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GENE EXPRESSION PATTERNS IN HUMAN ADIPOSE TISSUE IN RELATION TO FAT MASS AND ADIPOSE DEPOT

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To see a World in a Grain of Sand
And Heaven in a Wild Flower
Hold Infinity in the Palm of your Hand
And Eternity in an Hour

William Blake 1757-1827

To my family

ABSTRACT

Obesity, especially excess amount of abdominal fat, predisposes to a high risk for cardiovascular disease. Cancer cachexia is characterized by a specific loss of white adipose tissue (WAT) and skeletal muscle mass, and is associated with decreased survival and poor response to chemotherapy. We hypothesize that alterations in WAT function contribute to the negative metabolic consequences and disease outcome, respectively, of these two disorders. In this thesis we apply global transcriptome profiling on patient abdominal WAT biopsies to identify new genes and pathways of relevance for WAT function. In particular we explore gene expression in relation to (i) WAT depot, (ii) a dietary intervention study, and (iv) cancer with or without cachexia. In addition, we select one gene from microarray, Follistatin, for detailed expression profiling and functional evaluation in human fat cells.

In the first study, we successfully set up Representational difference analysis to identify a handful of genes differentially expressed between subcutaneous and visceral WAT, e.g. Adipsin, a component in the complement system, and Phospholipids transfer protein (PLPT), which is involved in transfer of phospholipids between lipoproteins. ***Our second study*** was part of a large consortium which compared the effects of a ten week intervention with a low-fat, high-carbohydrate hypoenergetic diet versus a moderate-fat, moderate-carbohydrate hypoenergetic diet. Both diets produced similar weight loss and beneficial changes in blood chemistry parameters. We performed abdominal subcutaneous WAT global transcriptome profiling on a subgroup of patients before and after the dietary intervention. The expression of 96 genes was significantly influenced by hypocaloric diet. Expression of genes involved in the synthesis of polyunsaturated fatty acids was downregulated, and CIDEA was up-regulated by hypocaloric diet. The pattern of gene expression response was almost identical between the two diets. ***In the third study*** we report that subcutaneous WAT Follistatin mRNA decreases with increasing weight, and that weight loss restores Follistatin levels. Furthermore, Follistatin is primarily produced by cells of the stroma vascular fraction in WAT. We show that WAT secretes Follistatin in vitro. Treating precursor cells with Follistatin in vitro stimulates adipogeneisis. We cotreated precursor cells with Follistatin and Myostatin under adipogenic conditions, and found that cotreatment reversed the inhibitory effect of Myostatin on adipogenesis. ***The fourth study*** compared cancer patients with or without cachexia. Global transcriptome profiling revealed that genes downregulated by cachexia were overrepresented in pathways related to extracellular matrix, actin cytoskeleton and focal adhesion. By contrast, genes upregulated in cachexia were overrepresented in pathways related to energy turnover, e.g. fatty acid degradation, and oxidative phosphorylation.

In conclusion, variation in WAT size is associated with changes in tissue morphology, fat cell number and metabolism, as well as adipokine secretion. We provide support that the dietary energy intake, and not the macronutrient composition, is associated with changes in WAT gene expression, and highlight the role of CIDEA, which subsequently has been shown to be an important regulator of metabolic switch in fat cells. We identify Follistatin as a new adipokine. Insufficient Follistatin in obesity could possibly contribute to a hypertrophic WAT with large insulin resistant fat cells. We provide support that cachexia is associated with changes in WAT remodeling, which could be involved in WAT loss in this clinical condition.

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II. Dahlman I*, **Linder K***, Arvidsson Nordstrom E, Andersson I, Liden J, Verdich C, Sorensen TIA, Arner P,and NUGENOB. **Changes in adipose tissue gene expression with energy-restricted diets in obese women.** Am J Clin Nutr 2005: 81:1275-85
*These authors contributed equally

III. Flanagan JN, **Linder K**, Mejhert N, Dungner E, Wahlen K, Decaunes P, Rydén M, Björklund P, Arver S, Bhasin S, Bouloumie A, Arner P, and Dahlman I. **Role of Follistatin in promoting adipogenesis in women.** J Clin Endocrinol Metab. 2009: 94:3003-9

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CONTENTS

LIST OF ABBREVIATIONS

AMI	Acute Myocardial Infarction
BMI	Body Mass Index
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary Heart Disease
CIDEA	Cell death-Inducing DFFA Effector A
CT	Computer Tomography
DXA	Dual Energy X-Ray Absorptiometry
ECM	Extra-cellular matrix
FDR	False discovery rate
FFA	Free Fatty Acid
FSTL3	Follistatin like 3
GDR	Glucose Disposal Rate
GI	Glycemic Index
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
HDL	High Density Lipoprotein
hMSC	Human bone marrow-derived Mesenchymal Stem Cells
HSL	Hormone sensitive lipase
LCD	Low Calorie Diet
MUFA	Monounsaturated Fatty Acid
MRI	Magnetic Resonance Imaging
PAI1	Plasminogen Activator Inhibitor 1
PGC1A	Peroxisome proliferator-activated receptor coactivator 1-alpha
PUFA	Polyunsaturated Fatty Acid
RDA	Representational Difference Analysis
RNA	Ribonucleic Acid
RT-q-PCR	Real-Time Quantitative Polymerase Chain Reaction
SAM	Significance Analysis of Microarrays
SFA	Saturated Fatty Acid
SVF	Stroma Vascular Fraction
TAG	Triacylglycerides
T2D	Diabetes Mellitus type 2
TGFb	Transforming Growth Factor Beta
TNF- α	Tumor necrosis factor-α
VLCD	Very Low Calorie Diet
WAT	White Adipose Tissue
WHR	Waist Hip Ratio
ZAG	Zinc-Alpha2 Glycoprotein

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

The ongoing world-wide obesity epidemic has spurred research to investigate the function of white adipose tissue (WAT). WAT, which previously was regarded as an inert store of energy, is now considered an active participant in regulation of whole body metabolism, which signals to other organs by secreting hormones and metabolites (Wajchenberg et al, 2009). Furthermore, WAT is extremely plastic – no other organ can change its volume as dramatically as WAT. Such change in fat mass is associated with large alterations in WAT cellular phenotypes. The regulation of WAT cellular function, depot differences and how WAT is influenced by two states of fat mass loss, i.e. hypocaloric diet and cancer cachexia, is the focus of this thesis. Below is given a brief introduction to obesity, human WAT function, approaches to voluntary weight loss by caloric restriction, and cancer cachexia.

1.2 OBESITY AND COMORBIDITIES

Obesity is a worldwide epidemic and predisposes to a high risk for premature mortality. Especially abdominal obesity is a well-known risk factor for the occurrence of cardiovascular disease (Yusuf et al, 2005) and premature mortality (Pischon et al, 2008); it is related to metabolic changes, such as dyslipidemia, hypertension, type 2 diabetes mellitus (T2D), inflammation and hypercoagulability. An easy approximative appreciation of abdominal obesity can be obtained by measuring waist circumference or waist hip ratio (WHR) (Shuster et al, 2012). There is an association between waist circumference or WHR and mortality, even in normal weight persons (Pischon et al, 2008; Ruderman et al, 1998). Also several malignancies are associated to obesity such as prostate, breast and colorectal cancer (Basen-Engquist & Chang, 2011; Kaidar-Person et al, 2011; Trinchieri, 2011).

It is widely accepted that the manifestations of the metabolic syndrome cluster of diseases depends of continued exposure to environmental factors, mainly diet and lack of exercise, modulated by genetic factors (Dahlman & Arner, 2010). Recent studies reveal also possible epigenetic influence (Herrera et al, 2011; Steger et al, 2010). Obesity is highly correlated with insulin resistance (Gregor & Hotamisligil, 2011), but both insulin resistant lean individuals and metabolically healthy obese individuals (Wildman, 2009) are well recognized by the clinician. A recent study comparing insulin resistant individuals with insulin sensitive subjects with the same BMI showed far more gene expression changes in adipose than in muscle suggesting a more important role for altered adipocyte transcription in impaired insulin action (Elbein et al, 2011).

1.3 WHITE ADIPOSE TISSUE CHARACTERISTICS

1.3.1 Cellular composition

With the escalating incidence of obesity a better understanding of adipose metabolism and techniques to quantify and characterize obesity are necessary (Shuster et al, 2012). WAT is a heterogeneous organ composed of mature adipocytes and the stromavascular

fraction (SVF), which includes mostly precursor cells, endothelial cells, fibroblasts, macrophages and lymphocytes. Each cell type may contribute to secretion from WAT. Adipose tissue growth results from two processes: the increase in number of adipocytes (hyperplasia) that develop from precursor cells, and the growth of individual fat cells (hypertrophy) due to incorporation of triacylglycerides (TAGs). The risk of metabolic complications is increased not only by the amount and location of WAT, but also by the size of the fat cells. Human fat cells can change ~20-fold in diameter and several thousand-fold in volume. Enlargement of subcutaneous abdominal adipocytes is associated with insulin resistance and is an independent predictor of T2D (Weyer et al, 2000). It is also shown that hypertrophic adipose cells display distinct gene expression profile compared to small cells (Jernas et al, 2006). Gene expression profile in adipocytes has also been compared to that of the SVF (Cancello et al, 2005). Adipocytes transcripts have well known metabolic and secretory properties while genes from SVF often belong to 'inflammatory' or 'immune' processes.

Spalding et al have recently shown that the total number of adipocytes increases only in childhood and adolescence and stays constant in adults. The annual turnover was appreciated to be approximately 10% (Spalding et al, 2008). Low generation rates of new adipocytes associate with WAT hypertrophy, whereas high generation rates associate with adipose hyperplasia (Arner et al, 2010). In morbid obesity, WAT hyperplasia with many small adipocytes is mainly associated with better glucose, insulin, and lipid profiles as compared to WAT hypertrophy with large adipocytes (Hoffstedt et al, 2010).

In the last years much attention has been focused on the WAT macrophages whose accumulation is correlated with extent of obesity and insulin resistance (Anderson et al, 2010; Weisberg et al, 2003). The metabolic syndrome, visceral adiposity and T2D are characterized by a low grade chronic inflammation in WAT, which is associated with altered lipoprotein metabolism and insulin resistance in the liver (Cancello et al, 2006). WAT from obese insulin resistant rodents and humans are characterized by increase in inflammatory macrophages and also fibrosis and increases in components of extra cellular matrix (Henegar et al, 2008). Obese insulin-resistant subjects have fewer capillaries and more large vessels in WAT as compared with lean subjects (Spencer et al, 2011). Hypothetically the extracellular matrix of adipose tissue may play an important role in regulating the expandability as well as angiogenesis of WAT.

1.3.2 Adipose Lipid turnover

WAT has a unique ability to adapt its size in response to calorie excess or depletion. Adipocytes are well equipped for storing energy as TAG in a large lipid droplet. Lipolysis is the breakdown of TAG into free fatty acids and glycerol (Arner, 2005). Adipose triglyceride lipase catalyzes the first step (formation of diglycerides), and hormone-sensitive lipase (HSL) catalyzes further hydrolysis to generate the end products fatty acids and glycerol. Free fatty acids and glycerol can be used as energy rich fuels in catabolic situations. There is a low basal lipolysis in adipocytes, that is a continuous breakdown of TAG. The major physiological stimuli of lipolysis are catecholamines and the major inhibitor insulin. Catecholamines bind to stimulatory beta adrenoreceptors on the adipocytes, which via a G-protein mediated pathway

phosphorylates HSL which is the rate-limiting step in lipolysis. Catecholamines can also bind inhibitory alpha adrenoreceptors.
Abdominal subcutaneous adipocytes are more sensitive to insulin, and not as lipolytically active as the visceral WAT depot. The beta adrenoreceptors are more active in the visceral adipocytes, while the inhibitory alpha 2 adrenoreceptor and insulin are more active in the subcutaneous adipocytes (Arner, 1995). The differences in lipolytic activity are even more pronounced in obesity.

Lipogenesis is the process of synthesizing TAGs. Fatty acids either originate from previous lipolysis in adipocytes, or are taken up from the blood. In addition, glucose needs to be taken up from the blood to form the glycerol backbone. It can also be used to synthesize fatty acids.

Mitochondria are cell organelles with a central role in energy turnover. In the mitochondria the beta oxidation of fatty acids, the tricarboxylic acid cycle (TCA), and oxidative phosphorylation take place. The beta oxidation and glycolysis converge onto the TCA, by which energy rich electron donors are formed. Subsequently, these energy rich donors are used in the electron transport chain, or oxidative phosphorylation pathway, to synthesize energy rich adenosine triphosphate.

It is established that mitochondrial function in skeletal muscle is impaired in human T2D individuals (Mootha et al, 2003; Petersen et al, 2003). This finding has evoked interest in WAT mitochondria function as well. It has been shown that glitazones increase mitochondria number and fatty acid oxidations in adipocytes (Bogacka et al, 2005) (Boden et al, 2005). An important transcriptional regulator of mitochondrial biogenesis and the enzymes of the electron transport chain is Peroxisome proliferator-activated receptor coactivator 1-alpha (PGC1A). Overexpression of PGC1A in human adipocytes increased the fatty acid oxidation capacity of the cells (Tiraby et al, 2003).

1.3.3 White adipose tissue secretory function

After the discovery of Leptin in 1994 (Zhang et al, 1994), WAT was no longer considered an energy reservoir only; the concept of WAT as a real endocrine organ was now established. Since then it has been accepted that WAT plays a vital role in regulating energy and glucose homeostasis (Kahn & Flier, 2000) by producing a number of biologically active proteins that are called adipokines. These adipokines have functional roles in diverse processes including glucose homeostasis, lipid homeostasis, appetite regulation, immune function, hormonal processes, angiogenesis and blood pressure control (Trayhurn & Wood, 2004). True adipokines are secreted from adipocytes but in WAT there are also other cell types secreting peptides.

In 1993 it was reported that Tumor necrosis factor-α (TNF-α) is overexpressed in the WAT of obese mice, which provided the first clear link between obesity, diabetes and chronic inflammation (Hotamisligil et al, 1995). In 2003, it was recognized that increased inflammation and macrophage infiltration accompany the expansion of WAT (Weisberg et al, 2003) and since then numerous studies showing obesity-related inflammation have been published. Many suggest an association between the inflammation and insulin resistance (Arner, 2007; Wajchenberg et al, 2009). Activated

macrophages in WAT secreting Interleukin 6 and TNF-α may lead to endothelial dysfunction and insulin resistance in parallel with accelerated arteriosclerosis (Anderson et al, 2010; Vykoukal & Davies, 2011). WAT macrophages and inflammation have been associated with obesity-related co-morbidities such as cardiovascular disease (Apovian et al, 2008), liver steathosis (Cancello et al, 2006) and T2D (Donath & Shoelson, 2011). The hereditary component of WAT inflammation seems to be strong, since even in young nonobese healthy first degree relatives of T2D patients a low grade inflammation is measurable and is aggravated by physical inactivity (Hojbjerre et al, 2011).

Recent extensive studies of secreted proteins in human adipose shows that there are still numerous new adipokines to characterize (Lehr et al, 2012). There is a continuous crosstalk between different tissues in the body where adipocytes can secrete substances acting locally or systemically on e.g. myocytes to reduce insulin sensitivity and myokines can effect adipose tissue (Trayhurn et al, 2011).

1.3.4 Subcutaneous and omental white adipose tissue

WAT from different regions of the body expresses different subsets of genes and displays distinct metabolic properties (Arner, 1998; Lafontan & Girard, 2008). WAT from intraabdominal depots (omental and mesenteric) is regarded different from subcutaneous WAT (femoral, gluteal, abdominal). Lipolytic rates in omental and subcutaneous WAT are different; omental WAT displays a low basal lipolysis while the catecholamine-stimulated lipolysis is increased compared with subcutaneous WAT (Arner, 1995). Recently it was shown that the catecolamine-stimulated lipolysis in omental adipose is 40% higher in metabolic syndrome than in healthy obese women (Andersson et al, 2011). Omental tissue is more extensively vascularized than subcutaneous WAT (Arner, 1995; Dicker et al, 2009). Adipocyte size has been reported to be larger in visceral than in subcutaneous WAT but opposing reports exist (Villaret et al, 2010).

Many, but not all, studies show a stronger association between visceral than subcutaneous WAT and metabolic disease. The Health, Aging and Body composition study (Nicklas et al, 2004) on 2500 elderly persons found an association in women between visceral adipose area and incidence of acute myocardial infarction. The Framingham Heart Study (Fox et al, 2007) reported that volumetric CT measures of both subcutaneous and visceral WAT were correlated with multiple metabolic risk factors, although risk factor correlations with visceral WAT were consistently significantly stronger than those for subcutaneous WAT. There was also a stronger association in women between metabolic risk factors and visceral WAT. A recent study by Smith (Smith et al, 2012) showed that visceral, but not subcutaneous, abdominal adiposity is strongly related to cardiometabolic risk factors and to the prevalence of cardiovascular disease, independent of presence of diabetes. Visceral WAT is a stronger correlate of insulin resistance than subcutaneous WAT (Preis et al, 2010). Coronary artery disease patients was recently shown to have an 25% increased risk for vascular mortality and 15 % increased risk of all-couse mortality with 1 cm increase in intraabdominal WAT (Kanhai et al, 2011).

A number of mechanisms have been proposed to explain the association between visceral WAT and metabolic disease. Omental WAT is drained by the portal venous system so adipokines, cytokines and free fatty acids from this fat depot directly impact liver metabolism. Omental WAT is more extensively vascularized than subcutaneous WAT and has a higher capillary network density (Villaret et al, 2010). Recent studies (Gealekman et al, 2011; Ibrahim, 2010) propose that subcutaneous WAT has a higher capacity to expand its capillary network than visceral WAT, but this capacity decreases with morbid obesity. The decrease correlates with insulin resistance, suggesting that impairment of subcutaneous WAT angiogenesis may contribute to metabolic disease pathogenesis. Investigations of adipocyte precursors from different depots suggest that the differences exist from embryogenesis through adipogenesis to the functional differences of mature adipocytes. Each depot has its unique pattern of developmental gene expression (Gesta et al, 2007; Tchkonia et al, 2007).

Furthermore, Harman-Boehm and others have shown that elevated macrophage infiltration in omental versus subcutaneous WAT exists in lean subjects and is exaggerated by obesity, particularly if predominantly intraabdominal (Bruun et al, 2005; Cancello et al, 2006; Dahlman et al, 2005). The number of omental macrophages correlates with waist circumference and metabolic syndrome parameters (Harman-Boehm et al, 2007), as well as hepatic fibroinflammatory changes (Cancello et al, 2006) potentially linking central adiposity with increased risk of diabetes and coronary heart disease (CHD).

1.3.5 Gender impact on white adipose tissue

There are gender differences in regional fat distribution. Already in 1947 Jean Vague presented detailed studies of the differences in WAT distribution between men and women (Vague, 1947; Vague, 1956). Based on these differences he could classify obesity into an android and a gynoid type. The android type has more central, abdominal fat whereas the gynoid type has a generalized fat distribution with more gluteofemoral WAT at the hips. Vague couples android obesity with diabetes and cardiovascular disease. Later studies have confirmed and extended his observations that premenopausal women have more subcutaneous WAT whereas men have more visceral fat. (Lemieux et al, 1994; Nedungadi & Clegg, 2009; Wajchenberg et al, 2002). This may be one reason why the incidence of obesity-related metabolic disorders is much lower in premenopausal women than men. A Dutch study examining body composition in 2506 individuals found sex-specific genetic effects underlying several body composition traits (Zillikens et al, 2008). Furthermore, there are differences in both subcutaneous and visceral adipocyte lipolysis between genders (Lofgren et al, 2002; Lonnqvist et al, 1997)

Interestingly, in a recent large metaanalysis of genome wide association studies many genetic loci associated with WHR displayed a gender specific effect (Heid et al, 2010). Some gene expression studies have shown gender differences in gene expression for the same fat depot suggesting hormonal control of these genes (Klaus & Keijer, 2004). One example is the extracellular matrix glycoprotein THBS1. THBS1 is an activator of the transforming growth factor, which in turn is an inducer of the inhibitor of

plasminogen activator inhibitor 1 (PAI-1). THBS1 was overexpressed in visceral fat only in males (Ramis et al, 2002).

1.4 IMPACT OF DIET ON WAT

While the beneficial effects of weight loss in obesity are evident, regulatory mechanisms in WAT that stand behind these effects are not well established. The search for patho-physiological mechanisms has been stimulated by the finding that obese states are likely to be low-grade inflammatory states. Voluntary weight loss can be achieved by life style changes, e.g. increased physical activity, reduced energy intake, pharmacological agents, liposuction, and bariatric surgery. Long term successful weight loss remains a challenge (Turk et al, 2009). However, it is clear that weight loss has beneficial impact on health and survival (Anderson & Konz, 2001; Tsigos et al, 2008). This thesis is limited to study the effects of hypocaloric diet and macronutrient content of diet, which are described in more detail.

1.4.1 Hypocaloric diets

The importance of the composition and amount of energy restriction of a diet for the prevention and management of obesity is debated. Several studies have been published regarding the effect of diet macronutrient composition on weight reduction. Ad libitum consumption of low fat diets result in short-term weight loss (Astrup et al, 2000) and low-carbohydrate, high-protein and high-fat diets may give substantial weight loss as compared with other diets (Gardner, 2007). Because of their satiating and thermogenic properties, high-protein diets have been prescribed for both body weight loss and maintenance (Brinkworth et al, 2004; Paddon-Jones et al, 2008). Recently, Sachs (Sacks, 2009) in a large study showed no difference between dietary fat, protein, or carbohydrate content on weight loss with the main effect caused by energy restriction.

In parallel, research has also focused on the type of carbohydrates in diets. The glycemic index (GI) refers to the postprandial blood glucose response expressed as a percentage of the response to an equivalent carbohydrate portion of a reference food, such as white bread or glucose (Wolever et al, 1991). Reducing the insulin response by lowering the GI in diets may lead to an improved blood glucose profile over the late postprandial period (Brand-Miller et al, 2002), which may be translated into a better appetite control and possibly different metabolic consequences. A Cochrane review of randomized controlled trials with low GI-diets showed that overweight or obese people on a low GI diet lost more weight than overweight or obese people on control diets (Thomas et al, 2007). Low GI combined with higher intake of unavailable carbohydrates leads to lower fasting glucose, higher insulin sensitivity and lower HbA1c in individuals at risk for obesity, T2D, and CHD (Livesey et al, 2008).

Also the prospects for weight maintenance has to be taken into account (Barte et al, 2010; Turk et al, 2009; van Dam & Seidell, 2007). Recently Marquez-Quinon (Marquez-Quinones et al, 2010) identified subcutaneous WAT gene expression patterns that discriminated successful weight control from weight regain over a 6-month period in weight-reduced obese women.

Very low calory diet (VLCD) and low calory diet (LCD) are common prescriptions in weight loss programs. VLCD is often a liquid formula supplying 800 kcal/day. It has since long been known to improve metabolic profile (Hainer et al, 1992). LCD is the individuals energy requirement minus approximately 600 kcal/day.

1.4.2 Impact of voluntary weight loss on WAT function

Since WAT is the organ most strongly affected by weight loss, it is likely that changes in WAT function are key mediators of the beneficial effect of weight loss on health and survival. Effects of weight loss, by either bariatric surgery or hypocaloric diet have been reported in a number of studies recently reviewed by Klimcakova (Klimcakova et al, 2010). The catabolic state associated with hypocaloric diet is, as expected, associated with increased lipolysis as free fatty acids are mobilized from WAT (Stich et al, 1997). By contrast, Löfgren et al has shown that adipocyte lipolysis are lower than in weight matched controls in the late weight stable phase following intense weight loss by behavioural modifications or bariatric surgery(Lofgren et al, 2005b). Insulin sensitivity return to the same level as in controls. Adipose tissue hypercellularity (many small fat cells) and low Leptin production were observed in the postobese state (Lofgren et al, 2005a).

As regards gene expression in WAT, Clement et al used gene expression profiling using 43000 cDNA clones to investigate changes in subcutaneous WAT expression during a 4-week VLCD (Clement et al, 2004). 10 obese subjects were investigated before and after VLCD. Six nonobese were used as controls. Expression of 170 genes changed by VLCD, most of them (60%) belonging to the metabolism gene ontology category. There was also 100 inflammation related genes that were affected by weight loss. Examining isolated adipocytes and the SVF showed that most of the inflammation related genes were expressed in the SVF in resident activated macrophages. The improving effect of weight reduction on WAT gene expression seems to be rapid. Two weeks of VLCD in obese patients with and without T2D decreased expression of chemotactic and proinflammatory factors in WAT but even faster and more pronounced in peripheral monocytes (Mraz et al, 2011). Follow up studies in other populations have found that hypocaloric diet and weight loss are accompanied by a reduction of macrophages in WAT (Kovacikova et al, 2011).

In addition, the temporal response of WAT gene expression to weight loss has been investigated. Capel F et al has shown that a period of energy restriction by VLCD is accompanied by reduced expression of metabolic genes and increased expression of inflammatory genes (Capel et al, 2009). This pattern is reversed after a period of weight stabilization. When they compare the patients before VLCD, and 4 months after in weight maintenance phase, there is no changes in metabolic genes expression but decreased inflammatory profile. This possibly indicates that the patients' have reached a new metabolic steady state with a lower weight, which results in less inflammation in WAT. Not surprising it becomes evident that the timepoint is important when studying WAT gene expression changes.
An exhaustive review of dietary intervention studies investigating adipokines at mRNA and protein level between 1996-2009 was published by Klimcakova 2010. The overall finding is that dietary interventions leading to 5-10% weight loss modulate production

of certain adipokines and generally induce improvements of clinical parameters, e.g. insulin sensitivity.

1.4.3 Impact of macronutrients on WAT function

The macronutrient composition of diet determines the risk of cardiovascular risk in humans. However, there is insufficient data as to whether the macronutrient composition of diet influences WAT phenotypes in humans. Diets rich in whole-grain cereals and food with a low glycemic index may protect against T2D (Schulze et al, 2004). It is also found that dietary resistant starch may improve insulin sensitivity (Robertson et al, 2005).The FUNGENUT Study compared in patients with the metabolic syndrome dietary carbohydrate modification with increased intake of rye and pasta with a low GI, as compared to oat wheat and potato with a high GI. The diet rich in rye and pasta with a low GI improved early insulin response(Laaksonen et al, 2005) and modulated gene expression in subcutaneous WAT, even in the absence of weight loss(Kallio et al, 2007). Genes regulating insulin signaling and apoptosis were downregulated during the ryepasta diet, and genes mainly related to metabolic stress were upregulated during the oat-wheat-potato diet.

In abdominally obese patients an eight week trial comparing two diets differing in fatty acid composition but with normal energy content was performed measuring impact on metabolic parameters and subcutaneous WAT gene expression profile(van Dijk et al, 2009). Consumption of a diet rich in satuarated fatty acids (SFA) resulted in a proinflammatory "obesity-linked" gene expression profile , whereas consumption of a diet rich is monounsaturated fatty acids (MUFA) caused a more anti-inflammatory profile in spite of unchanged adipose morphology and insulin sensitivity.

1.5 CANCER CACHEXIA

Cachexia is derived from the Greek words kakos and hexis meaning "bad condition". It is a syndrome with weight loss, reductions in skeletal muscle and WAT mass, anorexia and weakness, and is frequently associated with inflammation and insulin resistance. It usually occurs in chronic diseases such as cancer, chronic heart failure, chronic obstructive lung disease and end stage renal failure. Cancer cachexia is a wasting disorder seen in half of untreated cancer patients (Blum et al, 2011; Deans & Wigmore, 2005; Fearon & Moses, 2002).The diagnosis cancer cachexia involves involuntary weight loss >5% over the past 6 months or BMI <20 and weight loss >2% (Fearon et al, 2011). Cancer cachexia is associated with a specific loss of WAT and skeletal muscle mass, decreased survival, and poor response to chemotherapy (Esper & Harb, 2005). Hyper-metabolism can only partly explain the loss of weight (Bosaeus et al, 2001). The mechanisms behind tissue loss in cancer cachexia are unknown, but are thought to derive from multiple metabolic alterations, such as reduced appetite, increased energy expenditure and tissue breakdown. The main affected tissues are skeletal muscle and WAT.

1.5.1 Cancer cachexia in white adipose tissue

Recent studies have begun to explore the mechanisms that contribute to WAT loss in cancer cachexia. Increased lipolysis and fatty acid oxidation have been demonstrated in WAT of patients with cancer cachexia (Agustsson et al, 2007). The lipolysis increases via raised levels of HSL, which is the rate limiting step in lipolysis (Ryden et al, 2008). Increased fatty acid oxidation is partly mediated by Cell death-Inducing DFFA Effector

A (CIDEA) (Laurencikiene et al, 2008b).(Laurencikiene et al, 2008b). Systemic inflammation is found in human cachexia; however, WAT levels of cytokines, or leukocyte and macrophage markers are not altered (Ryden et al, 2008). In muscle of cancer cachexia patients an increased expression of genes regulating the ubiquitin-proteasome pathway has been observed (Bossola et al, 2001; Williams et al, 1999); this pathway has to our knowledge not been investigated in WAT in conjunction with cancer cachexia. Secretion of Zinc-α2-glycoprotein (ZAG) from human WAT is increased in cancer cachectic subjects (Mracek et al, 2011; Tisdale, 2009). ZAG is an abundant circulating protein that enhances the lipolytic effect of catecholamines on fat cells. It is produced by the host and by certain tumors. Given its lipid-mobilizing effect, ZAG may contribute to WAT atrophy associated with cancer cachexia in human beings. However, ZAG secretion from WAT increased significantly in obese subjects on a VLCD (Ryden et al, 2011). Thus, WAT-secreted ZAG correlates with nutritional status, i.e.catabolism, in both cancer and nonmalignant conditions.

2 AIMS

WAT is the organ most strongly affected by obesity and cachexia. We hypothesize that alterations in WAT function contribute to the negative metabolic consequences and disease outcome, respectively, of these two disorders. In this thesis we apply global transcriptome profiling to generate new hypothesis on molecules and pathways of relevance for WAT function.

The specific aims are

1) to search for differentially expressed genes in human omental and subcutaneous WAT

2) to elucidate diet-regulated gene expression changes in subcutaneous WAT of obese women

3) to establish whether different macronutrient content of food gives different gene expression changes in WAT

4) to elucidate the role of Follistatin in WAT

5) to study gene expression changes and gene regulation in WAT in cancer cachexia

3 METHODOLOGICAL CONSIDERATIONS

3.1 GENERAL COMMENTS CONCERNING SUBJECTS

All studies were performed in humans. There are differences in the distribution of WAT depots and metabolism between human and rodent. The clinical relevance of studies in rodent WAT is therefore unclear.

There are gender differences in WAT function (Wajchenberg, 2000). To get optimal power it is therefore advantageous to study one gender at a time. In studies of obesity in humans it is often easier to recruit women and therefore we have often focused on female WAT in our studies. However, for the Representational difference analysis (RDA) in study 1 we choose the individual with the largest visceral adipose area, not surprisingly a man. Sometimes we had to mix genders to get significant results. In the cancer cachexia project more men than women were included. We evaluated all patients scheduled for gastrointestinal cancer operation for four years at our hospital for inclusion into the study. For practical reasons, e.g. the WAT biopsies, it was not possible to recruit subjects from other hospitals. To get sufficiently large groups for analyses, we included both women and men in the analysis. In this project, we did not observe any gender differences in the analysis of the results. This could possibly in part be due to the age of the population, mean age was 65 years, and gender differences is most pronounced between premenopausal women compared to men.

To further minimize confounders in our obesity studies, we tried to minimize variation in baseline BMI and age in studied cohorts. We limited the studies to healthy individuals except obesity and almost always excluded those with medication. We usually study Caucasians living in Stockholm to avoid the influence of ethnicity on metabolism. Ethnic differences in adipose gene expression in insulin resistant states have been described (Elbein et al, 2011).

A body mass index (BMI) greater than 30 kg/m^2 was used as a definition of obesity. In study 2 and 3, most subjects were recruited by advertisement. We do not know if obese subjects recruited this way differ from obese in the general population.

In general, in our studies, sample size was calculated with the aim of getting some new interesting finding, i.e. identify some new genes that were differentially expressed between the compared clinical conditions. The aim was not to define the whole spectrum of WAT differentially regulated genes. Given the heterogeneity of obesity, as shown in recent genome scans (Speliotes et al, 2010), we expect there to be large variation in WAT gene expression within the obese population that require very large sample sizes to be defined. van Beek showed 2007 (van Beek et al, 2007) that the variation in gene expression between multiple WAT biopsies from the same individual and depot is small but the variation between individuals is considerable, also highlighting the importance of large sample size in human WAT studies.

The hospital´s committee of ethics approved each study.

3.2 DESCRIPTION OF STUDY COHORTS

Study 1: The obese subjects (7 males and 11 females) included were members of a subgroup of patients participating in a clinical trial of adjustable gastric banding (Thorne et al, 2002). From these subjects, it was possible to obtain adipose specimens from omental as well as subcutaneous WAT. We selected the subject that had the greatest ratio of intraabdominal to subcutaneous WAT for RDA. His proportion of visceral versus total WAT area was 53% (426 cm^2 visceral WAT area, 352 cm^2 subcutaneous WAT area) and WHR was 1.10. To verify differential gene expression in other obese subjects, we used a pool of patient samples. We had to pool RNA since the microarray hybridizations at that time required more RNA than was available from each subject. WAT from 6 males and 11 females was used to prepare female and male visceral and subcutaneous RNA pools, respectively. If we had performed the study today, the modern methods which need less RNA would have permitted us to investigate all samples individually showing the inter individual variation which probably is considerable (van Beek et al, 2007).

Study 2: This study was part of the European multicenter study termed Nutrient-Gene Interactions in Human Obesity-Implications for Dietary Guidelines (NUGENOB www.nugenob.org) within which the interaction between two hypoenergetic diets and genes was examined in 771 European patients. The present study was performed at the local center at Karolinska University Hospital, Huddinge, Sweden. 100 obese subjects (women and men) were recruited at the hospital and randomly assigned to either a low-fat, high-carbohydrate hypoenergetic diet or a moderate-fat, moderate-carbohydrate hypoenergetic diet for 10 weeks.

Dietary intervention: Energy intake during both diets was only moderately reduced, 600 kcal/day less than the individually estimated energy requirement. The aimed macronutrient composition was for the low-fat diet 20–25% of total energy from fat, 15% from protein and 60–65% from carbohydrate, and for the moderate-fat diet 40–45% of total energy from fat, 15% from protein and 40–45% from carbohydrate. When the study was planned low fat diets were claimed to be superior to high fat diet in producing weight loss and possibly also for weight maintenance. It was thought that carbohydrates are more satiating than fat and that overeating might be more likely with a high-fat diet because of the higher energy density and greater sensory pleasure. Dietary composition might affect risk factors for CHD and T2D by altering blood lipids or insulin resistance, therefore these were monitored. Compliance with diet was evaluated by weighed-food records and was within or close to the target ranges.

From the first 40 women out of the 100 subjects recruited at Huddinge, fine needle abdominal subcutaneous WAT biopsies were obtained before and after the diet period for investigations of WAT in relation to diet. We were able to isolate high quality RNA from WAT samples before and after intervention from 23 of the 40 women in order to perform global transcriptome profiling; n=10 of these women belonged to the low-fat, high-carbohydrate hypoenergetic diet and n=13 women to the moderate-fat, moderate-carbohydrate hypoenergetic diet. This subgroup did not differ from the whole group in baseline clinical measurements.

Study 3: To study the role of Follistatin in WAT, four cohorts comprising 10-35 subjects each were investigated. The sample sizes were chosen based on known pattern of expression of Follistatin mRNA in WAT in relation to obesity according to a microarray study (Dahlman et al, 2005). We took into account that microarray tends to squeeze the overall variation in expression of a gene.

Study 4: All patients in Karolinska scheduled for gastrointestinal cancer operation between March 2004 and March 2008 (about 500 patients) were evaluated for the study and patients who (a) were fit in spite of their cancer, (b) had not received prior anticancer treatment, (c) did not have clinical evidence of gastrointestinal obstruction and (d) were willing to participate were included (n=53). The remaining ones were excluded due to severe illness, evidence of gastrointestinal obstruction, disinclination, communication problems and logistic reasons. Patients were divided into three groups based on diagnosis after surgery. One group had cancer cachexia (n=13), which was defined as gastrointestinal cancer with unintentional weight loss of >5% of the habitual weight during the previous three months or >10% unintentional weight loss during the previous six months (Agustsson et al, 2007). The primary location of cancer was pancreas (n=11), stomach (n=1) and colon (n=1). The second group (n=14) consisted of patients with gastrointestinal cancer who reported no important weight change during the last year. The localization of malignancy was pancreas (n=6), esophagus (n=1), stomach (n=1), colon (n=3), gall bladder (n=1) and liver (n=2). The remaining 26 patients were excluded either because, although pre-diagnosed with gastrointestinal cancer, they did not have a malignancy according to final histological evaluations (n=8), or because we did not obtain adequate amounts of WAT for a complete investigation (at least 0.9 g).

We used the method by Liu to determine the appropriate sample size for microarray experiments in the cachexia study (Liu & Hwang, 2007). Assuming that significant differences in gene expression between the two studied groups were defined with a false discovery rate (FDR) of 5%, the proportion of non-differentially expressed genes between groups was 0.95. Assuming a standard deviation for effect size of 1, a population consisting of two groups composed of 15 subjects each yields a power of approximately 90% to detect differentially expressed genes. This is close to the actual number of 13 cachectic and 14 weight stable patients used in the present analysis.

We compared the changes in WAT gene expression associated with cachexia with changes in gene expression associated with obesity in two microarray studies reported elsewhere (Dahlman et al, 2005; Mutch et al, 2009). One study included Affymetrix HGU95Av2 expression profiles on 17 non-obese and 20 obese healthy Swedish women (Dahlman et al, 2005). The second study described Agilent 4x44K gene expression profiles in surgically-obtained subcutaneous adipose tissue biopsies from 9 obese and 10 lean age-matched subjects (Mutch et al, 2009). Differences in gene expression between isolated adipocytes and stroma vascular cells were examined in subcutaneous WAT in another group of overweight women (n = 9), as previously described(Clement et al, 2004).

3.3 ADIPOSE SAMPLING

All subjects were investigated in the morning after a night's fast. This is important since changes in nutritional status has acute effect on WAT expression.(Clement et al, 2004). Abdominal subcutaneous WAT biopsies were obtained by needle aspiration under local anesthesia as described earlier (Large et al, 1997). When both subcutaneous and omental WAT were studied we used biopsies obtained in conjunction with surgery. The WAT was taken from the surgical incision and omental WAT at start of surgery. None of the operated patients showed signs of general inflammation. Subcutaneous WAT samples obtained by needle and surgery were never included in the same comparison. WAT obtained by these two approaches are shown to differ slightly, the surgery biopsy containing more stroma vascular cells (Mutch et al, 2009). Fine needle biopsies are also shown to give higher variation of gene expression, possibly due to disruption and possible contamination of the tissue with blood (van Beek et al, 2007). Therefore we tried to rinse the WAT from blood prior to freezing.

3.4 BODY COMPOSITION AND FAT DISTRIBUTION

There are different methods to assess fat mass and adiposity in humans. Body mass index (BMI) and waist hip ratio (WHR) are common measures to describe if you are overweight and to appreciate fat distribution. BMI >18.5 and <25 and WHR <1 is considered normal for men. BMI >18.5 and <25 and WHR <0,8 are the normal values for women. Waist circumference is becoming more widespread as a measurement of visceral adiposity, where the International Diabetes Federation recommends >94 cm for men and >80 cm for women as cutoffs for abdominal obesity (Alberti et al, 2009). Abdominal CT scan was used in study 1 to assess intra-abdominal and subcutaneous adipose areas at L4-L5. There is so far no clear recommendation of normal levels of WAT areas when assessed by CT, but international obesity and diabetes federations are aiming for it. Several earlier studies have found an increased cardiovascular disease risk when visceral fat area exceeds 100-130 cm^2 (Wajchenberg, 2000). Higher levels of visceral WAT(>140 cm^2 for males and >114 cm^2 for women) were associated with greater prevalence of several clinical cardiovascular manifestations in individuals both with and without T2D in a study of 4144 individuals from 28 countries (Smith et al, 2012). Modern CT techniques for fat mass evaluations measuring WAT areas volumetrically will probably increase accuracy further**.** Dual energy x-ray absorptiometry (DXA) can measure total fat and lean body mass. The radiation dose is much less than CT. The new software to evaluate visceral WAT area or volume by DXA was not available when our studies were performed. In study 4 body composition was estimated by bio-impedance using Quad Scan 4000 which estimates % body fat and lean body mass. MRI gives good quality measurements but so far the MRI equipment is expensive and often prioritized for other usage.

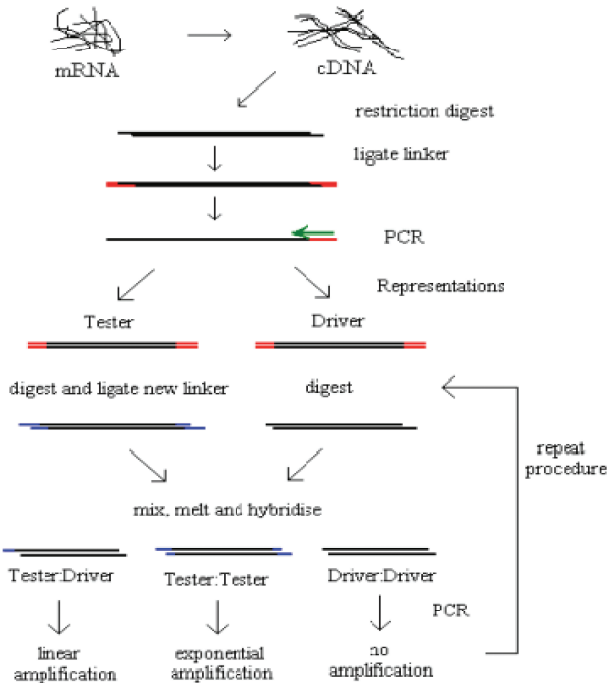
3.5 ADIPOSE RNA PREPARATION

In paper 1 subcutaneous and omental WAT from individual subjects were homogenized and total RNA was isolated using Trizol reagent. The quality was ascertained on denaturing agarose gels and concentration determined spectrophotometrically. For the verification of RDA results equal amounts of total RNA were mixed to generate female (n=11) and male (n=6) RNA pools.

In general it is difficult to isolate RNA from adipose tissue with high yield due to the high lipid content of the tissue. More recently, however, premanufactured kits that can be used to efficiently isolate RNA also from WAT have been developed. These kits simplify the laboratory work. In study 2 and study 3 frozen subcutaneous WAT from each subject was crushed under liquid nitrogen, and total RNA was prepared using the RNeasy mini kit (Quiagen, Hilden,Germany). In Study 4 we used the Nucleospin RNA11 kit (Macherey-Nagel, Duren, Germany).The advantage with Nucleospin is that it allows preparation of RNA and DNA in parallel from the same tissue piece. We used DNA preparation for quantification of mitochondrial DNA (mtDNA). RNA-concentrations were measured spectrophotometrically to check for integrity and purity (A260/A280 ratio). The quality was confirmed by an Agilent 2100 Bioanalyzer, which uses a microfluidics-based chip for eletrophoresis and size separation .

3.6 CDNA-RDA

cDNA representational difference analysis (cDNA RDA) is a PCR-based subtractive enrichment procedure for the cloning of differentially expressed genes. During the 90ies, before the era of microarrays, several PCR-based methods were developed to facilitate rapid and sensitive analysis of differential gene expression. Among these counted differential display, RNA fingerprinting by rapidly primed PCR, SAGE and RDA for cDNA. RDA was originally developed by Lisitsyn and coworkers (Lisitsyn & Wigler, 1993) for analysis of differences between closely related genomes, and further developed for cDNA-analysis by Hubank and Schatz (Hubank & Schatz, 1994). Compared to differential display, RDA minimizes the risk of isolating false positives. Also, it has the potential for analysis of differences in gene expression on different levels through modulation of driver:tester ratios (Odeberg et al, 2000). Thirdly, unwanted clones can be included in the driver, thereby inhibiting their amplification. Our RDA project was initiated before the era of global transcriptome analysis by microarray (Venter et al, 2001). We choose to use cDNA RDA to search for differences in omental versus subcutaneous WAT mRNA expression. With this method we hoped to get a non-hypothesis based screening method for identification of new genes involved in the complications of obesity and to isolate the expression differences between the two tissues. Below is a picture describing the procedure of RDA.



Repeated subtraction and amplification rounds yield an efficient depletion of ubiquitous gene fragments from both cDNA populations, thereby cloning the genes that are most differentially expressed. The products from the RDA were visualized on agarose gels, excised and cloned into vectors. These can be sequenced and in our case we also PCR-amplified them for later printing on cDNA microarrays.

After the arrival of cheap genome-wide microarrays and the sequencing of the human genome, the RDA-method is seldom used in human studies but publications in other fields, like honey bee research (Humann & Hartfelder, 2011) and seedless grapevine development (Costenaro-da-Silva et al, 2010) are still emerging.

3.7 HIGH THROUGHPUT OMICS METHODOLOGIES

Since the end of the 1990ies there has been a rapid evolution of high throughput technologies and we are now able to generate large-scale data on DNA, RNA and protein level (genomics, transcriptomics, proteomics and epigenomics), as well as on lipids and metabolites (lipidomics and metabolomics).

Microarray technology is a multiplexed hybridization-based process, which allows simultaneous analysis of a large number of nucleic acid transcripts. Nucleic acid based microarrays can be used for gene expression profiling, single-nucleotide polymorphism (SNP) detection, array-comparative genomic hybridizations, comparisons of DNA-methylation status, and microRNA evaluation. Gene arrays are used in a diversity of fields and with the help of more and more sophisticated computational tools data sets are nowadays integrated to enhance the extraction of information. It is called a systems biology approach and aims at modeling biological processes as interconnected and

regulated networks (Schadt, 2009). We are moving from the search for a disease-specific gene to the understanding of the biochemical and molecular functioning of a variety of genes whose disrupted interaction in complicated networks can lead to a disease state (Trachtenberg et al, 2012). Transcriptomics, the large scale measuring of gene expression, is one of important pieces to reach the goal of functional characterization.

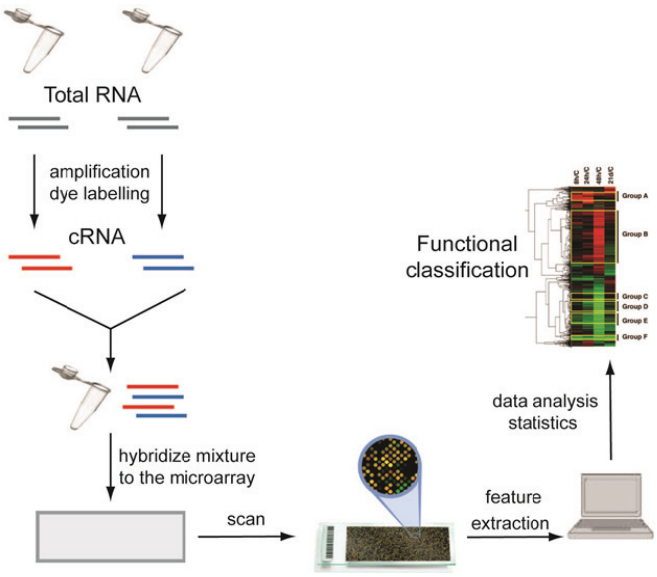
3.7.1 cDNA microarray

The RDA-study was planned and performed before microarrays for inferring the global transcriptome were widespread commercially available. We set up our own cDNA array facility to verify the RDA-derived results using a new set of samples. In order to verify many clones, which is often the result of the RDA-procedure used in study 1, we amplified the RDA products by PCRamplification and then printed them on amino-silane coated slides, where the amino-silane coat provides hydroxyl groups which allows the spotted cDNA to covalently bind the surface (Andersson et al, 2003)

Spotted or printed microarrays are prepared by printing probes of oligonucleotides or cDNA, that is prepared from known, selected probes. The probes hybridize to cDNA or cRNA derived from experimental samples. For gene expression analysis, the transcriptome of the sample is analyzed through hybridization with the complementary probes present on the array. It is important to note that the query sample needs to be labeled to allow signal detection by scanning in confocal microscopy. A signal is detected when the target hybridizes to its corresponding probe sequence on the array. Labeling of RNA samples can be achieved through a reverse transcription reaction by direct incorporation of nucleotides linked to fluorochromes, typically Cy3 or Cy5. The resulting cDNA can then be hybridized to the array under stringent conditions. After incubation, the array is washed to remove any nonspecific hybridization and to reduce background signals. Laser confocal devices are next used to scan the arrays and acquire images and spot intensities. By measuring the amount of cDNA bound to each spot (fluorescence intensity) it is possible to determine expression of that particular gene.

Printed arrays (cDNA and long oligo arrays) allow the contemporaneous hybridization of the two different samples, labeled with different fluorochromes, so that the test and control can be directly compared on the same slide. For this reason, printed arrays are also called “two-color arrays”. Direct comparison of the samples on the same array reduces artifacts due to uneven hybridization, local background differences, and slide-to-slide variations; on the other hand, dye bias requires performing dye-swap replicates. Experimental design is important using two-color array, especially if you are not just comparing two situations like before and during treatment.

Image analysis was performed as described in the RDA-study using Genepix Pro software. The cut-off for selection of upregulated and downregulated genes was set to 1.5 due to earlier experience in our group confirming our cDNA-array results with RT-q-PCR.



3.7.2 Oligonucleotide arrays

A number of commercial providers of oligonucleotide-based arrays are available today. In study 2 and 4 we have used arrays from Affymetrix, the first commercial company synthesizing gene-specific probes onto a silicon surface. The oligonucleotides of interest are synthesized directly on the array surface via a series of chemical coupling reactions. The procedure utilizes a solid support derivatized with a covalent linker molecule, which terminates with a photolabile protection group. Ultraviolet light causes deprotection and activates these molecules for chemical coupling with new protected nucleotides. With this method only relatively small, 25-30 bp, probes are synthesized. To overcome low specificity multiple probes are present for the same gene.

Short-oligo arrays support the hybridization of only one sample per chip; therefore they are also called one-color arrays. In this case, the intensity of each spot is measured and the comparison of different samples is achieved by comparing data from different chips. For this reason, consistency between sample preparation and hybridization procedures needs to be under tight control. Furthermore, there are algorithms to normalize data on each chip, to allow comparison between arrays. We used an all-probeset scaling to allow subsequent comparison of individual gene expression between microarrays.

A big advantage is that high density arrays can be made with up to 500.000 probes/cm^2. Another advantage of industrial short-oligo arrays is their reproducibility and the standardization of the array, as well as the small amount of RNA needed in each experiment. The main disadvantage has been the considerable costs and the difficulty to customize arrays based on specific researchers' needs, but the development is overcoming these problems. Another disadvantage with microarrays is that only known transcripts, represented on the arrays, are measured.

Another array platform is the Illumina array technology. The Illumina process involves using standard oligonucleotide synthesis methods as is used for spotted long-oligonucleotides arrays. However, on Illumina arrays the oligonucleotides are attached to microbeads which are then put onto microarrays using a random self-assembly mechanism. The probe selection and design procedure differs from Affymetrix and also the packaging. In the current packaging, multiple Illumina arrays are placed on the same physical substrate, meaning that hybridization and other steps are performed in a parallel manner. The Affymetrix arrays that we have used are processed separately.

3.8 REAL-TIME QUANTITATIVE PCR

For individual gene expression measurements, for example to confirm results from microarray studies, real-time quantitative PCR (RT-q-PCR) is the method most commonly used today (Lutfalla & Uze, 2006). First cDNA is reverse transcribed from sample RNA, and then gene-specific amplification by RT-q-PRC is performed. With the use of fluorescent molecules, each cycle of replication in RT-q-PCR can be monitored by sensors. There are differerent approaches in real-time quantification. We have applied Taqman and SYBR Green technology. The Taqman technique uses a gene-specific probe which has a fluorophore on one end and a quencher on the other end. Close proximity of the fluorophore and quencher prevents signal emission. Upon amplification of the specific gene, the polymerase will digest the bound probe and the fluorophore will be detected by its specific wavelength.

An alternative and less costly method uses SYBR Green. SYBR Green is a molecule that binds to doublestranded DNA. During PCR amplification, doublestranded DNA is produced, and SYBR Green binds to it and can then be detected. Since SYBR Green binds to all doublestranded DNA there is a risk of false positives from primer dimers and unspecific products. This risk can be minimized by careful primer design, the use of dissociation curves, and the use of electrophoresis gels which should show one single amplified product. RT-q-PCR in this thesis uses mostly the SYBR Green technique but in study 2 and 3 some genes were analyzed with Taqman probes. We have purchased commercial Taqman-kits, and tried to choose kits covering all known splice variants of the gene. Concerning primer design we excluded repeat regions by Repeat masker and used the software Primer3 to design the primers. We used bioinformatics to check that the predicted amplicon covered exon-exon borders to avoid amplification of genomic DNA We also checked that primers did not cross-react with other genome sequences using BLAST search.

To ensure that equal amounts of cDNAfrom different samples are compared , it is essential to determine mRNA levels of reference genes. These “housekeeping”-genes, are genes that are not affected by the conditions studied. Our use of reference genes has changed over time as we have learnt about new genes that display less variation between samples and experimental conditions. In study 2 we used GAPDH and in study 3 and 4 18S rRNA. After the completion of our studies Gabrielsson published a comparison of reference genes in WAT and found that LRP10 had the least variation (Gabrielsson et al, 2005). The optimal solution is probably to include several reference genes. Different genes might be suitable in different situations depending on experimental setup. A direct comparative method was used for data analysis,

i.e. $2^{(\text{Ct target gene calibrator} - \text{Ct target gene sample})}/2^{(\text{Ct 18S calibrator} - \text{Ct 18S sample})}$. As an alternative a standard curve can be used to compare mRNA levels between samples.

3.9 BIOINFORMATICS

High throughput omics technologies generate massive amounts of data that require extensive data analysis, as well as data storage. In the last decade numerous new software have been developed and new possibilities arise as computers and software can cope with increasing amounts of data at a higher speed (Trachtenberg et al, 2012).

Microarray data analysis starts with normalization of raw signals on the arrays, whereafter signals from different arrays can be compared. After microarray normalization, the raw data is further analyzed in order to identify genes differentially expressed between groups. For microarrays this analysis involves the simultaneous analysis of thousands of genes. With a nominal p value of 0.05 there will therefore be many false positive. Different approaches to adjust significance thresholds have been proposed. In study 2 and 4 we used the Significance Analysis of Microarrays (SAM) (Tusher et al, 2001) statistical program especially designed for microarray analysis. SAM adjusts for the multiple comparisons caused by the presence of thousands of genes on the microarrays. A score is assigned to each transcript based on its change in gene transcription in relation to the standard deviation of replicated measurements for that particular gene. SAM estimates the false discovery rate (FDR), which is the percentage of genes expected to be wrongly identified as differentially expressed. Each gene is assigned a q-value that indicates the minimum FDR at which the differential expression of the gene is called significant. SAM was used in our array studies and gave lists of differently expressed genes. There are additional approaches to identify differentially expressed genes, such as T test with Benjamini-Hochberg adjustment. However, our experience is that SAM is efficient to detect differentially expressed genes. Furthermore, we can confirm results obtained with SAM by RT- q-PCR, even with fold change as small as 1.2, and we therefore continue to use SAM.

To further understand the gene changes we used several software that aim at categorizing the genes in pathways and gene ontology categories. I will briefly mention some of them below. Many more software are available. However, since the software we have used produce similar results, we do not think the use of additional ones will improve our interpretation of results. Rather it is our belief that the limiting factor in pathway analysis is the annotation of genes, and the complex physiology. That is, too little is known about the function of many genes to allow them to be grouped into more detailed pathways.

FunNet integrates experimental gene expression data and knowledge about transcript´s biological roles, available in genomic annotation systems (Prifti et al, 2008). FunNet performs a functional profiling of gene expression data to identify a set of significant (i.e. overrepresented) biological themes characterizing the analyzed transcripts, i.e. cellular processes, pathways, or molecular functions in which the analyzed transcripts are involved. Then, based on the results of the functional analysis, a two-layer abstraction model is built to integrate the two types of transcriptional information: expression levels and transcripts' biological roles. This model is further used to derive a

measure of proximity between significant biological themes based on the similarity of the expression profiles of their annotated transcripts. In the end, themes demonstrating a significant relationship in the transcriptional expression space are associated to build transcriptional modules. FunNet relies on genomic annotations provided by the Gene Ontology consortium and the Kyoto Encyclopedia of Genes and Genomes.

Gene Ontology Tree Machine (Zhang et al, 2004) is a web-based platform for interpreting sets of interesting genes using Gene Ontology hierarchies. A tree-like structure is created to illustrate changes in expression of Gene Ontologies. Statistical analysis helps users to identify the most important Gene Ontology categories from the input gene sets and suggests biological areas that warrant further study. We have based the input gene list on genes that are differentially expressed between groups according to SAM.

Gene Set Enrichment Analysis (GSEA) (Subramanian et al, 2005) is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states even though the individual genes in the gene sets may not be significantly differentially expressed between groups. GSEA cannot be used for paired analysis.

SIMCA-P (Umetrics AB, Umea, Sweden) is a software used in multivariate analysis to get an exploratory overview of your data including assessment of the quality of your samples. Principal component analysis evaluates the principal components of a data set. Partial least square analysis can optimize the separation between groups. We have used SIMCA to get an overview of our datasets, before proceeding to other analysis.

An unbiased approach to detect functional connection between genes is cluster analysis that detects groups of genes that display similar expression between samples. We have successfully applied cluster analysis on our microarray results. However, genes in detected coexpressed clusters did not display any obvious functional connection. We therefore do not report the results of this analysis.

3.10 PROTEIN DETECTION

Enzyme-linked immunoassay was used to examine *in vitro* secretion of Follistatin in study 3. Western blot was used for protein measurements in study 3 and 4. Western blot in study 3 was used to confirm the size of the detected protein.

3.11 ISOLATION OF WHITE ADIPOSE TISSUE CELLS

In study 2-4 WAT pieces immediately underwent collagenase treatment followed by determination of fat cell volume and weight of isolated fat cells as described (Large et al, 1997)(Rodbell & Krishna, 1974). Lipid content was assessed by AdipoRed assay reagent. AdipoRed is a Nile Red fluorescent reagent that enables quantification of intracellular lipid droplets.

The different cell types of the SVF were separated with antibody coupled microbeads and subsequently identified by fluorescence activated cell sorter analysis (Curat et al, 2004; Sengenes et al, 2005): adipocytes, blood capillary endothelial cells, progenitor

cells, macrophages, lymphatic and blood vessel endothelial cells, and a fraction with lymphocytes and mast cells.

3.12 IN VITRO ADIPOGENIC DIFFERENTIATION

The non-adipose cells harvested from the SVF of WAT that was obtained after collagenase treatment was used for *in vitro* differentiation of preadipocytes as described (van Harmelen et al, 2002)(Hauner et al, 1995).

In study 3 we also used another primary progenitor cell type; human bone marrow-derived mesenchymal stem cells (hMSC). For adipogenesis experiments these cells were grown to 100% confluence and then induced with adipogenic medium. They were treated according to the specific experiment and cells were analyzed for lipid content, differentiation and RNA expression. Different types of human fat cell cultures display some differences in phenotype, primarily in gene expression. Which cell type that is used in different projects depends on which is the most appropriate fat cell type for the specific experiment, but also on the availability of specific fat cell types in different laboratories. The stem cell derived fat cells may better mimic the primary phenotypes of adipocytes, whereas mature adipocytes isolated from donors may display secondary changes due to obesity etc.

4 RESULTS AND DISCUSSION

In this section I will summarize and discuss on the results from the individual papers, All details about the results, p-values, graphs and tables can be found in Papers I-IV.

4.1 PAPER 1

There are since long ago known structural and functional differences between subcutaneous and visceral WAT (Wajchenberg, 2000). It is still unknown whether molecular changes in visceral or omental WAT can explain the increased mortality and risk for T2D and cardiovascular complications associated with visceral WAT that many prospective studies have shown (Wajchenberg et al, 2009). This study was planned and initiated in the late 90s before whole genome microarray technology was available. cDNA RDA was applied to search for and clone differences between omental and subcutaneous WAT mRNA. With this method we hoped to get a non-hypothesis based screening method for identification of new obesity genes and to isolate the expression differences between the two tissues.

We chose to clone the differentially expressed RNA between subcutaneous and omental WAT from one individual; a man with an extreme phenotype of prominent visceral obesity. His proportion of visceral adipose was 53% of total adipose mass, compared to the normal amount of 10-20% in men. His visceral area was enormous, 426 cm^2 and exceeded the subcutaneous area (352 cm^2). A recent European study of visceral adipose area in 28 countries (Smith et al, 2012) found that areas exceeding 140 cm^2 in men predicted increased CVD-risk and T2D. Our patient's WHR was 1,10 and BMI 39 km/m^2. Since it is shown that each cm (measured by ultrasound) increase of intraabdominal WAT increases the risk for CVD (Kanhai et al, 2011) this man ought to be a high-risk individual. There are of course drawbacks by our approach of using a single patient as material for cloning. We know that there are considerable inter-individual variations in WAT gene expression (van Beek et al, 2007; Zhang et al, 2007) and by investigating only one individual, expression differences that might exist in other subjects and not in our subject will not be found. Since our patient material of extremely abdominally obese individuals was limited we could not use a group of abdominally obese subjects but chose instead to clone only the most extreme subject and then to verify his expression differences in another group of obese subjects.

After cloning the mRNA differences between the patient´s subcutaneous and omental WAT, sequencing 768 clones, and excluding redundant clones, resulted in the 44 unique gene sequences presented in Table 2 in the paper. Many of these genes are previously been shown to be expressed in WAT, e.g. Adipsin, Properdin, Serum Amyloid A, Secreted Protein, Acidic and Rich in Cysteine (SPARC), Phospholipid transfer protein (PLPT) and several ribosomal proteins. We have compared the 44 gene sequences with genes differentially expressed between subcutaneous and visceral WAT in lean, obese and T2D-patients in a more recent study by Dahlman et al (Dahlman et al, 2006). 23 genes were also found on the wholegenome microarray used by Dahlman and 17 of these were differentially regulated. Of these eight were ribosomal proteins which all were overexpressed in omental WAT. SPARC was more common in

subcutaneous WAT as reported earlier and Annexin A8 overexpressed by omental adipose. S100A6 was not differentially regulated on the microarray.

In differential cloning procedures, one may pick up false positives, i.e. genes that are not differentially expressed. In this study we choose to evaluate this and simultaneously test if the patient's expression differences were valid in other obese individuals by testing a new set of male patients. In addition, we attempted to search for sex differences in expression by analyzing a group of obese females. To enable a rapid screening, we used cDNA microarray technology. The 44 RDA clones were spotted in triplicate onto glass slides and hybridized to fluorescently labeled cDNA from the above-mentioned male and female RNA pools. 16 (36%) of the cloned genes were detected in the pools of male or female subcutaneous or omental RNA. Four clones were differentially expressed in female pool, and five in the male pool between subcutaneous and visceral WAT. The relatively low frequency of detected clones could indicate biological differences between the RDA patient and the pools but we must also consider methodological explanations. The cDNA-technology has limited sensitivity. In our case, we used RDA clones which had lengths between 75 and 500 bp, which differs from commercial cDNAarrays. Using the same approach as ours, Boeuf et al (Boeuf et al, 2001) confirmed differential expression for 30% of obtained RDA-clones by microarray validation. Concerning biological differences the RDA-patient with prominent metabolic syndrome differs compared to the pools where the patients were extremely obese but with relatively modest clinical characteristics. Recent studies show that the gene expression differences are larger with worsening metabolic disease (Klimcakova et al, 2011).

The choice to pool RNA to a female and male pool can be criticized. Our cDNA-array technology demanded quite large RNA-amounts and we had to either pool individual RNAs or to amplify them. Since the experience with RNA amplification at that time was limited we chose not to amplify. Because of inter individual differences in gene expression pooling probably decreases the chance of finding differentially expressed genes. Repeating the study today I would have chosen to measure individual samples.

Three genes were found differentially regulated with higher expression in subcutaneous WAT in our male and female pool, i.e. Adipsin, Calcyclin, and a PAC clone. Adipsin and Properdin, which was cloned in the RDA but not proven to be differentially expressed in the pools, are components of the alternative complement system. High expression of complement components in omental WAT of obese men were described by Gabrielsson (Gabrielsson et al, 2003) who also found that Adipsin was overexpressed in subcutaneous WAT in line with our result. In addition to their roles in innate immunity, a dual role for complement has been suggested in adipocyte biology where Properdin is proposed to act as a switch between immune versus metabolic drives (Gauvreau et al, 2012). It is present in adipocyte cell surface membranes and may interfere with fatty acid uptake and esterification. Calcyclin, or S100A6 is a calcium binding protein whose expression is upregulated in proliferating and differentiating cells. We found an overexpression in subcutaneous WAT. This protein may function in stimulation of Ca2+-dependent insulin release. Its serum levels correlates with advanced cardiovascular disease in rats (Cai et al, 2011). There is an increasing interest in the adipokines as mediators in atherosclerosis (Zhang et al, 2010)

and S100A6 could be a candidate. The release from fat cells is stimulated by FFA and this release can be inhibited by insulin. A recent study by Cai (Cai et al, 2011) showed an association between serum levels of among other S100-proteins S100A6 and acute coronary syndrome.

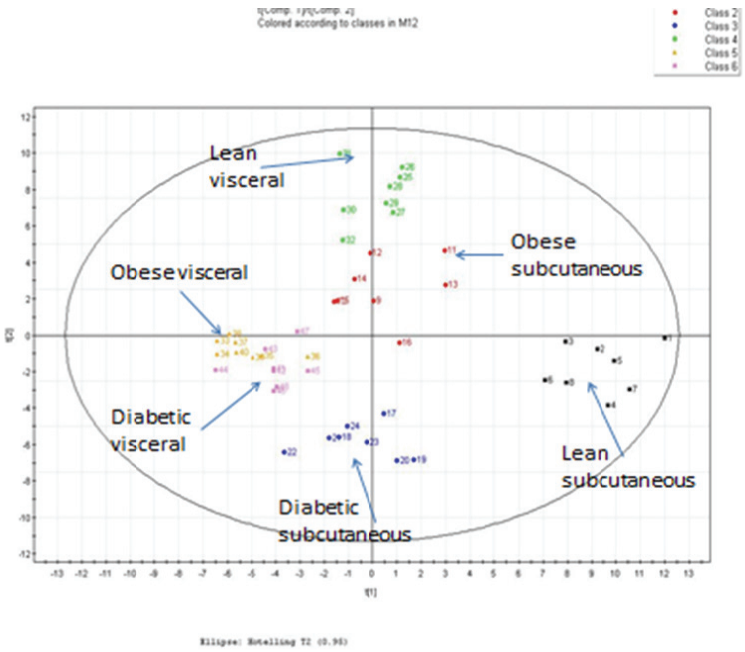
We cloned several ESTs of unknown function but only one of them fulfilled our criteria for differential expression and interestingly only in the male pool where it was more expressed in subcutaneous WAT. The gene product codes for a voltage-dependent calcium channel subunit alpha-2/delta-3 precursor. The classical roles of α2δ proteins are as accessory calcium channel subunits, enhancing channel trafficking (Bauer et al, 2010). We do not know the function of this subunit in male WAT. It is interesting to speculate about gender specific differences which so far are not widely investigated in WAT gene expression. Wolfs (Wolfs et al, 2010) found a module of gender related genes that were differentially expressed but the individual genes were not reported.

Two genes were overexpressed in omental WAT of both the male and female pool, PLPT and RAS, involved in lipid metabolism and signal transduction. PLPT transfers phospholipids from TAG-rich lipoproteins to high density lipoprotein (HDL). In addition to regulating the size of HDL particles, this protein may be involved in maintenance of efficient cellular cholesterol efflux by local expression in the vascular wall. The earlier plausible positive antiatherogenic role has recently been challenged by another picture: systemic PLTP act as a pro-atherogenic factor by increasing the production of apolipoprotein B-containing lipoproteins to decrease their antioxidative protection and to trigger inflammation. PLPT and related Cholesteryl transfer protein are secreted proteins and WAT is an important contributor to the systemic pools of these two proteins. PLTP activity and mass have been reported to be abnormally elevated in T2D and insulin-resistant states, and this elevation is frequently associated with hypertriglyceridemia and obesity. Haplotypes in the PLPT gene are associated with obesity-related phenotypes as shown by the the Québec Family Study (Bosse et al, 2005).The RAS gene encodes a member of the Rho family of small GTPases which function as molecular switches in signal transduction cascades. Rho proteins promote reorganization of the actin cytoskeleton and regulate cell shape, attachment, and motility. Its expression is widespread and e.g. fibroblasts and endothelial cells express RAS. We found RAS expression to be higher in omental than subcutaneous WAT which could be due to a higher vascular density and endothelial cell abundance in omental WAT as described by Villaret (Villaret et al, 2010).

There are some clones obtained by the RDA that were not found in the other subjects but that deserve mentioning because of biological interest. The cloning of SPARC from our abdominally obese man is interesting since it has gained interest as a plausible key player in obesity related complications (Kos & Wilding, 2010). SPARC was originally found in bone as Osteonectin, is expressed ubiquitously, but predominantly in adipocytes. Others have shown higher expression in subcutaneous compared to omental WAT (Vohl et al, 2004) (Klimcakova et al, 2011; van Beek et al, 2007) and recently also in larger adipocytes (Kos et al, 2009). As a regulator of extracellular matrix, SPARC contributes to WAT fibrosis. Fibrosis of subcutaneous WAT may restrict accumulation of TAGs, which are therefore deposited as ectopic lipids in other tissues such as liver or muscle, and may predispose to insulin resistance in these organs. The

plasma level of SPARC correlates with BMI, CRP and coronary artery disease (Takahashi et al, 2001). SPARC is correlated to fasting insulin levels and insulin resistance (Kos et al, 2009). SPARC has also been shown to inhibit adipogenesis by its enhancement of beta catenin signaling(Nie & Sage, 2009). Raised SPARC-concentrations are associated with the development of diabetes-associated complications like nephropathy and retinopathy, possibly due to profibrotic and angiogenic effects (Taneda et al, 2003; Watanabe et al, 2009). SPARC was not detected in our male and female cohorts. We might speculate that the extreme abdominal obesity in the RDA-patient is coupled to extreme insulin resistance and therefore gives higher levels of SPARC than in the other less abdominally obese subjects. One must of course consider that our microarray has low sensitivity and might give false negative results. Acute phase Serum Amyloid A (A-SAA) is highly expressed in WAT adipocytes even during non acute-phase conditions. Weight reduction reduces A-SAA. A-SAA is a known risk factor for coronary artery disease. SAA is considered a marker of adiposity-induced low-grade inflammation but not of the metabolic status of obese subjects (Poitou et al, 2006).

During the last years a number of microarray-studies have been published which compare visceral and subcutaneous WAT gene expression. Our group have used Affymetrix whole genome microarrays to study omental and subcutaneous biopsies from 8 lean, 8 healthy obese and 8 obese T2D women (Dahlman et al, 2006).
To complete the picture concerning depotrelated gene expression changes we would like to report some unpublished results from the above mentioned study. Using SIMCA-P to separate the differentially expressed genes we found a good separation between the diseases and depots by using only the 200 most discriminating genes.



Score Scatter plot – patients separated based on 200 genes

Comparing subcutaneous and omental WAT we found overexpression of genes dealing with GO-categories complement activation, protein biosynthesis and koagulation in omental adipose and lipid metabolism, transcription, energy pathways and phosphate transport in subcutaneous WAT. A larger number of genes displayed significant differences in expression between patient groups in the omental than in subcutaneous WAT. We have compared the results from these microarrays with the cloned genes from the RDA-study and found that 23 of the 44 RDA-clones were expressed on the whole genome microarray and 17 of these were differentially expressed between the WAT depots. Of these eight were ribosomal proteins which were all overexpressed in omental WAT. SPARC was verified as significantly higher expressed in subcutaneous WAT.

In another study, Vohl performed wholegenome arrays of visceral and subcutaneous adipose in 10 obese men (Vohl et al, 2004). 131 genes were higher expressed in subcutaneous WAT and 216 in visceral WAT. Genes from the CEBPs and Wnt signaling pathways were higher expressed in omental fat and HOX genes in subcutaneous WAT, which may suggest differences in differentiation and adipogenic capacities. Reanalyzing these arrays Zhang (Zhang et al, 2007) measured interindividual variation between depots. More than 90% of clones showed a coefficient of variation of lower than 23,6 and 21,7% in omental and subcutaneous WAT. Among the highly regulated genes, 18 transcripts in subcutaneous WAT were involved in lipid metabolism and 28 transcripts in omental WAT were involved in cell death.

An impressive study by Wolfs et al (Wolfs et al, 2010) performed whole genome arrays on subcutaneous and omental WAT from 75 massively obese individuals. The individuals were by blood parameters and BMI categorized as healthy obese, T2D, Nonalcoholic steatohepatitis (NASH)-patients and individuals with both NASH and T2D. They could show that 1344 genes were significantly upregulated in subcutaneous WAT, of these 609 with more than 1.5 fold change. 1246 genes were significantly overexpressed in omental WAT, and 909 more than 1.5 fold increased. A large number of genes had more than 10-fold higher expression in omental WAT. These genes were involved in signal transduction, cell adhesion, cell communication and developmental processes. Only 20 genes were >10-fold overexpressed in subcutaneous WAT. They were highly enriched in homeobox transcription factors. Groups of co-expressed genes were identified that correlated with lipid and glucose metabolism parameters; they were enriched with immune genes.

In a recent study by Klimcakova (Klimcakova et al, 2011) whole genome gene expression arrays were used to investigate genes differentially expressed between subcutaneous and visceral WAT in 24 subjects with differing degree of obesity and metabolic syndrome. The depots were compared and also correlated with degree of obesity, visceral fat accumulation, insulin resistance and metabolic syndrome. Comparing visceral and subcutaneous gene expression identified 492 genes with higher expression in subcutaneous WAT and 560 genes with omental overexpression. The increase in adiposity and the worsening of metabolic status were associated with a coordinated down-regulation of metabolism-related and an up-regulation of immune

response-related gene expression. Molecular adaptations in subcutaneous WAT proved as discriminating as those in visceral WAT in differing lean from metabolic syndrome.

Summarizing these large microarray studies it is evident that there are large expression differences between omental and subcutaneous WAT. Omental WAT seems to have more differentially expressed genes in relation to adiposity. Our RDA study underestimated the changes in gene expression between fat depots for several reasons. The cDNA RDA methodology do not pick up all differentially expressed changes. During the cDNA-RDA-procedure, lack of Sau 3A1 restriction sites in the mRNA might generate less than 100% coverage in the representations. Large representated fragments may not be efficiently amplified by PCR. We found 44 clones and would probably have found some more if more clones had been sequenced. Importantly, most of our cloned differentially expressed genes are verified in the large microarray studies (analysis not shown). The studies by Klimcacova and Wolfs clearly shows that expression changes between the subcutaneous and visceral WAT depots are more pronounced in subjects with severe metabolic syndrome and /or T2D than in the healthy obese. This encourages us to think that expression changes between these depots can guide us to find the metabolic changes leading to these diseases.

4.2 PAPER 2

The aim of paper 2 was to define dietary effects on WAT gene expression and to search for gene expression differences due to macronutrient composition. Genes and gene pathways responsive to dietary treatment and energy restriction may contribute to the development of obesity complications.

NUGENOB
The study was part of a European cooperation project called NUGENOB. 771 patients from eight European countries were randomized to either of two hypocaloric diets with different fat/carbohydrate ratios. The main clinical results were reported 2006 (Petersen et al, 2006). The low-fat diet produced similar mean weight loss as the moderate-fat diet, but resulted in more subjects losing >10% of initial body weight and fewer dropouts. Both diets produced favorable changes in fasting blood lipids, insulin and glucose.

The clinical results have subsequently been confirmed. One of the larger studies comparing diets in 811 patients was performed by Sachs 2009 (Sacks, 2009) and showed consistent with our study a modest weight reduction, which was the same for all diets. Regardless of dietary composition the weight loss improved blood parameters and insulin resistance similarly. The study included the same diet composition as ours, but also two other diets, i.e. low carbohydrate and high protein diets.

Our study within the NUGENOB consortium focused on WAT gene expression in relation to diet. We expected rather small changes in gene expression because of;

- The mild energy restriction (aim -600 kcal/day) which according to the dietary registrations in our group of patients was even less, -500 kcal/day.

- The relatively long time period, 10 weeks, where the body might have adapted to the hypocaloric situation.
- Differences in diet compositions was rather small and not far from our Swedish nutrition recommendations.

In our subgroup of patients the total amount of calories from fat during the intervention was significantly different from baseline and was within the targeted 40–45 E% in the moderate-fat diet group (42+/-3 E%) and close to the targeted 20–25 E% in the low-fat diet group (28+/-4 E%). The reduction in energy intake was almost identical in the two groups (-556 vs 503 kcal/day).There was a marked difference in the percentage of total calories from carbohydrate and fat between the groups and also in the amount of dietary fiber. The percentage of total calories from protein also differed somewhat between the groups. The reported intake for the moderate- and low-fat diets, respectively, were for carbohydrates: 40 vs. 51 E%; dietary fiber: 13 vs 19 g/day; and protein: 19 vs. 21 E%.

The decrease in body weight was 7,5% in both groups. Fat cell volume decreased and insulin sensitivity increased with weight reduction. We found no significant differences between the diet groups in fat cell volume, plasma insulin and glucose. Protein secretion in these patients were measured and reported by Arvidsson (Arvidsson et al, 2004) and showed no differences between diets. The secretion rate of Leptin decreased by approximately 40%, as did that of TNF-alpha. Interleukin 6 and -8 decreased by 25-30%, whereas the secretion of Plasminogen activator inhibitor 1 (PAI-1) and Adiponectin did not show any changes. PAI-1 levels in plasma increased, probably indicating that other sources such as liver or endothelial cells produced more PAI-1 in response to energy restriction. As expected S-Leptin decreased.

Our global transcriptome analysis showed that 3746 of the 8500 genes represented on the microarray were expressed in WAT of more than half of the patients. Of these, expression of 96 genes, that is 2.5%, was significantly changed by the intervention with hypocaloric diet. Fold change (expression after diet divided by before diet) among these genes varied between 1.10 and 1.46 for upregulated genes, and between 0.35 and 0.90 for down-regulated genes, respectively. The pattern of response was almost identical between the two diets.

Our study was one of the first to use microarray to study effects of diet on gene expression in WAT. A few other microarray studies on dietary impact on subcutaneous WAT gene expression have been published (Capel et al, 2009; Capel et al, 2008; Clement et al, 2004; Marquez-Quinones et al, 2010; Rizkalla et al, 2012) and our finding that there are no major macronutrient effects on WAT gene expression is still predominant. It is the energy restriction or decreased fat mass that induces WAT gene expression changes.

Two other studies have examined WAT gene expression in the NUGENOB study. Viguerie performed RT-q-PCR on selected genes in two groups of 25 women each, (none overlapping with patients in our study) (Viguerie et al, 2005). 38 genes were studied. None of the genes was differentially regulated according to fat or carbohydrate content of diet. 10 genes were regulated by caloric restriction. Consistent with our data Viguerie showed a downregulation of genes involved in lipid metabolism. Only two of

the genes regulated by energy restriction in our study overlapped with the ones measured by Viguerie et al, FASN and CIDEA, which were not regulated by diet in their study. The discrepancy could depend on different genetic and cultural background, limited power, or on different probes for mRNA detection.

Also part of the NUGENOB study Capel et al (Capel et al, 2008) performed global transcriptome profiling by microarrays on 47 women on the NUGENOB study diet. None of the women overlapped with our group. Concerning the method, unlike us Capel used RNA amplification before cDNA synthesis, and hybridized to cDNA arrays with 24469 Unigene clusters. We used Affymetrix oligonucleotide arrays with 8793 genes, Our arrays did not cover as many transcripts as the ones used by Capel so we cannot expect to find as many differentially expressed genes or pathways. The two different microarray platforms might also give different results due to different principles in array design. The cDNA technology with longer clones might have different specificity.

In accordance with our results, Capel et al showed that the majority of genes were downregulated by diet. Fatty acid metabolism was one of the downregulated pathways and Stearoyl-CoA desaturase 1 (SCD-1) was one of the most downregulated genes. Capel also observed a decrease in genes regulating triacylglyceride storage capacity, carbohydrate metabolism, electron transport, fatty acid and steroid metabolism. Interestingly, using two gene classification programs combined with the array results, and RT-q-PCR on a larger group of patients they could show five genes with differential expression between the two diets. Sirtuin 3 (SIRT3), Fatty acid binding protein 4 (FABP4) and the Glucocorticoid hormone receptor NR3C1 were among the genes that increased by the moderate fat diet. NR3C1 expression correlated with total and low-density lipoprotein cholesterol. Probably the considerably larger cohort with 48 patients for the array analysis and 96 patients for RT-q-PCR analysis contributed to the more extended findings by Capel as compared to our results.

Other dietary intervention studies applying WAT global transcriptome profiling
When interpreting our results in NUGENOB it is important to remember that energy restriction and weight reduction may regulate mRNA levels via different mechanisms. An acute negative energy balance upregulates certain pathways whereas the same pathways can be unchanged or even downregulated after longer moderately hypocaloric diet. This is illustrated in the study by Capel et al (Capel et al, 2009). They measured insulin sensitivity by glucose disposal rate (GDR), and global transcriptome WAT gene expression profiles before and after 1 month of VLCD diet and again after a 4 month weight maintenance program. During VLCD clusters of genes were associated with GDR. Of interest and in total agreement with our results, the genes associated with synthesis of unsaturated fatty acids were downregulated by VLCD and associated with changes in GDR. Furthermore, CIDEA, which was the most upregulated gene in our study, was on their list of gene predictors of insulin sensitivity using partial least square regression analysis.

Overall the changes in adipose gene expression in our study was modest which is in accordance with other array-studies on human WAT in relation to hypocaloric diet e.g. Kohlemainen (Kolehmainen et al, 2008) found fold changes ranging between 0.67 and

1.68, which is very close to our 0.35-1.46. Kohlemainen performed a comparison of WAT gene expression before and after a 33 week dietary intervention program, where the first 12 weeks were moderate energy restriction which was followed by a weight maintenance phase. The weight reduction was comparable to our study. Weight loss or a lower fat mass resulted in decreased expression in a small subset of the measured genes. i.e. 105 of the approximately 22.000 annotated genes on the array. Pathway analysis revealed mainly changes in genes related to extracellular matrix and cell death. These pathways, which were not observed in our study, could be due to Kolehmainen investigating WAT expression in the weight maintenance phase when the metabolic situation is in steady state and changes mostly depend on the actual weight, while we were looking at a catabolic state with ongoing moderate energy restriction.

The DiOGenes program is a randomized, controlled dietary intervention study investigating the effects of dietary protein and glycaemic index on weight (re)gain, and metabolic and cardiovascular risk factors in obese and overweight families in eight European centres (Larsen et al, 2010). After an initial eight week LCD, the weight-reduced obese subjects were assigned to ad libitum low-fat diets with different protein amounts and glycemic index. One report from the Diogenes trial (Marquez-Quinones et al, 2010) investigated subcutaneous WAT mRNA to identify gene biomarkers of weight control during the weight maintenance follow-up period. The authors concluded that the main differences in WAT gene expression were due to weight variations rather than to differences in dietary macronutrient composition. There was no gene expression patterns associated with macronutrient diet composition.

It could be that the macronutrient impact on WAT gene expression is overrun by the influence of reduced fat mass in intervention studies which in parallel studies hypocaloric diet. Normocaloric studies, comparing different diets, are therefore of interest. Kallio (Kallio et al, 2007) evaluated for the FUNGENUT study the effect of two different normocaloric carbohydrate diets in patients with metabolic syndrome. Kallio could show that a low GI diet composed of rye, pasta and wholegrain cereals could improve the insulinogenic index and downregulate genes linked to insulin signaling and apoptosis in WAT, whereas the oat, wheat and potato-diet upregulated genes related to stress, cytokine-chemokine-mediated immunity and interleukin pathways.

The association between gene expression changes in WAT and modification of adipocyte size was explored recently by Rizkalla (Rizkalla et al, 2012) comparing a low calorie conventional diet with a low calorie diet with low GI and more protein and soluble fiber (LC-P-LGI). Rizkalla could show that the diets were similarly effective in reducing weight in moderately obese participants. Like in our NUGENOB study, that global WAT gene expression changes did not differ between diets. Unexpectedly there was a greater reduction in adipocyte size after the LC-P-LGI diet than after the low calorie conventional diet. The reduction in adipocyte size correlated with several parameters of importance for CHD-risk. Smaller adipocytes correlated with some WAT gene expression pathways, i.e. inhibition of adipogenesis, cell migration, adhesion, and angiogenesis, but increased expression of apoptosis genes. Altogether they could show a WAT gene expression pattern that correlated with adipocyte diameter, which

stimulates further studies concerning local remodeling changes in human adipose tissue and their association with diet and systemic improvement of metabolic variables.

It is evident from our, Viguerie's and Capel's WAT gene expression studies on the NUGENOB cohort that 10 weeks of moderate energy restriction reducing weight 7-8 kg is not enough to reduce inflammatory genes in adipose. Clement who studied a cohort of women before and after 4 weeks of VLCD described the modulation of several inflammatory genes induced by energy restriction When comparing WAT gene expression studies we should remember that all WAT samples from the above mentioned diet studies were obtained by needle biopsy, which poorly aspirates the fibrotic fraction of subcutaneous WAT, in turn resulting in an underrepresentation of the SVF (Mutch et al, 2009) . Therefore, gene expression in WAT in our study reflects mostly changes in adipocytes and to a lesser extent those in the SVF.

SCD1 and CIDEA

One of the most pronounced gene-nutrient interactions in our study was the downregulation of Stearoyl-CoA desaturase 1 (SCD1). SCD1 is a delta-9 fatty acid desaturase that converts saturated fatty acids (SFA) into monounsaturated fatty acids (MUFA). This activity is elevated by dietary carbohydrates (Flowers & Ntambi, 2009). Parallel activation of de novo lipogenesis and Stearoyl-CoA desaturase activity is shown in humans after three days of high-carbohydrate feeding (Chong et al, 2008). Thus, because SCD1 is the rate limiting step in the conversion of MUFAs, it is important in the regulation of the endogenous SFA pool caused by diet and de novo lipogenesis (Flowers, 2009). Elevated SCD1 activity within adipose tissue is closely coupled to the development of insulin resistance in humans (Sjogren et al, 2008). According to experimental studies, Scd-1 appears to play an important role in maintaining a balance in lipid profiles that, when deregulated, can contribute to inflammation, atherosclerosis, hypertriglyceridemia, and metabolic syndrome (Merino et al, 2010). It is thus possible that the reduced SCD1 expression in our patients is related to the improved insulin resistance.

Our NUGENOB study, showing upregulation of CIDEA after energy restriction, evoke the interest for CIDEA in humans. Before our study, CIDEA was known as an apoptosis gene, and a mouse knockout model showed that mice deficient in CIDEA exhibited a lean phenotype. A study from our group (Nordstrom et al, 2005) extended the knowledge of human CIDEA expression by showing that CIDEA expression was decreased twofold in obese humans and normalized after weight reduction. This was later confirmed by others (Gummesson et al, 2007). Nordstrom showed that low WAT CIDEA expression was associated with several features of the metabolic syndrome. Human adipocyte depletion of CIDEA by RNA interference stimulated lipolysis and increased TNF-alpha secretion by a posttranscriptional effect. Recent studies show that CIDEA and other lipid droplet proteins define a highly regulated pathway of triacylglyceride deposition in human WAT. CIDEA colocalizes around lipid droplets (Christianson et al, 2010) with Perilipin, a regulator of lipolysis. Failure of this pathway results in ectopic lipid accumulation, insulin resistance, and is associated comorbidities in humans (Puri et al, 2008).

There are several limitations to our study. First of all, we studied no more than around 8500 genes and the investigated group of patients was quite small. Since our study was one of the first microarray studies of diet-regulated WAT-changes we had limited information of the expected size of gene expression effects. Furthermore, the simultaneous analysis of hypocaloric diets and diets differing in macronutrient content may have limited the opportunity to identify the influence of macronutrients on WAT. Since we have investigated tissue biopsies of WAT, our gene expression results could be due to mRNA expression from any other of the WAT cells, i.e. stroma cells or adipocytes. We also should not forget that investigating gene expression data may not reflect actual changes in protein expression. Post-transcriptional and posttranslational activity in the WAT might change the result and subsequent metabolic effect.

4.3 PAPER 3

Follistatin is an extracellular glycoprotein that was originally identified as an inhibitor of pituitary Follicular stimulating hormone secretion (Ueno et al, 1987) and later has been shown to be produced by many tissues. Follistatin functions as an antagonistic binding protein that neutralizes the bioactivity of the members of the Transforming growth factor beta (TGFβ) superfamily (Mather et al, 1997). Thus, Follistatin prevents ligand-receptor interaction of Activin, Myostatin, and bone morphogenetic proteins (Bessa et al; Lee & McPherron, 2001; Nakamura et al, 1990). Activin and Myostatin signal by binding to type II serine/threonine kinase receptor (activin type IIB) that partners with type I receptor (TGFβR1).

We found Follistatin in a microarray study (Dahlman et al, 2005) comparing subcutaneous WAT gene expression between obese and lean persons. Since Follistatin in human WAT was not studied before we wanted to elucidate its role in human WAT and obesity. We showed that subcutaneous WAT Follistatin mRNA decreases with increasing weight, and that weight loss restores Follistatin levels. RT-q-PCR detected more Follistatin in subcutaneous than visceral WAT. The SVF of WAT seems to be the major source. Highest levels of Follistatin were observed in CD34+/CD31- cells, which contain adipocyte progenitor cells, and in the capillary endothelial cell fraction. Isolated adipocytes had very low levels of Follistatin. We could by ELISA show that WAT secretes follistatin *in vitro*. In accordance with the mRNA-measurements in adipocytes there was no secretion of Follistatin from isolated fat cell cultures. Follistatin acts through Activin/Myostatin receptors ACVRIIB and TGFbetaR1. We quantified mRNA of these receptors and could show that they are expressed in WAT and isolated adipocytes, so the necessary machinery for Activin, Myostatin, and Follistatin signaling is present within WAT.

Human WAT Follistatin is probably acting locally in WAT, because circulating Follistatin levels do not seem to be affected by obesity (Eldar-Geva et al, 2001). Beyond this, Follistatin has hardly been studied in humans in relation to obesity and related metabolic disease. By contrast, several animal studies describe changes in metabolic parameters like visceral fat mass, insulin sensitivity and pancreas mass that are of potential clinical interest. To understand potential Follistatin effects, knowledge concerning Myostatin and Activin effects is mandatory. Myostatin acts as a negative regulator of muscle growth (McPherron et al, 1997). Loss of myostatin expression

results in increased muscle mass and reduced adiposity (Gonzalez-Cadavid & Bhasin, 2004) (Schuelke et al, 2004), whereas overexpression of Myostatin leads to severe reduction of both muscle and adipose mass, along with cachexia (Reisz-Porszasz et al, 2003; Zimmers et al, 2002)). Knock-out-studies by Tu 2009 showed that inactivation of Myostatin protects against the development of insulin resistance, proatherogenic dyslipidemia, and aortic atherogenesis in Ldlr−/− mice (Tu et al, 2009). A very recent study (McPherron et al, 2012) reduced the expectation of Myostatin inhibitors as treatment for obesity since mice treated with pharmacologic Myostatin inhibition displayed increased lean mass without loss of WAT and no effect on glucose metabolism. Opposing this is another recent study in which rats were subjected to exercise, which resulted in expression changes in Myostatin, its receptors and Follistatin in both muscle and WAT (Dutra et al, 2012) highlighting the probable importance of this system in muscle and WAT physiology, and in metabolic disorders like T2D. Probably the Myostatin and Activin-inhibition will have to be tissue specific since recent studies (Guo et al, 2009) indicate different effects in different organs.

As regards Activin, mice with a targetet deletion of the gene display developmental defects and early neonatal death, whereas Activin overexpression results in cancer, liver necrosis and cachexia (Lee & Glass, 2011; Lee et al, 2005; Matzuk et al, 1994). Finally, different mice knockout models have tried to elucidate the role of Follistatin. Deletion of Follistatin results in a number of developmental defects and early neonatal death (Matzuk et al, 1995). Follistatin like 3 (FSTL3) is a member of the Follistatin-gene family. Nakatani (Nakatani et al, 2011) describes the FSTL3-KO mice. The FSTL3-KO mice have reduced visceral fat mass, enhanced glucose tolerance and increased insulin sensitivity. The reduced visceral fat mass observed in FSTL3 KO mice may be a primary action of excess Myostatin bioactivity resulting from loss of its antagonist. A Follistatin-derived peptide FS I-I that blocks Myostatin but does not neutralize Activin is described (Nakatani et al, 2011). FS I-I transgenic mice exhibit reduced fat accumulation. Adipocytes are smaller and the mice are resistant to high fat diet-induced obesity and hepatic steatosis.

Follistatin and adipogenesis

It has been shown that Follistatin reverses Activin and Myostatin inhibition of bovine adipogenesis (Hirai et al, 2007) but so far there are no human studies. To examine the effect of recombinant human Follistatin in human WAT we treated two human primary progenitor cell types with Follistatin for 21 days. Adipogenic differentiated hMSCs had 50% more lipid content compared with controls efter 21 days of Follistatin treatment. Similar effects were also obtained with differentiated human preadipocytes. To examine Follistatin neutralizing activity of TGF beta family members we cotreated hMSCs with Follistatin and Myostatin under adipogenic conditions, and found that cotreatment appeared to reverse Myostatin inhibitory effect on adipogenesis. To further investigate Follistatin promotion of adipogenesis we assessed mRNA levels of some adipogenic markers. We investigated mRNA levels of two initiating transcription factors of adipogenesis, CEBP alpha and Peroxisome proliferator activator receptor gamma (PPARG), and one late marker, FABP4, after 21 days of chronic Follistatin treatment. All adipogenesis markers were increased compared to adipogenic control cells. Also lipid content was increased. To summarize recombinant Follistatin

promoted adipogenic differentiation of progenitor cells and neutralized the inhibitory action of myostatin on differentiation *in vitro*.

Targeting preadipocyte to adipocyte conversion may be an effective way to regulate adipose mass. Interestingly, the expression of a Follistatin ligand, Activin B, was shown to be reduced by diet-induced weight loss in subcutaneous WAT from obese subjects (Sjoholm et al, 2006). These data suggest that obesity decreases the Follistatin to Activin ratio.

To summarize down-regulation of Follistatin in obesity could aim to limit adiposity by inhibiting adipogenesis but, by failing to limit fat accumulation, indirectly contribute to hypertrophic obesity. Low Follistatin levels may result in enlargement of existing adipocytes at the expense of new fat cell formation. One limitation of our study is that we do not know if Follistatin regulates adipogenesis in humans *in vivo*. Furthermore, we cannot rule out that changes in the degree of vascularization in WAT that accompanies the development of obesity might account for the changes in Follistatin expression in relation to obesity.

4.4 PAPER 4

Although the cancer cachexia syndrome has received growing attention in the last few decades, its cause remains unknown and no known therapy is available to reverse its detrimental effects. In this study we aimed to search for regulatory pathways that accompany loss of WAT in cancer cachexia. We chose to investigate patients in a relatively early stage of cancer disease when the cachexia causes WAT loss and lean tissue is still preserved (Fouladiun et al, 2005) to avoid secondary changes that could exist in a more advanced metabolic situation like refractory cachexia. Our primary hypothesis was that changes in pathways involved in the regulation of energy metabolism would accompany the loss of adipose tissue.

Examining the study groups it is evident that the cachexia group has a clearly reduced body fat mass compared to weight stable cancer patients while lean mass was preserved. There were signs of systemic inflammation, a higher CRP and Interleukin 6, in the cachexia group. Glycerol/kg bodyweight and FFA/kg bodyweight were doubled in the cachexia group indicating increased lipolysis. Fat cell volume was decreased by cachexia. All together the metabolic changes reported in earlier studies exist also in our patients.

We performed global transcriptome profiling by microarrays and showed that 364 genes were downregulated and 61 genes were upregulated in subcutaneous WAT of cachexia patients versus weight-stable cancer patients using an FDR of 5%. By comparing with previously published array results from Clement (Clement et al, 2004) on adipocyte vs SVF expression we could see that there was a fairly even distribution of the regulated genes between SVF and adipocytes. The use of needle biopsy subcutaneous WAT, which has less SVF than surgical biopsies probably made us underestimate the SVF-enriched genes.

Pathway analysis revealed that genes downregulated by cachexia were overrepresented in pathways related to extracellular matrix, actin cytoskeleton and focal adhesion.

These changes are similar to the changes observed during voluntary weight reduction by hypocaloric diet in obese subjects as reported by Henegar and Kolehmainen (Henegar et al, 2008; Kolehmainen et al, 2008). The extracellular matrix has gained a lot of interest during the last years and increased extracellular matrix and fibrosis is observed in obesity. One theory suggests that adipocyte hypertrophy in obesity is limited by WAT extracellular matrix components. Excessive lipids then have to be stored ectopically in the liver and muscle and might increase insulin resistance. We could not find any enrichment of inflammatory genes among cachectic patients. A probable explanation might be that the systemic inflammation originates from other tissues than WAT. Also the amount of inflammatory genes, which in obesity is mostly found in the SVF, might be lower because of the needle biopsy technique.

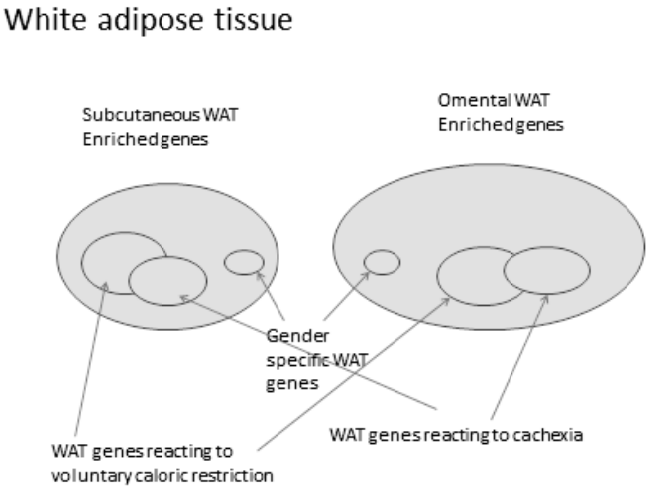
Genes in pathways regulating energy turnover were upregulated in cachectic patients, that is electron transport, fatty acid degradation, oxidative phosphorylation and the Krebs TCA cycle. These findings are consistent with the physiological finding of increased lipolysis and lipid oxidation in cachexia patients. Fat oxidation in the adipocytes could be one of several ways to get rid of the excess fatty acids generated by lipolysis. This would favor the metabolism of fatty acids to acetyl CoA over glucose, which fits with the observation that glucose administration suppresses lipid mobilization in normal individuals, but there is an impaired suppression in patients with malignant disease and continued oxidation of fatty acids (Edmonson, 1966). A potential mechanism to this old observation might be CIDEA, which we originally found in study 2 as a gene upregulated during dietary energy restriction. Our group has shown that CIDEA is also involved in WAT loss in cancer cachexia and this may, at least in part, be due to its ability to inactivate the pyruvate dehydrogenate complex, thereby switching substrate oxidation in human fat cells from glucose to fatty acids (Laurencikiene et al, 2008a).(Laurencikiene et al, 2008a). CIDEA was not differentially regulated by cachexia in our study which might depend on the small study group and the lower sensitivity of microarrays compared to RT-q-PCR, which was used by Laurencikiene et al.

The changes in the abovementioned pathways were compared with two studies using whole genome microarrays to compare subcutaneous WAT gene expression between lean and obese individuals (Dahlman et al, 2005) (Mutch et al, 2009). Interestingly the changes were reciprocal as shown in table 2.

In summary, examining WAT gene expression in human cancer cachexia reveals marked changes in pathways regulating energy turnover, extracellular matrix and cytoskeleton. The pathways are in many cases a mirror image to those observed with obesity but similar to those seen with intentional weight loss.

5 SUMMARY AND GENERAL DISCUSSION

We chose cDNA-RDA and genome-wide expression analysis to study gene expression changes in human subcutaneous and omental adipose tissue. The goal was to find new genes and pathways related to abdominal obesity, voluntary caloric restriction and cancer cachexia. We also examined a new adipokine, Follistatin and its effects on WAT and obesity.



Gene products that are enriched genes in omental and subcutaneous fat can be illustrated as shown in the figure and depending on the definition of enriched genes (i.e. fold difference) each compartment can represent a few to several thousands of genes. The present thesis along with more recent large genomewide expression studies have contributed to name and add some gene differences between omental and subcutaneous WAT. This, together with our studies describing the reaction of subcutaneous WAT to caloric restriction and cachexia contribute to an increased understanding of the underlying metabolic changes.

DEPOT SPECIFIC GENE EXPRESSION DIFFERENCES

Abdominal obesity - the most prevalent manifestation of metabolic syndrome - is a marker of 'dysfunctional adipose tissue', and is of central importance in clinical diagnosis. Increased understanding of the underlying metabolic disturbances will hopefully lead to development of new treatment strategies including physical activity, operation and/or new drugs.

In study 1 we cloned 44 unique sequences that differed between subcutaneous and omental WAT in an extremely abdominally obese man. Many of these genes were not

earlier shown to be expressed in WAT, but since the publication of our study in 2004 a few large genome wide expression studies comparing subcutaneous and omental WAT gene and protein expression have verified some of the results. Adipsin, Properdin, Serum Amyloid A, SPARC, phospholipid transfer protein (PLPT) and several ribosomal proteins were differentially expressed in the RDA-patient. These genes belong to protein synthesis pathways, immune response, cell structure, signal transduction and lipid, carbohydrate and amino acid metabolism pathways. Several of these pathways are discussed in the large studies by Wolfs (Wolfs et al, 2010) and Klimcakova (Klimcakova et al, 2011).

To summarize the results from study 1, we can conclude that the differentially expressed gene products from the RDA-study that were verified also in female and male obese patients showed depot related expression in 5 genes involved in gene pathways in immunity (Adipsin), metabolism (PLPT) and signal transduction (Ras, Calcyclin). These pathways and genes have later been verified by genome wide expression studies to be differentially expressed between depots. An unknown gene product was verified only in the male patients. The gene product codes for a voltage-dependent calcium channel subunit alpha-2/delta-3 precursor. The classical roles of α2δ proteins are as accessory calcium channel subunits, enhancing channel trafficking. This gene is not studied in WAT. The finding could imply that gender differences exist in adipose gene expression.

Our cloning of 44 differentially expressed genes in the man with extreme metabolic syndrome but verification of only 5 of these as differentially expressed in other obese patients is intriguing. Of course the sensitivity of our home-made cDNA arrays could be limited but other explanations are also possible. Our RDA patient probably had more exaggerated expression changes than our pooled healthy obese patients. This is supported by very interesting recent observations by Klimcacova and Wolfs concerning the relation between advancing metabolic disorder and gene expression changes in WAT. Both Klimcakova (Klimcakova et al, 2011) and Wolfs (Wolfs et al, 2010) could show gene expression changes that correlated with worsening metabolic traits and these changes were observed both in subcutaneous and omental adipose even though they were more prominent in omental WAT. Their findings that it is possible to some extent to mirror the changes in omental WAT by subcutaneous adipose biopsies is positive since the subcutaneous biopsies are much easier to obtain.

A question we must ask is if these gene expression changes propagate to changes in protein expression. Perez (Perez-Perez et al, 2009) demonstrated that tissue-specific molecular differences in the protein secretion patterns of subcutaneous and omental fat depots mainly related to metabolic processes such as glucose and lipid metabolism, lipid transport, protein synthesis, protein folding and response to stress and inflammation. They suggest higher metabolic activity as well as increased cell stress in the omental compared to the subcutaneous fat. We can conclude that the pathways reported are similar to the ones mentioned above meaning that the gene expression changes induce protein changes and thereby probably metabolic effects.

We must also consider which cell type we study. Whole WAT samples were used, and it is possible that some differentially expressed genes were isolated from other cell

types found within adipose tissue, such as endothelial cells, fibroblasts, or macrophages. Differential expression observed may be the result of differences in these cells or their proportion within a given fat depot. Studies using isolated cell fractions and culture techniques will be required to elucidate this question. Peinado (Peinado et al, 2010) showed 2010 that the protein fingerprints of isolated adipocytes from visceral WAT and subcutaneous WAT of lean individuals were strikingly similar, whereas significant differences were observed between the SVF of both fat depots in terms of their protein expression patterns. We did not study adipocytes and SVF separately in study 1 so we cannot conclude which cell type is responsible for our results.

Role of Follistatin in human WAT

We found Follistatin in a microarray study (Dahlman et al, 2005) comparing subcutaneous WAT gene expression between obese and lean persons. Since Follistatin in human WAT was not studied before we wanted to elucidate its role in human WAT and obesity. We showed that subcutaneous WAT Follistatin mRNA decreases with increasing weight, and that weight loss restores Follistatin levels. RT-q-PCR detected more Follistatin in subcutaneous than visceral WAT. The SVF of WAT seems to be the major source. Highest levels of Follistatin were observed in CD34+/CD31- cells, which contain adipocyte progenitor cells, and in the capillary endothelial cell fraction.We could speculate that Follistatin has a role in angiogenesis. Recombinant Follistatin promoted adipogenic differentiation of progenitor cells and neutralized the inhibitory action of Myostatin on differentiation *in vitro.*

We believe that down-regulation of Follistatin in obesity could aim to limit adiposity by inhibiting adipogenesis but, by failing to limit fat accumulation, indirectly contribute to hypertrophic obesity. Low Follistatin levels may result in enlargement of existing adipocytes at the expense of new fat cell formation. This might increase insulin resistance leading to a worse metabolic profile. Treatments interacting with these pathways could possibly be used in metabolic syndrome and T2D but so far only murine studies exist and they show contradicting results. Probably there is a need to block or interact with these pathways only in selected tissues.

Gene expression in subcutaneous WAT of obese women

- impact of hypocaloric diet and macronutrient content

The effects of energy restriction and different diets on weight loss is widely studied by researchers as described in section 1.4 and there is an ongoing debate in public. Also the effects of diet on gene expression have evoked much interest and there is a hunt for the optimal diet that both reduces weight and decreases risk for the metabolic complications of obesity.

Our second study was part of a large EU-financed multicenter study which compared the effects of a ten week intervention with low-fat, high-carbohydrate hypoenergetic diet with a moderate-fat, moderate-carbohydrate hypoenergetic diet. Both diets produced similar weight loss and beneficial changes in blood chemistry parameters. We

performed abdominal subcutaneous WAT global transcriptome profiling on a subgroup of patients before and after the dietary intervention. The expression of 96 genes was significantly influenced by hypocaloric diet. Expression of genes involved in the synthesis of polyunsaturated fatty acids (PUFA) was downregulated, and CIDEA was upregulated by hypocaloric diet. The pattern of gene expression response was almost identical between the two diets.

We can conclude that dietary macronutrient composition is of little or no importance for the gene expression in human subcutaneous WAT. It is the energy restriction *per se* that is important. This finding is confirmed by others as reported in section 4.2.

GENE EXPRESSION CHANGES AND GENE REGULATION IN WAT IN CANCER CACHEXIA

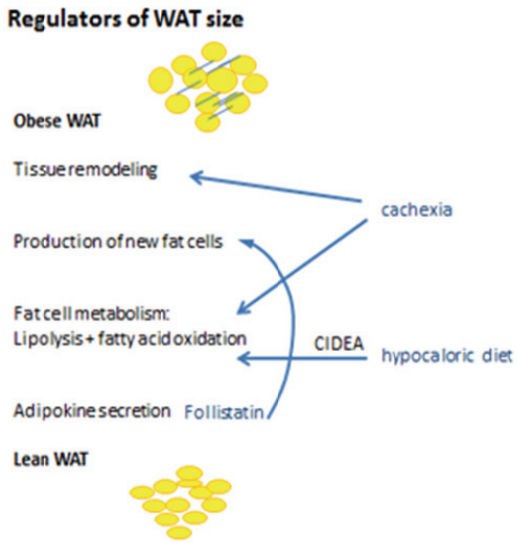
The fourth study compared cancer patients with or without cachexia. The former group displayed reduced body fat mass, signs of systemic inflammation, and increased levels of circulating free fatty acids. Global transcriptome profiling followed by pathway analysis revealed that genes downregulated by cachexia were overrepresented in pathways related to extracellular matrix, actin cytoskeleton and focal adhesion. By contrast, genes upregulated in cachexia were overrepresented in pathways related to energy turnover, e.g. fatty acid degradation, and oxidative phosphorylation.

We did not find differential expression of CIDEA, the gene that was most upregulated by hypocaloric diet in study 2. Our group has continued to elucidate the roles of CIDEA in metabolism and used RT-PCR in a larger group of cancer patients with and without cachexia. They could show that CIDEA was involved in WAT loss in cancer cachexia and this may, at least in part, be due to its ability to inactivate the pyruvate dehydrogenate complex, thereby switching substrate oxidation in human fat cells from glucose to fatty acids (Laurencikiene et al, 2008a). Probably the smaller group and the lower sensitivity of the microarray explain the different results.

In summary, examining WAT gene expression in human cancer cachexia reveals marked changes in pathways regulating energy turnover, extracellular matrix and cytoskeleton. The pathways are in many cases a mirror image to those observed with obesity but similar to those seen with intentional weight loss.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

We hypothesize that alterations in WAT function contribute to the negative metabolic consequences and disease outcome, respectively, of abdominal obesity and cancer cachexia. This work presents some new molecules and pathways of relevance for WAT function.



i. In a man with pronounced metabolic syndrome we cloned 44 genes differentially expressed between omental and subcutaneous WAT. They belonged to pathways involved in immune response, lipid, carbohydrate and aminoacid metabolism, protein synthesis, signal transduction and cell structure. 5 genes were confirmed differentially expressed in other obese patients.

ii. Two hypocaloric diets differing concerning fat and carbohydrate content were compared by subcutaneous WAT transcriptome profiles. The expression of 96 genes was significantly influenced by hypocaloric diet. Expression of genes in involved in the synthesis of polyunsaturated fatty acids was downregulated, and CIDEA was upregulated by hypocaloric diet. Macronutrient content gave no difference in the pattern of gene expression

iii. Follistatin is identified as a new adipokine. It decreases with obesity and is restored by weight reduction. It is produced by SVF. In vitro Follistatin is secreted and it stimulates adipogenesis, possibly by inhibiting myostatin.

iv. Subcutaneous WAT in cancer cachexia was investigated by global transcriptome profiles. Cachexia downregulated genes in pathways related to extracellular matrix, actin cytoskeleton and focal adhesion. By contrast, genes

upregulated in cachexia were overrepresented in pathways related to energy turnover, e.g. fatty acid degradation, and oxidative phosphorylation.

How to proceed? Large genome-wide studies of WAT in different metabolic situations and different depots are beginning to clarify human WAT metabolism. So far many transcriptomic studies are too small and the patient phenotype not carefully described to give good answers to the questions asked. We need more studies of large patient materials of carefully phenotyped individuals studied with a systems biology approach, i.e at all levels including genomic, transcriptomic, proteomic, epigenomic, lipidomic and metabolomic level. Qualified bioinformatic handling of data and computational skills is obligate. When combined with adequate clinical questions and follow-up I think we can anticipate that this will be of large clinical importance and utility.
This approach could be used to extend the knowledge both concerning diet effects in WAT, but also concerning metabolic syndrome and T2D effects on WAT with a future goal to provide individualized advice and treatments.

Smaller laboratories could take advantage of converging genomics which proposes to combine the results of whole genome-scans and whole genome expression profiling by searching for genes differentially expressed within known regions of linkage (Bouchard et al, 2007). This approach has the advantage to provide a rationale when selecting potential candidate genes for further detailed molecular investigation.

Our studies have shown changes in WAT pathways dealing with ECM, lipid metabolism, immune response and energy metabolism. A prominent role for subcutaneous WAT lipid metabolism in insulin sensitivity as recently shown stimulates further research (Elbein et al, 2011). The studies in this thesis is mostly descriptive. More functional knowledge concerning individual pathways or genes can be gained by using *in vitro* studies of human preadipocytes or hMSC using e.g. si-RNA. An important consideration when making WAT studies in the future is the mix of cell types in WAT. Analysis of the separate cellular components and their gene and protein expression is important, with the aim of identifying the basic features and the contribution of each component to different adipose tissue-associated pathologies (Peinado et al, 2012). The role of endothelial cells and angiogenesis in expanding adipose is established but so far not extensively studied (Lemoine et al, 2012).

Insufficient Follistatin in obesity could possibly contribute to a hypertrophic WAT with large insulin resistant fat cells. Further development of tissue specific Myostatin inhibitors or compounds that induce Follistatin effects might be useful for treatment of metabolic syndrome. Further characterization by e.g. human *in vitro* studies are needed, to be followed by human *in vivo* trials.

7 SAMMANFATTNING PÅ SVENSKA

Fetma, särskilt bukfetma, ökar risken för hjärt-kärlsjukdom och för tidig död. Cancer-associerad kakexi karakteriseras av en ofrivillig specifik förlust av vit fettväv och skelettmuskel och är kopplad till sämre överlevnad och dåligt svar på kemoterapi. Vi tror att förändringar i fettvävens funktion bidrar till de negativa metabola konsekvenserna vid fetma, och dåligt sjukdomsutfall vid cancerkakexi. I denna avhandling har vi använt global transkriptionsprofilering (mätning av alla aktiva gener) på bukfettvävsbiopsier från patienter för att identifiera nya arvsanlag och signalvägar av betydelse för fettvävsfunktionen. Fettväven utsöndrar tusentals substanser, sk adipokiner som kan ha effekter både lokalt och via cirkulationen generellt i vår kropp. Vi har utforskat genuttryck i relation till (i) fettvävsdepot, (ii) två olika lågkalori-dieter, (iii) cancerkakexi jämfört med viktstabila cancerpatienter. Härutöver har vi valt ett arvsanlag som är nedreglerat i fettväv från feta, Follistatin, och gjort en detaljerad beskrivning av dess roll i human fettväv.

I den första studien satte vi upp en metod kallad ”Representational difference analysis” och identiferade en handfull arvsanlag som var olika uttryckta i subkutant bukfett jämfört med intraabdominell fettväv, t ex Adipsin, som ingår i komplementsystemet, och Fosfolipid transfer protein (PLPT), som flyttar fosfolipider mellan lipoproteiner.

Vår nästa studie ingick i ett stort konsortium som jämförde effekten av en tio veckors lågkaloridiet med antingen lite fett-mycket kolhydrater eller måttligt med fett-måttligt med kolhydrater. Båda dieterna åstadkom liknande viktreduktion och positiv effekt på blodfetter mm. Vi gjorde global transkriptionsprofilering på en subgrupp av patienter före och efter interventionsstudien. Uttrycket av 96 arvsanlag påverkades signifikant av lågkaloridiet. Det var inga skillnader i förändring i genexpression mellan de båda dieterna. Uttrycket av gener som reglerar syntes av fleromättade fettsyror nedreglerades och en gen kallad CIDEA uppreglerades av lågkaloridiet.

I den tredje studien visar vi att Follistatin i fettväven nedregleras vid fetma och uppregleras av viktreduktion. Fettväven utsöndrar Follistatin in vitro. Follistatin in vitro stimulerar utvecklingen till mogna fettceller. Follistatin verkar genom att hämma gener i TGF beta familjen. Vi behandlade omogna fettceller parallellt med Follistatin och Myostatin och kunde då visa att Follistatin hämmade Myostatins effekt på fettceller. Otillräckligt med Follistatin vid fetma kan möjligen vara en orsak till hypertrof fettväv med stora insulinresistenta fettceller.

Den fjärde studien jämförde cancerpatienter med och utan kakexi. Den förra gruppen uppvisade mindre fettmassa, systemisk inflammation, och ökade nivåer av cirkulerande fria fettsyror. Global transkriptionsprofilering och bioinformatisk analys visade att gener som var nedreglerade vid kakexi var överrepresenterade i signalvägar relaterade till extracellulär matrix, aktin cytoskeleton, och fokal adhesion. Gener som var uppreglerade vid kakexi var överrepresenterade i signalvägar relaterade till energiomsättning, t ex fettsyreoxidation och oxidativ fosforylering.

Sammanfattningsvis associerar förändringar i fettvävens storlek med förändringar i fettvävsmorfologi, fettcellsantal och metabolism, samt adipokinsekretion. Mot denna bakgrund ger våra resultat stöd för att födans energiinnehåll, men inte dess sammansättning av näringsämnen, påverkar fettvävens genuttryck. Vi identifierade CIDEA, som sedermera har visat sig vara en viktig reglerare av ämnesomsättningen i

fettväven. Vi identifierade Follistatin som en ny adipokin som stimulerar fettcellsdifferentiering och som är nedreglerad vid fetma. Denna kunskap kan stimulera forskning kring behandlingar mot insulinokänslighet och diabetes. Våra resultat ger stöd för att cancerkakexi är associerad med remodulering i fettväven, vilket skulle kunna vara involverat i och bidra till fettvävsförlusten vid kakexi.

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