significantly between adipose depots. Therefore alterations that unfavourably alter adipose tissue function, such as inflammation, impaired insulin signaling or lipolysis in the adipocyte, may facilitate the development of insulin resistance and cardiovascular disease.

2.2 Specific Aims

- Paper I and II. The mechanisms by which dysfunctional adipose tissue contributes to the development of insulin resistance: To investigate the role of ceramides as candidate mediators of inflammation in subcutaneous, intra-abdominal and mediastinal adipose tissue
- Paper II and III. The neglected mediastinal depot: Friend or foe? To evaluate whether mediastinal adipose tissue – one of the thoracic visceral depots – has an inflammatory and insulin resistant-pheonotype or a brown-like and fatty acid oxidation-promoting phenotype
- Paper IV. Dysfunctional adipose tissue and cardiovascular risk in complex diseases: To examine whether uremic adipose tissue shows signs of dysfunctionality, thereby potentially contributing to the increased risk of CVD in patients with kidney failure

3 METHODOLOGICAL CONSIDERATIONS

Individual papers contain more detailed descriptions of both study cohorts and methods. This Chapter intends to put them in a broader perspective, and therefore contains less detailed information.

3.1 Study material

In all papers human adipose tissue biopsies were collected from different adipose depots, according to the purpose of the study. Additionally, in Paper I liver biopsies were also collected. In Paper I adipose depots included the subcutaneous and the intra-abdominal depots, in Paper II and III – subcutaneous and mediastinal, in Paper IV – subcutaneous fat was used. The biopsies were collected during different surgical procedures (described in the Methods sections in individual papers) and usually consisted of approximately 200mg of tissue that was immediately cleaned from blood or excess connective tissue, divided (if needed) and treated according to the method they were intended for. For gene expression analyses the tissue (both adipose tissue and liver) was either stored in RNA Later® or was snap-frozen and stored in -80°C, for lipidomics and Western Blot the tissue biopsies were snap-frozen. For proteomic analyses the tissue was stored in All Protect Reagent® at -20°C and for immunohistochemistry it was collected in 4% zinc formaldehyde and stored in ethanol until paraffin embedding.

In Paper III murine adipose tissue was used in addition to human samples. The biopsies were inguinal white adipose tissue and the interscapular depot of brown fat collected from 9-week old outbred Naval Medical Research Institute mice acclimatized for 3 weeks to either 30°C or 4°C.

3.2 Study subjects

The subjects involved in the studies described in this thesis consisted of different cohorts of adult individuals and were enrolled in different research centers. However, all studies were approved by the relevant ethics committee, the potential risks related to the participation in the study were explained to the patients and written consents were obtained.

The study groups described in Paper I involve 20 obese women (BMI 30-42kg/m^2) and 8 morbidly obese individuals (2 women, 6 men, BMI 52.6±2.0kg/m^2) recruited in Helsinki University Central Hospital, Finland. The first group was divided into 2 subgroups (n=10 in

each group) according to the liver fat content (low liver fat – 1-3.5% or high liver fat – 6-35%). An additional group (n=23, non-obese men and women) was recruited in Karolinska University Hospital, Stockholm, Sweden, as a part of the Advanced Study of the Aortic Aneurysm (ASAP).

Patients enrolled in the ASAP study formed also the study cohorts used in Paper II and Paper III (overweight men and women, n=10 in Paper II and n=10+23+25 in Paper III). All ASAP patients used in the investigations described in this thesis were referred for open-heart aortic valve surgery and/or aortic aneurysm surgery and had no documented carotid artery disease.

In Paper IV two study groups were described - one consisted of normal- or slightly overweight men and women recruited at Karolinska University Hospital, Huddinge and the second one consisted of morbidly obese patients admitted to King's College Hospital, London, UK. The group recruited at Karolinska University Hospital, Huddinge involved 17 patients suffering from kidney failure and referred to the kidney transplant surgery and 11 healthy kidney donors. The group recruited at King's College was comprised of 9 morbidly obese CKD patients (stage 3-5).

3.3 Methods

3.3.1 Gene expression analyses

Quantification of gene expression is one of the most routine analyses used in research in order to estimate the relative or absolute number of a gene transcript. Two different approaches of gene expression analysis were taken. In Paper I and III the mRNA expression of selected genes was quantified by real-time PCR using gene-specific assays (inventoried, Invitrogen). This technique is most useful in a "candidate gene approach", where a response from one or several selected genes is expected. In Paper I, II and III global gene expression analyses with Affymetrix GeneChip Human Exon 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) were performed. This method measures the expression of a large number (thousands) of genes simultaneously and therefore can be very useful in screening for genes or pathways that are responsive to a treatment, for instance. This approach however creates a very large amount of data which analysis often requires multivariate statistics (discussed in section 3.3.6).

The RNA isolation from biopsies taken in Finland and used in Paper I was done with the RNA-STAT60 reagent. RNA was purified with RNeasy kit and the concentration was

measured using RiboGreen fluorescent nucleic acid staining. cDNA synthesis was performed using M-MLV reverse transcriptase and oligo(dT)$_{12-18}$ primers.

From adipose biopsies used in Paper II and III and liver biopsies used in Paper I total RNA was extracted using Trizol Reagent (Invitrogen) using the FastPrep Homogenizer (Qbiogene). RNA was purified using RNeasy Mini Kits (Qiagen) following the manufacturer's protocol. RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo) and the quality was analyzed with Agilent Bioanalyzer 2100 (Agilent Technologies). The cDNA synthesis was performed using Superscript III reverse transcriptase and oligo(dT)$_{12-18}$ primers. A pooled cDNA obtained from human adipose tissue samples served as an inter-plate control and was used to create a standard curve relative to which all individual levels were expressed in arbitrary units. Gene expression was normalized relative to the housekeeping genes.

For global gene expression, RNA samples with integrity number (RIN) value of at least 5.0 were used. Samples were hybridized and scanned at Karolinska Institutet's microarray core facility and Affymetrix GeneChip Human Exon 1.0 ST protocols were used. The raw Cel files were preprocessed and log2 transformed using robust multiple-array average (RMA) as implemented in the Affymetrix Power Tools 1.10.2 package apt-probeset-summarize. All investigations were performed on the *core* sets of meta probes provided by Affymetrix.

3.3.2 Immunohistochemical staining (IHC)

Immunostaining methods allow detection and visualization of antigens in cells via immunological and chemical reactions. The principle of this technique relates to the high affinity of antibodies towards antigens and the visualization can be achieved by coupling antibodies to a variety of enzymatic or fluorescent labels. The popularity of IHC depends on many advantages of this method – it is relatively inexpensive, quick, sensitive and specific, allows detection on the protein level and enables detection of a variety of antigens in multiple species in relation to the tissue structure (more than one antigen can be detected and visualized in one staining). Care should be taken however to avoid certain common problems related for example to unspecific binding and high background staining.

In the articles presented in this thesis staining was performed on formalin-fixed paraffin sections of human (Paper I, III and IV) and murine (Paper III) adipose tissue. The sections were incubated with primary antibodies followed by incubation with biotinylated secondary antibody and the staining was visualized using avidin-biotin peroxidase complex (Vector Laboratories) followed by 3,3'-diaminobenzidine tetrachloride (DAB, Vector Laboratories).

All sections were counterstained with Harris hematoxyline (Histolab) and analyzed with light microscopy.

In Paper I and IV collagen fibers were stained with Sirius Red. In linear polarized light collagen fibers appear as orange, yellow or green filaments, depending on their thickness, alignment and packing. The sections were analysed with Leica QWin software and collagen content (thresholded chromogen area) was calculated as the percentage of the total section area (Paper IV).

3.3.3 Protein analyses

Similar to gene expression analysis, proteins can also be quantified in different ways. One of the most traditional ways is quantifying the amount of a single (or several) "candidate" proteins by Western blotting, where a mixture of proteins from a sample is loaded on a gel and subjected to electrophoretic separation, transferred to a membrane and stained with antibodies allowing detection and subsequent quantification of a specific protein. Western blotting can be a straight-forward and sensitive method that quickly provides information about the level of protein expression, however some problems are related to this technique, such as lack of signal or too strong background as well as uneven distribution of bands.

For Western blot analyses human and murine adipose tissue protein extracts were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% milk and incubated with a primary antibody at 4 °C for 12 h. Incubation with the secondary biotin-conjugated antibody was performed for 1h at room temperature. The signal was then detected with ECL plus Western Blotting Detection System (GE Healthcare) with Amersham Hyperfilm ECL (GE Healthcare) (Paper III) or with X-ray film Agfa CP-BU new (Agfa Healthcare, Paper IV).

Another approach for protein analysis that is useful for quantification of large numbers of proteins is two-dimensional gel electrophoresis. This technique is used in Paper IV and allows more precise separation of proteins, first according to their isoelectric point (first dimension) and next according to their molecular weight (second dimension). Labelled proteins form a spotted pattern on the gel where they were loaded and separated and the spot volume corresponds to the amount of the protein present in the sample. The spots can be then analyzed by mass spectrometry in order to identify the proteins. This method combined with multivariate data analysis was extremely valuable in the project in Paper IV,

where adipose proteins with differential expression levels between patients and controls were looked for – a goal that cannot easily be achieved with traditional Western blot.

3.3.4 Ceramide quantification

Lipids are extremely interesting from a metabolic point of view, since they serve as potent signaling molecules. In Paper I and II adipose tissue and plasma lipids were extracted and measured. In Paper I lipidomics was introduced – a large-scale profiling comprising 154 lipids including ceramide (Cerd18:1/16:0, Cerd18:0/22:0 and Cerd18:1/24:1) and sphingomyelin (SMd18:1/16:0, SMd18:1/18:0, SMd18:1/20:0, SMd18:1/22:0, SMd18:1/22:1, SMd18:1/24:1, SMd18:1/24:2) species measured by Ultra Performance Liquid Chromatography coupled to time-of-flight mass spectrometry (UPLC-QTOFMS). In Paper II ceramides were separated and purified using straight-phase HPLC and analyzed using reversed-phase HPLC coupled to a triple quadrupole mass spectrometer. Six ceramides species (Cerd18:1/16:0; Cerd18:1/18:0; Cerd18:1/20:0; Cerd18:1/22:0; Cerd18:1/24:0 and Cerd18:1/24:1) were quantified using external standards.

3.3.5 Adipocyte size measurement

As discussed briefly in the Introduction, adipocyte size varies between depots and can be a feature of dysfunctional adipose tissue. Therefore estimating the size of adipocytes provides an additional parameter in the assessment of tissue function. One way of measuring the adipocyte diameter/volume is isolating mature cells and calculating their size in suspension in the light microscope, where the cells are well separated and round in shape. However this requires a relatively large biopsy that can be freshly processed and still one needs to account for the loss of many of these fragile cells. An alternative approach was used in Paper III and IV where adipocyte area was calculated on paraffin-fixed and hematoxylin stained adipose sections using an open source scientific image processing program (ImageJ) allowing the accurate measurement of the whole cell area, even when irregular in shape. Briefly, the background threshold was set for optimal visualization of adipocyte plasma membranes. Images were then flattened to binary images and “dilated” to ensure best separation of adipocytes. “Fill holes” function was applied to remove debris and optical artefacts. Adipocyte area was measured using “Analyze particles” function with parameters: particle size 100-50000um^2, circularity 0-1.00, exclude on edges.

3.3.6 Cell culture

Two types of experiments involving cell culture were used in our as yet unpublished data. One included human primary monocytes isolated from buffy coats with the Ficoll-Percoll method. Cells were seeded in 6-well plates at a density of $1.5x10^6$ cells/well and grown in RPMI 1640 supplemented with 1% antibiotics (penicillin-streptomycin) and 10% heat inactivated fetal bovine serum. The cells were stimulated towards macrophage differentiation with human macrophage colony-stimulating factor at concentration 50ng/mL.

The second cell culture included the 3T3 L1 cell line purchased from the European Collection of Authenticated Cell Cultures (ECACC). The cells are mouse embryo fibroblasts that were stimulated to differentiate into adipocytes by first growing the cells to confluency using Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% serum. Two days after confluency differentiation was initiated by adding 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25µM dexamethasone and 1µg/ml insulin in DMEM with 10% Foetal Bovine Serum (FBS). After 2 days the IBMX and dexamethasone were removed but insulin was maintained for another 2 days. On day 4 and thereafter, the cells were cultured in DMEM with 10% FBS only. The majority of cells had an adipocyte-like phenotype after 14 days of differentiation, with multiple visible lipid droplets. The cells used for the experiment were at passage 16.

C6 ceramide was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 100mM. Prior to each experiment ceramide was coupled to fatty acid-free bovine serum albumin and sonicated. Fresh 10µM ceramide solution was used immediately for cell culture experiments.

3.3.7 Statistical analyses

The statistical analyses of data presented in this thesis were performed with StatView software (Paper I), Statistica (Paper II and IV), R Bioconductor packages (Paper III) and Simca 13.0.3 (Paper II and IV). Most of the comparisons were made with a non-parametric Mann-Whitney test and Wilcoxon signed rank test, and the relationships between variables were assessed with Spearman's rank correlation. Non-parametric statistics were preferable in most of the cases, since they do not depend on normal data distribution, which is often difficult to achieve with low number of observations. Additionally, non-parametric tests are not sensitive to extreme values, thus lowering their impact on data interpretation.

In Paper II and IV multivariate data analysis was necessary in order to extract the required information from large data sets. The approach described here was based on projection methods, where observations are presented as points in a K-dimensional space (where K is a number of variables) and then projected to a lower-dimensional (2-dimensional) plane. This system of analyzing data sets is a relatively simple way of summarizing it, looks at all the variables at the same time thus eliminating the problem of false discovery rate related to multiple testing and – most importantly – it reveals quantitative relationships between variables, even if the data is noisy. The projection methods can also handle data with many more variables than observations, which is of importance in particular in Paper II. The analytical tool used in Paper II and IV is called Orthogonal Projections to Latent Structures (OPLS). It connects information from two data sets or matrices (X and Y) with the ability of separating the part of the information in X that is related (orthogonal) to Y. In other words – it separates variation in data set X into two parts – one that is linearly related (predictive) to Y and one that is not (orthogonal), improving transparency and interpretability of the data.

4 RESULTS

4.1 Paper I – Expression of ceramide-metabolizing enzymes in subcutaneous and intra-abdominal human adipose tissue

Ceramide has been shown to be a potent molecule involved in the pathogenesis of insulin resistance, however we still know very little about its role in human adipose tissue. The aim of this study was to investigate which pathways are responsible for the increased ceramide content previously observed in inflamed adipose tissue (101).

The expression levels of genes involved in ceramide de novo synthesis and metabolism were measured in subcutaneous adipose tissue of obese women (n=20). The subjects were divided into 2 groups depending on their liver fat content – normal liver fat (NLF = 2.3% ± 0.3%, n=10) or high liver fat (HLF = 14.4 ± 2.9%, n=10). The HLF group had a more adverse blood lipid profile and more inflamed adipose tissue with a higher ceramide and sphingomyelin content than the NLF group (101). The expression of genes involved in de novo ceramide synthesis (SPTLC1, SPTLC2, DEGS1, LASS1, LASS4 and LASS6) did not differ between the groups (**Table 1**). However several genes involved in ceramide metabolism were expressed at higher levels in the HLF group as compared to the NLF group, namely ASAH1, SPHK1, SMPD1, SMPD3 and SMPD2 and CERK had a strong tendency to be greater in HLF group (**Table 1**). The expression of SMPD1-3 was also compared between subcutaneous and intra-abdominal depots of non-obese (n=23) and morbidly obese (n=8) subjects. SMPD3 was higher in the intra-abdominal depot of both groups, while SMPD1 and SMPD2 did not show consistent differences in their expression pattern (**Figure 2**). The adipose tissue localization of SMPD1-3 by immunohistochemical staining revealed that these enzymes are present in macrophages and adipocytes, with the strongest staining present in and around blood vessels (**Figure 3**). The presence of sphingomyelinases in blood vessels and higher expression of these enzymes in HLF individuals led to an attractive hypothesis, that the sphingomyelin used as a substrate for sphingomyelinases to produce ceramide could come from circulating lipoproteins, secreted in abundance from fatty liver. Therefore additional apolipoprotein B staining of both obese and non-obese subjects was also performed and positive staining was found in blood vessels and CD68-rich areas of adipose tissue (**Figure 4**).

Table 1. Expression in subcutaneous adipose tissue of ceramide-metabolising enzymes in relation to adipose tissue inflammation and liver fat content.

Gene	Less inflamed adipose tissue, normal liver fat n=10	More inflamed adipose tissue, high liver fat n=10	*P*
SPTLC1	0.99±0.04	1.05±0.06	0.20
SPTLC2	0.97±0.07	1.00±0.05	0.39
DEGS1	0.99±0.05	1.07±0.05	0.11
LASS1	9.07±3.12	9.22±1.63	0.48
LASS4	1.08±0.10	1.07±0.16	0.49
LASS6	0.87±0.07	0.89±0.07	0.42
ASAH1	**1.16±0.10**	**1.47±0.13**	**0.03**
UGCG	0.91±0.08	0.96±0.06	0.34
SGMS1	1.01±0.05	1.10±0.06	0.12
SGMS2	2.09±0.27	2.57±0.32	0.13
SMPD1	**1.51±0.10**	**1.85±0.10**	**0.01**[a]
SMPD2	1.13±0.06	1.26±0.06	0.08[a]
SMPD3	**1.33±0.13**	**1.76±0.18**	**0.05**[a]
SMPD4	1.57±0.09	1.52±0.09	0.35
CERK	1.57±0.15	1.87±0.10	0.06
SPHK1	**12.18±1.49**	**17.64±1.65**	**0.01**
CGT	4.45±1.29	3.45±0.52	0.24

Relative gene expression levels of genes involved in ceramide and sphingomyelin metabolism in subcutaneous adipose tissue of obese women with different degrees of adipose tissue inflammation are given. SPTLC: serine palmitoyl transferase long-chain; DEGS: dihydroceramide desaturase; LASS: LAG1 homolog (ceramide synthase); ASAH: N-acylsphingosine amidohydrolase (ceramidase); UGCG: UDP-glucose ceramide glucosyltransferase; SGMS: sphingomyelin synthase; SMPD: sphingomyelin phosphodiesterase (sphingomyelinase); CERK: ceramide kinase; SPHK: sphingosine kinase; CGT: ceramide glycosyl transferase. Gene expression was normalized to housekeeping genes RPLP0 and TBP. Values are expressed as mean ± SEM. [a] Reported previously (101).

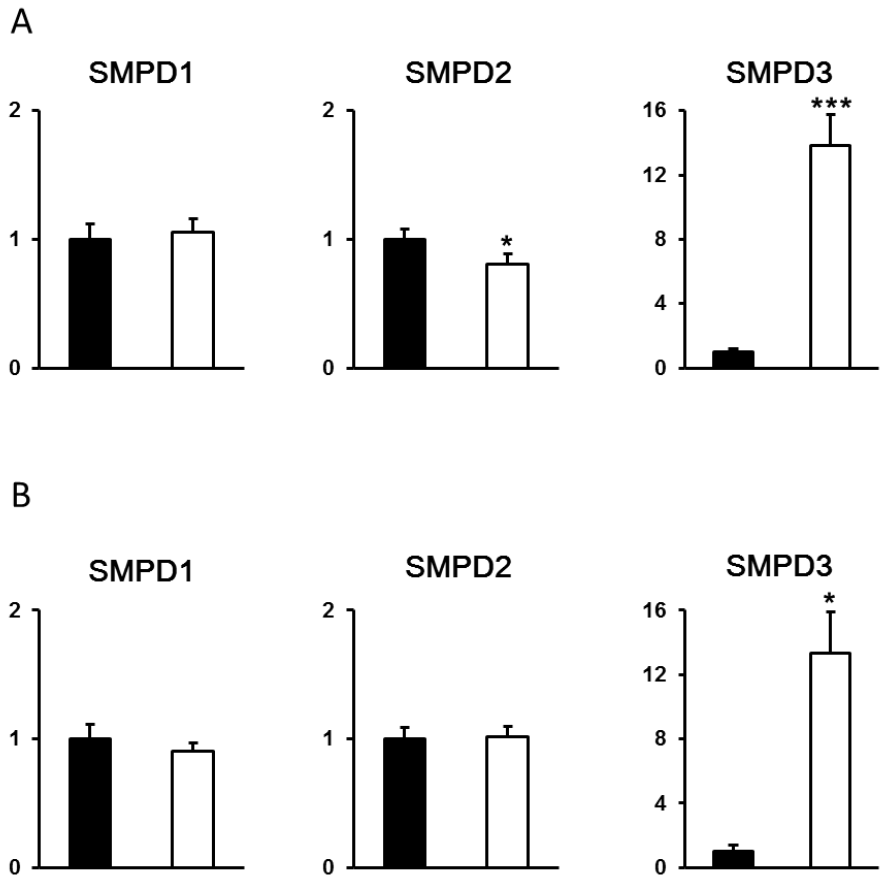


Figure 2. Gene expression levels of SMPD1-3 in subcutaneous (black bars) and intra-abdominal (white bars) adipose tissue from 23 non-obese (**A**) and 8 obese (**B**) individuals. Expression is in arbitrary units normalized to housekeeping genes RPLP0 and TBP, and set to 1 for the subcutaneous depot. *p<0.05, ***p<0.01 compared to subcutaneous tissue.

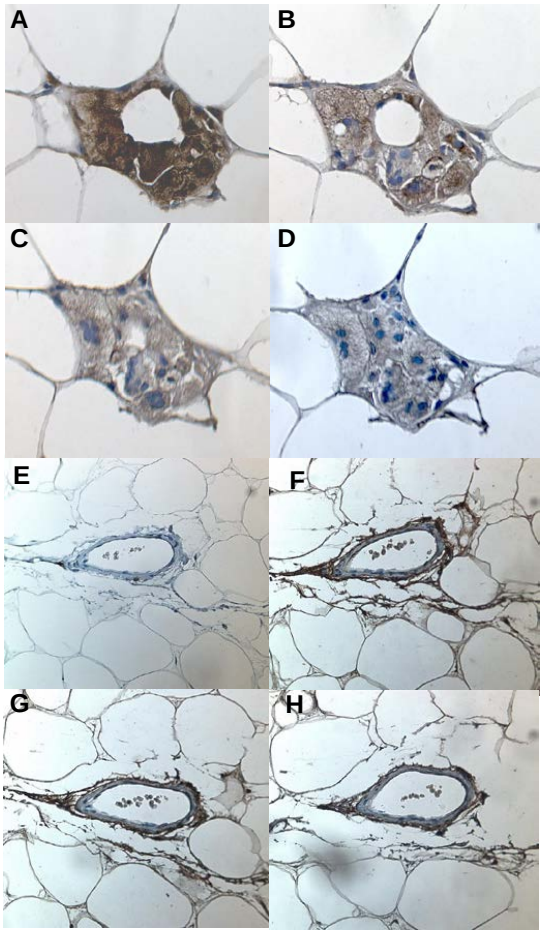


Figure 3. Immunohistochemical localization of CD68 (**A, E**), SMPD1 (**B, F**), SMPD2 (**C, G**) and SMPD3 (**D, H**) in macrophages (**A-D**) and blood vessels (**E-H**) in subcutaneous adipose tissue from an obese woman. Positive staining is coloured brown. All sections were counterstained with hematoxylin.

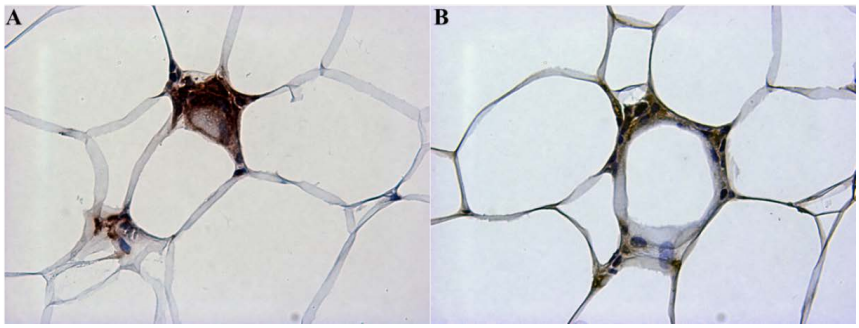


Figure 4. Localization of CD68 (**A**) and apoB (**B**) in a serial section of human subcutaneous adipose tissue from an obese woman.

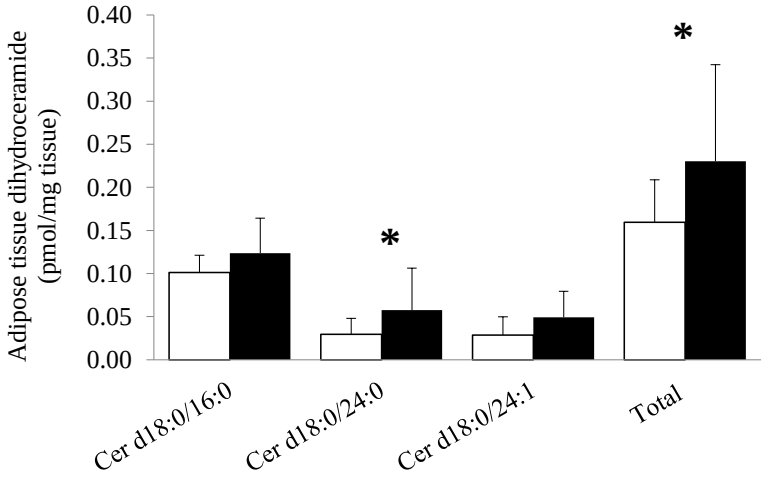
4.2 Paper II – Ceramides are associated with inflammatory processes in human mediastinal adipose tissue

It is now recognized that different adipose depots contribute differently to metabolic dysregulation, therefore we wondered whether these differences are reflected also in their ceramide content. The aim of this study was to investigate ceramide concentrations of several ceramide species in human subcutaneous and mediastinal adipose tissue and to determine associations between adipose tissue ceramides and global gene expression profiles.

Concentrations of 3 dihydroceramide (**Figure 5A**) and 6 ceramide species (**Figure 5B**) were evaluated in subcutaneous and mediastinal adipose depots from 10 patients. Ceramide with fatty acid length 16:0 was the dominant species in both depots. One dihydroceramide and all 6 ceramide species were significantly more abundant in the mediastinal as compared to the subcutaneous depot (**Figure 5**).

In addition to ceramide quantification, global gene expression profiling was performed in both adipose depots. We decided to assess relationships between ceramides and depot-specific gene expression profile with the OPLS technique. The model quality parameters were satisfactory only in the mediastinal tissue, thus only this depot was analyzed further. In the OPLS analysis of the mediastinal fat 2398 genes contributed significantly to the model, among which 1986 correlated positively and 412 negatively with ceramide. These genes were then subjected to gene ontology analysis in order to identify biological processes related to ceramide concentration (**Figure 6**). Genes that correlated positively with ceramide were categorized into 165 biological processes, of which 59 were related to immune or inflammatory processes (**Figure 6A**). Genes that correlated negatively with ceramide were classified into 42 biological processes, of which 13 were related to carbohydrate and 11 to lipid (predominantly fatty acid/cholesterol) metabolism (**Figure 6B**).

A



B

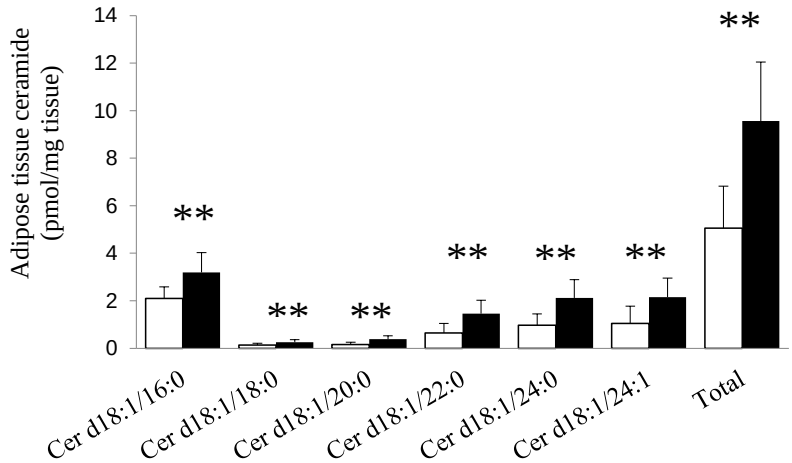
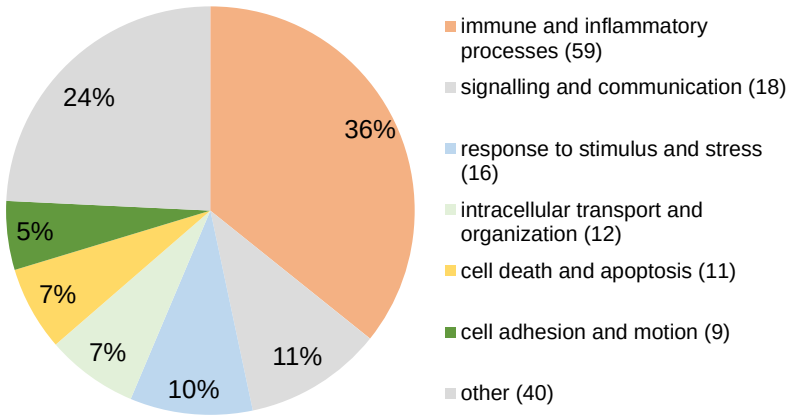


Figure 5. Dihydroceramide (A) and ceramide (B) concentrations (µmol/mg of tissue) in subcutaneous (white bars) and mediastinal (black bars) adipose tissue from 10 subjects. Data expressed as mean ± SD, *p<0.05 and **p<0.01 for differences between depots.

A



B

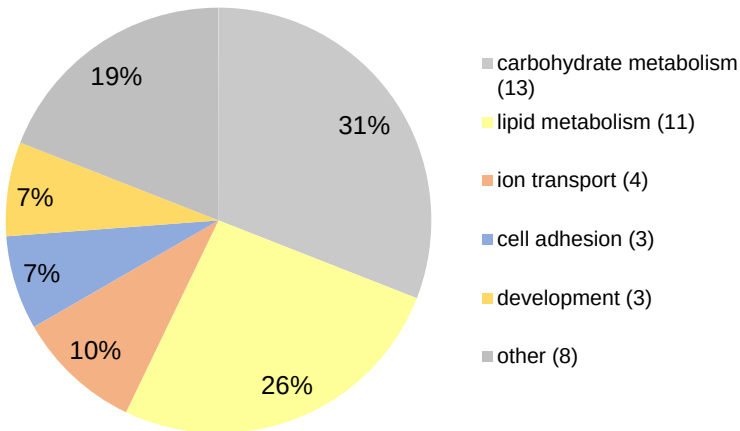


Figure 6. Distribution of the biological processes (listed clockwise) identified by GO analysis of the 1986 genes correlating positively (A) and the 412 genes correlating negatively (B) with ceramide concentrations in human mediastinal adipose tissue in OPLS analysis. The number of GO categories in each group is indicated.

4.3 Ceramide induces an inflammatory response from human monocyte-derived macrophages and 3T3-L1 cells (unpublished data)

In Paper II we clearly demonstrated associations between adipose tissue ceramides and inflammation. However, taking into account the complexity of adipose tissue and the different cell types that this tissue is composed of, we wished to further evaluate whether cells in adipose tissue (with particular interest in adipocytes and macrophages) responded in an inflammatory manner to ceramide and whether these cells produce more ceramide in response to inflammation. However, to date we have only started investigating the first question. Moreover, these results are very preliminary and should be considered as such (n=3 for macrophage experiments and n=1 for adipocyte experiment). Due to difficulties obtaining adipose tissue biopsies large enough to isolate the required number of primary cells we started evaluating the effect of ceramide on human primary monocyte-derived macrophages and on differentiated 3T3 L1 cells.

Monocytes were isolated from buffy coats and stimulated with 50ng/mL of human macrophage colony-stimulating factor (hMCSF) for 5 days. The cells were then treated with 10µM C6 ceramide or DMSO for 0, 3, 6 or 24h and harvested for mRNA expression analysis of selected inflammatory cytokines. C6 ceramide evoked a very strong increase in the expression of IL6, TNF and interleukin 1B (IL1B) (**Figure 7**) after 3 and 6 hours, with the effect returning towards control values after 24h (**Figure 7**).

Differentiated 3T3 L1 cells were treated with 10µM C6 ceramide or DMSO for 0, 3, 6 or 24h and then harvested for mRNA expression analysis of selected inflammatory cytokines. To date only one experiment has been performed where IL6 and MCP1 expression levels were measured, therefore no statistical analysis has been performed. Ceramide treatment evoked a multiple fold-change in the expression levels of both cytokines after 3h, which returned towards control levels after 24h (**Figure 8**).

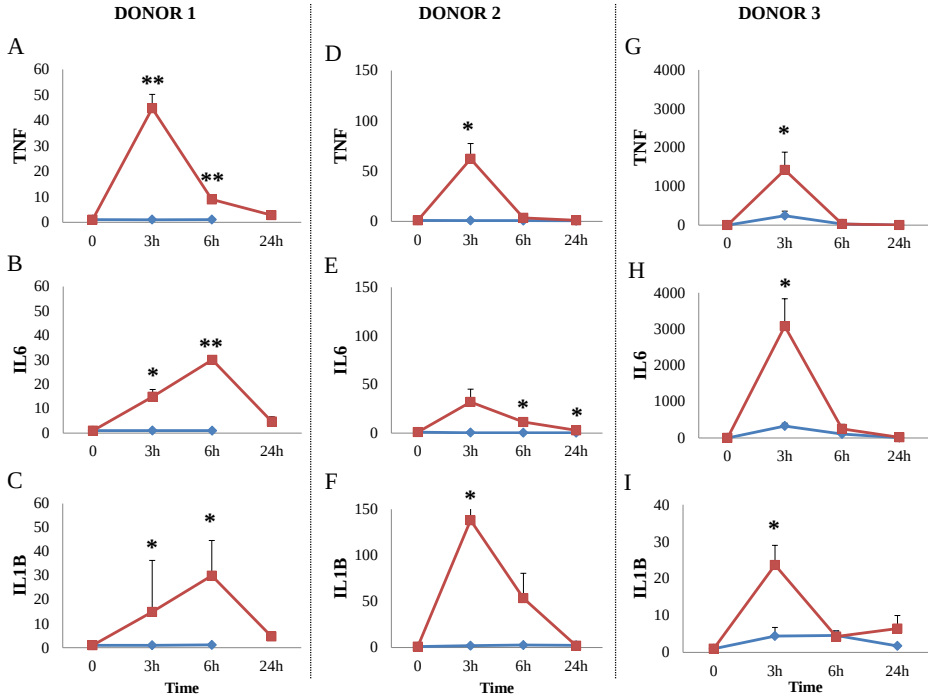


Figure 7. mRNA expression levels of TNF (A, D, G), IL6 (B, E, H) and IL1B (C, F, I) in human monocyte-derived macrophages from 3 donors (donor 1 – A, B, C; donor 2 – D, E, F; donor 3 – G, H, I) following ceramide treatment. Data are expressed as fold change of the expression levels relative to time 0. Data are expressed as mean ± SD. Control experiments (DMSO treatment) are coloured blue and 10μM ceramide-treatment experiments are coloured red. Differences between the groups were analysed with paired t-test. *p<0.05, **p<0.01.

A B

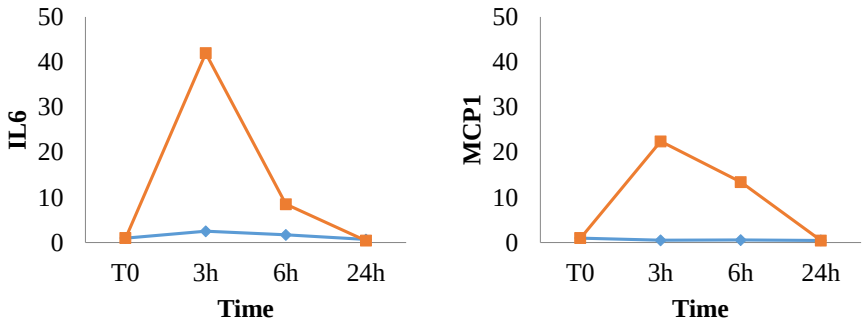


Figure 8. mRNA expression levels of IL6 (A) and MCP1 (B) in differentiated 3T3-L1 cells treated with 10µM C6-ceramide for 0, 3, 6 and 12 hours (n=1). Data Are expressed as fold change of the expression levels relative to time 0. The control experiment (DMSO treatment) is coloured blue and the ceramide experiment is coloured orange.

4.4 Paper III – Human mediastinal adipose tissue displays certain characteristics of brown fat.

The mediastinal adipose depot has been associated with CAD (103) but other studies discuss the possibility of this depot to display features of brown fat (14). If this depot truly contains brown adipocytes, an exciting possibility opens of redirecting excess fatty acids into heat production and thus decreasing the CVD risk. The goal of this study was to examine possible brown-fat characteristics of human mediastinal depot in comparison to the subcutaneous depot.

In the initial analysis we compared subcutaneous and mediastinal expression levels (measured by microarrays) of 33 genes previously described as markers of brown or white adipose tissue in humans, animals as well as cell models (see Table 2 in Paper III). Seven out of the first 33 screened genes (UCP1, PPARGC1A, COBL, CIDEA, PRDM16, HOXC8 and SHOX2) were chosen for confirmation in a separate group of 23 subjects and were measured by qPCR. UCP1 and PPARGC1A showed higher and SHOX2 and HOXC8 showed lower expression in the mediastinal depot as compared to the subcutaneous (**Figure 9**). Gene ontology analysis revealed that the mediastinal depot was enriched in mitochondrial gene sets related to mitochondrial matrix, mitochondrial inner membrane, respiratory chain complex or fatty acid oxidation. We also performed microscopic evaluation of the mediastinal depot and found that the adipocytes were smaller than in the subcutaneous fat, and in 2 out of 10 examined patients multilocular cells that stained positively for UCP1 could be found (**Figure 10**).

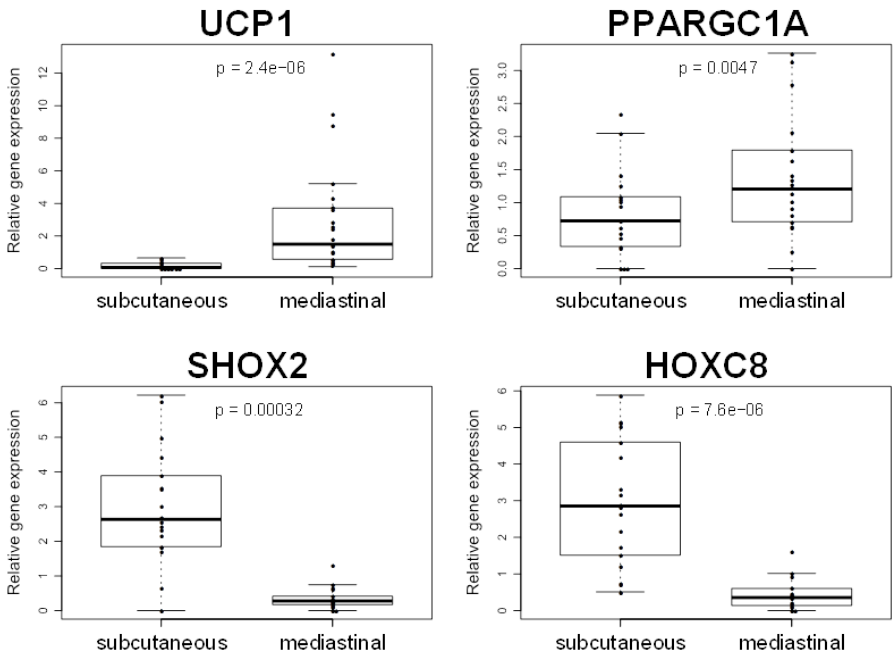


Figure 9. Comparison of gene expression levels of four (UCP1, PPARGC1A, SHOX2, HOXC8) selected genes in human subcutaneous and mediastinal adipose tissue (n=23). Box plots represent median (thick black middle line), first and third quartile (box outlines) and the interquartile range (whiskers). Gene expression was normalized to reference gene PPIA. P values were calculated using Wilcoxon paired sample test.

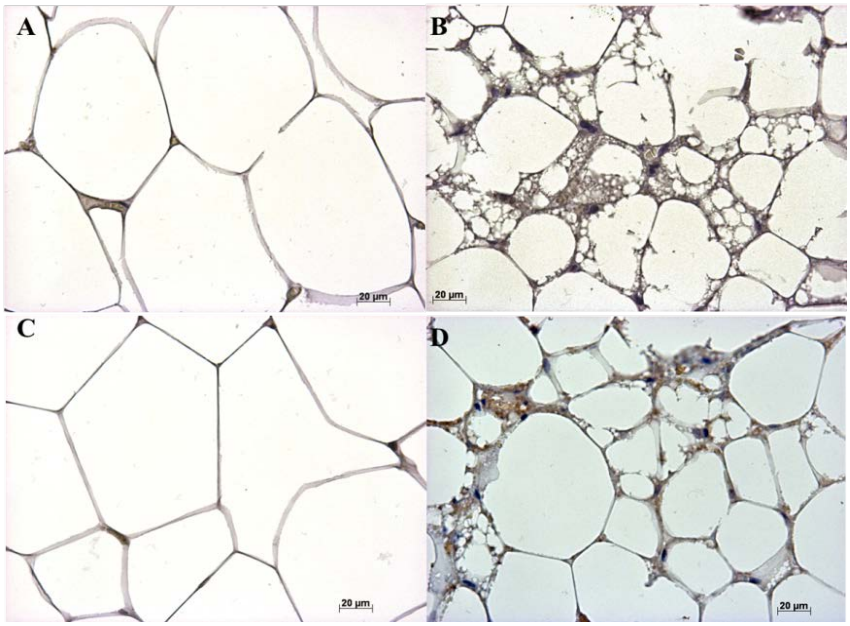


Figure 10. Microscopic comparison of paired subcutaneous (A, C) and mediastinal (B, D) adipose depots from 2 subjects. Multilocular adipocytes were visible in mediastinal (B, D) but not in subcutaneous (A, C) depots. The sections were stained for brown fat marker UCP1.

4.5 Paper IV – Proteomic analysis reveals an altered protein composition of subcutaneous adipose tissue in patients with chronic kidney disease

CVD is the main cause of death in CKD patients. In spite of a well-known contribution of adipose tissue to the development of CVD it remains unknown whether or how uremic fat could increase the CVD risk in those patients. The hypothesis of this study was that adipose tissue in patients suffering from kidney failure is dysfunctional and contributes to the high risk of cardiovascular disease observed in this group of patients.

Subcutaneous adipose tissue from patients with stage 5 CKD was compared to the same depot from healthy individuals. The tissue of the kidney disease patients had more inflammation expressed as the number of CD68-positive cells (**Figure 11A**), but no difference in the degree of fibrosis (**Figure 11B**). The patients also had significantly smaller adipocytes than healthy individuals (**Figure 11C**). Two-dimensional electrophoresis of protein extracts of subcutaneous adipose tissue was performed and the data analysed using multivariate techniques. This analysis revealed a very good separation of the two groups (CKD patients and healthy controls) indicating significant differences between them in terms of their adipose tissue protein composition (**Figure 12A**). The proteins that differed most significantly between the groups and that were further identified by mass spectrometry were vimentin and alpha-1-microglobulin/bikunin precursor (AMBP) (**Figure 12B**). The differences in the expression levels of vimentin and AMBP were also confirmed by Western blot (**Figure 13**).

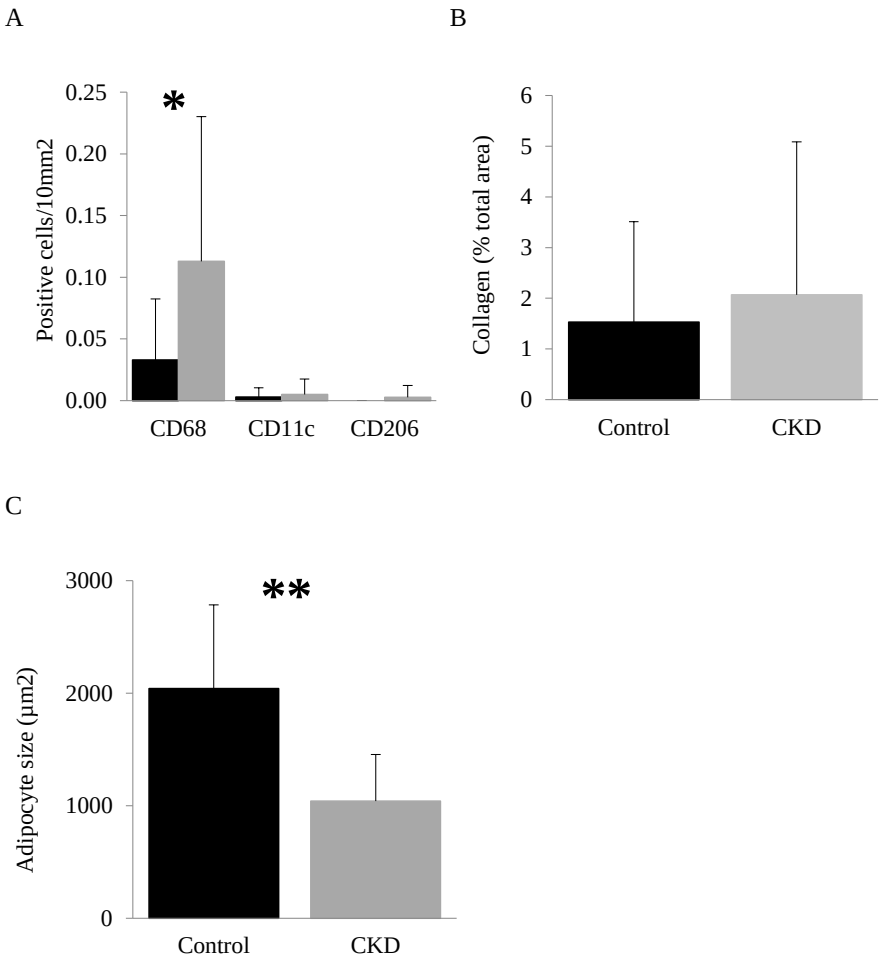


Figure 11. (A) Infiltration of inflammatory cells (number of CD68, CD11c and CD206 positive cells per $10mm^2$ tissue area) in adipose tissue of healthy subjects (black bars) and patients with CKD (grey bars). Data expressed as mean ± SD. *$p<0.05$ for differences between groups. (B) Comparison of collagen content in adipose tissue of healthy subjects (black bars) and patients with CKD (grey bars). Data expressed as mean ± SD. (C) Adipocyte size comparison between healthy subjects (black bars) and patients with CKD (grey bars). Data expressed as mean ± SD. **$p<0.01$ for differences between groups.

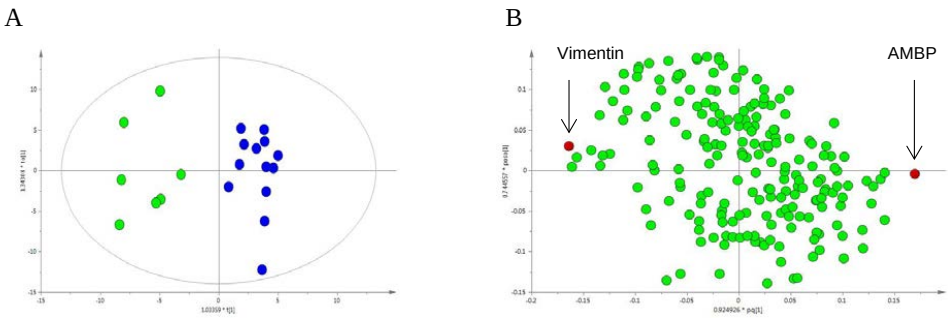


Figure 12. OPLS-DA analysis of protein spot volumes of adipose tissue from healthy controls and patients with CKD. The presence or absence of CKD was used as the Y vector. The analysis was performed on 7 control individuals, 13 CKD patients and 202 protein spots. The Hotelling's T2 (based on 95% confidence level) tolerance ellipse is shown in the score plot (A), which shows all the individuals analyzed (control individuals colour-coded green, CKD patients colour-coded blue). Predictive loadings representing the analyzed protein spots are plotted as a scatter plot (B).

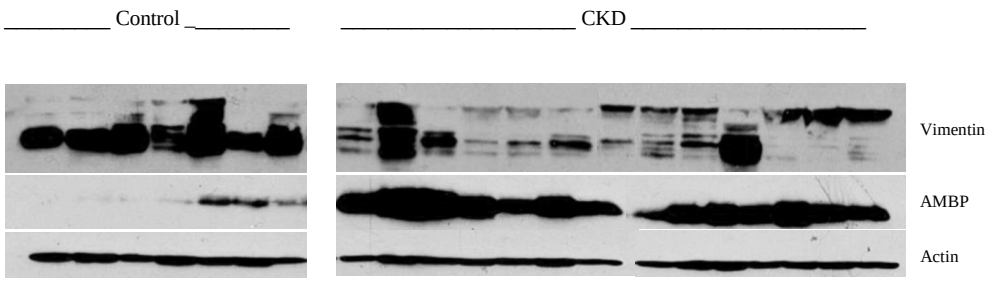


Figure 13. Western blot analysis of AMBP and vimentin in control individuals and CKD patients. Beta actin was used as loading control.

5 DISCUSSION

5.1 Paper I and II – adipose tissue ceramides: local production, global effects

In spite of relatively well-studied involvement of ceramides in the pathogenesis of insulin resistance in skeletal muscle, not much has been reported on ceramides in human adipose tissue. Several studies measured ceramide content in different adipose depots and plasma (101, 102, 104-106). Ceramide content is greater in visceral (intra-abdominal and epicardial) as compared to the subcutaneous depot (104, 105), but confounding results were reported comparing obese and lean subjects, since both increased and decreased ceramide content was shown (102, 105). However, it appears that there is more to define adipose ceramide levels than only BMI, since it seems that the presence of diabetes may increase the adipose ceramide content (102, 105) and higher ceramide concentrations have been observed in adipose tissue from obese women with high liver fat content as compared to equally obese women with normal liver fat (101).

The major results from Papers I and II can be summarized in several points – ceramide levels differ between adipose depots, where ceramide species show little correlation with systemic concentrations of ceramides, and that there are clear associations between ceramides and inflammatory processes within adipose tissue. The mechanisms responsible for the increased ceramide production in the inflamed fat are likely to be related to the formation of ceramide from sphingomyelin by the action of sphingomyelinases rather than from *de novo* ceramide synthesis. Immunohistochemical staining revealed the localization of apoB in areas rich in inflammatory cells (CD68-positive) and within blood vessels, where sphingomyelins are also present, indicating the possibility of direct access of adipose tissue sphingomyelinases to sphingomyelin-rich lipoproteins. In fact, it has been reported previously that the percentage of adipose tissue ceramides is not related to the percent of tissue sphingomyelin (104), which could support the hypothesis that the sphingomyelin used by adipose sphingomyelinases comes from circulating lipoproteins rather than adipose tissue itself. This mechanism would also explain previous findings that obese subjects with high liver fat have more ceramide in their adipose tissue (101), linking the local (adipose) ceramide production to features that have been well documented as promoting insulin resistance (fatty liver) and atherosclerosis development (adverse lipoprotein profile). This illustrates very well the integrative role of adipose tissue as an organ that can sense signals from the periphery and respond at local as well as systemic levels. A response in the form of increased ceramide production (from sphingomyelin hydrolysis) within adipose tissue is one of the local effects and leads to the

induction of insulin resistance and the expression of inflammatory genes within adipose tissue. These however may have systemic consequences, since some of the ceramide-induced cytokines, such as IL6, will be released into circulation and contribute to further impairment of insulin sensitivity. We have shown in our unpublished data that both macrophages as well as adipocytes are capable of a rapid inflammatory response when treated with exogenous ceramide and it remains to be established to what extent inflammatory treatment affects the ceramide content in these cells. These data will be extremely interesting in order to evaluate the cause and effect nature of the relationship between ceramide and inflammatory pathways.

5.2 Paper II and III – the mediastinal adipose depot – friend or foe?

Mediastinal adipose tissue belongs to the visceral depots located within the thoracic cavity, together with pericardial and epicardial fat. Epicardial fat is located between the outer myocardium and the visceral layer of the pericardium and is therefore in direct contact with the heart, the two tissues sharing microcirculation. Pericardial fat is located between the visceral and the parietal layers of the pericardium. The mediastinal depot is situated outside the pericardium. Intrathoracic fat depots may serve as a site for NEFA uptake that can be stored and quickly released to supply the heart with its energy demands. Large amounts of these depots however could lead to excess lipolysis, inflammation and increased adipokine release, affecting coronary vessels with accelerated atherosclerosis (107). In the current studies we sampled only the mediastinal depot, not the epicardial or pericardial depots, taking into consideration potential risks and the patients' well-being. Mediastinal fat volume has been previously associated to CAD (107) and certain adverse features of this depot, such as elevated number of inflammatory cells, have been also associated with the elevated risk of coronary atherosclerosis (103). Moreover, a gene expression comparison of inflammatory markers revealed that many of them were expressed at similar (IL1B, MCP1, PAI1, CD68) or higher (TNF, IL6) levels in the mediastinal as compared to the epicardial fat (108). In Paper II we also show that the mediastinal depot is characterized by higher ceramide content and higher expression of inflammatory cell markers than the subcutaneous fat and that ceramide content in this depot is positively associated with inflammatory processes. These results suggest a rather detrimental (promoting development of insulin resistance and/or CAD role of mediastinal adipose tissue and indicate that this ceramide-rich and inflamed depot could have other properties similar to intra-abdominal fat, such as a high lipolysis rate, however this requires more functional studies. On the other hand, several reports showed brown fat activity in the mediastinal region (14, 109-111). The presence of active brown adipose tissue in this region would provide a beneficial situation, where the excess of fatty acids is used to generate

heat. However, in spite of higher UCP1 mRNA expression levels in the mediastinal as compared to the subcutaneous depot, multilocular adipocytes in the mediastinal depot could only be visualised in 2 out of 10 patients and the UCP1 staining was scarce, indicating that the functional protein may not be widely expressed in the tissue. These results could indicate that the brown-like cells observed scattered between the white adipocytes may be beige rather than classically brown, taking into account the very low UCP1 expression. One could speculate that from the evolutionary point of view the presence of brown/beige adipose tissue around the heart would have practical implications, protecting this vital organ during prolonged and cold winters. Nowadays we are rarely exposed to such extreme conditions, but it is tempting to wonder whether our current observations could be related to the remnants of thermogenic capability and more brown-like properties of the mediastinal adipose tissue. With no need for heat generation and a constant oversupply of energy, the mediastinal depot may have turned away its friendly face, being more of an enemy today, though the metabolic relevance of this depot is definitely worth further investigations.

5.3 Paper IV – uremic fat – more than meets the eye

The characterization of subcutaneous adipose tissue from patients with CKD revealed several differences as compared to healthy subjects. The tissue from kidney failure patients has smaller adipocytes, is enriched in CD68-positive cells and displays a differential protein pattern, with the greatest alterations in expression levels of vimentin and AMBP protein. Taken together these results indicate that adipose tissue in the uremic milieu is disturbed and therefore its proper function is compromised. The most interesting part of this study was the proteomic analysis, since, to the best of our knowledge, no such comparisons of the protein profiles between uremic and healthy adipose tissues have been reported previously. Lower expression of vimentin in CKD patients was observed. Vimentin is one of the intermediate filament proteins present in mesenchymal cells and several studies reported the contribution of vimentin to the regulation of lipolysis, where it is present as afunctional partner of HSL facilitating the lipolytic process (112). Vimentin knock-out results in about 40% reduction in adipocyte lipolysis (113) and small adipocytes (114). Vimentin has been also shown to be one of the lipid droplet-associated proteins (115, 116) interacting with perilipin during lipid droplet formation (117) and primary human adipocytes cultured with uremic serum show decreased perilipin expression as well as increased basal lipolysis rates (63) pointing at altered lipid droplet metabolism in these conditions. Moreover vimentin protein expression in human adipose tissue has been shown to increase and correlate with proteins involved in GLUT4 transport and fusion after rosiglitazone treatment (118) indicating that higher

vimentin expression is a hallmark of more insulin-sensitive adipose tissue. Thus vimentin seems to be involved in some of the basic adipocyte functions, therefore alterations in vimentin expression may have adverse consequences for adipose tissue metabolism. On the contrary to vimentin, which was expressed at lower levels in CKD patients, AMBP was the most significant of the proteins expressed higher in this group. This finding fits with data showing that circulating concentrations of AMBP are elevated in CKD patients (119, 120). Alpha-1-microglobulin, one of the components of AMBP, binds and degrades free radicals protecting tissues from oxidizing agents, in particular free hemoglobin. Since oxidative stress is one of the features of disturbed adipose tissue and has been linked to inflammation, diabetes and obesity (121, 122), high AMBP levels in CKD patients could indicate that the tissues are exposed to the detrimental effects of free radicals, which in turn could oxidize lipids and proteins leading to cell stress and death. In summary, our results indicate that uremic adipose tissue is exposed to oxidative stress and presents with perturbed lipid droplet metabolism, together with increased phagocytic cell number, supporting the hypothesis that in uremic conditions adipose tissue function is disturbed, which could result in elevated CVD risk.

5.4 General discussion and conclusions

All four articles presented in this thesis are an attempt to characterize different aspects of dysfunctionality within human adipose tissue. They encompass different adipose depots, body compositions and health statuses. However they all focus on changes within the adipose tissue that may be a cause or consequence of more complex metabolic conditions, in particular insulin resistance and cardiovascular disease. The forms of dysfunctionality that were evaluated include the size of adipocytes, presence of fibrosis, inflammation, ceramide content as well as expression profiles of genes and proteins – all in light of their plausible contribution to the development of metabolic diseases. Taken together - the main findings of our investigations can be summarized as follows:

- Increased ceramide content in human adipose tissue observed in relation to fatty liver is most probably due to accelerated hydrolysis of sphingomyelins, possibly from circulating lipoproteins, rather than *de novo* synthesis
- Compared to the subcutaneous adipose tissue, the mediastinal depot is characterised by increased ceramide content, with ceramide concentrations within the depot being positively associated with genes involved in immune and inflammatory processes and negatively with lipid and carbohydrate metabolism
- Mediastinal adipose tissue displays certain features of brown fat, such as scarce multilocular cells, expression of certain BAT markers, like UCP1, and enrichment in genes related to mitochondrial function, however the metabolic properties of this depot (for instance fatty acid metabolism) are yet to be established
- Uremic adipose tissue shows signs of dysfunction, such as higher number of phagocytic cells and expression of proteins that are involved in altered lipid droplet metabolism and lipolysis rate as well as oxidative stress, supporting the hypothesis that in CKD disturbed adipose tissue may contribute to the high risk of CVD

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