Obesity-associated Inflammation in Adipose Tissue

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To my family

TABLE OF CONTENTS

TABLE OF CONTENTS **i**
ABSTRACT **iii**
LIST OF PAPERS **iv**
ABBREVIATIONS **v**
SAMMANFATTNING PÅ SVENSKA **vii**
INTRODUCTION **1**
Adipose tissue as a metabolic and endocrine organ *1*
Adipose tissue distribution and depots *2*
Menopause *4*
Adipose tissue inflammation 5
Mechanical stress *5*
Hypoxia *5*
Endoplasmic reticulum stress *6*
Fatty acid flux *6*
Adipose tissue macrophages *6*
Cytokines & chemokines *8*
Tumor necrosis factor α *8*
Monocyte chemoattractant protein 1 / C-C chemokine receptor 2 *8*
Interleukin 8 *9*
Interleukin 6 *9*
Macrophage migration inhibitory factor *10*
Fatty acids and Toll-like receptors *10*
Adipose tissue as the origin of chronic low-grade inflammation *11*
Reducing & maintaining body weight 12
Lifestyle modification *12*
Dieting *12*
Physical activity *13*
Pharmacological therapy *14*
Bariatric surgery *14*
AIMS **15**
SUBJECTS & METHODS **16**
Study participants 16
Study I *16*
Study II *16*
Normal weight premenopausal and postmenopausal women *16*
Obese women before and after gastric bypass surgery *16*
Study III *17*
Methodological issues 18
Anthropometrics *18*
Body composition and fat distribution *18*

Blood chemistry *18*
Adipose tissue biopsies *18*
RNA extraction, reverse transcription, and real-time polymerase chain reaction *19*
Statistical analysis 19
Study I *19*
Study II *19*
Study III *20*
RESULTS & DISCUSSION **21**
Study I *21*
Subject characteristics *21*
The visceral adipose tissue depot has a unique inflammatory profile *21*
Macrophage migration inhibitory factor and C-C chemokine receptor 2 in adipose tissue inflammation *23*
Study II *23*
Anthropometric and biochemical characteristics *24*
Massively reduced low-grade inflammation following gastric bypass surgery *24*
Increased circulating cytokine levels after menopause *25*
Study III *26*
Subject characteristics *26*
Improved inflammatory profile after weight reduction *27*
Changes in TLR4 and MIF expression may favor fat storage in subcutaneous adipose tissue *27*
Reductions in inflammatory parameters and associations with characteristics *28*
GENERAL DISCUSSION **31**
Fat distribution & metabolic risk *31*
Menopausal status, aging & adiposity *32*
Weight reduction *33*
SUMMARY & CONCLUSIONS **35**
ACKNOWLEDGEMENTS **36**
REFERENCES **38**

ABSTRACT

Background: Excess body fat, particularly in the visceral depot, is linked to increased mortality and morbidity, including the development of diseases such as type 2 diabetes, cardiovascular disease, and cancer. Chronic low-grade inflammation in adipose tissue may be a key mediator of obesity-associated diseases. Importantly, specific pro-inflammatory cytokines have been shown to influence adipose tissue function and could therefore be a link to metabolic disorders. Circulating cytokine levels may also be increased in obesity and metabolic diseases. However, although fat distribution and inflammation are clearly linked to metabolic disorders, inflammatory gene expression in the different abdominal adipose depots has not been investigated in detail. The menopausal transition is followed by a centralization of body fat and increased adiposity. Notably, inflammatory changes in fat during the menopausal transition have not been characterized. Finally, there is a lack of studies investigating the long-term effects of weight loss on low-grade inflammation. The aim of this thesis was to characterize differences between fat depots and investigate putative changes in low-grade inflammation in adipose tissue and circulation following menopause or weight loss. ***Materials & Methods:*** The expression of inflammation-related genes was investigated in abdominal adipose tissue depots obtained from women with varying adiposity, before and after menopause or weight loss induced by surgery or dietary intervention. Circulating cytokine levels were analyzed using immunoassays. ***Results:*** Visceral fat displayed a distinct and adverse inflammatory profile compared with subcutaneous adipose tissues, and the higher gene expression in visceral fat was associated with adiposity. Postmenopausal women exhibited a higher expression of pro-inflammatory genes than premenopausal women that associated with central fat accumulation. There was also a menopause-related increase in circulating cytokine levels in postmenopausal women. After surgery-induced weight loss, there was a dramatic reduction in inflammatory gene expression followed by increased insulin sensitivity. We observed no alterations in circulating cytokine levels. Long-term dietary intervention, associated with weight loss, had favorable effects on inflammation in both adipose tissue and serum. ***Conclusion:*** Fat accumulation is linked to low-grade inflammation in abdominal adipose tissue. The unique inflammatory pattern of visceral fat suggests a distinct role in adipose tissue inflammation that is aggravated with increasing adiposity. In postmenopausal women, the adverse adipose inflammatory profile was associated with central fat accumulation, while higher circulating cytokine levels correlated with menopausal state/age. Our data from severely obese women undergoing surgery-induced weight loss clearly supports a link between adipose inflammation and insulin resistance. The long-term beneficial effects of weight loss were also demonstrated by the improved inflammatory profile after dietary intervention. In summary, excess body fat is clearly linked to adipose tissue inflammation. Long-term weight loss is accompanied by improved metabolic profile and reduced low-grade inflammation in fat.

Keywords: adipose tissue, inflammation, pro-inflammatory cytokines, serum, obesity, weight loss, menopause.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by the indicated Roman numerals:

I. **Alvehus M,** Burén J, Sjöström M, Goedecke J, Olsson T. 2010 The human visceral fat depot has a unique inflammatory profile. *Obesity 18(5):879-883*

II. **Alvehus M,** Simonyte K, Andersson T, Söderström I, Burén J, Rask E, Mattsson C, Olsson T. 2012 Adipose tissue IL-8 is increased in normal weight women after menopause and reduced after gastric bypass surgery in obese women. *Clinical Endocrinology, Epub ahead of print, In press*

III. **Alvehus M,** Ryberg M, Blomquist C, Larsson C, Lindahl B, Sandberg S, Söderström I, Burén J, Olsson, T. Decreased TLR4 and increased MIF adipose gene expression following long-term dietary intervention. *Manuscript*

ABBREVIATIONS

ATM	Adipose tissue macrophage
BMI	Body mass index
CCR2	C-C chemokine receptor 2
CRP	C-reactive protein
CVD	Cardiovascular disease
CT	Computed tomography
DXA	Dual energy x-ray absorptiometry
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FFA	Free fatty acid
GBP	Gastric bypass
GEE	Generalized estimating equation
GI	Glycemic index
GLUT4	Glucose transporter 4
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
IL	Interleukin
IRS	Insulin receptor substrate
JNK	JUN N-terminal kinase
LCD	Low-calorie diet
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
L4/5	Lumbar vertebrata 4/5
MCP-1	Monocyte chemoattractant protein 1
MIF	Macrophage migration inhibitory factor
mRNA	Messenger ribonucleic acid

MUFA	Monounsaturated fatty acid
NNR	Nordic nutrition recommendations
NF-κB	Nuclear factor-κB
PD	Paleolithic diet
PLS-DA	Partial least squares discriminant analysis
PPARγ	Peroxisome proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acid
SAT	Subcutaneous adipose tissue
SD	Standard deviation
SFA	Saturated fatty acid
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TNF-αR	Tumor necrosis factor α receptor
VAT	Visceral adipose tissue
VLCD	Very-low-calorie diet
WHR	Waist-to-hip ratio

SAMMANFATTNING PÅ SVENSKA

De senaste årtiondena har förekomsten av övervikt och fetma ökat dramatiskt i Sverige. Fetma är en välkänd riskfaktor för utveckling av nedsatt insulinkänslighet, typ 2 diabetes och hjärt-kärlsjukdom. Var fettet lagras har stor betydelse och bukfetma är starkt kopplat till ökad risk att utveckla metabol sjukdom. Efter klimakteriet förändras fördelningen av kroppsfett hos kvinnor med ökad bukfetma, vilket kan vara en bidragande orsak till utveckling av hjärt-kärlsjukdom och diabetes hos äldre kvinnor. Runt buken finns också underhudsfett som är kroppens främsta depå för fettinlagring med nästan obegränsad kapacitet att expandera. Upptag och inlagring samt frisättning av triglycerider sker till stor del i underhudsfettet runt buken. Underhudsfettet runt buken kan delas in i ett ytligt och ett djupare lager som delvis har olika egenskaper. I likhet med bukfetma har man sett att inlagring av djupt underhudsfett är kopplat till nedsatt insulinkänslighet.

Vid kronisk övervikt och fetma förekommer förhöjda nivåer av inflammatoriska proteiner i blodet och i fettväven har man noterat ett ökat uttryck av inflammationsrelaterade gener. Dessa inflammatoriska ämnen, så kallade cytokiner, påverkar kroppens förmåga att ta upp och lagra glukos och fetter som vi får i oss via kosten. Förhöjda cytokinnivåer i fettväven och blodet skulle därför kunna vara en starkt bidragande orsak till metabola störningar såsom diabetes och hjärt-kärlsjukdom. Dessutom har en ökad ansamling av cytokinproducerande makrofager noterats i fettväv hos patienter med typ 2 diabetes vilket tyder på en koppling mellan låggradig inflammation och metabol sjuklighet.

I studie I undersökte vi genuttryck av inflammatoriska cytokiner och makrofagmarkörer i fettväv taget från tre olika depåer i buken. Kvinnorna som ingick i studien var från normalviktiga till feta. Vi fann att det ytliga och djupa underhudsfettet uppvisade liknande nivåer av inflammationsrelaterade gener. Däremot utskilde sig det djupt liggande bukfettet som visade högre uttryck av ett par gener som har kopplats samman med utveckling av bl a insulinresistens och hjärt-kärl sjukdom. Uttrycket av dessa gener var dessutom associerat till fetmarelaterade mått. Resultaten tyder på att bukfett har en starkare koppling till låggradig inflammation än underhudsfett.

I studie II jämförde vi inflammationsrelaterade markörer i underhudsfett och serum hos normalviktiga kvinnor före och efter klimakteriet (pre- och postmenopausala) samt i en grupp kraftigt feta kvinnor före och två år efter kirurgisk magsäcksförminskning, s.k. gastric bypass. De äldre postmenopausala kvinnorna hade mer fett ansamlat runt buken och högre blodfetter än de premenopausala kvinnorna. Genuttrycket av vissa inflammatoriska cytokiner i fettväven var högre hos postmenopausala kvinnor och det relaterade till den ökade fettansamlingen runt buken. Postmenopausala kvinnor hade även högre serumnivåer av hjärt-kärlsjukdomsmarkörer. Resultaten tyder på att en ökad låggradig inflammation hos postmenopausala kvinnor kan bero på förändringar i kroppsfettsfördelning samt ökad risk för hjärt-kärlsjukdom som följer med klimakteriet/åldern.

Kraftigt överviktiga kvinnorna som genomgått gastric bypass operation gick ner i genomsnitt 40 kg i vikt och fick avsevärt bättre insulinkänslighet. Nivån av inflammationsmarkörer i fettväven minskade dramatiskt. Resultaten visar på ett troligt samband mellan kraftig fetma, låggradig inflammation och metabol sjuklighet.

I studie III ingick 70 överviktiga/feta kvinnor som randomiserats till att följa en kost enligt Nordiska näringsrekommendationer (NNR) eller en modifierad stenålderskost (Paleolitisk kost, PD) i två år. Förutom olika antropometriska mått togs blodprover samt fettbiopsier från underhudsfett vid studiens början, efter 6 månader och vid studiens slut efter 24 månader. Efter 6 månader hade kvinnorna i båda kostgrupperna minskat ordentligt i vikt, med en mer uttalad nedgång i PD gruppen och efter 24 månader kvarstod viktminskningen i båda grupperna. Förändringar i genuttryck och proteinnivåer tyder på minskad inflammation i fettväv och serum efter 6 och 24 månader. Vissa inflammationsmarkörer minskade inte förrän efter 24 månader, vilket visar på betydelsen av långtidsstudier. Viktnedgång i sig, snarare än kostsammansättningen, verkar vara av störst betydelse för den minskade låggradiga inflammationen.

Sammanfattningsvis är övervikt och fetma kopplat till låggradig inflammation i fettväven. En ökad ansamling bukfett kan ha ogynnsamma inflammatoriska och metabola effekter. Däremot är viktnedgång associerat med minskad låggradig inflammation och metabola förbättringar. Resultaten ökar förståelsen om fettfördelningens betydelse och förändringar i samband med klimakteriet. Studierna ökar också kunskapen om förändringar i fettväven vid viktnedgång som kan vara av betydelse för fetmarelaterad sjukdom.

INTRODUCTION

The global prevalence of obesity has more than doubled during the last 30 years, and today approximately 10% of all adults are affected. In 2008, 1.5 billion adults were considered overweight; of those, over 200 million men and nearly 300 million women were obese[1]. The recent rise in overweight and obesity makes excess body fat more related to death than underweight[2].

The definitions of overweight and obesity are based on the body mass index (BMI), which is calculated as one's weight in kilograms divided by one's height in meters squared (kg/m^2): a BMI of 18.5-24.9 denotes "normal weight", BMI ≥ 25 denotes "overweight", and BMI ≥ 30 denotes "obesity". Obesity has been further separated into subclasses: a BMI ≥ 35 is designated as "severe obesity" and a BMI ≥ 40 is designated as "morbid obesity".

The rising rate of overweight and obesity brings on a great burden that affects both the individual and society. Obese individuals are more frequently afflicted by cancer, diabetes, and cardiovascular disease, and their overall mortality is higher[3]. Although body fat content is certainly influenced by several factors, including genetics, the recent surge in obesity must be ascribed to changes in lifestyle. Increased energy intake combined with low levels of physical activity predisposes to obesity and its related comorbidities[4-7].

Adipose tissue as a metabolic and endocrine organ

Adipose tissue is a highly active metabolic and endocrine organ. Excess energy intake is stored as triglycerides in lipid droplets within the adipocytes, and these lipids are mobilized and used as energy in times of negative energy balance. The capacity of adipose tissue to store triglycerides is almost unlimited, with individual adipocytes increasing in size as they accumulate more triglycerides[8]. In addition to adipocytes, adipose tissue consists of a stromal vascular fraction of fibroblasts, preadipocytes, endothelial cells, and immune cells, surrounded by an extracellular matrix (ECM)[9].

The endocrine function of adipose tissue comprises the production and secretion of a variety of compounds, including sex steroids and bioactive peptides, acting in a local (autocrine/paracrine) or systemic (endocrine) manner[10] (Figure 1). Adipokines, including leptin and adiponectin, have endocrine effects and are almost exclusively synthesized and secreted by adipocytes. Cytokines (i.e., peptides with an immunomodulatory function) are also produced in the adipose tissue[11]; however, in contrast to adipokines,

cytokines are synthesized by cells in the stromal vascular fraction, as well as by adipocytes[12]. Excess body fat is linked to disturbances in the metabolic and endocrine function of adipose tissue, and these shifts may cause metabolic dysfunction and the development of obesity-related diseases[13].

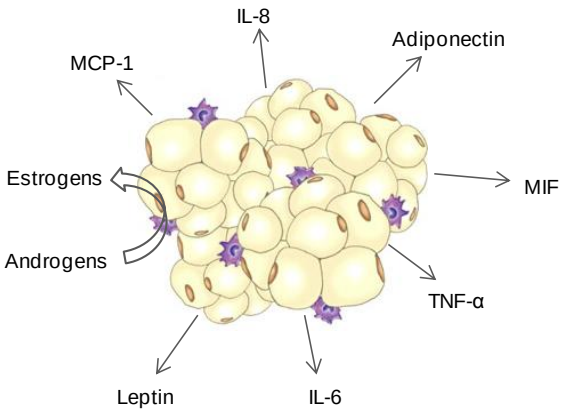

Figure 1. The endocrine functions of adipose tissue.

Adipose tissue distribution and depots

In 1947, J Vague described the relation between body fat distribution and metabolic outcome. He observed that upper-body (android) obesity was associated with disorders like diabetes and atherosclerosis, while lower-body (gynoid) fat accumulation seemed to have a protective effect[14].

The subcutaneous adipose tissue (SAT) depot is the largest fat storage site and is distributed throughout the body. Abdominal SAT is the primary source of systemic free fatty acids (FFAs)[15], and appears to work as a "metabolic sink" for the clearance and storage of excess lipids. Subcutaneous abdominal fat can be subdivided into two distinct compartments: superficial and deep SAT, which are separated by Scarpa's fascia. The two SAT depots have several distinct characteristics, including morphological and metabolic features. Deep SAT adipocytes are smaller in size than superficial subcutaneous fat cells[16]. The organization of the fat lobules also differs: superficial SAT has small, tightly packed lobules, while the lobules of the deep SAT are bigger and more irregularly distributed[17]. Moreover, deep SAT has been suggested to be more lipolytically active than superficial SAT[18], and also associated with insulin resistance[19, 20]. Indeed, the relationship between

deep SAT accumulation and insulin resistance is strong and comparable to that of visceral adipose tissue (VAT), while there is no association between the superficial SAT depot size and insulin resistance[19, 20]. Thus, to some extent the deep SAT mirrors the characteristics of VAT rather than superficial SAT.

Visceral fat is located intra-abdominally and surrounds the organs. Figure 2 shows a computed tomography (CT) scan representing the different fat depots. Accumulation of VAT is linked to detrimental alterations in glucose and lipid metabolism, manifested by disorders such as dyslipidemia, insulin resistance, and atherosclerosis[21]. In addition, the anti-lipolytic effect of insulin is weaker in VAT[22], which may exacerbate the impact of visceral obesity. The harmful effects of excess VAT may be explained by its direct access to the liver. VAT is drained by the portal vein, and consequently lipids, cytokines, and adipokines released from the visceral depot are directed straight to the liver.

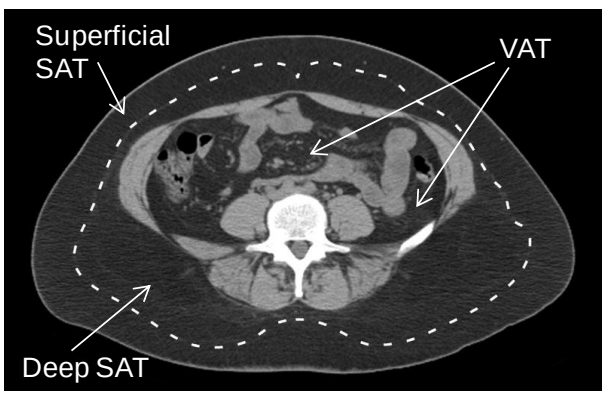

Figure 2. A computed tomography scan depicting the superficial and deep subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) depots. The broken line represents Scarpa's fascia[23].
Reprinted with permission from Oxford University Press.

Menopause

The menopausal transition in women starts with declining estrogen levels and is followed by significant changes in body composition and distribution. Body fat is redistributed from peripheral (e.g., gluteo-femoral depots in premenopausal women) to a more central accumulation after menopause (Figure 3). A centralized fat distribution, characterized by a larger visceral depot, exposes postmenopausal women to metabolic disorders, while a peripheral fat deposition appears to have a protective role[24]. Aside from the menopause-related increase in VAT, there is also a tendency toward general weight gain in mid-life women that is most likely related to additional factors, such as aging and changes in eating and exercise habits[25, 26]. Additionally, a change in lipid metabolism linked to an adverse blood lipid profile follows menopause[27, 28]. The effect on glucose metabolism is debated, with some studies indicating reduced insulin sensitivity after menopause[29, 30] while other groups report no change[31-33]. The menopausal transition has long been associated with an enhanced risk of cardiovascular disease; however, this view is questioned by more recent reports[34-36]. In all, postmenopausal women have a less favorable metabolic profile than premenopausal women, caused by factors other than menopause *per se*.

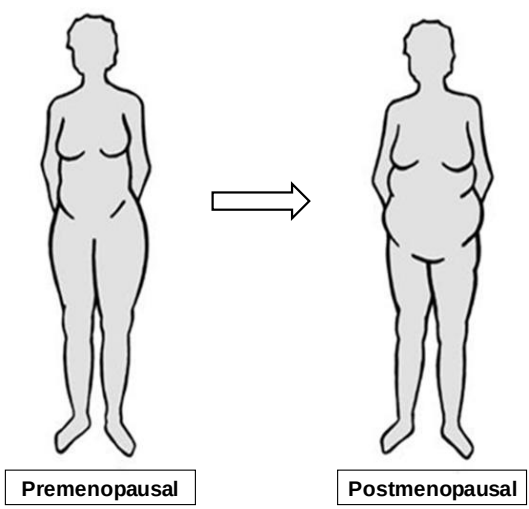

Figure 3. Redistribution of body fat during the menopausal transition. Illustration: Anton Grenholm.

Adipose tissue inflammation

Metabolism and immunity are two fundamental systems for survival, and are closely linked to each other. Overeating and obesity are associated with immune activation and inflammatory disease, while malnutrition can lead to immunosuppression and enhanced risk of infections[37]. The intersecting and interdependent pathways and partly overlapping characteristics of macrophages and adipocytes are also of interest[37].

Adipose tissue expansion can occur either through hyperplasia (i.e., increasing number of adipocytes) or by hypertrophy (i.e., increasing adipocyte size)[38]. The number of fat cells is mainly set during childhood and adolescence, and remains constant during adulthood in both lean and obese subjects. Consequently, enlargement of fat mass is primarily due to hypertrophy in adults; similarly, weight loss is associated with decreasing adipocyte volume[39]. This is important because hypertrophic adipocytes display distinct expression and secretion of cytokines and adipokines[40, 41]. Moreover, enlarged adipocytes exhibit elevated lipolysis and are more resistant to the anti-lipolytic effect of insulin[42]. Hence, hypertrophy appears to be central in adipose tissue dysfunction and inflammation. A number of mechanisms implicated in the initiation and progress of adipose tissue inflammation have been described, and some are reviewed below and presented in Figure 4.

Mechanical stress

Adipose tissue remodeling is an ongoing process that intensifies in response to fat mass expansion. The ECM provides mechanical support for adipocytes, and an upregulation of ECM genes, as observed in the onset of obesity, may restrict fat cell enlargement[43, 44]. Thus, when adipocytes accumulate lipids and increase in size, the strain from the expanding intracellular lipid-droplet concurrently with the rigid surrounding ECM causes shear stress on the cell membrane. This activates inflammatory pathways and induces pro-inflammatory cytokine expression[44].

Hypoxia

Hypoxia is another concern for hypertrophic adipocytes[11]. Indeed, obese rodent adipose tissue is poorly oxygenated[45], and exposure to hypoxia evokes an inflammatory response in adipose tissue as well as in adipocytes. Poor oxygenation has also been demonstrated in adipocytes derived from humans and mice, as well as in 3T3-L1 adipocytes, in which hypoxia treatment induced the expression and release of interleukin (IL)-6 and macrophage migration inhibitory factor (MIF)[45, 46].

Endoplasmic reticulum stress
Endoplasmic reticulum (ER) stress is induced by the overflow of energy and nutrients. Folding and trafficking of proteins take place in the ER, and the capacity of the ER may be exceeded during abnormal energy fluxes in the cell. This ER stress leads to activation of inflammatory and stress signaling pathways (e.g., JUN N-terminal kinase [JNK]) and subsequent suppression of insulin action[47].

Fatty acid flux
Fatty acid release from enlarged adipocytes is elevated, and obese individuals are characterized by high circulating FFA levels. FFAs inhibit the anti-lipolytic action of insulin, thereby leading to additional FFA release. A rise in circulating FFAs can activate toll-like receptors (TLRs), which in turn trigger the nuclear factor-κB (NF-κB) pathway and induce downstream expression of pro-inflammatory cytokines[42]. High fatty acid flux also contributes to ER stress and can induce macrophage recruitment to the adipose tissue[48].

A common finding among the mechanisms described above is the activation of signaling pathways and subsequent expression of pro-inflammatory cytokines. Cytokines are small signaling proteins with immunomodulatory activity, and affect the inflammatory and metabolic functions of adipose tissue. Some cytokines, known as chemokines, are chemotactic (i.e., induce the infiltration of immune cells into adipose tissue).

Adipose tissue macrophages

Chemokines are important in the inflammatory process and crucial in recruiting macrophages to adipose tissue. Accumulation of macrophages is a hallmark of adipose tissue inflammation[49, 50]; the chemokine monocyte chemoattractant protein (MCP)-1 is central to this process[51, 52] and is induced prior to macrophage infiltration[50]. The production of inflammatory proteins rises after macrophage infiltration, and adipose tissue macrophages (ATMs) are the primary source of inflammatory cytokines and chemokines in excess fat[53-55]. Hence, recruited ATMs can attract additional macrophages in a feed-forward manner. As a matter of fact, macrophage accumulation in human and rodent fat increases proportionally to adiposity; in the adipose tissue of obese individuals, ATMs can represent nearly 40% of the cells[49]. The increased ATM density most likely has a great impact on adipose function, as macrophage-secreted factors evoke an inflammatory response in adipocytes along with increased lipolysis and reduced glucose uptake[56]. Adipose expression of macrophage- and inflammation-related genes has been shown

to accelerate at the onset of hyperinsulinemia[50]. This finding indicates that ATMs are central to the development of metabolic disease.

In addition to increased density, there is a phenotypic switch in ATMs following obesity. ATMs from obese mice have a pro-inflammatory phenotype with, for example, high tumor necrosis factor (TNF)-α expression, while ATMs from lean animals are characterized by anti-inflammatory cytokine expression[57]. A substantial increase in adipocyte death related to the size of fat cells is observed in the obese state. The necrotic-like dead adipocytes are surrounded by macrophages of a pro-inflammatory phenotype that aggregates in crown-like structures to scavenge the residual debris from lipid droplets and cells[58, 59]. Interestingly, MCP-1 and its receptor, C-C chemokine receptor (CCR) 2, appear crucial in macrophage infiltration and selective recruitment to dead adipocytes[59].

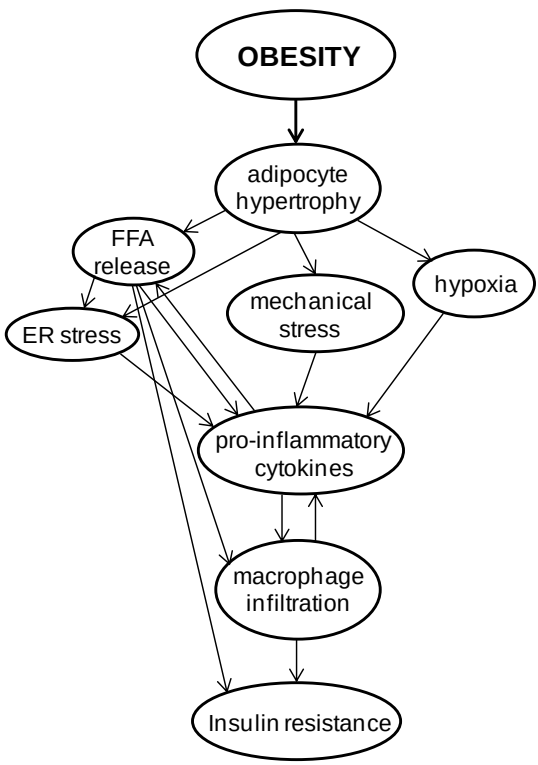

Figure 4. Mechanisms involved in obesity-associated adipose inflammation, and links to insulin resistance.

Cytokines & chemokines

Cytokines and chemokines share several signaling pathways and mechanisms, and have partially overlapping functions. Consequently, the network of cytokines and chemokines is highly complex. Selected cytokines and chemokines of particular importance to this thesis are presented in more detail below.

Tumor necrosis factor α

TNF-α was the first cytokine linked to increased adipose tissue expression and metabolic dysregulation[60]. It influences both adipocyte glucose and lipid metabolism through several mechanisms. For example, TNF-α downregulates lipoprotein lipase and perilipin expression, and thus contributes to hypertriglyceridemia and lipotoxicity[61]. The interaction between TNF-α and insulin resistance has been extensively studied, and occurs through both transcriptional and signaling mechanisms. TNF-α reduces the expression of insulin receptor, insulin receptor substrate (IRS)-1, and glucose transporter (GLUT)4, and also stimulates the inhibitory phosphorylation of IRS-1[61]. Another complex mechanism is the suppression of mRNA expression and transcriptional activity of peroxisome proliferator-activated receptor gamma (PPARγ), a central regulator of glucose and lipid metabolism, as well as inflammation[61]. Adipose TNF-α expression does not differ between fat depots, but is higher in obese than lean individuals[62, 63]. Although circulating TNF-α has also been associated with metabolic disorders, systemic levels are not directly linked to adipose production because there is no net release of TNF-α from fat[64]. However, circulating TNF-α as an inflammatory marker has been questioned, as its half-life in serum is short. Instead, the more serum-stable soluble TNF-α receptors (TNF-αR) I and particularly TNF-αRII have been suggested to better indicate the activity of the TNF-α system[65].

Monocyte chemoattractant protein 1 / C-C chemokine receptor 2

MCP-1 (also named CCL2) and its receptor CCR2 are centrally involved in obesity-associated inflammation and diseases. MCP-1 is overexpressed in subcutaneous fat in obese rodents and humans[66, 67], and MCP-1 and CCR2 are crucial for macrophage recruitment and linked to metabolic disorders like insulin resistance and hepatic steatosis[51, 52, 68]. *In vitro* studies support these results and demonstrate decreased insulin-stimulated glucose uptake in 3T3-L1 adipocytes after MCP-1 exposure, as well as decreased expression of adipogenic genes, including lipoprotein lipase, GLUT4, and PPARγ[67]. Serum MCP-1 levels are increased in individuals with type 2 diabetes and coronary artery disease[69-71]; however, the elevated levels appear not to directly derive from fat[66]. Within the arterial wall, a number of cell types can

produce MCP-1, including macrophages, endothelial cells, and smooth muscle cells[72] and MCP-1 expression is high in macrophage-rich atherosclerotic lesions[73]. In addition, monocyte CCR2 expression is increased in women with hypercholesterolemia. The elevated CCR2 expression increases the MCP-1-induced chemotaxis of monocytes, and may facilitate macrophage accumulation in the vascular wall[74]. Finally, elevated MCP-1 concentrations have been linked to sex hormone deficiency in postmenopausal women[75] and aging[76].

Interleukin 8

IL-8 was first identified as a neutrophil attractant, and has also turned out to be a major chemokine in monocyte recruitment[77]. A variety of cells, including macrophages, endothelial cells, smooth muscle cells, and adipocytes, are potential sources of IL-8[78, 79]. In adipose tissue, the majority of IL-8 derives from nonfat cells[80]. Both circulating and adipose IL-8 are involved in obesity and its related comorbidities, and the release of IL-8 from SAT correlates with BMI[80]. Interestingly, IL-8 appears to act in insulin resistance, as IL-8 overexpression has been revealed in visceral fat[81] and adipocytes[82] from insulin-resistant individuals. Indeed, experimental studies demonstrate that IL-8 inhibits insulin action in human adipocytes[83]. Moreover, elevated serum IL-8 levels are associated with visceral obesity[84], diabetes[85], and increased cardiovascular risk[86-88].

Interleukin 6

IL-6 is a multifunctional cytokine produced by various immune cells and non-immune tissues. IL-6 triggers the induction of acute-phase proteins from the liver such as C-reactive protein (CRP), which can be elevated 1000-fold in acute inflammatory processes and slightly increased during low-grade inflammatory conditions[89]. In addition to its immunomodulating function, IL-6 impairs adipocyte differentiation and interfere with glucose and lipid metabolism[90]. Adipose tissue is an important source of IL-6, and IL-6 production increases with fat mass; in fact, approximately 30% of systemic IL-6 levels are derived from fat[64]. Skeletal muscle is another source of IL-6, and circulating IL-6 levels can increase substantially during exercise[91]. The influence of IL-6 on metabolic function is somewhat controversial. IL-6 deficiency has reportedly been followed by either increased or unaltered body weight and insulin resistance[92-95]; in contrast, elevated adipose and serum IL-6 are associated with obesity and impaired insulin action[96]. Subcutaneous fat cells from insulin-resistant individuals overexpress IL-6 mRNA, and *in vitro* studies have revealed that IL-6 reduces IRS-1 and GLUT4 expression, and therefore reduces insulin-stimulated glucose uptake[82]. Moreover, IL-6 influences lipid metabolism, and IL-6 exposure *in vivo* and in cultured adipose tissue results in increased lipolysis[97, 98].

Macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF), the first cytokine to be described, is a pro-inflammatory chemotactic cytokine that is expressed by many cell types and participates in several acute and chronic inflammatory disorders, including atherosclerosis, arthritis, and sepsis[99, 100]. Although it is not classified as a chemokine, MIF has chemokine-like functions and can interact with chemokine receptors[101], and the MIF-induced recruitment of monocytes/macrophages involves the MCP-1/CCR2 pathway[102]. In the vessel wall, endothelial cells and macrophages in atherosclerotic plaques express MIF and thus contribute to lesion progression, macrophage recruitment, and inflammation[103].

MIF is secreted from adipose tissue and adipocytes[12, 104], and the adipocyte secretion rate is associated with donor BMI[105]. In addition, MIF expression is related to adipocyte size[106, 107], and *in vitro* studies have revealed the involvement of MIF in adipogenesis and triglyceride accumulation[108]. Pro-inflammatory cytokines typically inhibit adipogenesis and lipid storage, indicating distinctive features of MIF in adipose function.

However, MIF seems to play a pro-inflammatory role in adipose tissue inflammation and ensuing diseases, as MIF deficiency reduces ATM density and atherosclerosis and improves insulin sensitivity without affecting fat mass[107]. Moreover, circulating MIF levels have been associated with inflammatory vascular disease[103], obesity[109, 110], and type 2 diabetes[111].

Fatty acids and Toll-like receptors

Fatty acids are usually stored in adipocytes as triglycerides, which consist of three fatty acids linked to a glycerol backbone. Fatty acids are divided into unsaturated fatty acids with one (monounsaturated) or more (polyunsaturated) double bonds, and saturated fatty acids (SFAs), which lack double bonds. When fatty acids are released by lipolysis, they can be re-esterified into triglycerides and transported by lipoprotein particles, or circulate in non-esterified form (i.e., as FFAs) bound to albumin in the serum[112].

FFAs appear as active modulators of metabolism and inflammation in adipose tissue, as well as at more distant sites, such as the liver, pancreas, skeletal muscle, and vessel wall[42]. Chronically elevated circulating FFAs can induce insulin resistance in obese individuals, and reducing FFA levels may enhance insulin sensitivity in obese non-diabetics and type 2 diabetics[113].

Replacing dietary SFAs with monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) is associated with reduced insulin resistance and cardiovascular risk[114]. SFAs have the capacity to provoke an inflammatory response in target cells; one known mechanism is via activation of the cell-surface receptors TLR2 and TLR4. TLRs are a family of

pattern recognition receptors with a central function in innate immunity, and are expressed in both macrophages and adipocytes[115]. In adipocytes, TLR2 and TLR4 stimulation induce NF-κB activation and subsequent pro-inflammatory cytokine release. TLR4 appears as the most abundantly expressed TLR in human adipose tissue[116]. Both TLR2 and TLR4 are upregulated in excess adipose tissue, and inactivation of either TLR2 or TLR4 protects against adipose inflammation, macrophage infiltration, insulin resistance, and fatty liver induced by high-fat diet or lipid infusions[117-120].

Some of the beneficial effects of PUFA consumption may be mediated by TLR4. *In vitro* studies have demonstrated that long-chain PUFAs blunt the pro-inflammatory response generated by SFAs[118]. High intake of long-chain PUFAs also prevents the activation of TLR4 by its natural agonist lipopolysaccharide (LPS) in human blood monocytes[121].

In addition to pro-inflammatory responses, LPS-induced TLR4 activation evokes lipolysis from adipose tissue, as well as from adipocytes *in vivo* and *in vitro*[122]. Further support for a role for TLR4 in lipolysis was obtained from TLR4 mutant mice, which were protected from elevated serum FFA levels when maintained on a high-fat diet[123].

Adipose tissue as the origin of chronic low-grade inflammation

Several models have been described that support a fundamental link between obesity, immune activation, and metabolic dysregulation. In obese animals, high-dose salicylate treatment targeting an upstream activator of NF-κB abolished obesity and diet-induced insulin resistance[124]. Salicylate treatment in patients with type 2 diabetes improved glycemic control[125, 126], and ER stress was alleviated when cultured human abdominal subcutaneous adipocytes were treated with salicylate[127]. Lipid infusions are able to induce insulin resistance, even in the absence of obesity[128]. TNF-α expression was upregulated in adipose tissue from obese mice, while expression in other tissues, including liver and skeletal muscle, was undetectable in both lean and obese mice[60]. Neutralization of TNF-α protein[60] or genetic deficiency[129] enhanced insulin action and sensitivity in obese rodents. Furthermore, lack of MCP-1[52] or CCR2[68] results in reduced ATM levels, reduced inflammatory gene expression, and reversal of insulin resistance and hepatic steatosis. Similarly, macrophage depletion reduced ATM density in VAT and ameliorates insulin activity and steatosis[130]. In severely obese individuals, macrophage markers decreased in adipose tissue after weight reduction, but remained unaltered in skeletal muscle[53].

Taken together, it seems reasonable that obesity-associated inflammation initiates in the adipose tissue, as excess energy is stored in adipocytes,

leading to hypertrophy and FFA release and subsequent induction of stress and inflammatory pathways. In the context of chronic adiposity, macrophages are recruited and may accelerate the production of cytokines by adipose tissue. Cytokines can also be produced by cells in the vascular wall and may be released into circulation[72, 78, 103]. Ultimately, if these processes continue, systemic cytokine and FFA levels may rise, in turn affecting additional tissues and organs, and possibly resulting in ectopic fat deposition (i.e., inappropriate storage of fat in non-adipose tissues), inflammation and subsequent development of insulin resistance and cardiovascular complications[131].

Reducing & maintaining body weight

Obesity is caused by an imbalance between energy intake and energy expenditure. Excess caloric consumption combined with low physical activity will inevitably lead to increased body fatness and considerable risk of developing associated disorders, such as type 2 diabetes, cardiovascular disease, sleep apnea, and cancer[112]. Thus, obesity prevention should be a major concern that begins at early ages.

A shift towards negative energy balance and ensuing short-term weight loss can be achieved by various regimens. Conversely, strategies for the long-term maintenance of lowered body weight have been less successful, but are crucial to avoid consequences associated with chronic obesity. Clearly, the development of strategies to reduce weight and sustain weight loss is of profound importance.

At present, dieting, physical activity, pharmacological therapy, and bariatric surgery are the dominating strategies to treat obesity.

Lifestyle modification

Lifestyle modification, comprising diet and exercise treatment, is the primary strategy to eliminate excess body fat. In long-term lifestyle intervention studies, there is usually an initial phase of weight loss followed by weight regain. However, individualized treatment and regular personal guidance and support appear to facilitate long-term body weight control[132].

Dieting

There is an abundance of dietary regimens that focus on losing weight. Personal preferences and individual physiologic response to a particular diet are of vital importance for managing weight loss. In the 1980s, very-low-calorie diets (VLCDs) providing <800 kcal/day and low-calorie diets (LCDs)

providing 800-1800 kcal/day became very popular. VLCDs can induce a weight loss of 16% after a mean of 3 months, while weight reduction on LCDs is approximately 10%. Despite the superior short-term effect of VLCD, the long-term outcomes appear to be similar in both diets, with a reduction of roughly 5% from initial weight after a mean of 2 years[133].

The impact of dietary macronutrient content has been in focus more recently, and the potential beneficial effects of fat or carbohydrate restriction have been extensively debated. In a long-term (2 years) study, a low-carbohydrate diet appeared to induce greater weight loss and have an advantageous effect on blood lipids compared with a low-fat diet, while the two diets had comparable effects on blood pressure[134]. Similarly, a low-carbohydrate diet had more favorable effects on insulin sensitivity and cardiovascular disease (CVD) risk factors than a low-fat diet after a 12-week intervention[135]. Moderate-protein (30%) diets, which replace a part of the carbohydrate content with proteins, are associated with greater weight loss and favor visceral fat reduciton[136]. The beneficial effect of increased protein intake appears to be due to its satiating capacity, which results in reduced calorie consumption. Diets high in protein may also help preserve lean mass during weight loss and increase thermogenesis (and therefore energy expenditure)[137]. The glycemic index (GI) is another factor that has been considered important for the outcome of dietary modulation. Intake of low-GI foods, such as whole grains, vegetables, and legumes, enhances weight control and has positive effects on insulin secretion and sensitivity[136]. Dietary fatty acid content also seems to be important, and a moderate-fat diet enriched in MUFA improves blood lipids and insulin sensitivity. Further, intake of omega-3 fatty acids, with fish as the central source, is associated with reduced inflammation and CVD risk, and may also influence appetite and satiety[136]. Indeed, a Mediterranean diet, which is moderate in fat but enriched in MUFAs and PUFAs, has been suggested to have favorable effects on cardiovascular risk factors and improve glycemic control[134, 138, 139].

Aside from dietary composition, energy restriction vs. unrestricted (*ad libitum*) food intake must be considered in a weight loss program. *Ad libitum* food intake may facilitate adherence to the diet, long-term weight loss, and maintenance of the reduced body weight. By contrast, energy restriction induces weight loss and improves health, but may cause feelings of hunger, thus discouraging compliance with the weight reduction program and durable weight loss[136].

Physical activity

Exercise is a central factor in obesity treatment, and regular physical activity also has beneficial effects independent of weight loss. Although physical activity alone generally induces a modest weight loss of 2-3 kg, exercise is fundamental in obesity management because it helps to prevent weight

regain and maintain lean body mass[140]. It is recommended that healthy adults is to perform physical activity of moderate intensity for at least 150 minutes/week to prevent weight gain and related comorbidities[141].

Pharmacological therapy

When it is not possible to achieve sufficient weight loss solely by changing diet and exercise habits, pharmacological therapy can be applied in combination with lifestyle modification. The only available drug today is the pancreatic lipase inhibitor orlistat, which inhibits the hydrolysis of ingested triglycerides, and thereby reduces fatty acid and glycerol absorption by approximately 30%. Pharmacological therapy normally generates a moderate weight loss of <5 kg after 1 year when compared with placebo, and is associated with gastrointestinal side-effects[142].

Bariatric surgery

When lifestyle changes and pharmacological therapy have failed to reduce body weight, bariatric surgery may be an option for individuals suffering from severe obesity. Generally, individuals who are authorized to undergo surgery have a BMI >40, or a BMI >35 in combination with comorbidities, including diabetes. There are a number of different surgical procedures (e.g., vertical banding, gastric bypass, and biliopancreatic bypass with duodenal switch), all of which aim to induce weight loss by reducing stomach size and consequently limiting food intake. Gastric bypass (Roux-en-Y gastric bypass) is the most common procedure: in addition to reduced stomach volume, the first segment of the small intestine is bypassed. The Swedish Obese Subjects (SOS) study demonstrated that gastric bypass induces greater weight loss both short-term and 15 years after surgery compared with vertical-banded gastroplasty and banding[143]. Individuals who undergo obesity surgery experience lower incidence of CVD and cancer and lower overall mortality compared with control participants[143, 144]. Importantly, bariatric surgery is very efficient in resolving type 2 diabetes, with a reversal rate of 83% after gastric bypass and 62% after gastric banding[145].

It was recently claimed that the benefits from bariatric surgery are unrelated to weight loss, and therefore that BMI alone should not be used as a criterion for obesity treatment[146]. Individuals with a peripheral fat distribution can have a high BMI without developing comorbidities. Therefore, fat distribution (rather than total body fatness) is the main determinant of health outcome. According to the report, indications for bariatric surgery should be based on an individual's risk factor profile, and persons without weight-related complications should generally not undergo surgery[146].

AIMS

The overall aim of this thesis was to characterize adipose tissue inflammation and relate it to anthropometric, metabolic, and circulating inflammation-related parameters.

The specific aims were:

- To compare the expression of inflammation-related genes in different abdominal adipose tissue depots.

- To investigate the influence of menopause on adipose tissue and whole-body inflammation, as well as metabolic parameters in healthy women of normal weight.

- To study whether obesity-induced low-grade inflammation is reversible after weight loss as a result of gastric bypass surgery.

- To investigate the long-term effects of dietary intervention, and compare the outcomes of two different diets regarding obesity-related inflammation in adipose tissue and circulation.

SUBJECTS & METHODS

Study participants

This section briefly describes the study cohorts and methods included in this thesis. Details are included in the respective papers. Written informed consent was obtained from the included participants. All studies were approved by the local Ethical Committees.

Study I

In the first study, 17 South African women (six black and eleven white) who underwent abdominal open surgery for various benign (mainly gynecological) conditions were enrolled. The women were apparently healthy, and BMIs ranged from 21.5 to 38.8 kg/m^2. No differences in anthropometric or gene expression data were observed when black and white women were compared; therefore, no adjustments were made for ethnicity. Anthropometric data and blood samples were collected before surgery. Adipose tissue biopsies from the superficial subcutaneous, deep subcutaneous, and visceral depots were collected during the surgery.

Study II

Normal weight premenopausal and postmenopausal women
Forty-six premenopausal and postmenopausal women were recruited to the study. The women also underwent examinations of cortisol metabolism in adipose tissue and liver[147]. The women were healthy and of normal weight; none used tobacco or hormonal contraceptives. Anthropometric data were collected as a primary health control. Postmenopausal women had not menstruated during the previous 12 months. Premenopausal women were examined during either the follicular or luteal phase of the menstrual cycle; however, because there were no significant differences in any of the parameters included, the data were analyzed as a single group. After an overnight fast, venous blood samples were drawn and subcutaneous adipose tissue biopsies were collected.

Obese women before and after gastric bypass surgery
Twenty-seven women undergoing Roux-en-Y gastric bypass surgery to treat severe obesity (i.e., BMI >35) at Örebro University Hospital were included in the study. This study cohort was also included in an earlier examination of

cortisol metabolism before and after surgery-induced weight loss[148]. Exclusion criteria were body weight >160 kg, untreated endocrine disorder, and pregnancy. Four individuals were diagnosed with type 2 diabetes and one additional subject was insulin-resistant. One woman was postmenopausal. Some women used hormonal contraceptives, and a few had irregular menstrual cycles due to obesity. Anthropometric data, venous blood samples, and subcutaneous adipose tissue biopsies were collected 2 weeks before and 2 years after the surgery.

Study III

Seventy overweight or obese postmenopausal women were recruited to a 24-month dietary intervention study. After baseline measurements, the women were randomized to either a diet based on Nordic Nutrition Recommendations (NNR) or a Paleolithic diet (PD). Food intake was *ad libitum*. Data included in the present study were collected at baseline, at 6 months, and at the end of the study after 24 months (Figure 5). Normal fasting plasma glucose and non-smoking were required for inclusion. Postmenopausal status was defined as no menstrual periods during the previous 12 months. The individuals participated in group education led by a dietician to practice cooking according to their respective diet. Adherence to diet was evaluated by self-reported food records during four days at baseline, 6 months, and 24 months, and protein intake was assessed by analyses of nitrogen excretion in urine.

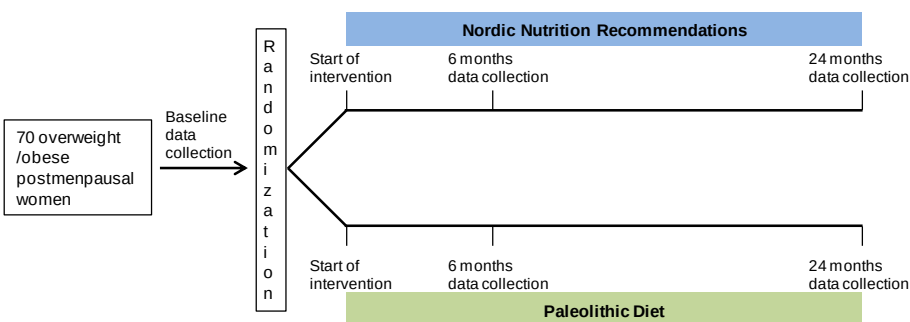

Figure 5. Design of study III

Methodological issues

Anthropometrics
Waist circumference was measured to the nearest 0.5 cm at the level of the umbilicus in study I, and between the lowest rib and the iliac crest in studies II and III. Hip circumference was measured at the largest gluteal area. Height was determined to the nearest 0.5 cm and weight to the nearest 0.1 kg in all individuals. Blood pressure was measured in a sitting position in study I and in normal weight premenopausal and postmenopausal women in study II. Blood pressure was determined in the supine position among the obese women included in study II, as well as in study III.

Body composition and fat distribution
Body composition was analyzed using dual energy x-ray absorptiometry (DXA) in study I, in the obese women in study II, and in study III. The normal weight premenopausal and postmenopausal women in study II underwent bioelectric impedance analysis to determine body composition. In study I, regional body fat distribution was determined at the L4/L5 level with a CT scan.

Blood chemistry
Serum levels of inflammation-related proteins were determined using commercially available immunoassay kits. Estrogen levels were assessed by ultrasensitive radioimmunoassay. Levels of IL-6, MCP-1, IL-8, and MIF in study III were analyzed using multiplex detection kits, and TNF-αRII levels were measured using a single plex detection kit. Serum CRP levels were analyzed using an automated high-sensitivity CRP detection method. All other blood parameters were determined using standard laboratory measurements.

Adipose tissue biopsies
In study I, adipose tissue biopsies were collected during open abdominal surgery under general anesthesia. The adipose tissue samples were taken below the umbilicus. Fascia superficialis discriminated the two subcutaneous depots: superficial SAT was located above the fascia, and deep SAT was located below it. In study II, superficial SAT biopsies were obtained from the peri-umbilical region, with open biopsies under local anesthesia in both normal weight and overweight/obese women. In study III, superficial SAT biopsies were collected using needle aspiration under local anesthesia.

All adipose tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C until analysis.

RNA extraction, reverse transcription, and real-time polymerase chain reaction

Total RNA was extracted from adipose tissue and cDNA synthesis was performed by reverse transcription. Real-time polymerase chain reactions were run using pre-designed commercially available gene expression assays. Each gene expression was relatively quantified using the standard curve method (studies I and II) or the $2^{-\Delta\Delta Ct}$ method (studies II and III). Expression of each target gene was normalized to a control gene that was selected based on a previous evaluation of endogenous control genes from human adipose tissue[149]. To compare the selected control genes, the coefficient of variation (studies I-III) and the NormFinder algorithm (study III) were used.

Statistical analysis

Study I

The non-parametric Friedman analysis of variance was performed to compare gene expression levels in the three different fat depots, along with a post-hoc Wilcoxon signed ranks test. The significance level was corrected for multiple comparisons. Pearson correlation coefficients were used to analyze bivariate correlation between different variables. When required, data were ln-transformed to achieve normal distribution. In the multivariate data analysis, a partial least squares discriminant analysis (PLS-DA) regression model was used. In the PLS-DA model, all gene expression data from the three different depots were evaluated simultaneously to find out whether the depots discriminated from each other and which genes contributed to the discrimination.

Study II

The non-parametric Mann-Whitney U-test was used to compare premenopausal and postmenopausal women, and the Wilcoxon signed ranks test was used for the obese women. Linear regression analysis was performed to determine independent associations between cytokine levels, waist circumference, and menopausal state. In these analyses, skewed data were ln-transformed to achieve normal distribution. The Spearman correlation coefficient was used to analyze association in the obese cohort.

Study III

In this prospective study, a generalized estimating equation (GEE) was used to explore time effects, diet effects, and interactions between time and diet. All gene expression and serum protein data are presented as geometrical means because of the skewed distribution of the data.

RESULTS & DISCUSSION

Detailed descriptions of the results are included in the respective papers.

Study I

In the first study, we aimed to characterize adipose tissue inflammation in three distinct abdominal fat depots. Because low-grade fat inflammation and deep SAT accumulation are associated with insulin resistance[19, 20], we hypothesized that the expression of inflammation-related genes would be higher in deep vs. superficial SAT. Superficial and deep SAT and VAT were obtained from apparently healthy women, and the expression of inflammation-related genes in the different depots was investigated.

Subject characteristics
The BMIs of the individuals ranged from normal weight to severely obese. The volume of the VAT depot was significantly smaller than those of the superficial and deep SAT depots ($p<0.001$ for both). The volumes of the superficial and deep SAT depots did not differ from each other.

The visceral adipose tissue depot has a unique inflammatory profile
Multivariate data analysis revealed that VAT differed from both superficial and deep SAT and appeared as a distinct depot according to gene expression data. In contrast, the two SAT depots did not differ from each other (Figure 6A). Although previous studies investigating low-grade inflammation in the superficial and deep SAT depots are sparse, in accordance with our results, TNF-α mRNA and protein levels were reportedly similarly expressed in the two SAT depots[150]. However, a very recent study in morbidly obese individuals described higher IL-6 expression and ATM density in deep vs. superficial SAT. In addition, individuals with hepatic steatosis exhibited elevated ATM accumulation in deep SAT and VAT, but not superficial SAT, compared with individuals without fatty liver[16]. In sharp contrast to our study, these findings clearly indicate a distinction between the two SAT depots. Yet, it is important to keep in mind that these observations were made in morbidly obese individuals, and several participants suffered from type 2 diabetes.

Multivariate data analysis revealed which genes contributed most strongly to the difference between the depots. MIF and CCR2 were the main factors, with higher expression in the VAT depot (Figure 6B). Univariate

analysis demonstrated that the MIF expression in both SAT depots was 53% of the MIF expression in the VAT depot ($p<0.001$ for both). CCR2 expression in superficial and deep SAT was 43% and 49% of the expression level observed in VAT, respectively ($p<0.01$ for both).

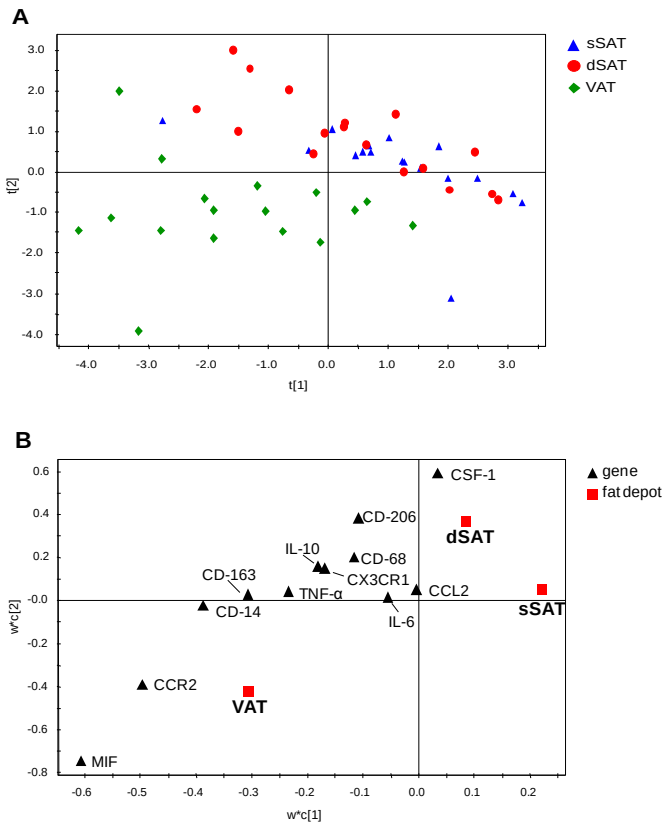

*Figure 6. A multivariate model (PLS-DA) comparing 12 gene expression variables in superficial subcutaneous adipose tissue (sSAT), deep SAT (dSAT), and visceral adipose tissue (VAT). In (A), the PLS-DA scores (t[1]/t[2]) displays VAT as significantly different from the sSAT and dSAT depots. (B) The PLS-DA weight plot (w*c[1]/w*c[2]) reveals the contributions of the different gene variables to the scores in **A** for the fat depots. MIF and CCR2 are the main contributors to the difference between VAT and the two SAT depots.*

Macrophage migration inhibitory factor and C-C chemokine receptor 2 in adipose tissue inflammation

Gene expression of MIF was higher in the VAT depot in every individual, suggesting that MIF is upregulated in the more metabolically harmful intra-abdominal fat, irrespective of adiposity. We observed a positive association between body fat percentage and MIF expression in VAT. Although earlier studies regarding MIF in human VAT are missing, prior reports of MIF secretion and expression in subcutaneous adipocytes demonstrate correlations with BMI[105] and cell size[106]. The elevated MIF expression in VAT may be of particular importance because MIF appear to promote triglyceride accumulation[108]. It is therefore possible that the higher MIF expression facilitates the storage of body fat in the metabolically detrimental visceral depot. In a mouse model, MIF deficiency reduced fat cell size and adipose tissue inflammation, and improved insulin sensitivity[107], indicating a role for MIF in obesity-associated adipose tissue inflammation and metabolic dysfunction.

The chemokine receptor CCR2 was reportedly more highly expressed in VAT than SAT in humans, and CCR2 expression also increases with obesity[151]. Our result, which shows a positive correlation between CCR2 mRNA levels and BMI in VAT, is in agreement with these findings. While CCR2 has been linked to glucose homeostasis in rodents[68], neither Huber et al.[151] or we observed any parallel association between insulin sensitivity and CCR2 expression in human VAT. In contrast, we observed an association between CCR2 expression and HOMA2 in deep SAT ($r=0.57$, $p=0.022$). This finding might indicate a putative link between deep SAT and insulin resistance, as suggested previously[19, 20]. Additional studies of the putative role of deep SAT in obesity-associated inflammation would be interesting.

In summary, the human VAT depot displays a unique inflammatory pattern characterized by increased expression of MIF and CCR2. This finding suggests these pro-inflammatory factors may play a key role in low-grade VAT inflammation.

Study II

In the second study, we aimed to discover whether there is a difference in inflammatory status in the adipose tissue and circulation of healthy premenopausal and postmenopausal women of normal weight. A cohort of severely obese women undergoing gastric bypass (GBP) surgery to reduce weight was also included. We investigated changes in inflammatory parameters in fat and serum before surgery and 2 years after surgery.

Anthropometric and biochemical characteristics

Although the BMIs of premenopausal and postmenopausal women were similar, their body composition and distribution differed. Postmenopausal women exhibited a higher body fat percentage ($p<0.001$) and waist-to-hip ratio (WHR) ($p<0.01$), indicating a more central fat accumulation. In addition, cardiovascular risk factors like blood lipids (except HDL) and systolic blood pressure were higher in postmenopausal women. One contradictive finding was lower HOMA-IR, suggesting higher insulin sensitivity after menopause. This finding was due to lower fasting insulin levels among the postmenopausal women, and may be partly explained by reduced insulin secretion after menopause[152].

Women undergoing GBP surgery experienced dramatic reductions in adiposity measurements 2 years after surgery, including BMI, waist circumference, and body fat percentage ($p<0.001$ for all). The mean body weight of this cohort before GBP surgery was 124 ± 15 kg; afterwards, mean body weight had decreased to 83 ± 14 kg ($p<0.001$) and was followed by greatly improved insulin sensitivity ($p<0.001$).

Massively reduced low-grade inflammation following gastric bypass surgery

We observed higher IL-8 expression ($p<0.05$) in subcutaneous fat after menopause and an association between IL-8 expression and waist circumference in both premenopausal and postmenopausal women. In obese women, we observed a 90% decrease in IL-8 expression after GBP-induced weight loss. This finding clearly suggests that adipose IL-8 expression is linked to fat accumulation, and agrees with a previous report of a correlation between BMI and IL-8 release from SAT explants[80].

Expression of IL-6 was also significantly increased in postmenopausal compared with premenopausal women ($p<0.01$), and was also independently associated with waist circumference and menopausal state/age. This finding is interesting because an adipocyte-specific rise in IL-6 release has been associated with older age in male mice[153], while another study observed elevated IL-6 production in ATMs and stromal vascular cells in old mice[154]. The results of these studies suggest an age-related increase in adipose IL-6 levels. Hence, the age difference between premenopausal and postmenopausal women may contribute to our findings. Although adipose IL-6 is associated with excess body fat, the decrease in IL-6 expression after GBP surgery did not reach significance. This finding can likely be explained by the limited number of individuals and the large degree of inter-individual variation in gene expression. Yet, the expression of TNF-α and MCP-1 mRNAs were significantly reduced to 63% and 72% of presurgical levels, respectively, after surgery, indicating an improved inflammatory profile following weight loss.

Because there is a link between obesity, adipose inflammation, and metabolic disorders, we correlated our gene expression data with serum insulin levels and observed significant associations in severely obese women before surgery (r_s=0.59-0.71, $p<0.05$). Two years after surgery, when the women had decreased in body weight and were more insulin sensitive, there were no such associations. Thus, our data are in agreement with the relationship between obesity-associated low-grade inflammation and metabolic disease.

Increased circulating cytokine levels after menopause

Despite a great reduction of body fat after GBP surgery, we did not observe any change in circulating MCP-1 levels. Studies of serum MCP-1 levels after surgery-induced weight loss show varying results; a 47% reduction of MCP-1 concentration approximately 2 years after surgery has been reported[155], and a recent study describes a significant 11% MCP-1 reduction after 6 months[156], while another study observed unaltered MCP-1 levels roughly 1 year after GBP[157]. In those studies, the included participants were solely or a majority of women, and therefore the divergent results cannot only be explained by possible sex differences. Menopausal status affects circulating MCP-1 levels, and although menopausal state was not reported, most of the women included in these studies were relatively young. Therefore, the diverse results concerning surgery-induced weight loss effects on MCP-1 levels remain ambiguous.

In contrast, there was a distinct difference between premenopausal and postmenopausal women, with twofold higher MCP-1 levels after menopause ($p<0.001$). Sex hormones are well-known modulators of MCP-1 levels[75, 158], which most likely explains much of the difference between the premenopausal and postmenopausal women. In addition to MCP-1, we observed higher IL-8 levels after menopause ($p<0.01$). This is an interesting finding because the cardiovascular risk is higher among postmenopausal than premenopausal women, and elevated MCP-1 and IL-8 levels are linked to the risk of coronary artery disease[70, 87]. Aside from menopause, aging *per se* contributes to increased systemic inflammatory protein levels[159, 160].

The menopausal transition is generally considered to be associated with increased risk of experiencing cardiovascular events; however, that conception has been challenged by a report showing that mortality in ischemic heart disease increased gradually over all ages in adult women, with no acceleration at menopausal ages[35]. We were unable to distinguish between menopause and age-related effects in our study, and can merely conclude that postmenopausal women have higher circulating levels of cardiovascular risk factors, including blood lipids and cytokines, than premenopausal women.

In summary, we observed higher SAT gene expression and circulating protein levels of certain cytokines in normal weight postmenopausal vs. premenopausal women. This difference appears to be linked to adiposity as well as menopausal state and/or age. We can also conclude that significant weight loss in severely obese women was associated with a remarkable reduction in adipose low-grade inflammation in parallel with improved insulin sensitivity.

Study III

In this study, we aimed to investigate the effects of diet-induced weight loss and compare the outcomes of two different dietary regimens (PD and NNR) on low-grade inflammation in adipose tissue and serum. The study was part of a 2-year dietary intervention study.

Subject characteristics
Both dietary groups had decreased significantly in body weight at 6 months and 24 months compared with baseline ($p<0.001$ for both). During the first 6 months, weight loss was 9.7% in the PD group and 5.4% in the NNR group. Decreased adiposity was also demonstrated by a reduction in abdominal fat, measured by waist circumference and decreased fat mass. Although the weight loss mainly occurred during the beginning of the intervention period, the participants managed to maintain their reduced body weight throughout the study. At the end of the study (24 months), the average weight loss was 10.7% in the PD group and 7.7% in the NNR group. The greater weight loss in the PD group was demonstrated by a significant diet-by-time interaction for body weight at baseline vs. 6 months ($p<0.001$) and vs. 24 months ($p=0.042$).

Total cholesterol levels decreased in both dietary groups after 6 months, but returned to baseline levels after 24 months. Triglycerides were differentially affected by diet, with decreased levels after 6 and 24 months compared with baseline in the PD group, but not in the NNR group. The reduced intake of carbohydrates in the PD group may be the reason for this change, since carbohydrate restriction seems to favor the reduction of triglyceride levels[134, 161]. HDL cholesterol levels were significantly higher in the PD group at baseline, and remained so during the intervention period.

The change in insulin sensitivity was affected by time, with a slight increase in insulin resistance (HOMA-IR) after 24 months in the NNR group, compared with after 6 months ($p=0.022$). Systolic and diastolic blood pressure decreased after 6 months in both dietary groups ($p<0.001$), but increased slightly at the end of the study period in the PD group and returned to baseline levels in the NNR group.

Improved inflammatory profile after weight reduction

The collection of fat biopsies during the study provided a unique opportunity to investigate longitudinal changes in adipose gene expression after dietary intervention. As expected, inflammation-related genes and serum proteins generally decreased with time during the intervention period, although there was no effect of diet *per se*. However, there was a significant diet-by-time interaction: serum IL-6 decreased more in the PD group between baseline and 24 months. The diet-by-time interaction was also significant for TLR2 expression, with slightly increasing levels between 6 and 24 months in the PD group, but not in the NNR group, and for MIF expression, which increased more in the NNR group after 24 months compared with baseline and 6 months.

Changes in TLR4 and MIF expression may favor fat storage in subcutaneous adipose tissue

TLR4 expression did not change after 6 months in either group, although a significant body weight reduction occurred. However, after 24 months of intervention, when the participants were weight stable, significant downregulation of TLR4 expression was observed in both dietary groups vs. baseline and 6 months ($p<0.001$ for all) (Figure 7). Because TLR4 signaling involves the NF-κB pathway and downstream TNF-α induction[118], we analyzed gene expression of the transcriptionally active p65 subunit (RelA) of the NF-κB pathway and TNF-α. We observed no significant changes in expression levels of either p65/RelA or TNF-α. Previously, a study in human TLR4-stimulated adipocytes demonstrated activation of NF-κB signaling as verified by p65 nuclear translocation, as well as by elevated mRNA and protein levels[116]. Moreover, TNF-α and IL-6 secretion were induced after TLR4 stimulation[116]. The results of our study indicate additional modulators of adipose tissue inflammation, such as the protein kinase JNK1[162].

Similar to TLR4, MIF mRNA expression was unaltered in both dietary groups after 6 months of intervention. In contrast to TLR4, MIF expression was significantly upregulated in both dietary groups at the end of the intervention period compared with baseline and 6 months ($p<0.001$ for all). MIF appears to be involved in adipose inflammation[107]; however, in contrast to most pro-inflammatory cytokines, MIF also promotes adipogenesis and triglyceride accumulation[108], which may be the key to our results. During a state of negative energy balance (i.e., weight loss), adipocyte lipolysis is elevated, resulting in decreasing cell size. Smaller adipocytes have an increased fat-filling drive[163] and thus, after 24 months (when weight stabilization has occurred), MIF may be induced to facilitate lipid uptake.

TLR4 expression is also in agreement with this finding. TLR4 activation has been linked to lipolysis, and may influence adipocyte fat storage[120, 122, 123]. The lipolytic rate is higher in the context of obesity and during weight loss,

which may explain why TLR4 expression did not decrease until after 24 months of dietary intervention. The increased MIF and reduced TLR4 expression at 24 months may be related to each other and may mutually enhance lipid storage in SAT, preventing lipotoxicity and ectopic fat deposition.

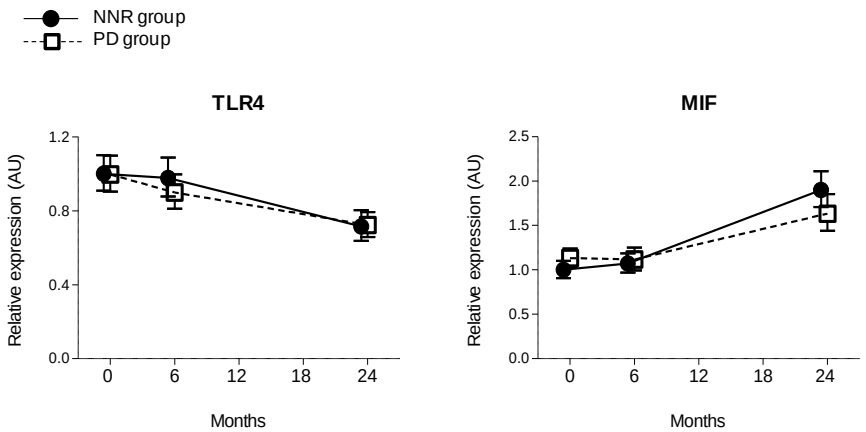

Figure 7. TLR4 and MIF gene expression in SAT during 2 years of dietary intervention. TLR4 expression did not change during the first 6 months, but decreased after 24 months compared with baseline and 6 months in both dietary groups (p<0.001 for all). MIF expression did not change after 6 months in either dietary group. At 24 months, a significant increase in MIF expression compared with baseline and 6 months was observed in both dietary groups (p<0.001 for all).

Reductions in inflammatory parameters and associations with characteristics

At the end of the intervention period, there was a 46% reduction in MCP-1 gene expression in the PD group and a statistically non-significant 23% reduction in the NNR group compared with baseline. The decrease in IL-6 expression between baseline and 24 months was 50% in the PD group (p<0.001) and 35% in the NNR group (p=0.002). Given that we observed no dietary effects, the greater reduction in IL-6 and MCP-1 expression is likely due to greater weight loss in the PD group. Similar long-term results were observed for circulating IL-6 levels, which decreased 45% in the PD group and 23% in the NNR group (significant for both groups) between baseline and 24 months. The observed change is reasonable because circulating IL-6 levels derive in part from fat[64]. However, the slight (but statistically

significant) increase in serum IL-6 levels after 6 months in the NNR group was somewhat unexpected. Circulating MCP-1 levels did not change during the intervention. This result is in contrast to the findings of another long-term dietary intervention study in which MCP-1 concentration decreased after 6 months and returned to baseline levels after 24 months[164]. This study suggests that changes in MCP-1 reflect the changes in body weight, since their study participants regained part of their initial weight loss[164]. Although the BMIs of their study cohort were comparable to those of our cohort, most of their participants were men while our cohort included only women. Nevertheless, a study investigating changes in circulating inflammatory markers in women during a 24-week weight loss intervention concludes that MCP-1 concentrations are not associated with indices of adiposity[165].

Hepatic production of the acute phase protein CRP is induced by IL-6, and CRP concentration is associated with excess body fat, inflammation, and metabolic risk factors[89]. We observed a significant decrease in CRP levels after 6 months, and at 24 months the reduction was 31% compared with baseline levels in the PD group. In the NNR group, the decrease in CRP levels was 14% after 24 months and statistically non-significant. This finding is in agreement with the greater reduction in serum IL-6 in the PD group.

Serum TNF-αRII decreased after 6 months, although the change did not reach significance in the NNR group. At the end of the intervention period, serum TNF-αRII in the NNR group was slightly but significantly elevated compared with 6-month levels. Serum levels of TNF-αRII are associated with BMI[166, 167], as indicated by the reduced levels at 6 months. The increase in TNF-αRII concentration in the NNR group at 24 months may be related to the slight decrease in insulin sensitivity, given that an earlier study demonstrated an association between those parameters[166].

Circulating MIF concentrations decreased significantly after 6 months and were 33% reduced in the PD and 37% in the NNR group. MIF levels were unaltered at 24 months, with no significant change compared with either baseline or 6 months. MIF is implicated in the development of atherosclerosis[103], and the changes in MIF concentration in our study correlated with changes in atherosclerotic risk factors, such as blood pressure, LDL cholesterol, and total cholesterol.

Adipose IL-8 gene expression decreased significantly after 6 months in both dietary groups; however, at 24 months there was only a significant decrease in the NNR group compared with baseline. The reduced IL-8 expression is in agreement with a previous short-term intervention study in which weight loss was followed by reduced levels of inflammatory markers, including IL-8[53]. Unfortunately, more than one-third of the samples in the serum IL-8 analysis were below the level of detection, and therefore we were unable to draw any major conclusions regarding putative changes in IL-8

levels. Nevertheless, statistical analyses on the remaining samples indicate that IL-8 levels did not change as a result of either time or diet.

In summary, we observed diminished low-grade inflammation in adipose tissue and circulation 2 years after diet-induced weight loss. Our findings suggest that weight reduction, rather than dietary composition, is central to improving the inflammatory profile in fat and serum. Additionally, our study highlights the importance of long-term interventions, as some alterations first appeared after a period of weight maintenance.

GENERAL DISCUSSION

The recent rise in overweight and obesity can primarily be ascribed to lifestyle changes, with genetic factors predisposing individual susceptibility to gain body fat[168]. A variety of factors, including cytokines, adipokines, diet, hormones, age, and fat distribution, may influence the risk of developing obesity-related comorbidities and underscore the complexity of the issue.

Fat distribution & metabolic risk

Body fat distribution is a main determinant of metabolic outcome, with visceral fat accumulation being strongly linked to the development of insulin resistance and cardiovascular disease[21]. In this thesis, we demonstrate that VAT has a more pro-inflammatory profile, which in turn is related to adiposity; however, we observed no major differences in gene expression levels between the two SAT depots. The impact of VAT is shown in morbid obesity, in which insulin resistance is associated with VAT accumulation and inflammation[81, 169]. Additional support for VAT accumulation as the main determinant of metabolic outcome is demonstrated by the fact that surgical removal of SAT had no impact on diabetes and CVD risk factors[170]. However, the SAT depots are important for dietary fatty acid uptake[171], and it has been proposed that a failure to induce postprandial lipid storage, as observed in abdominal obesity, is the root of visceral adiposity[99] and ectopic fat deposition[172]. Thus, normal SAT function appears to be crucial for whole-body metabolism and may affect the risk of developing disease.

Despite the suggested link between insulin action and low-grade inflammation and the association between deep SAT accumulation and insulin resistance, the inflammatory profile of deep SAT has not been characterized before. We observed no major differences in gene expression between the two SAT depots in our cohort of apparently healthy women. Nevertheless, recent findings in morbidly obese individuals, including diabetic individuals, suggest a link between deep SAT inflammation and comorbidities[16]. Clearly, more studies of the VAT depot and the two SAT depots in insulin-sensitive and insulin-resistant individuals matched for body fatness will be of interest.

Menopausal status, aging & adiposity

Cardiovascular mortality increases with age, although it is not entirely clear to what extent the menopausal transition or aging *per se* influence this development. In our study of healthy normal weight women before and after menopause, we observed higher serum MCP-1 and IL-8 levels and higher SAT IL-8 and IL-6 gene expression in postmenopausal women. Some serum proteins increase with aging as well as with elevated CVD risk, and in our study of premenopausal and postmenopausal women we cannot conclude whether the difference in body composition, menopausal status, or age is the main reason underlying the varying protein concentrations[86, 159, 160, 173]. Postmenopausal women exhibited higher body fat percentage and presumably more VAT (as indicated by higher WHR) than premenopausal women, predisposing them to higher cardiovascular risk. In young individuals of normal weight, even a modest increase in visceral fat has been linked to impaired endothelial function, which is predictive for cardiovascular events[174]. Thus, it would have been of interest to include a premenopausal group that had been matched against postmenopausal women for adiposity, and preferably for visceral fat mass.

There are several factors that may induce adipose tissue inflammation after menopause. These include hypertrophic adipocytes due to increased adiposity, decreased blood flow[175], metabolic alterations[27], and hormone levels. In addition to systemic concentrations, local estrogen levels may have effects on adipose function. SAT expression of the estrogen-producing enzyme aromatase is higher in postmenopausal women than in premenopausal women[176], with a putative discrepancy between local adipose and peripheral estrogen levels.

Despite the relationships between menopause and adiposity and between adiposity and adipose inflammation, the present study is the first to compare inflammatory parameters in adipose tissue of premenopausal and postmenopausal women. Earlier studies that used rodent models to mimic ovarian failure propose increased adipocyte size, adipose inflammation, and oxidative stress after estrogen deficiency[177, 178]. However, lack of estrogen in rodents results in increased adiposity; consequently, it is unclear whether the estrogenic effects are direct or indirect. Results from *in vitro* studies support the latter possibility, because estrogen incubation did not alter cytokine levels in adipose tissue fragments[177]. Furthermore, in women suffering from polycystic ovarian syndrome, adiposity seems to have a greater impact on adipose inflammation than the altered sex hormone levels that have been observed in this syndrome[179].

Aging appears to be an additional confounder in low-grade adipose inflammation. In mice, a switch towards more pro-inflammatory

macrophages and altered cytokine production are associated with older age[153, 154].

Thus, differences in adipose expression of inflammatory cytokines between premenopausal and postmenopausal women appear to be influenced, directly or indirectly, by several factors, including fat mass, sex hormones, and age.

Weight reduction

Although a majority of overweight/obese individuals are able to lose weight during short-term interventions, very few manage to maintain reduced body weight for long periods of time. Certainly, lifestyle changes are difficult to sustain, and adipose tissue itself counteracts loss of fat mass[163]. Clearly, prevention and long-term treatment of chronic obesity are cornerstones for obesity guidelines. In cases of severe obesity, bariatric surgery is an efficient treatment that results in durable weight loss and improved health and life expectancy[143, 144]. We showed remarkable long-term reductions in adipose inflammation-related gene expression concomitant with beneficial effects on insulin sensitivity, clearly demonstrating the beneficial effects of GBP surgery-induced weight loss on severe obesity. However, circulating cytokine levels did not change despite significant weight loss. Additional analysis of, for example, high-sensitivity CRP and detailed estimations of insulin sensitivity, would also have been of interest.

Diets restricted in carbohydrates or fat, often combined with high protein intake and a Mediterranean diet enriched in PUFAs and MUFAs, have become popular and frequently used of late. A few studies have investigated the long-term outcomes of these diets on circulating inflammatory proteins. A so-called Paleolithic diet (PD), rich in lean meat, fish, fruits, and nuts, turned out to have favorable effects on diabetes and CVD risk factors in earlier short-term interventions[180, 181], but has not been evaluated during extended periods before.

As a group, overweight/obese postmenopausal women are at high risk of developing metabolic comorbidities. Therefore, in study III we compared the outcomes of a PD and an NNR diet during a 2-year intervention. We investigated short-term and long-term changes in inflammatory parameters in adipose tissue and serum. In agreement with the GBP surgery study, we observed long-term attenuation of low-grade inflammation in adipose tissue after weight loss. We also observed lowered serum levels of inflammation-related proteins during the intervention. Nonetheless, it is noteworthy that we observed unaltered MCP-1 and IL-8 levels after both diet-induced and GBP surgery-induced weight loss. There were no direct dietary effects in our cohort of relatively healthy women, and therefore reduced body weight appeared to be the main factor attenuating low-grade inflammation.

However, it should be considered that the women in both dietary groups most likely changed to a healthier diet when starting the intervention. The inclusion of an observational group would have been of interest, and may have contributed to further elucidation of the results.

In contrast to a majority of weight reduction studies, the postmenopausal women in our study managed to preserve their decreased body weight over a longer time period. A highly motivated study group and unrestricted energy intake may have facilitated adherence to the intervention, and thus contributed to durable weight loss.

SUMMARY & CONCLUSIONS

- Visceral fat has an adverse inflammatory profile, characterized by higher CCR2 and MIF gene expression, compared with superficial and deep subcutaneous adipose tissue. The association between CCR2 and MIF expression and adiposity measurements suggests that weight gain is related to aggravated visceral adipose inflammation.

- Postmenopausal women exhibited increased adipose expression of certain pro-inflammatory cytokines that were linked to abdominal fat accumulation. There was also an increase in circulating inflammation-related protein levels in postmenopausal compared with premenopausal women. Elevated serum cytokine concentrations were associated with menopausal state/age independent of abdominal fat.

- Weight loss induced by gastric bypass surgery was followed by a remarkable reduction of inflammatory gene expression in subcutaneous fat and improved insulin sensitivity. Associations between the expression of various genes and insulin levels before surgery support a link between low-grade inflammation and metabolic disease.

- Diet-induced weight loss was accompanied by long-term attenuated whole-body and adipose inflammation. There was no significant dietary effect; therefore, reduced body weight, rather than dietary composition, is suggested to be the main reason for the improved inflammatory profile.

In conclusion, our data suggest an association between low-grade adipose inflammation and fat accumulation. Visceral adiposity is linked to unfavorable changes in inflammatory profile. Long-term weight loss is followed by beneficial alterations in adipose tissue and, to some extent, circulating inflammation-related markers, as well as improved metabolic profile.

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