Microarray analysis of gene expression in human adipocytes and adipose tissue

Margareta Jernås

GÖTEBORGS
UNIVERSITET

Institute of Medicine
Department of Molecular and Clinical Medicine
Sahlgrenska Academy
Göteborg University

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Abstract

Obesity has reached epidemic proportions worldwide and is associated with several serious conditions such as insulin resistance, type 2 diabetes, hyperlipidemia and atherosclerosis. Adipose tissue exerts important endocrine and immune functions through the release of adipokines. Adipokines are involved in the regulation of adipose tissue metabolism and associated with alterations in insulin resistance. The aim of this thesis was to identify genes, expressed in adipose tissue and adipocytes, that may contribute to insulin resistance and metabolic diseases related to obesity.

Enlarged adipocytes are associated with insulin resistance and type 2 diabetes. A technique to separate human adipocytes from an adipose tissue biopsy into populations of small and large adipocytes was developed and the expression profiles of the populations were compared. This showed that serum amyloid A (SAA) and NAD(P)H:quinone oxidoreductase 1 (NQO1) were higher expressed in large versus small adipocytes. The expression of both SAA and NQO1 correlated to adipocyte size. SAA has been implicated in inflammation and insulin resistance and NQO1 is known to be involved in oxidative stress suggesting that these findings may provide novel insights into the connection between hypertrophic obesity and insulin resistance/type 2 diabetes. SAA, NQO1 and also the cell death-inducing DFFA-like effector A (CIDE-A) were predominantly expressed in human adipocytes as compared to a panel of 32 other human tissues and cell types. During diet-induced weight loss in obese subjects, adipose tissue expression of NQO1 was reduced and CIDE-A was elevated. NQO1 expression correlated to measures of adiposity, insulin and the markers of liver dysfunction, AST and ALT. These findings indicate a role for NQO1 in the metabolic complications of human obesity. CIDE-A expression was inversely associated with basal metabolic rate independently of body composition, age, and gender. These data suggest that human CIDE-A plays a role in adipose tissue energy balance.

Adipokines may play a key role in the rapid development of insulin resistance during critical illness. We identified gene expression changes in human adipose tissue in subjects with subarachnoidal hemorrhage during intensive care. Zinc-alpha2-glycoprotein (ZAG) was the only adipokine that was increased in adipose tissue during critical illness, and this increase was accompanied by elevated plasma ZAG levels. Plasma levels of SAA and CRP were increased and adiponectin levels decreased of during intensive care.

In summary, gene expression profiling of human adipocytes and adipose tissue during different conditions suggest that SAA, NQO1, CIDE-A and ZAG may be implicated in human obesity-related metabolic disease. During intensive care, increased plasma levels of ZAG, SAA, and CRP together with decreased levels of adiponectin may be involved in the decrease in insulin sensitivity.

List of publications

This thesis is based upon the following papers:

Paper I

Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression.

Jernås M, Palming J, Sjöholm K, Jennische E, Svensson PA, Gabrielsson BG, Levin M, Sjögren A, Rudemo M, Lystig TC, Carlsson B, Carlsson LM, Lönn M.

FASEB J. 2006 Jul;20(9):1540-2.

Paper II

The expression of NAD(P)H:quinone oxidoreductase 1 is high in human adipose tissue, reduced by weight loss, and correlates with adiposity, insulin sensitivity, and markers of liver dysfunction.

Palming J, Sjöholm K, Jernås M, Lystig TC, Gummesson A, Romeo S, Lönn L, Lönn M, Carlsson B, Carlsson LMS.

J Clin Endocrinol Metab. 2007 Jun;92(6):2346-52.

Paper III

Relations of Adipose Tissue CIDEA Gene Expression to Basal Metabolic Rate, Energy Restriction, and Obesity: Population-Based and Dietary Intervention Studies.

Gummesson A, Jernås M, Svensson PA, Larsson I, Glad CA, Schéle E, Gripeteg L, Sjöholm K, Lystig TC, Sjöström L, Carlsson B, Fagerberg B, Carlsson LM.

J Clin Endocrinol Metab. 2007 Dec;92(12):4759-65.

Paper IV

Changes in adipose tissue gene expression and plasma adipokine levels in patients with critical illness.

Jernås M, Olsson B, Sjöholm K, Sjögren A, Rudemo M, Nellgård B, Carlsson LMS, Sjöström CD.

Submitted.

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Abbreviations

AD	Average difference
ADRP	Adipose differentiation-related protein
ALT	Alanine aminotransferase
APACHE	Acute Physiology and Chronic Health Evaluation
ASP	Acetylation stimulating protein
AST	Aspartate aminotransferase
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
BF	Body fat
BMC	Bone mineral content
BMI	Body mass index
BMP	Bone morphogenic protein
BMR	Basal Metabolic Rate
BSA	Bovine serum albumine
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
CIDE	Cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector
cRNA	Complementary ribonucleic acid
CT	Computed tomography
CXCL	Chemokine (C-X-C motif) ligand 2
DEXA	Dual Energy X-ray Absorptiometry
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FATP	Fatty acid transport protein
FFA	Free fatty acids
FFM	Free fat mass
FTO	Fat mass and obesity associated
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter 4
HDL	High Density Lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMW	High molecular weight
HPA	Hypothalamic-pituitary-adrenal
Hs-CRP	High sensitivity C-reactive protein
HSL	Hormone sensitive lipase
IGF-	Insulin-like growth factor
IGT	Impaired glucose tolerance
IL-	Interleukin
INSIG2	Insulin induced gene 2

IRS	Insulin resistance syndrome
LDL	Low density lipoprotein
LTM	Lean tissue mass
MAS	Micro array suite
MC4R	Melanocortin receptor 4
MCP-1	Monocyte chemoattractant protein 1
MEM	Minimum Essential Medium
MIP-1	Macrophage inflammatory protein 1
mRNA	messenger Ribonucleic acid
MT	Metallothionein
NAFLD	Non-alcoholic steatohepatitis
NCEP	National Cholesterol Education program
NICU	Neurosurgical intensive care unit
NQO1	NAD(P)H dehydrogenase, quinone 1
OGTT	Oral glucose tolerance test
PAI-1	Plasminogen activator 1
PCK	Phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PLA2G2A	Phospholipase A2, group IIA (platelets, synovial fluid)
RBP4	Retinol-binding protein 4
RMA	Robust Multiarray Average
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAA	Serum amyloid A
SAH	Subarachnoidal hemorrhage
SD	Standard deviations
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
SOS	Swedish obese subjects
TIMP-1	Tissue inhibitor of metalloproteinases
TM4SF1	Transmembrane 4 L six family member 1
TNF-alpha	Tumour Necrosis Factor alpha
UCP-1	Uncoupling protein
WAME	Weighted Analysis of Microarray Experiments
WAT	White adipose tissue
WHO	World Health Organization
WHR	Waist-hip ratio
VLCD	Very low caloric diet
ZAG	Zink-alpha2-glycoprotein

Introduction

Obesity

The prevalence of obesity and type 2 diabetes is rapidly increasing in all parts of the world and this is thought to be mainly due to a lifestyle with excessive food consumption and insufficient physical activity. Worldwide, more than 1.3 billion people are either overweight or obese, whereas only about 800 million people are underweight. The shift from undernutrition to overnutrition has occurred in less than a generation and these statistics are diverging rapidly. In the U.S., 26% of the population aged 15 years or older are overweight or obese[1]. In many developing countries, the frequency of obesity now competes with the U.S and other welfare states.

Obesity develops when energy intake exceeds energy expenditure[2] and World Health Organization (WHO) defines obesity as a body mass index (BMI, weight in kg/(height in metre)^2) equal to, or greater than 30 kg/m^2.

Classification	BMI kg/m2
Underweight	< 18.5
Normal	18.5-25
Overweight	≥25
Obese	≥30
Obese class I	30-35
Obese class II	35-40
Obese class III	≥40

Table 1. WHO classification of adults according to BMI.

Genetic predisposition clearly contributes to the development of obesity. Studies of families, adoptees, twins and adopted twins[3,4] have demonstrated that heritable factors are responsible for 45–75% of the inter-individual variation in BMI[5-8]. Monogenic causes of human obesity are very rare[9]. The most common known cause of monogenic obesity is mutations in the melanocortin receptor 4 gene (MC4R). Different studies have found a prevalence of 2.5-6% of MC4R mutations in obese subjects[10-12]. Common obesity, also called polygenic obesity, depends upon complex interactions between genetic, social, behavioural, and environmental factors, all capable of influencing the obese phenotype[13]. The genetic influences seem to operate through susceptibility genes[14] and the first susceptibility genes for common obesity, INSIG2 (insulin induced gene)[15] and FTO (fat mass and obesity associated)[16,17] have recently been identified by genome wide approaches.

Obesity, in particular abdominal obesity, is associated with several complications such as glucose intolerance, insulin resistance, dyslipidemia, type 2 diabetes, hypertension, atherosclerosis, stroke, depression, sleep apnea, cancer

and fatty liver[18]. In addition to the increased morbidity, obesity is also associated with increased mortality[14,19].

Conventional treatment such as diet, exercise and behaviour modifications lead to weight loss, however the long-term effects are often unsatisfactory[20]. There are anti-obesity drugs such as Sibutramine (Meridia), which acts as an appetite suppressant, and Orlistat (Xenical) that prevents the absorption of fat in the intestines. However, these drugs cause side effects and the average weight loss is modest. Bariatric surgery remains the treatment of choice for the extremely obese patient since is it associated with long-term weight loss and decreased overall mortality[21]. However, it is not possible or desirable to treat all obese subjects with bariatric surgery. Increased understanding of the mechanisms involved in the regulation of body weight and metabolism are therefore urgently needed to identify targets for drug development.

Insulin resistance and the metabolic syndrome

Insulin was discovered 1921 and is the most important anabolic hormone with profound effects on glucose and lipid metabolism. Insulin is released from the beta-cell in the pancreas. It stimulates glucose up-take in adipose tissue and skeletal muscle, and suppresses the endogenous glucose production in the liver[22]. Insulin resistance is an inability of peripheral target tissues, e.g. adipose tissue, liver and muscle to respond properly to insulin stimulation[23]. This was described in the 1930s, with the conclusion that diabetes could be separated into two types; insulin sensitive (type 1) and insulin insensitive (type 2)[24].

Insulin resistance induces several metabolic alterations, such as hyperglycemia, dyslipidaemia and hypertension, and subjects with the metabolic syndrome are at an elevated risk for both the development of type 2 diabetes[25,26] and cardiovascular disease[27-29]. It is estimated that by the year 2020, there will be approximately 250 million people affected by type 2 diabetes worldwide[30].

In the 1920s, the physician Eskil Kylin from Göteborg in Sweden described a "hypertension-hyperglycaemia-hyperuricaemia" syndrome[31]. The metabolic syndrome was more than 60 years later described by Gerald M Reaven as "Syndrome X"[32]. The metabolic syndrome is also known as "the insulin resistance syndrome" (IRS)[33] or the deadly quartet[34], and has been described as insulin resistance in combination with a cluster of metabolic risk factors that create a predisposition to cardiovascular disease[32]. Even though the term "metabolic syndrome" is widely accepted, the definition of this syndrome has been extensively discussed. There is not yet a universal agreement of the name and there are several published definitions of the condition[35-37]. In this thesis, the term "metabolic syndrome" is used according to the definition of WHO[38]. The WHO definition of the metabolic syndrome includes glucose

intolerance, impaired glucose tolerance (IGT) or type 2 diabetes and/or insulin resistance, in combination with two or more criteria of obesity and/or elevated waist hip ratio (WHR), triglyceridaemia and/or low HDL (high-density lipoproteins) cholesterol, hypertension or microalbuminuria.

Component	
Diabetes, impaired glucose tolerance, impaired fasting glucose or insulin resistance and two of the following criteria;	
Hypertension	SBP ≥ 160 / DBP ≥ 90 mm Hg
Dyslipidemia	Plasma triglycerides ≥ 1.7 mmol/L and/or HDL cholesterol ≤ 0.9 mmol/L (men), ≤1.0 mmol/L (women)
Abdominal obesity	WHR≥0.9 (men) or ≥0.85 (women) or BMI ≥30
Microalbuminuria	Urinary albumin excretion rate ≥20 µg/min

Table 2. WHO definition of the metabolic syndrome[38]. SBP; systolic blood pressure, DBP; diastolic blood, pressure, HDL; high density lipoprotein, WHR, waist-hip-ratio.

Multiple mechanisms contribute to the development of insulin resistance, such as impaired insulin signalling, glucose storage and glucose oxidation[39,40]. When insulin resistance is accompanied by insufficient pancreatic beta-cell function a failure to control blood glucose levels results. Abnormalities in beta-cell function are therefore critical in defining the risk and development of type 2 diabetes[40]. Adipokines have been proposed to regulate insulin sensitivity[40]. TNF-alpha (tumor necrosis factor-alpha), IL-6 (interleukin-6) and resistin are insulin resistance-inducing factors, whereas adiponectin and leptin are considered to protect against insulin resistance[40,41].

"Diabetes of injury"

Hyperglycemia is common in critically ill patients and associated with insulin resistance, also in those with no history of diabetes[42]. It is well known that any type of acute severe illness, injury or surgery, results in insulin resistance, glucose intolerance and hyperglycemia, a condition termed *diabetes of injury*[22]. Hyperglycemia is a predictor for increased morbidity and mortality in patients admitted for acute coronary syndrome, cerbrovascular accidents, surgery and trauma[43]. Evidence for altered glucose homeostasis during stress was reported over a century ago.[44] However, this is most likely just one aspect of the total homeostatic derangements after surgery or injury, which also includes hypothalamic-pituitary axis dysfunction and a systemic inflammatory response syndrome[45,46]. Metabolic alterations during critical illness also include protein catabolism and abnormal serum lipid profiles[47-49].

Adipose tissue distribution

Adipose tissue is a heterogeneous tissue consisting of different depots with varying biological functions[50-52]. Both the distribution of adipose tissue and the amount of body fat differ between women and men. In the male, or android, type of obesity, excess fat is located in the central/ abdominal region (the apple shape). The female, or gynoid, type of obesity is characterized by adipose tissue accumulation on thighs, buttocks and legs (the pear shape). Visceral adipose tissue accumulation (upper-body obesity) shows a stronger connection with metabolic and cardiovascular disease, than gluteofemoral adipose tissue accumulation (lower-body obesity)[34,53,54]. Gluteofemoral adipose tissue accumulation does not appear to increase the risk of cardiovascular disease. The mechanism for this gender difference is not fully understood, but sex hormones may be involved[55-57].

Subcutaneous adipose tissue contains about 80% of all body fat, and the major subcutaneous depots are deep abdominal, superficial abdominal, and gluteofemoral adipose tissue. Abdominal adipose tissue is either subcutaneous or visceral, surrounding the abdominal organs. The visceral adipose tissue can anatomically be divided into omental, mesenteric, and retroperitoneal depots, and constitutes approximately 6 and 20% of total body fat in women and men, respectively[58,59]. The retroperitonal depot is located behind the abdominal cavity.

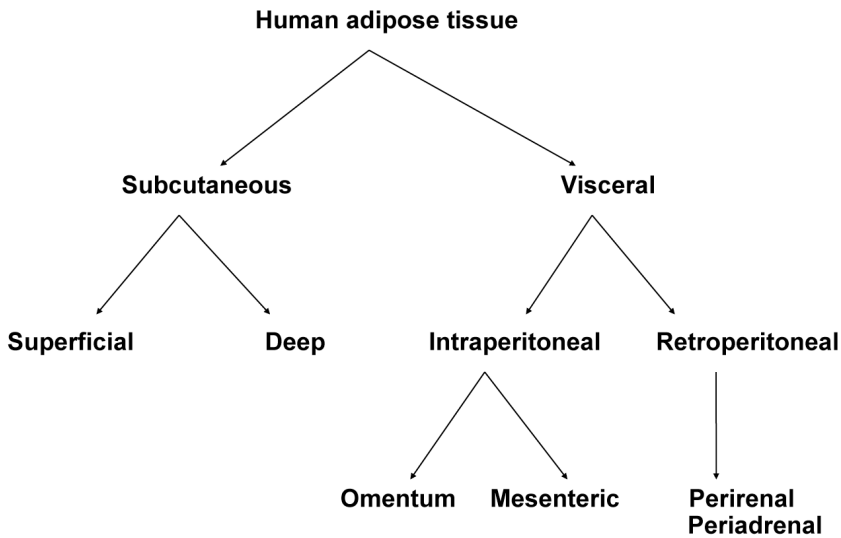

Fig 1. Subcutaneous and visceral adipose tissue depots in human adipose tissue.

In 1947 Vague described upper-body adiposity as the type of obesity that is commonly associated with the metabolic abnormalities found in type 2 diabetes and cardiovascular disease. In the early 80's, Björntorp and colleagues

used anthropometric variables such as waist and hip circumferences to develop a simple index of body fat distribution, the waist to hip ratio (WHR)[55,60,61]. The proportion of abdominal adipose tissue, as roughly estimated by the WHR, is an independent risk factor for the development of cardiovascular diseases and diabetes[60,61]. Anthropometric measurements that are currently used to describe regional obesity include circumferences of waist and hip, the ratio of waist and hip circumferences (WHR) and sagittal diameter[62]. Several studies indicate that visceral adipose tissue is strongly associated with insulin resistance and type 2 diabetes[62-65]. Adipocytes from the visceral adipose depot have been reported to have higher lipolytic activity and lower sensitivity to the antilipolitic effects of insulin than subcutaneous adipocytes[66-69]. Visceral adipose tissue, except the retroperitoneal depot, is drained by the portal vein, and this adipose tissue depot therefore has direct contact with the liver. This is thought contribute to the development of insulin resistance[70].

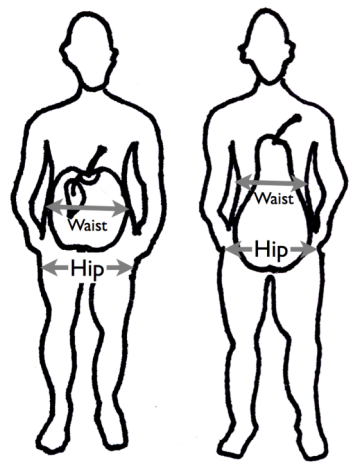

Figure 2. Android (apple) and gynoid (pear) adipose tissue deposition patterns in men and women. The waist-to-hip ratio is the circumference of the waist divided by the circumference of the hips.

Although the cause-and-effect association has not been definitively established, the available evidence indicates that visceral fat is an important link between obesity and the metabolic syndrome[71]. However, the discussion of body composition and insulin sensitivity is controversial[72-74]. It has been reported that abdominal subcutaneous fat, as determined by magnetic resonance imaging and CT, was at least as strongly correlated with insulin sensitivity as visceral fat and retained independent significance after adjusting for visceral fat[75]. Whether visceral adipose tissue has a uniquely strong association with insulin resistance or if subcutaneous abdominal fat shares this link, need further investigation.

Disease related risks are depending on both content and distribution of body components. The body consists of several components i.e. water, muscle, adipose tissue, bone, nerve tissue, and each component has a different density. Traditionally, a two-compartment model has described body composition, where body fat (BF) and fat-free mass (FFM), together constitute the total body weight. Adult men normally have 15 to 20% body fat, while women have 25 to 30% body fat[76] In obese subjects, the fat content is increased and levels in the order of 50% have been reported[77-79]. In non-obese subjects, about 70 to 80% of the body weight is FFM, and the FFM is negatively related to age[80-82] Low levels of FFM may be related to risk for chronic disease[83] and mortality[84,85].

Increases in caloric intake, reductions in energy expenditure, or both, result in excess energy being stored in the fat depots and excessive weight being gained. There are three principal components of human energy expenditure: basal metabolic rate (BMR), the diet-induced thermogenesis and energy expenditure of physical activity[86]. BMR is the minimum energy requirement to sustain vital functions during absolute rest and accounts for approximately 50 to 70 % of daily total energy expenditure[87]. Variation in BMR between individuals is usually small and is essentially a function of body composition i.e, fat-free mass[86,88]. Measurement or estimation of energy expenditure is important in relation to the determination of energy requirements in health and disease[87].

Adipose tissue and adipocytes

Adipose tissue is comprised of many cell types including adipocytes, adipose precursor cells, blood cells, endothelial cells, fibroblasts, and monocytes/macrophages[89]. The major constituent of adipose tissue is the adipocyte and this cell type is proposed to share a common precursor with osteoblasts, chondrocytes and myocytes[90]. The fat cell is adapted for its main function, to store and release energy. Surplus energy is stored as triglycerides in the lipid droplet. The adipocytes are surrounded by a basement membrane, composed of collagen, lamini and heparane sulfate proteoglycans[91].

Mature adipocytes are among the largest cells of the body and can increase in size by incorporating more triglycerides. Human adipocytes can change about 20-fold in diameter and several thousand-fold in volume and as adipocytes grow larger, they become dysfunctional[92-94]. While the smaller adipocytes are insulin sensitive, large adipocytes become insulin resistant and contribute to the metabolic problems associated with obesity[55,92]. Adipose tissue can expand in two ways: adipocytes can increase in volume (hypertrophy) and they can increase in number (hyperplasia). It has been demonstrated in *in vitro*

studies that there is a capacity for human adipocyte differentiation throughout life, indicating a continuous formation of fat cells[95].

Macrophages have been shown to infiltrate adipose tissue of obese subjects and[96,97] have been demonstrated to produce most of the TNF-alpha in the adipose tissue[96]. Recently, more extensive macrophage infiltration was observed in visceral fat compared with subcutaneous adipose tissue[97,98]. This difference may contribute to the differences in the proinflammatory state observed between these two adipose tissue depots[97,99]

Mesothelial cells line the chest cavity, the abdominal cavity, and the cavity around the heart. They also cover the outer surface of most internal organs. In adipose tissue mesothelial cells are present in the omental and mesenteric depots but are absent in subcutaneous adipose tissue. Mestothelial cells in visceral adipose tissue express inflammatory-related factors, in particular the proinflammatory cytokine IL-18, and mesothelial cells appear to be involved in obesity-associated low-grade inflammation[100].

Brown and white adipose tissue

There are two main types of adipose tissue in mammals, brown adipose tissue (BAT) and white adipose tissue (WAT). There are several differences between WAT and BAT, e.g. the structure and the function of the fat cells as well as regulation and anatomical distribution of the tissue[101].

Morphologically, white adipocytes consist of a large lipid droplet, with the nucleus displaced to the periphery and relatively few mitochondria, while brown adipocytes contain several small lipid droplets and high numbers of mitochondria. BAT is highly vascularized and innervated by the sympathetic nervous system (SNS)[102]. The colour difference between WAT and BAT is due to the differences in lipid content and mitochondrial abundance in white and brown adipocytes, as well as the increased vascularization of BAT.

The developmental relationship between white and brown adipocytes is not clear. Several studies have examined whether they are derived from common or separate precursor cells or if a white adipocyte, through transdifferentiation, can transform into a brown adipocyte and vice versa[103,104]. However, even though data suggests that most white adipocytes do not derive from BAT, further studies are requisited.

BAT has a thermogenic function through mitochondrial uncoupling protein 1 (UCP-1). UCP-1 is exclusively expressed in brown adipocytes and enables the generation of heat in response to cold.

In rodents, BAT is located interscapularly[105]. Human depots of BAT are found in the supraclavicular and the neck regions with some additional paravertebral, mediastinal, para-aortic, and suprarenal localizations[106].

Within WAT depots in adult humans, islets of brown adipocytes may be found, and UCP1 mRNA is detectable[102,107-109].

In rodents, BAT depots last into adulthood. In newborn higher mammals BAT is helping the newborn maintain body temperature. However, shortly after birth, BAT is replaced by WAT[110-115]. The prevalence of active BAT in adult man can only be indirectly estimated[106]. The activity of BAT in man is acutely cold induced and stimulated via the sympathetic nervous system[106,116,117].

In rodents, defective BAT function has been associated with obesity[118,119], whereas less is known about the clinical importance of BAT in humans.

Adipose tissue as an endocrine organ

White adipose tissue is an active endocrine organ that secretes a large number of bioactive molecules, so called adipokines or adipocytokines. WAT is communicating both with the brain and peripheral tissues through the adipokines[120]. Examples of adipokines are; acylation stimulating protein (ASP), adiponectin, adipsin, angiotensinogen, bone morphogenic protein (BMP), estrogen, insulin-like growth factor-1 (IGF-1), various IGF binding proteins, interleukins (ILs), leptin, monocyte chemoattractant protein 1 (MCP-1), plasminogen activator I (PAI-1), resistin, TNF-alpha, transforming growth factor-beta (TGF-beta) and various prostaglandins[121-128]. Adipokines have been implicated in a wide variety of processes, such as lipid metabolism, haemostasis, appetite and energy balance, immunity, insulin sensitivity, angiogenesis, inflammation and blood pressure regulation. For the more recently discovered adipokines, however, such as VASPIN[129] and chemerin[130], function and mode of action are yet to be elucidated.

Leptin and adiponectin, two of the most studied adipokines, are secreted by the adipocytes. However, over 90% of the bioactive molecules referred to as adipokines are in fact produced by the stromal-vascular cells. Secretion of adipokines varies between different regions of white adipose tissue[131,132]. For example, IL-6 release is increased from the visceral adipose tissue, while leptin is mainly secreted by the subcutaneous depot. Six adipokines were recently identified as secretory products of visceral adipose tissue, three chemokines (growth-related oncogen factor, RANTES, macrophage inflammatory protein-1), one interleukin (IL-7), one tissue inhibitor of metalloproteinases (TIMP-1), and one growth factor (thrombopoietin)[133]. Adipocyte size and number may also influence adipokine secretion. Several clinical studies have observed that circulating adiponectin levels are reduced in obesity, in which mean adipocyte size is increased, and elevated in lean individuals, with smaller adipocyte[134]. This could underlie the association of adipocyte size to obesity-related complications, such as insulin resistance and the increased risk for coronary heart disease[135].

Adiponectin

Adiponectin[136] is an adipocytokine that is produced very abundantly in adipocytes and exists as a full-lenght protein, as well as as a proteolytic cleavage fragment, consisting of the globular C-terminal domain. Full-length adiponectin exists as; a trimer, known as low molecular weight oligomers, a hexamer, which consist of two trimers linked by a disulphid bond, known as middle-molecular weight[137-139] adiponectin, and a high molecular weight multimer. The relative distribution of adiponectin multimers seems to differ between the adipose tissue and the circulation. The larger HMW forms is dominating in plasma[140], whereas the presence of the globular fragment in human plasma has been questioned[141,142].

Although secreted by adipocytes, plasma concentrations of adiponectin are reduced in obese compared with lean subjects[143] and the mRNA expression of adiponectin is reduced in adipose tissue from both obese mice and humans[144]. Adiponectin inhibits vascular smooth muscle proliferation and may modulate coronary artery disease risk by altering expression of various adhesion molecules[145,146].

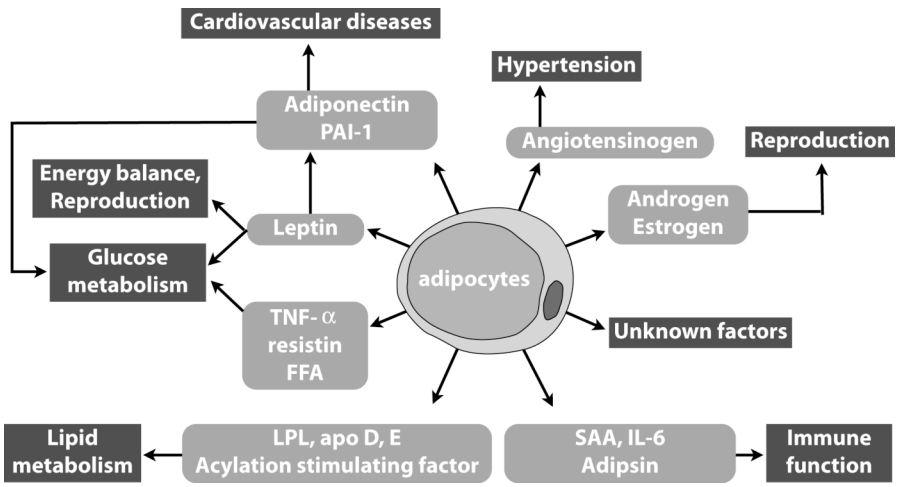

Figure 3. The cross talk between white adipose tissue and other organs and metabolic systems through various adipokines. Altered production of adipokines is considered to be important in the development of obesity-related diseases, particularly Type II diabetes and the metabolic syndrome.

SAA

It is well established that serum amyloid A (SAA) is an acute-phase protein, produced by the liver in response to inflammatory stimuli[147]. The expression may be induced up to 1,000-fold, in response to inflammation, infection and injury, primarily as a result of a 200-fold increase in the rate of SAA gene transcription[148]. However, recent studies have shown that adipose tissue is the major site of SAA production during non acute-phase[149,150] and that SAA is a proinflammatory and lipolytic adipokine in humans[151].

SAA1 and SAA2 are two major isoforms of SAA, collectively called acute-phase SAA (A-SAA). SAA is an apolipoprotein associated with HDL[152]. This SAA-HDL interaction may impair the function of HDL as an antiatherogenic molecule. Little is known about the isoform SAA3 and its function and regulation in adipocytes, and most studies refer to human SAA3 as pseudogene[153]. SAA4 is constitutively expressed and referred to as a reference gene[154]. SAA negatively regulates insulin sensitivity and increased level in the circulation is associated with obesity and insulin resistance[151].

The elevated expression of SAA by adipocytes in obesity suggests that it may be a link between obesity and inflammation, insulin resistance and cardiovascular disease.

RBP-4

Serum retinol-binding protein 4 (RBP-4) is the principal transport protein for retinol (vitamin A) in the circulation[155-157]. A large proportion of circulating RBP4 is produced by hepatocytes[158], however, RBP-4 was recently described as an adipokine[159] produced in rat [160] and human adipocytes[161].

Increased levels of serum RBP4 have been shown to be the signal for the development of systemic insulin resistance both in experimental animals and in humans[162-164]. Elevated serum RBP4 levels were shown to be an independent predictive biomarker at early stages of insulin resistance and identified individuals at risk of developing diabetes [162-165]. However, several reports have demonstrated that RBP4 gene expression in humans is not associated with insulin resistance[166-168]. Hence, the role of RBP4 in humans is under debate. In mouse models of insulin resistance, adipose tissue RBP4 expression and circulating levels are elevated, and increased circulating RBP4 elevate blood glucose by upregulating gluconeogenesis and inhibiting insulin signalling in skeletal muscle[159].

ZAG

Zink–alpha2-glycoprotein (ZAG) is a novel adipokine that may act locally to influence adipose tissue metabolism[169]. The function of ZAG probably lies in regulation of lipid storage homeostasis[170]. ZAG is a member of the major histocompatibility complex (MHC) class I family of proteins[171,172] and it is identical in amino acid sequence to a tumor-derived lipid-mobilizing factor

associated with cachexia in cancer patients[173,174]. ZAG was isolated from plasma and other body fluids[175,176] and is produced in the liver, prostate, kidney, salivary gland, mammary gland and sweat gland[176] and also by white and brown adipose tissue[177]. The induction of cachexia is followed by major increases in ZAG mRNA and protein levels in both brown and white adipose tissue[178]. Recently, ZAG -/- mice were found to have increased body weight and reduced lipolysis in adipose tissue[179], while the function of ZAG in man has to be further elucidated.

Adipose tissue and inflammation

It is well established that obesity is associated with low-grade inflammation[180]. Increased adiposity has been related to elevated systemic inflammation, in both clinical and experimental settings[138]. The acute-phase protein C-reactive protein (CRP) is a non-specific marker of inflammation secreted by the liver[181]. The circulating level of CRP is higher in obese compared with nonobese subjects[181] and also correlated to BMI and adiposity[182,183]. It is well known that elevated levels of CRP and SAA are associated with insulin resistance[184,185] and cardiovascular disease[186,187]. However, it was recently demonstrated in a mouse model, that human CRP was not proatherogenic, instead it appeared to be atheroprotective[188]. Furthermore, several other inflammation markers in plasma, such as haptoglobin, alpha-1 antitrypsin, and alpha-1 acid-glycoprotein or orosomucoid are also associated with insulin resistance[189] and cardiovascular disease[190,191].

Adipose tissue in obese subjects is often characterized by adipocyte hypertrophy and macrophage infiltration[192]. Proinflammatory TNF-alpha, IL-6 and hs-CRP levels are positively correlated with adipocyte size while anti-inflammatory adiponectin is negatively correlated with adipocyte size[193]. We (Paper I) and others[194] have shown that the hypertrophic adipocytes show an increased expression of many immune-related molecules. Several studies indicate an increased number of macrophages in white adipose tissue of obese humans and rodents compared with lean controls[96,195-198]. Furthermore, several studies have found that macrophages in adipose tissue, together with the adipocytes, are responsible for the majority of the proinflammatory adipokine production in WAT in the obese state[96,124,195,199]. Proinflammatory adipokines, such as IL-1, IL-6, MCP-1 and TNF-alpha are secreted and released from macrophages[195,200] and adipocytes[96,195,201,202]. Furthermore, macrophages present in WAT from obese subjects may be the source of adipokines that induce the hepatic expression of acute-phase proteins, such as CRP and SAA [200]. Inflammation plays an important role in the pathogenesis of atherosclerosis[203] and these observations suggest a potential mechanism linking obesity, inflammation and atherosclerosis.

Lipolysis

Adipocytes play a critical role in regulating energy balance by storing energy in the form of triglycerides during periods of energy excess. During conditions of fasting, hypocaloric diets and exercise, lipolysis becomes crucial, releasing energy-rich free fatty acids (FFAs) and glycerol[204]. The major inhibitor of lipolysis is insulin, and its antilipolytic effect is mediated through a decrease in the phosphorylation of hormone sensitive lipase (HSL)[205-207]. Until recently, HSL was considered to be the only important regulator of fat cell lipolysis. An other lipase, called adipose triglyceride lipase (ATGL) has now been identified. Few human ATGL studies have been undertaken. However, it appears that HSL is more important than ATGL in regulating hormone-stimulated lipolysis, whereas both lipases are important for the control of basal lipolysis in human fat cells[212]. Other important proteins involved in regulation of lipolysis are perilipin and adipophilin (ADRP)[213,214].

Catecholamines, i.e. noradrenaline and adrenaline, are powerful stimulators of lipolysis, and like insulin, they regulate HSL[208-210]. Other hormones involved in the regulation of lipolysis include glucocorticoids, sex hormones, thyroid hormones, growth hormones and possibly glucagon[211]. Thus, a complex network of hormones influence lipolysis, however, the exact mechanisms are not fully understood.

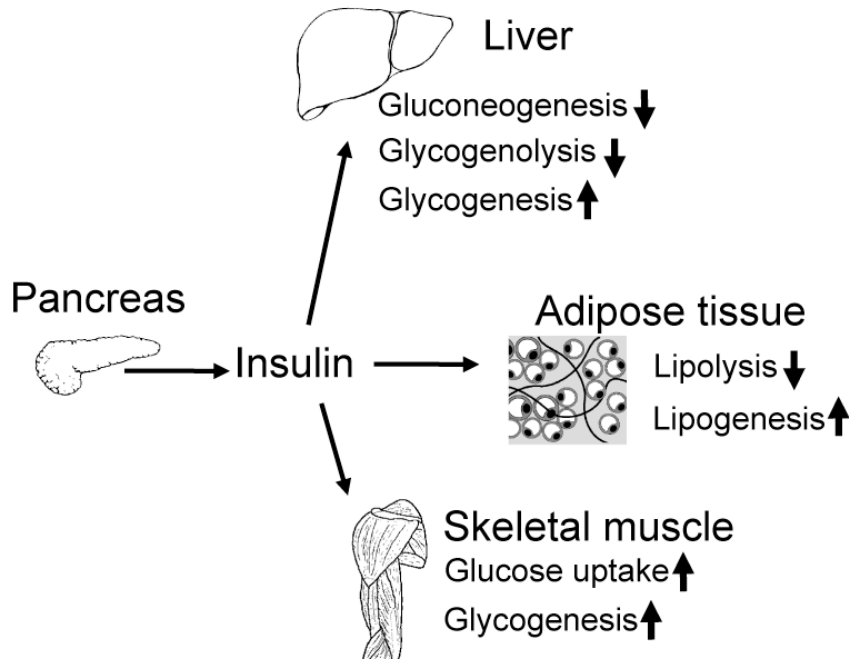

Fig 4. Effects of insulin on adipose tissue, liver, and skeletal muscle.

Elevated levels of FFAs in the circulation are often present in obesity[215,216]. Under normal conditions, adipocytes are able to store excess triglycerides while in subjects with obesity, adipocytes become enlarged and may reach their fat storage capacity. Lipid surplus may instead accumulate ectopically, i.e. in non-adipose tissues, such as liver, skeletal muscle, heart, and the beta-cells of the pancreas, increasing insulin resistance and impairing insulin secretion[217].

Thus, when insulin resistance develops, the increased lipolysis from excess triglycerides in adipose tissue may produce higher levels of FFA and ectopic lipid accumulation.

Oxidative stress

Oxidative stress is an imbalance between tissue oxidants, i.e. free radicals or reactive oxygen species (ROS), and antioxidants[218]. ROS are constantly produced by the endothelium under normal circumstances, and antioxidant systems defend the body against ROS[219]. Low levels of ROS and reactive nitrogen species (RNS) in the cell are necessary for normal redox status and intracellular signaling[220]. However, in some disease states, elevated levels of ROS and RNS directly oxidize and damage DNA, proteins, carbohydrates and lipids, and may cause cell death[219]. Further, ROS and RNS indirectly induce damage to tissues by activating several cellular stress-sensitive pathways[221].

Elevated levels of ROS may play a role in the development of a variety of diseases, such as diabetes, cancer and atherosclerosis. Direct measurement of oxidative stress in vivo is complex, since ROS are present in low concentrations, are highly reactive and have a short lifetime. Therefore, the identification of a useful marker to assess the effect of antioxidants on oxidative stress would be valuable[222].

Hyperglycemia has been recognized as one of the main factors increasing the level of ROS as a result of autooxidation of glucose and protein glycation[223]. Several studies show that hyperglycemia, which occurs during diabetes (both type 1 and type 2), metabolic syndrome and insulin resistance, causes oxidative stress[224,225]. Continuous hyperglycemia may induce oxidative stress and contribute to beta-cell destruction in type 2 diabetes[224]. Hyperglycemia stimulates ROS production also in adipocytes, which leads to elevated secretion of proinflammatory cytokines[226]. A hyperglycemic condition causes excess production of ROS in mitochondria, resulting in oxidative damage and activation of inflammatory signalling cascades inside endothelial cells[227].

Obesity is associated with chronic oxidative and inflammatory stress[228,229]. Plasma levels of several products of oxidative stress are elevated in obese subjects compared to controls[221].

Critical illness, injury and surgery can drastically increase the production of ROS and lead to oxidative stress[219]. Critical illnesses produce a complex and strong inflammatory, immune, stress-hormonal and metabolic response[230]. Hyperglycaemia and insulin resistance is prevalent in critical care[231,232], and hyperglycemia might be a part of the mechanisms leading to oxidative stress in critically ill patients.

Aims

The overall aim of this thesis was to increase our understanding of the mechanisms involved in the development of obesity-related metabolic disease.

The specific aims were;

To separate small and large adipocytes from the same biopsy and identify genes differentially expressed in the two populations. Paper I.

To study genes expressed specifically in human adipocytes and to examine their association with metabolic dysfunction, energy expenditure and basal metabolic rate. Paper II and III.

To identify changes in human adipose tissue gene expression during critical illness. Paper IV.

Methods

Subjects and samples

This thesis included participants from the Very Low Caloric Diet 1 (VLCD-1) study, VLCD-2 study, Swedish Obese subjects (SOS) Reference study, Intergene study, Dallas Heart study, Mölndal Metabolic study and the neurosurgical intensive care unit (NICU) study. The Regional Ethical Review Board in Göteborg approved all studies, and all participants, or next of kin, gave written informed consent. In Papers II – IV, abdominal subcutaneous adipose tissue biopsies were obtained with needle aspirations and were immediately frozen in liquid nitrogen and stored at -80°C.

Paper I

Abdominal subcutaneous adipose tissue biopsies were obtained from 12 subjects (BMI 25.4± 2.0 kg/m2, 9 women, 3 men) for adipocyte isolation, size separation and gene expression analysis. One patient had type 2 diabetes. The biopsies were collected in MEM and immediately processed to isolate adipocytes. Aliquots of adipocytes were used for analysis using immunohistochemistry.

Paper II and III

Abdominal subcutaneous adipose tissue biopsies were obtained at four timepoints during VLCD-2 study. Abdominal subcutaneous adipose tissue biopsies were obtained from 9 subjects for isolation of small and large adipocytes (BMI 25.3± 2.0 kg/m^2, 6 women, 3 men, included in Paper I). Adipose tissue biopsies from the abdominal subcutaneous and omental depots respectively, were obtained from 11 subjects (BMI 44.9± 9.4 kg/m^2, 4women, 7 men). Blood samples from 189 lean subjects (BMI 21.9± 1.5 kg/m^2, 135 women, 54 men), 131 healthy obese subjects (BMI 38.1± 4.8 kg/m^2, 84 women, 47 men) and 250 dysmetabolic obese subjects (BMI 38.3± 3.6 kg/m^2, 171 women, 79 men) were used for genotyping.

Paper III

Abdominal subcutaneous adipose tissue biopsies and blood samples were obtained and determination of body composition was performed in 50 men and 50 women, aged either 27-31 years (BMI 24.0±2.5 men, 22.6±3.0 women) or 57-61 years (BMI 25.9±2.9 men, 25.2±3.3 women). Abdominal subcutaneous adipose tissue biopsies were obtained with needle aspirations and immediately frozen in liquid nitrogen and stored at -80°C.

Paper IV

Abdominal subcutaneous adipose tissue biopsies and blood samples were obtained from 4 men and 4 women, (mean age 54.7 ± 9.3 year, mean BMI 27.1 ± 3.6 kg/m^2) from the NICU study.

VLCD-1 study

In the VLCD-1 study, subjects were originally included in the SOS study[233]. A subgroup was recruited from the SOS study to evaluate the weight loss maintenance after very low caloric diet (VLCD), plus dietary and behavioural support[234,235]. From this subgroup, 14 obese subjects with the metabolic syndrome according to slightly modified WHO criteria[38] together with age-, sex-, and BMI-matched controls were selected for adipose tissue gene expression analysis by microarray analysis and real-time PCR. Table 3 shows the characteristics of the subjects. All of the subjects with the metabolic syndrome but none of the controls had type 2 diabetes. In addition, the subjects with metabolic syndrome also had elevated blood pressure and/or dyslipidemia as defined by WHO. Evaluations of microalbumineria had not been performed why this parameter was excluded in the classification. Gene expression analysis with real-time PCR from subjects included in the VLCD-1 study, were used in Papers II and III.

VLCD-1 study	Subjects with the metabolic syndrome			Subjects without the metabolic syndrome		
Characteristic	Week 0	Week 8	Week 18	Week0	Week8	Week18
n (men/women)	14 (5/9)			14 (5/9)		
Age (years)	47.0±10.1			46.7±9.4		
BMI (kg/m2)	40.5±9.1	35.9±8.3	34.7±8.7	40.0±8.9	35.4±8.7	33.2±9.9
WHR	1.0±0.1			1.0±0.1		
Sagittal diameter (cm)	29.4±4.7			29.0±3.7		
SBP mmHg	159±29	143±21	150±23	130±15	120±16	131±16
DBP mmHg	92±20	85±14	85±12	86±11	76±9	77±9
Insulin (pmol/L)	26.6±15.6	14.9±6.6	16.1±9.7	14.4±9.4	11.3±8.3	11.8±10.6
b-Glucose (mmol/L)	9.6±2.3	7.4±2.9	7.6±2.5	4.3±0.6	4.3±0.7	4.4±0.9
Triglycerides (mmol/L)	2.5±1.3	1.9±1.1	2.1±1.1	1.8±1.0	1.3±0.5	1.3±0.4
Cholesterol (mmol/L)	5.7±0.7	5.3±1.1	5.5±0.9	6.0±2.1	4.8±1.0	5.3±1.2
HDL-C (mmol/L)	1.1±0.4	1.1±0.4	1.2±0.3	1.3±0.5	1.1±0.3	1.3±0.3

Table 3. Characteristics of subjects from the VLCD-1 study used for expression analysis with DNA microarray. Obese subjects with and without the metabolic syndrome, matched for BMI, sex and aged were treated with a very low calorie diet for 16 weeks, followed by 2 weeks of gradual reintroduction of ordinary diet. The metabolic syndrome was diagnosed according to slightly modified WHO criteria[38]

VLCD-2 study	Subjects with the metabolic syndrome				Subjects without the metabolic syndrome			
Characteristic	Week 0	Week 8	Week16	Week 18	Week0	Week8	Week16	Week18
n (men/women)	21 (18/3)				19 (16/3)			
Age (years)	49.7±9.4				44.5±9.9			
BMI (kg/m2)	36.7±5.0	30.8±4.3	27.8±4.2	27.9±3.8	37.0±3.9	31.2±3.3	28.2±3.4	28.5±3.2
WHR	1.0±0.1	1.0±0.1	0.9±0.1	0.9±0.1	1.0±0.1	1.0±0.1	0.9±0.1	0.9±0.1
Sagittal diameter (cm)	29.7±2.8	23.9±2.6	21.7±2.3	22.0±2.5	29.8±3.7	24.8±3.2	22.3±2.7	22.3±2.9
SBP mmHg	143±17	122±15	118±11	127±14	129±14	118±11	115±15	116±13
DBP mmHg	89±14	74±11	71±10	76±11	86±11	76±11	72±11	74±0
Insulin (pmol/L)	19.5±7.8	5.4±2.1	4.1±1.6	6.5±3.3	14.9±7.9	7.5±4.8	4.4±2.7	7.1±4.4
p-Glucose (mmol/L)	6.6±1.4	4.6±0.8	4.6±0.8	5.3±1.1	5.1±0.5	4.5±0.6	4.3±0.4	4.6±0.4
Triglycerides (mmol/L)	2.5±1.2	1.1±0.2	0.9±0.2	1.3±0.4	1.4±0.5	1.0±0.3	1.0±0.3	1.1±0.5
Cholesterol (mmol/L)	5.9±1.0	3.9±0.9	4.3±0.8	4.9±0.8	5.4±1.1	4.0±0.9	4.6±0.6	4.8±0.7
HDL-C (mmol/L)	1.3±0.4	1.2±0.3	1.3±0.3	1.4±0.2	1.3±0.4	1.2±0.3	1.4±0.3	1.4±0.3
LDL-C (mmol/L)	3.6±0.8	2.3±0.9	2.5±0.7	3.0±0.7	3.4±1.0	2.3±0.7	2.7±0.5	2.9±0.6

Table 4. Characteristics of subjects from the VLCD-2 study used for expression analysis with DNA microarray. Obese subjects with and without the metabolic syndrome, matched for BMI, sex and aged were treated with a very low calorie diet for 16 weeks, followed by 2 weeks of gradual reintroduction of ordinary diet. The metabolic syndrome was diagnosed according to slightly modified WHO criteria[38]

VLCD-2 study

Forty subjects (34 men and 6 women) were recruited among patients treated at the Department of Body Composition and Metabolism, Sahlgrenska University Hospital and by advertisement in the local press. The criteria for inclusion were BMI>30 and age 25-60. Subjects were divided into two groups, consisting of individuals with the metabolic syndrome according to slightly modified WHO criteria[38] and age-, sex-, and BMI-matched controls. Exclusion criteria were medication (except antihypertensive therapy in the group with metabolic syndrome), pregnancy, breast feeding, type-1 diabetes mellitus, serious psychiatric disorder, established coronary heart disease, malignant arrhythmias, participation in any other ongoing weight reduction study, eating disorder, history of bariatric surgery or cancer treatment, drug abuse, insufficient compliance, other significant somatic disease, smoking or unwillingness to participate. The subjects with the metabolic syndrome had diabetes, impaired glucose tolerance, or impaired fasting glucose according to WHO[38], and at least one of the following risk factors: (i) elevated arterial (systolic/diastolic) pressure, >140/90 mm Hg (either value) or use of blood pressure medication; (ii) raised triglycerides (>1.7 mmol/L) and/or low HDL cholesterol (<0.9 mmol/L). Subjects were treated with VLCD during 16 weeks followed by two weeks gradual reintroduction of the ordinary diet. During this study period adipose tissue biopsies and blood samples were collected at week 0, 8, 16 and 18. At these time-points, anthropometric measurements and computed tomography investigations were also performed. Data from subjects included in the VLCD-2 study, were used in Paper II and III. Table 4 shows the characteristics of subjects with metabolic syndrome and controls.

Intergene study

The Intergene study was designed to investigate which candidate genes that could explain the hereditary part of coronary artery disease (CAD) in the population from the west part of Sweden. This study was also designed to investigate the interaction between susceptibility genes for CAD and external factors such as life style and environment as well as the function of the candidate genes in the pathogenesis. The study is a combined control and cohort study of two thousand consecutive patients with coronary artery disease from hospitals situated in the western part of Sweden. The control group was selected from relatives of the patients and approximately 10 000 healthy individuals randomly selected from the population. Subjects were between 25 and 75 years old and sampling took place between 2001 and 2004[236]. More information regarding the Intergene-study is available on http://www.sahlgrenska.gu.se/intergene/eng/index.jsp. Table 5 shows the characteristics of cases and controls from the Intergene study included in Paper II.

Integene study		
Characteristics	Case	Healthy subjects
n (men/women)	411 (296/115	411 (296/115)
Age (years)	61.2±8.5	61.3±8.5
BMI (kg/m2)	27.5±3.9	26.6±3.5
WHR	0.95±0.07	0.91±0.08
SBP mmHg	133±21	142±22
DBP mmHg	82±11	85±10
Glucose (mmol/L)	5.6±1.1	5.3±0.9
Triglycerides (mmol/L)	1.6±1.1	1.5±0.8
Cholesterol (mmol/L)	4.6±1.0	5.7±1.0
LDL-C (mmol/L)	2.6±0.9	3.6±0.9
HDL-C (mmol/L)	1.3±0.4	1.5±0.4

Table 5. Characteristics of subjects of the Intergene study divided into CAD-cases and healthy subjects and used in Paper II to study the possible association between polymorphism in the NQO1-gene.

Mölndal Metabolic Study

The Mölndal Metabolic study aims to elucidate the relation between body composition, energy expenditure, dietary intake, and risk factors for diabetes and cardiovascular diseases in two age-groups of 50 men and 50 women. Participants were recruited from a cross-sectional and population-based sample of inhabitants in the city of Mölndal in western Sweden, aged either 27-31 years (BMI 24.0±2.5 men, 22.6±3.0 women) or 57-61 years (BMI 25.9±2.9 men, 25.2±3.3 women). Examinations included anthropometry, blood pressure recording, blood sampling, oral glucose tolerance test (OGTT), Dual Energy X-ray Absorptiometry (DEXA), abdominal subcutaneous adipose tissue biopsy, and measurements of BMR in a chamber of indirect calorimetry.

NICU study

The participants in this study [four men and four women; mean age 54.7 ± 9.3 yr; mean body mass index (BMI) 27.1 ± 3.6 kg/m^2 (range 21.4–32.1 kg/m^2)] were recruited among patients with subarachnoidal hemorrhage at NICU, Sahlgrenska University Hospital. The participants were severely ill at admission with an average APACHE II (Acute Physiology and Chronic Health Evaluation II) score of 14.9 (range 10–23). The aneurysm related to the hemorrhage was verified with intracerebral angiography. Inclusion criteria included: admittance to the NICU within 2 days of hemorrhage, an expected NICU stay of one week, and permanent residency in the Göteborg area, Sweden, to allow follow-up sampling. Plasma glucose levels were maintained within normal limits (4–6 mmol/l) by a continuous insulin infusion for 3–15 days (mean 7.8 days). Initially the nutritional intake was 10–15

kcal/kg/24 h; this level was gradually increased to a maximum of 25 kcal/kg/24 h.

The participants remained in the NICU for 5–18 days (mean 11.5 days). The day of the subarachnoidal hemorrhage was set as day 0. Blood samples and subcutaneous abdominal adipose tissue biopsies were taken at three time-points. The first sampling during intensive care (IC1) was performed 1-2 days after subarachnoidal hemorrhage and the second sampling (IC2) was performed at 7-9 days after subarachnoidal hemorrhage. In order to obtain normal values for reference, a third fasting sampling was performed at complete recovery after on average 8 months.

Dallas Heart Study

The Dallas Heart Study (DHS) includes subjects from a multiethnic, (1830 African-Americans, 601 Hispanics, and 1045 European Americans) population-based probability sample of Dallas County, Texas[237,238].

Anthropometry adipose tissue distribution and body composition

Anthropometry and adipose tissue distribution

The anthropometric parameters used in this thesis include body weight, height and circumferences. Measurements were used to determine the size and proportions of the body, as well as to assess total and regional body composition. Body mass index (BMI) was calculated. Body circumferences (cm) e.g. waist and hip circumferences, can be used as independent measurements, but also as the waist-hip ratio to describe the regional fat distribution[239]. The sagittal abdominal diameter (cm) is an estimate of the amount of abdominal fat and can be used to determine the visceral fat mass using computed tomography (CT)-calibrated equations[240,241]

Body composition

Body composition can be examined in both absolute amounts and relative terms regarding muscle, fat, bone and other tissue of the body[242]. CT uses attenuation of X-rays in order to make detailed pictures of structures inside of the body allowing measures of tissue areas and volumes[243]. The image from the scan at the lumbar 4 vertebrae level was analyzed to determine the subcutaneous, visceral and total adipose tissue areas and as well as the sagittal diameter, Paper II and Paper III.

Dual Energy X-Ray Absorptiometry (DEXA) is an established technique for both regional and whole body composition measurements and it measures bone mineral content (BMC), lean tissue mass (LTM) and total body fat (BF)[243]. The fat-free mass (FFM) was calculated as LTM+BMC, Paper III.

Basal metabolic rate, BMR

Measurements of BMR were assessed in a chamber of indirect calorimeter, in which consumption of oxygen and production of carbon dioxide were measured. From these entities, energy expenditure was calculated[244].The BMR-measurement was conducted according to standardized procedures during 60 minutes. When analyzed, the BMR-values were extrapolated to 24 hours Paper III.

Adipocyte isolation and separation by size Paper I

A new technique to separate populations of small and large adipocytes, from a single adipose tissue sample was developed. Adipose tissue biopsies were cut into small pieces and digested in minimum essential medium, pH 7.4, containing 1.05 mg/ml collagenase, 4% bovine serum albumin (BSA), 25 mM HEPES, and 0,15 µM adenosine. Digestion was performed at 37°C in a gently shaking water bath for 60 minutes. The stromal-vascular fraction was separated from the adipocytes by filtration through a nylon mesh, pore size 250 µm. After a three step washing procedure adipocytes were suspended in fresh medium. The original adipocyte suspension was gently agitated, and cells that resurfaced within 30 seconds were transferred to new tubes, this procedure was repeated once. These more buoyant cells were then filtered through a 70 µm nylon mesh and cells not passing through were resuspended in medium as the final preparation of large adipocytes. The denser adipocytes that did not resurface within 30 sec were filtered with a 50 µm nylon mesh. Cells that passed through the mesh were considered the final preparation of small adipocytes. The medium and the adipocyte suspensions were maintained at 37°C during the separation.

Expression analysis with DNA microarray Paper I-IV

Microarray technology represents a powerful research tool for determination of the expression level of thousands of genes (transcripts) simultaneously. Synthetic 25-mer oligonucleotides (probes) of a defined sequence (transcript) are chemically synthesized on a coated quartz surface. Each probe set is composed of 12-16 probe pairs, randomly distributed over the microarray. A probe pair consists of a perfect match probe, which is complimentary to the sequence of the gene of interest and a mismatch probe with a single base mismatch in the middle of the oligonucleotide sequence. The mismatched probe is used as a control for non-specific binding to the perfectly matched probe.

For gene expression analysis, total RNA was extracted from the samples, and used for cDNA synthesis. The cDNA was in vitro transcribed into biotin-labeled cRNA fragmented and hybridized to the microarrays. Hybridized

fragments were detected using a fluorescent dye linked to streptavidin by washing and staining protocols, followed by scanning with a confocal laser scanner. Scanned microarrays were visually inspected for hybridization artifacts of the microarray, and then analyzed using Micro Array Suite (MAS) (Affymetrix). The quality of the data was evaluated to check for variations introduced by differences in target preparation, labeling, hybridization, and handling of individual samples. The noise and background levels and the range of percentage of probe sets called present, were within acceptable levels. All the exogenously added prokaryotic hybridization controls such as BioB, BioC and BioD of the *E. coli* biotin synthesis pathway showed signal intensities above threshold limits. The ratio of intensities of 3' probes to 5' probes for housekeeping genes such as GAPDH and β-actin, representing the efficiency and accuracy of target, were also included.

In Paper I and Paper IV, gene expression levels were calculated by the Robust Multiarray Average (RMA) method[245], and differentially expressed genes were identified using Weighted Analysis of paired Microarray Experiments (WAME)[246].

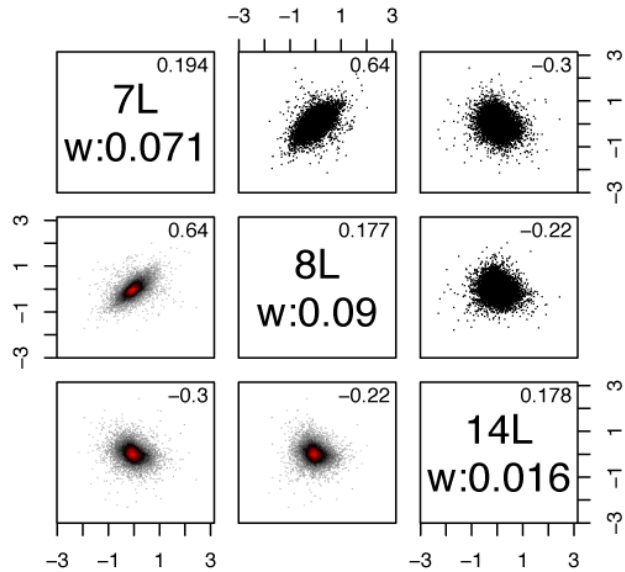

Figure 5. The figure, originally included in Weighted analysis of general microarray experiments, visualises baseline- subtracted gene expression values from a selection of the arrays in an analysed dataset. Such figures can be used to inspect the quality of the microarray experiment at hand. The upper triangle contains scatterplots. The lower triangle contains heatmaps, where the majority of the genes are in the centre portion of the plot, ideally revealing important trends inside the black clouds. Here, the diagonal centre clouds reveal correlations in the noise of different arrays, violating the assumptions behind e.g. the common F-test. Off-diagonal numbers show estimated correlations from WAME. Diagonal boxes contain sample names and weights used in the analysis, as well as estimated variances from WAME.[364]

WAME incorporates quality estimates of the different samples into the statistical analysis by using a model which allows for different precisions for different arrays and correlated noise between arrays (e.g. caused by shared sources of variation). WAME weights the samples in calculation of (geometric) signal means and P values for differential gene expression, giving lower weight to imprecise or positively correlated array Fig 5.

The microarray versions that were used in these studies were U133A, U95 and U133 Plus 2.0.

Adipocyte specific genes Paper I-III

Identification of genes predominantly expressed in omental adipocytes was performed using in house Hu95A microarray (Affymetrix) expression profiles from omental adipocytes[247], macrophages[248], T-cells[249] and nasal mucosa[250] as well as publicly available expression profiles from liver, whole blood, testis, prostate, ovary, uterus, lung, thymus, spleen, kidney, pancreas, thyroid, cerebellum, fetal brain, cortex, whole brain, trachea, amygdala, caudate nucleus, thalamus, corpus callosum, pituitary gland, spinal cord, dorsal root ganglia, salivary gland, adrenal gland, fetal liver, heart[251] and muscle[252].

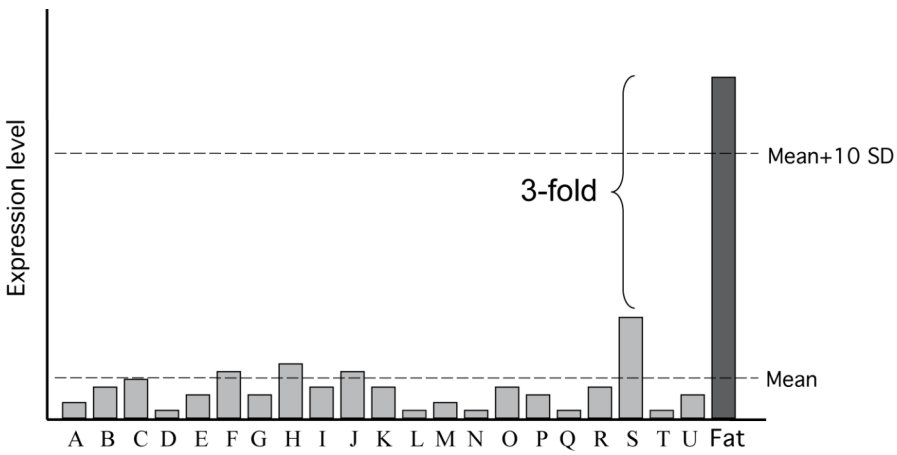

Figure 6. Selection criteria for genes predominantly expressed in human adipose tissue. A-U illustrates expression in other human tissues and cell types.

The average intensity of each array is multiplied by a scaling factor to bring it to an arbitrary Target Intensity value, set by the user. The public expression profiles were scaled to an average intensity of 200, and all microarrays were analyzed using the same intensity. The expression level of each gene was calculated using Affymetrix average difference (AD) algorithm This algorithm serves as a relative indicator of the level of expression of a transcript and it can

be used to determine the change in expression of a gene between experiments. An average AD was calculated for the replicates of each tissue or cell type and used for all analysis. Genes having an AD of less than 20 were given an AD of 20[253]. To identify genes predominantly expressed in omental adipocytes, two criteria were used;

1) The genes should have an expression level of 10 standard deviations (SD) higher than the mean expression of all the other tissues and 2) the genes should have at least 3-fold higher expression level than the tissue with the second highest expression (Fig 6). These two criteria allowed us to identify genes that had an expression level that was clearly separated from both the average expression level of all tissues, but also from any other highly expressing tissue or cell type.

Real-time PCR (Paper I-IV)

Real-time PCR is a sensitive method for quantification of specific mRNAs. Before the PCR reaction, total RNA is isolated and reverse transcribed into cDNA. The assay is based on the 5´nuclease activity of *Taq* DNA polymerase that cleave an oligonucleotide probe during PCR, thereby generating a detectable signal over time. For the gene of interest, a probe and a primer pair is designed that span an exon-exon boundary to allow amplification of cDNA but not genomic DNA. The probe is labelled with a fluorogenic reporter in the 5´end, and a nonfluorescent quencher in the 3´end, and is designed to anneal between the primers used in the assay. Specificity is here conferred at three levels: via two PCR primers and the probe. As long as the probe is intact, the quencher absorbs the fluorescens emitted from the reporter dye. When both primer and probe have annealed to the cDNA, the Taq polymerase cleaves the reporter dye from the non-extendable probe.

The fluorescence increases due to the extended distance between the reporter dye and the quencher, and it is thus possible to measure the amplified products of the target transcript in the starting mRNA during the exponential phase. The Threshold Cycle (Ct) is defined as the cycle number at which the reporter fluorescence reaches a certain level. There is a linear relationship between Ct and the log of the initial target copy number.

To ensure that equal amounts of cDNA in different samples, or different efficiencies of the reverse transcription reaction are compared, the determination of the mRNA levels of one or more reference genes or ”house-keeping” genes, i.e. genes that are not affected by the conditions studied, is essential. The relative expression value of the target gene is calculated using a standard curve where serial dilutions of the standard cDNA are plotted against the corresponding fluorescence signals at a chosen time-point. The calculated amounts from the gene of interest are then divided by amounts of reference gene for each sample.

Immunohistochemistry Paper I

Adipocytes were fixed in buffered paraformaldehyde, dehydrated and embedded in paraffin using standard procedures, before sections were prepared. Adipocyte sections were incubated with monoclonal antibodies against SAA and transmembrane 4 L six family member 1 (TM4SF1). All sections were counter stained with hematoxylin.

Results and Discussion

The data presented in this thesis are based on samples and observations in human subjects with obesity, obesity-related metabolic complications, or insulin resistance induced by critical illness. In this section I will summarize and comment on the results from the individual papers. All details about the individual results, P-values, graphs and tables can be found in Papers I-IV.

We have focused on genes predominantly expressed in large adipocytes (Paper I) and in subcutaneous and omental adipose tissue (Papers II-III). In Papers II-III, we also studied genes regulated in adipose tissue during diet-induced weight loss in obese subjects. Paper IV describes gene expression in adipose tissue during critical illness.

Adipocyte size Paper I

Adipose tissue produces several secreted bioactive molecules (e.g. lipid metabolites, cytokines and other proteins) and some of them are known as adipokines. The number of identified adipokines is constantly increasing and their potential role in obesity is under investigation[92]. The release of many adipokines from adipose tissue is related to the adipocyte size[194] and the amount of adipose tissue influences the circulating levels[254]. For example, the release of leptin and IL-6 from subcutaneous adipocytes is correlated to fat cell size[255-257].

The production of adipokines from enlarged adipocytes is thought to directly promote insulin resistance and to trigger inflammation[258]. This may, in turn, cause type 2 diabetes, cardiovascular disease, cancer and other obesity-associated problems[259]. Increased knowledge of adipocyte-derived factors might be of importance in the development of new therapies for obesity-associated diseases[138].

In several studies focusing on adipocyte size, adipocytes or adipose tissue biopsies with different mean adipocyte diameter were obtained from different tissue locations or from different donors[260-264]

Thus, differences in environmental conditions or genetic factors that affect adipocyte gene expression and metabolism could not be excluded. We therefore developed a technique to separate human adipocytes from a single adipose tissue biopsy, into significantly different populations of small and large cells. DNA microarray analysis of gene expression profiles from the populations revealed markedly higher expression of several genes in the large (hypertrophic) adipocytes. Fourteen genes had more than fourfold higher expression in large compared to small adipocytes. Classification by function, based on Gene Ontology definitions, revealed that five of the genes that were up-regulated in large adipocytes were immune-related; E-selectin, C1q receptor 1, IL-8, CXCL2 and SAA.

The acute-phase protein SAA, implicated in coronary artery disease and impairment of reverse cholesterol transport, has previously been reported to be produced mainly in the liver[154]. However, under non-acute phase conditions, the SAA gene is predominantly expressed in human adipocytes and the liver is the tissue with the second highest SAA expression[150].

Among the 14 genes with fourfold higher gene expression in large adipocytes, we also found TM4SF1, a gene with unknown function. Publicly available expression profiles of adipocytes from Pima Indians[265] revealed that TM4SF1 expression was higher in adipocytes from obese subjects as compared to adipocytes from non-obese subjects. Further, by comparing different adipose tissue depots, we found that TM4SF1 gene expression was higher in omental compared to subcutaneous adipose tissue. In relation to other human cells and tissue types, the expression of SAA and TM4SF1 were markedly higher in large adipocytes. Both SAA and TM4SF1 expression levels also correlated significantly to adipocyte size and using immunohistochemistry we found that both SAA and TM4SF1 expression was higher in large cells also at the protein level.

The adipocyte plays an important role in the metabolic homeostasis in obesity and related diseases, and several studies have investigated the connection between adipocyte size and adverse metabolic profiles[266]. The mechanisms behind the association between enlarged adipocyte size and insulin resistance is not entirely clear. However, abnormalities in the large fat cell function and production have been identified that may explain the alteration from a healthy adipocyte to a hypertrophic insulin resistant adipocyte and whole body insulin resistance.

When the amount of adipose tissue is increased, as seen in obesity, and the body is overloaded with lipids and glucose, the adipocyte continues to accumulate triglycerides and thereby increases in size. As discussed above, the production of many adipokines is altered when the amount of adipose tissue is increased, as seen in obesity and it is likely that some of them might be involved in the development of insulin resistance associated with obesity[267].

The association between obesity and inflammation is supported from studies, showing macrophage infiltration of adipose tissue[192]. The proportion of cells expressing the macrophage marker CD68, increases with increasing adipocyte size in subcutaneous adipose tissue[96]. SAA has been demonstrated to have cytokine-like properties by stimulating the production of proinflammatory cytokines from monocytic cell line, THP cells[268]. We found in our study, that IL-8 was highly expressed in large adipocytes, and this proinflammatory cytokine may act as a potential monocyte-recruiting factor in adipose tissue[269]. Increased number of hypertrophic adipocytes and macrophages in adipose tissue is likely to further increase the levels of inflammatory cytokines and thereby increase insulin resistance.

The lumen of the endoplasmic reticulum (ER) is a compartment specialized for protein folding; proteins enter the ER lumen unfolded, and only exit when correctly folded. Obesity causes ER stress[270], and in the enlarged adipocyte, which is filled with excess lipids, ER may be unable to properly fold proteins. This type of cellular stress might influence enlarged adipocytes to decrease production of molecules such as adiponectin, and to induce production of molecules such as TNF-alpha. This proinflammatory cytokine, TNF-alpha, is involved in systemic inflammation, and it might trigger insulin resistance in other cells[270]. In a recent study, enlarged adipocytes were associated with insulin resistance in non-diabetic subjects independently of BMI[271]. This was not seen in subjects with type 2 diabetes, indicating that after development of type 2 diabetes, factors other than adipocyte size are more important for promotion of insulin resistance, e.g. high FFA and glucose levels[271].

SAA may also play a role in lipid metabolism. When released into the circulation SAA is incorporated in HDL cholesterol[272,273] and may modulate its role in cholesterol transport[274]. The presence of SAA on HDL has been reported to both promote[275] and to reduce[276] cholesterol efflux, suggesting that SAA may alter cholesterol removal from cells. Enlarged adipocytes from obese rodents and humans, demonstrate altered cholesterol distribution that modifies adipocyte metabolism[277]. Within a particular fat pad in rodents, larger adipocytes have reduced membrane cholesterol concentrations compared with smaller adipocytes, indicating that altered cholesterol distribution is related to adipocyte hypertrophy *per se,* and this might serve as a link between fat cell size and adipocyte insulin resistance[277].

Substrate supply to and from the adipocyte must be transported via the systemic vasculature. Adipose tissue microvasculature is well developed and adipocytes are always associated with blood vessels. The degree of adipose tissue vascularity and vascular wall permeability is greater than that of skeletal muscle[278]. When the amount of adipose tissue is increased, as seen in obesity, adipose tissue grows by recruiting new adipocytes from the adipose precursor cell pool and by enlarging adipocytes. Recruitment of new adipocytes is always accompanied by development of blood vessels, whereas enlargement of adipocytes produces no changes in the vasculature and the capillary endothelial surface can thus become limiting for hypertrophy of the cell[278]. Several local effectors tightly regulate the growth and cellular composition of adipose tissue and subjects with a hypertrophic type of adipose tissue expansion will usually experience a greater risk of metabolic and cardiovascular complications than subjects with a hyperplastic tissue growth[279].

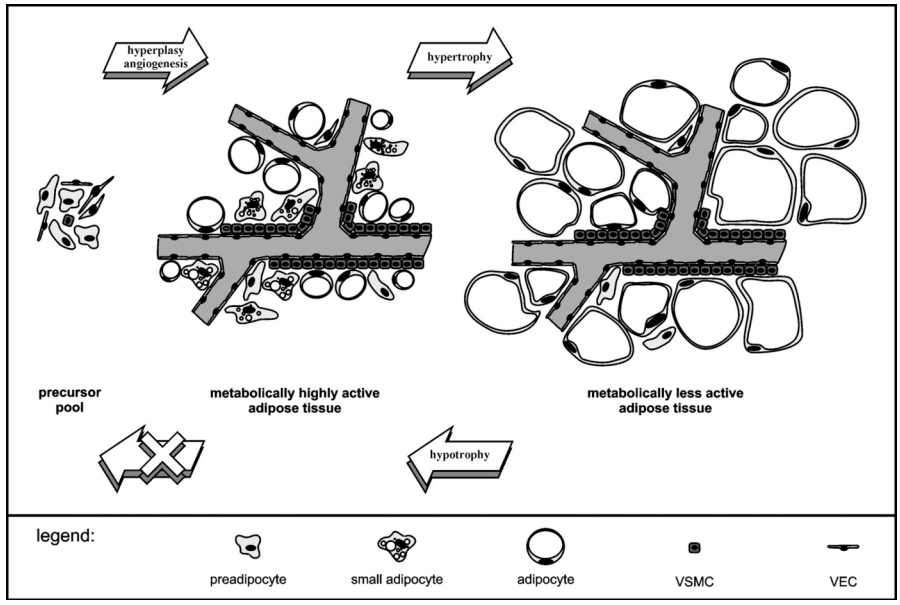

Figure 7. Changes in white adipose tissue mass and cellularity. Recruitment of new adipocytes is always accompanied by development of blood vessels, whereas enlargement of adipocytes produces no changes in the vasculature and the capillary endothelial surface can thus become limiting for hypertrophy of the cell. Used with permission.[279]
VSMC, vascular smooth muscle cells; VEC, vascular endothelial cells.

NQO1 in adipocytes and adipose tissue Paper II

Identification of genes predominantly expressed in adipocytes and adipose tissue can give us new insights into adipocyte and adipose tissue function, and may indicate pathways involved in the development of obesity and obesity-associated metabolic disease. It is well known that visceral adipose tissue is tightly linked to components of the metabolic syndrome[280]. To increase our understanding of the link between visceral adiposity and metabolic disease we therefore searched for genes predominantly expressed in human omental adipocytes using microarray analysis.

One of the identified genes, NAD(P)H:quinone oxidoreductase 1 (NQO1), showed the most abundant expression in omental adipocytes, followed by subcutaneous adipocytes, trachea and stomach. In our study with adipocytes separated by size (Paper I), NQO1 was markedly higher expressed in large versus small adipocytes, and this was verified with real-time PCR. We also observed that NQO1 expression levels correlated positively to adipocyte size.

NQO1 is thought to be part of an important cellular defence mechanism to obstruct electrophiles and oxidant damage[281], possibly by maintaining co-enzyme Q in a reduced, anti-oxidant state[282].

It is also a cancer preventive enzyme, which has been recognized for more than 40 years[283]. NQO1 is mainly a cytosolic enzyme although it has also been detected in small amounts in mitochondria, endoplasmic reticulum and the nucleus. In humans, high levels of NQO1 have been found in most tissues except for the liver[284].

Mice lacking a functional NQO1 gene (NQO1-/-) have lower blood glucose levels and higher levels of triglycerides as compared with wild-type mice. An insulin tolerance test demonstrated that the NQO1-/- mice are insulin resistant and have a significantly reduced amount of abdominal adipose tissue as compared with the wild-type mice[285]. These observations suggest that NQO1 could have important metabolic functions.

To study the regulation of NQO1 in human adipose tissue we analyzed expression of NQO1 during diet-induced weight loss in obese subjects in the VLCD-2 study. NQO1 expression was high at baseline and decreased markedly during weight reduction. Abdominal adipose tissue area as well as insulin and glucose levels correlated positively to NQO1 expression. However, there was no correlation between the NQO1 expression and serum triglycerides during diet-induced weight loss.

The NQO1-/- mice have elevated triglyceride content in the liver[285]. In humans, insulin resistance has been associated with fat liver and non-alcoholic steatohepatitis (NAFLD), which is characterized by excess triglyceride deposition[286]. A strong relationship between elevated serum levels of the liver enzyme, alanine aminotransferase (ALT), fatty infiltration of the liver, and insulin resistance was identified in the setting of obesity[286]. We found that NQO1 expression in adipose tissue correlated to ALT and aspartate aminotransferase (AST) during the VLCD-2 study. However, no correlation was seen between NQO1 expression and liver attenuation analyzed by computerized tomography (CT). Insulin resistance and obesity represent the most important risk factors for development of NAFLD[287]. Oxidative stress, invariably associated with NAFLD, may be a primary and necessary event for this disease progression[288]. The clinical presentation of NAFLD is variable and there are different methods used for diagnosis. Most patients with NAFLD have no symptoms, even though the most common clinical finding is hepatomegaly, with a prevalence of 75%. Using biochemical markers, such as aminotransferases, an elevation of ALT is usually greater than AST and both tend to decrease with weight loss. However, serum aminotransferase levels are not always helpful in establishing the diagnosis of NAFLD. Although most patients with NAFLD may have elevated liver enzyme levels, these levels can be normal, even in the presence of severe disease[237,289]. Imaging studies, such as computed tomography (CT) scan of the abdomen may have a low level of specificity and only reveal features that are suggestive of fatty infiltration of the liver. The clinical suspicion of NAFLD and its severity

can only be confirmed with a liver biopsy. Liver biopsy is thus the only diagnostic test that can reliably identify and quantify hepatic steatosis, inflammation, necrosis, and fibrosis[290].

ROS generation decreases dramatically in obese subjects during dietary restriction[221]. This is in line with our own results, the antioxidative enzyme NQO1 is highly expressed in obese subjects when the protein deactivates ROS. During a diet-induced weight loss, the expression of NQO1 is dramatically reduced. Oxidative stress in obesity may be improved by diet-induced weight loss, exercise and antioxidant rich diet[218].

Genetic polymorphisms have been suggested as an important contributor to overall human variability. Single nucleotide polymorphism, SNP, the most common type of genetic variation represents a difference in a single nucleotide. When SNPs occur within a gene or in a regulatory region near a gene, they may play a direct role in disease by affecting the gene's function. A C609T polymorphism in the coding region of NQO1 leads to an amino acid change from serine to proline at position 187, which results in loss of NQO1 activity in humans[291]. Individuals heterozygous for the T allele show significantly lower NQO1 protein levels, and individuals homozygous for the T allele have no detectable levels of NQO1 protein. This polymorphism also varies in frequency among ethnic groups (range 4–22%)[282].

We investigated the C609T polymorphism in three different studies; the SOS study, INTERGENE study, and Dallas Heart Study. We found an association between the CT/TT genotype and lower WHR in obese healthy subjects, a sub-population in the SOS-study. There was also an association between the CT/TT genotype and higher triglyceride levels in a sub-population of obese subjects with metabolic disease in the SOS study. However, we were not able to verify these associations in the Intergene study or Dallas Heart study.

CIDE-A in human adipose tissue Paper III

We identified cell death-inducing DFFA-like effector A (CIDE-A) as a gene predominantly expressed in human adipocytes and adipose tissue using microarray analysis. The expression of CIDE-A was markedly higher in isolated adipocytes compared to all other tissues and cell types investigated, and this was verified with real-time PCR. The implication of this finding is that CIDE-A is likely to have its main function in adipose tissue.

CIDE-A, a glycoprotein localized to the mitochondria, was initially identified based on sequence homology to the apoptotic 45 kDa subunit of the N-terminal region of DNA fragmentation factor (DFF)[292]. There are five CIDE domain containing proapoptotic proteins in humans and mice, DFF40, DFF45, FSP27, CIDE-A and CIDE-B[293].

In mice, CIDE-A is highly expressed in brown, but undetectable in white adipose tissue[294]. CIDE-A -/- mice are lean, and have much less white adipose tissue and lower leptin levels compared to wild type mice. CIDE-A -/- mice are resistant to high-fat-diet-induced obesity and diabetes, indicating that CIDE-A is important for regulation of adiposity[294]. CIDE-A deficient mice also have higher metabolic rate, higher lipolysis in BAT, and higher core body temperature when subjected to cold treatment compared to wild type mice, suggesting that CIDE-A plays a role in the regulation of energy expenditure[294].

In humans, CIDE-A is highly expressed in white adipose tissue[295,296], while no information is available regarding the expression of CIDE-A in human BAT. The finding that the CIDE-A gene V115 polymorphism is associated with BMI in a Swedish population[296] suggests that CIDE-A may play a role also in human obesity.

Due to the significantly higher metabolic rate in CIDE-A -/- mice compared to wild type mice and to the association between CIDE-A polymorphism and human obesity, we investigated the possible link between metabolic rate and CIDE-A expression in human white adipose tissue in the Mölndal Metabolic study. The major factor determining basal metabolic rate is FFM, which constitutes 70-80% of the body weight. The FFM is negatively related to age[80,297,298] and, in longitudinal studies, the decrease in FFM with increasing age is paralleled with an increase in body fat[81,299]. As expected, FFM was strongly associated with BMR in the Mölndal Metabolic study, whereas the association between BF and BMR was weaker. We observed a significant inverse association between CIDE-A expression in adipose tissue and BMR in the study subjects. Due to the strong influence of FFM on the BMR measurement and of BF on CIDE-A expression, the analysis was performed with adjustment for these body composition variables. CIDE-A expression and BMR remained significantly associated after adjustment for BF, FFM, age and gender, which is consistent with the concept that CIDE-A might function as a negative regulator in adipose tissue energy expenditure.

In the CIDE-A -/- mice the link between CIDE-A and BMR was suggested to depend on the inhibitory effect of CIDE-A on the heat generating uncoupling protein 1 (UCP1). We show that human UCP1 expression is up regulated during diet induced weight-loss. However, it has to be noted that the expression levels of UCP1 in human white adipose tissue are very low. The co-localisation of CIDE-A and UCP1 expression in mouse brown adipose tissue supports the suggestion that the increased metabolic rate observed in the CIDE-A -/- mice depend on the inhibitory effect of CIDE-A on UCP1. However, in humans the expression pattern of CIDE-A and UCP1 is not identical to that of mice. Also, the role for human UCP1 in human basal metabolic rate is also less established than that in mice[300,301]. This makes it

less likely that a direct inhibition of UCP1-mediated uncoupling by CIDE-A is the cause of the observed inverse association between CIDE-A expression in adipose tissue and the basal metabolic rate. The function of CIDE-A in human white adipocytes remains unclear and merits further investigation.

It has previously been reported that CIDE-A mRNA expression in adipose tissue is increased by weight-reduction after an energy-restricted diet[296] and also after bariatric surgery[295]. Low adipose tissue CIDE-A expression was associated with several features of the metabolic syndrome[295]. However, in another study[302], CIDE-A was not regulated during a moderately calorie-restricted diet. In the VLCD-2 study, CIDE-A was one of the genes that showed the most dramatic increase in expression in adipose tissue during the diet. In this study, regular food was gradually re-introduced between week 16 and 18, and during this period the average body weight was unchanged while CIDE-A expression significantly decreased. Previous reports have not discriminated between the effects of caloric restriction and weight loss. Our results reveal a rapid decrease of CIDE-A expression when regular food is re-introduced and caloric intake increases while body weight is stable. This indicates that during a diet-induced weight-loss, it is the caloric restriction per se, rather than the weight loss that influences the CIDE-A expression.

A previous study has shown that CIDE-A expression in adipose tissue is associated with BMI[296]. In the Mölndal metabolic study, body fat content, measured by DEXA, showed a strong and inverse correlation with CIDE-A expression in adipose tissue. We found negative associations between CIDE-A adipose tissue expression and fasting insulin and measurements of adiposity; BMI, waist circumference and WHR. However, there was no difference in CIDE-A expression levels between subjects with and without metabolic syndrome at any time-point during the VLCD-2 study. In the Mölndal Metabolic study, there were associations between CIDE-A expression and metabolic syndrome variables. However, after adjustment for body fat content, only association with serum insulin remained.

In humans, CIDE-A expression in white adipose tissue is inversely correlated with fat mass, shown in this study and others[295,296]. The negative energy balance produced by a hypocaloric diet is known to entirely modify adipocyte metabolism, particularly the lipolytic pathway[303]. During the VLCD-2 diet there is a limited calorie intake and consequently, energy is needed, and the oxidation of fatty acids is increased in adipose tissue to meet that need.

Adipose tissue during critical illness Paper IV

Increased blood glucose and decreased sensitivity to insulin frequently occur in critically ill patients without previous history of diabetes[304-307]. Several studies have showed that hyperglycemia worsen morbidity and mortality in

critically ill patients[308,309]. Many of the acute metabolic changes seen in critically ill patients are similar to those seen in patients with the metabolic syndrome, a condition that takes years to develop[310]. Adipose tissue is thought to play a key role in development of the metabolic syndrome via the secretion of adipokines[311]. Adipokines, such as leptin and adiponectin, can modulate insulin signalling and may promote insulin sensitivity, and TNF-alpha has insulin-antagonistic properties[312]. Whether adipose tissue and adipokines also contribute to the rapidly occurring insulin resistance during critical illness is not know. To address this issue, we performed a study in critically ill patients with subarachnoidal hemorrhage (SAH). SAH was chosen because it is a serious but homogenous injury that does not involve multiple organ failure.

The patients were admitted to the neurosurgical intensive care unit (NICU) within 2 days of hemorrhage. Consecutive blood samples and abdominal subcutaneous adipose tissue biopsies were taken at three timepoints, twice during intensive care treatment [1-2 days (IC1) and 7-9 days (IC2) after subarachnoidal hemorrhage] and once after complete recovery. The patients received a continuous insulin infusion to maintain adequate glucose levels, and hence the high plasma insulin levels we observed during intensive care reflect reduced insulin sensitivity.

DNA microarray analysis of adipose tissue gene expression identified 21 genes that were up-regulated and 52 genes that were down-regulated during intensive care. Among these regulated genes, ZAG was the only adipokine. Increased plasma ZAG levels in samples taken at the IC2 time point accompanied the increased ZAG mRNA levels during critical illness.

ZAG is a lipid-mobilizing factor[313], that is present at high concentrations in plasma and other body fluids[169]. ZAG is thought to be responsible for increased lipolysis in the adipose tissue of patients with cancer cachexia and in mice bearing a cachexia-inducing tumour[177]. The idea that ZAG could be involved in the development of insulin resistance is supported by the fact that ZAG levels are elevated in patients with cancer cachexia and such patients also frequently exhibit insulin resistance[314]. Increased lipolysis may contribute to the development of insulin resistance[210] and high levels of triglycerides and FFA play a key role in the development of insulin resistance in individuals with the metabolic syndrome[315]. Increased lipolysis occurs during critical illness but in our study circulating levels of FFA and triglycerides were not increased and could therefore not explain an effect of ZAG on insulin sensitivity. However, it is possible that ZAG also has other yet unknown effects that could influence insulin sensitivity without increasing the amount of FFA and triglycerides in the blood. Thus, ZAG could be involved in the development of insulin resistance by mechanisms unrelated to lipolysis or by a local elevation of FFA in adipose tissue without increased levels of FFA in the

circulation. Such local effects in adipose tissue could lead to systemic insulin resistance as has been shown in genetically modified mice with GLUT4 deficiency in adipose tissue[316]. Adiponectin is an adipokine that has been linked to insulin sensitivity both in animal models and in man. Adiponectin -/- mice are insulin resistant, and in humans, adiponectin levels correlate with insulin sensitivity[317]. Since adiponectin is an insulin-sensitizing hormone with anti-diabetic, anti-atherogenic and anti-inflammatory effects[318,319], low levels of adiponectin appear to be an important initiating factor of insulin resistance[319,320]. We observed reduced plasma levels of adiponectin at IC1. To our knowledge, this is a novel finding, and may indicate that adiponectin plays a key role in the development of insulin resistance during intensive care. The change in plasma adiponectin protein levels was not mirrored by changes in mRNA levels from subcutaneous adipose tissue. This is in line with our previous finding that it is difficult to relate adiponectin expression to circulating levels[321].

RBP-4 is a recently discovered adipokine that has been proposed to mediate systemic insulin resistance[159,165], and serum levels of RBP4 are increased in insulin-resistant states[322]. However, several reports have shown conflicting results, whether elevated levels of serum RBP4 is associated with insulin resistance and metabolic syndrome or not[166-168]. In an effort to determine which adipose tissue compartment that may be associated with elevated RBP4 levels in humans, several studies have reported results, however, they are diverging. Some studies show that RBP4 is more highly expressed in visceral than in subcutaneous adipose tissue and may be a marker of intra-abdominal fat mass[323,324], while others find that circulating RBP4 is not associated with the amount of total adipose tissue, or in the ectopic depots in muscle[325,326].

It has recently been suggested that shortcomings in methodology complicate measurements of RBP4[327]. Three commercially available multiwell immunoassays were compared with quantitative western blotting and in parallel measurements of RBP4 concentrations in serum, from both insulin-resistant subjects and from insulin-resistant subjects with impaired glucose tolerance or type 2 diabetes[327]. The assays yielded different absolute values and magnitudes of elevation of serum RBP4 and this might contribute to the diverging reports.

In our study, insulin resistance could not be explained by RBP-4 since RBP-4 levels were reduced at IC1. Severe calorie restriction has been shown to promote a reduction in adipose tissue expression and plasma levels of RBP-4[328]. The metabolism of critical illness is characterized by a combination of starvation and stress[329] and the decreased RBP-4 levels in our study might therefore reflect the effects of calorie restriction during intensive care. Although plasma RBP-4 levels were reduced during critical illness, we did not observe a parallel decrease in RBP-4 gene expression in adipose tissue. This

is in line with a previous report demonstrating that RBP-4 gene expression in adipose tissue correlates poorly with plasma levels[161]. Comparison of the expression levels in different tissues indicates that the liver may be the main site of RBP-4 production and this may explain the lack of correlation between circulating RBP-4 and adipose tissue RBP-4 mRNA levels

CRP and SAA are acute-phase proteins and their concentrations increase markedly within hours of trauma, infection or inflammation[330]. As expected, we observed high serum levels of CRP and SAA during intensive care. A recent study indicated that increased CRP levels lead to decreased adiponectin levels, and that CRP thereby influences insulin sensitivity[331]. During IC1, we observed markedly elevated levels of CRP and reduced levels of adiponectin in the circulation, and this may be a contributing factor in the development of insulin resistance.

In addition to its role as an acute phase protein, SAA is a proinflammatory and lipolytic adipokine in humans[151], and it is likely that it is involved in the regulation of insulin sensitivity. In our study, the increase in serum levels of SAA during intensive care was not matched by parallel changes in adipose tissue gene expression. Although human adipocytes are primary sources of SAA during non-acute phase[150], SAA probably originates from the liver during acute illness, explaining the lack of correlation between adipose tissue SAA expression and circulating SAA in our patients.

Although our primary aim was to identify adipokines that could be causing insulin resistance during critical illness, the microarray analysis also identified regulation of other genes expressed in adipose tissue. Among the 21 up-regulated genes from the DNA microarray analysis, phospholipase A2, group IIA (PLA2G2A) was the most highly regulated gene during intensive care. PLA2G2A is an acute-phase protein mediating decreased plasma HDL cholesterol and increased atherosclerosis[332]. This enzyme is a potent mediator of the inflammatory process and increased $sPLA_2$ plasma levels have been reported in patients with various acute and chronic inflammatory conditions[333,334].

The key enzyme of gluconeogenesis, phosphoenolpyruvat (PCK1) was also up-regulated during intensive care in our study. PCK1 is a cytosolic isozyme of phosphoenolpyruvate carboxykinase (PEPCK-C)[335]. Increased cortisol levels enhance the gluconeogenesis pathway, and it is well known that cortisol increases the transcription of PCK1[336]. High levels of PCK1 are sufficient to induce diabetes[337], while decreased PCK1 expression is sufficient to reverse hyperglycemia[338]. Mouse studies involving over-expression of PCK1 have resulted in type 2 diabetes, and it is possible that it has a similar function in man.

During intensive care, we also observed that the expression of the antioxidant metallothionein 1M (MT1M) was markedly up-regulated. MTs are a family

of intracellular metal-binding and cysteine-rich proteins, being highly inducible in many tissues[339,340]. MTs act as regulators of homeostasis of metals, such as zinc and copper, in tissues, and they have also been found to be potent antioxidant proteins protecting cells and tissues from oxidative stress[341]. A link between oxidative stress and insulin resistance has been shown[342] and free radicals may regulate adipocyte function. Hyperglycemia has been associated with oxidative stress since the early 1960s[343] and it has been suggested that hyperglycemia-induced oxidative stress contributes to the development of diabetic complications[344]. Oxidative stress impairs insulin action in adipocytes in vitro[345-347] and in vivo treatment with an antioxidant lipoic acid improves insulin-stimulated glucose disposal[348]. Recent studies suggest that MT can prevent both diabetes development and diabetic complications[341]. In diabetic experimental animals, over expression of MT in various metabolic organs has been shown to reduce hyperglycaemia-induced oxidative stress, organ specific diabetic complications, and DNA damage, and the results have been confirmed by studies in MT-knockout mice[349].

Acute and chronic stressful events initiate a physiological response to maintain homeostasis[350]. The central components of the stress response involve the hypothalamus and brain stem, while the peripheral components of the stress response include the hypothalamic-pituitary-adrenal (HPA) axis as well as the systemic and adrenomedullary sympathetic system[350].

Hormone	Mechanism
Adrenaline	Skeletal muscle insulin resistance, via altered postreceptor signaling Increased gluconeogenesis Increased skeletal muscle and hepatic glycogenolysis Increased lipolysis; increased FFA direct suppression of insulin secretion
Noradrenaline	Increased lipolysis Increased gluconeogenesis; marked hyperglycemia only at high concentrations
Glucocorticoids	Skeletal muscle insulin resistance Increased lipolysis Increased gluconeogenesis
Glucagon	Increased gluconeogenesis Increased hepatic glycogenolysis
Growth hormone	Skeletal muscle insulin resistance Increased lipolysis Increased gluconeogenesis

Table 6 Hormones involved in the hyperglycemic response to critical illness

The catabolic state, such as seen during trauma, sepsis or major surgery, is characterized by altered metabolism, leading to a rapid loss of lean body mass and a change in body composition. Injuries can initiate the stress response via the sympatic nervous system involving a complex cascade of mediators, such

as cortisol, insulin, glucagon, growth hormone, cytokines and lipid mediators.

During catabolism there is high energy expenditure, and body fat and skeletal muscle proteins are used as fuel[351]. Major patophysiological conditions underlying hyperglycemia in critical illness include enhanced hepatic gluconeogenesis, impaired insulin secretion and decreased insulin sensitivity due to anti-insulin effects of stress hormones and proinflammatory cytokines[305,352].

Decreased concentrations of total cholesterol, lipoproteins and lipoprotein cholesterols are seen early in the course of critical illness and may be due to decreased synthesis or increased catabolism of cholesterol[353]. The presence of hypocholesterolemia following trauma was first described in 1911, when Chauffard and co-workers reported decreased cholesterol levels in patients who were in 'very bad general condition' during the febrile phase of tuberculosis. In a study of critically ill trauma patients, mean cholesterol levels were significantly lower, with decreased concentrations of both low LDL (low-density lipoprotein) and HDL-cholesterol[354]. We obtained the same results in our study. Total cholesterol and HDL-cholesterol have been shown to be considerably reduced as a part of an acute-phase response to many disorders, such as infection, malignancy and surgery[355]. However, the physiologic significance of these decreased concentrations is to our knowledge largely unknown.

Increased levels of triglycerides and FFA are involved in the development of insulin resistance[315], however in our study, FFA and triglycerides were not changed during intensive care. Elevated levels of FFA results in enhanced hepatic triglyceride accumulation, and thus to fatty liver infiltration and hepatic failure, which is associated with increased incidence of sepsis and mortality[356,357]. During injury, increased peripheral lipolysis and decreased transporter proteins, such as LDL- and HDL cholesterol, may result in accumulation of triglycerides in the liver[356,357]. It has been shown that insulin administration decreases FFA and serum triglycerides after injury and this metabolic action of insulin represents an advantage, as liver failure and death due to increased fat accumulation could be prevented[358].

Future perspectives

The results of this thesis demonstrate that adipocyte size influences gene expression and suggest that the adipocyte specific genes NQO1 and CIDE-A may influence human metabolism. In addition, the changes in adipose tissue gene expression in critically ill patients are explored. Although the results add to current knowledge, they also lead to further questions and ideas. The studies included in this thesis are based on expression profiling using DNA microarrays. This is a very powerful method that could be used in many other situations. For example, the study of adipocytes of different sizes has so far been focused on genes that are overexpressed in large adipocytes. The rational behind this was the previously described association between enlarged adiocytes and metabolic disease. However, we can not exclude the possibility that there are genes that have increased expression in small adipocytes. Since large adipocytes seem to express genes that could cause metabolic disease, it is possible that genes over-expressed in small adipocytes could have a protective role. If so, identification of such genes may provide novel targets for drug development. Future studies will therefore investigate the genes more highly expressed in the small adipocytes.

The microarray technique is also very suitable for comparison of gene expression in different tissues. The importance of visceral adipose tissue as a risk factor for metabolic and cardiovascular disease is well recognized in both adults and children. The reason why the visceral adipose tissue is more dangerous than subcutaneous adipose tissue is largely unknown although some suggestions have been made [133,359-361]. Adipose tissue secrets many adipokines and depot differences in the secretion of some of these have been described.

Because of the known association between visceral adipose tissue and metabolic disturbances studies have been performed to compare gene expression in visceral adipose tissue and abdominal subcutaneous adipose tissue. Much less in known about gene expression and the secretion of adipokines from other adipose tissue depots. Future studies may reveal if there are differences in gene expression and the secretion of adipokines between abdominal and femoral-gluteal subcutaneous fat and if so, how this relate to metabolic disease. In addition to clarifying differences related to adipose tissue depots and cell size, future micorarray studies may also indicate novel proteins produced by adipocytes or by adipose tissue, which might influence metabolic function.

Inter-individual variation in gene expression of adipokine is well known from the literature[362,363] and also shown in my experience. This is probably due to a combination of genetic and environmental factors. For comparison of gene expression or adipokine secretion between different depots or between adiopo-

cytes of different size it is therefore important to use samples from the same subject. To understand the biological importance of expression differences between cells of different size or adipose tissue at different locations, the function and regulation of identified genes or adipokines must be studied. For example, even modest weight loss can reduce an individual's risk for obesity-related diseases. Identification of genes that are regulated in response to energy restriction and/or changes in BMI may therefore reveal important metabolic and physiologic mechanisms involved in the development of obesity-related disease. Furthermore, the function of such genes may be elucidated by over-expression or gene silencing in *in vitro* systems.

So far, all microarray based studies of adipose tissue gene expression in human adipose tissue has used white adipose tissue samples. Little is known about the clinical importance of BAT in humans, although recent studies suggest that this tissue is more abundant than previously thought also in adults. By using DNA microarray to study gene expression in human BAT in comparison with other human tissues, we may be able to identify pathways and regulatory systems of importance for thermogenesis, a function that could be of great importance for dissipation of excess energy and thus a target for anti-obesity drug development.

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