

Thesis for doctoral degree (Ph.D.)
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ROLE OF INFLAMMATORY AND MITOCHONDRIA GENES IN ADIPOSE TISSUE AND OBESITY

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Niels Kjaer

ABSTRACT

Obesity is rapidly increasing in prevalence around the world together with its complications such as dyslipidemia, cardiovascular disease (CVD) and insulin resistance, which eventually may lead to type 2 diabetes (T2DM). The excess of adipose tissue, the hallmark of obesity, led us to study the adipose tissue as a contributor to obesity and insulin resistance. Both systemic and local, that is in adipose tissue, inflammation have in the recent years emerged as complications to obesity. This inflammation contributes to the infiltration of macrophages and to the insulin resistance in adipose tissue.

We investigated the role of inflammatory genes in the regulation of obesity in subcutaneous adipose tissue pieces. We found that several cytokines were significantly upregulated in obesity and this led us to investigate the secretion of the corresponding proteins. We found that monocyte chemoattractant protein 1 (MCP1) was the only upregulated chemokine on gene level to be secreted from both adipose tissue and isolated adipocytes. MCP1, since it is not secreted from the adipose tissue into the general circulation, seems to be a local inflammatory mediator. Other studies have shown its ability to induce insulin resistance in adipose tissue.

The gene arachidonate 5-lipoxygenase activating protein (ALOX5AP) was first implicated in CVD in men. We aimed to investigate its role in obesity in adipose tissue. We found the mRNA expression to be increased in obesity and strongly associate to insulin resistance measured as homeostasis model assessment (HOMA). The ALOX5AP protein FLAP, as well as its head enzyme 5-lipoxygenase (5-LO), were detected in adipocytes. We propose an increased synthesis of 5-LO products in adipose tissue in the obese, leading to an inflammatory profile and insulin resistance.

Mitochondrial dysfunction has recently emerged as being associated with insulin resistance, firstly in skeletal muscle and subsequently in human adipose tissue. We found a significant downregulation in the expression of several electron transport chain genes selectively in T2DM in the visceral fat depot. We suggested this downregulation to be mediated by TNF-alpha and found that the cytokine downregulated the electron transport chain genes as well as significantly decreased the fatty acid oxidation in differentiated primary human adipocytes. We next investigated the amount of mitochondrial DNA/cell (mtDNA/cell) in adipose tissue in relation to clinical and experimental parameters of insulin sensitivity. We found an association between mtDNA/cell and basal and insulin stimulated lipogenesis, suggesting that adipose tissue mitochondria are local regulators of lipid metabolism.

We suggest a causal relationship between the inflammation present in adipose tissue in obesity with the downregulation of mitochondrial genes. Also we have found a potential new role for mitochondria in human adipose tissue as regulators of adipocyte metabolism. These findings add to the knowledge of the role of human adipose tissue in obesity and development of insulin resistance.

LIST OF PUBLICATIONS

- I. **A unique role of monocyte chemoattractant protein 1 among chemokines in adipose tissue of obese subjects.**
I Dahlman, M Kaaman, T Olsson, G D. Tan, A S. T. Bickerton, K Wåhlén, J Andersson, E Arvidsson Nordström, L Blomqvist, A Sjögren, M Forsgren, A Attersand and P Arner
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- II. **ALOX5AP expression, but not gene haplotypes, is associated with obesity and insulin resistance.**
M Kaaman, M Rydén, T Axelsson, E Nordström, A Sicard, A Bouloumié, D Langin, P Arner and I Dahlman
Int J Obes. 2006 30:447-52
- III. **Downregulation of Electron Transport Chain Genes in Visceral Adipose Tissue in Type 2 Diabetes Independent of Obesity and Possibly Involving Tumour Necrosis Factor-alpha.**
I Dahlman, M Forsgren, A Sjögren, E Arvidsson Nordström, M Kaaman, E Näslund, A Attersand and P Arner
Diabetes 2006 55:1792-9
- IV. **Strong association between mitochondrial DNA copy number and lipogenesis in human white adipose tissue**
M Kaaman, L M Sparks, V van Harmelen, S R Smith, E Sjölin, I Dahlman, P Arner
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LIST OF ABBREVIATIONS

AC	adenylate cyclase
ADP	adenosine disphosphate
AMP	adenosine monophosphate
ALOX5AP	arachidonate 5-lipoxygenase activating protein (gene)
AR	adrenoceptor
ASP	acid soluble products
ATP	adenosine triphosphate
ATP6	ATP synthase 6
B2MG	beta 2 microglobulin
BMI	body max index
cAMP	cyclic AMP
CCL2	C-C motif chemokine ligand-2, also known as MCP1
CCR2	C-C motif chemokine ligand-2 receptor
cDNA	complementary DNA
CNS	central nervous system
COX1	cytochrome c oxidase 1
CRP	C reactive protein
Ct	threshold cycle in qRT-PCR
CVD	cardiovascular disease
DAG	diacylglycerol
DDT	dithiothreitol
EE	energy expenditure
ELISA	enzyme linked immunosorbent assay
FABP	fatty acid binding protein
FADH2	flavin adenine dinucleotide (reduced form)
FATP	fatty acid transport protein
FFA	free fatty acid
FLAP	arachidonate 5-lipoxygenase activating protein
FTO	fat mass and obesity associated
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gi	inhibitory G-protein
GLUT4	glucose transporter 4
Gs	stimulatory G-protein
GSEA	gene set enrichment analysis
HDL	high density lipoprotein
HOMA	homeostasis model assessment
HSL	hormone sensitive lipase
IL6	interleukin 6
INSIG2	insulin induced gene 2
IOTF	International Obesity Task Force
IRS1	insulin receptor substrate 1
5-LO	5-lipoxygenase
LDL	low density lipoprotein
LPL	lipoprotein lipase

LTB4	leukotriene B4
MAG	monoacylglycerol
MAPK	mitogen-activated protein kinase
MCAD	medium-chain specific acyl-coenzyme A dehydrogenase
MCP1	monocyte chemoattractant protein 1, also known as CCL2
MCR4	melanocortin receptor 4 gene
MIF	macrophage migration inhibitory factor
mRNA	messenger RNA
NADH	nicotinamide adenine dinucleotide, reduced form
ND1	NADH dehydrogenase subunit 1
NDUFB4	NADH dehydrogenase 1 β subcomplex 4
OXPHOS	oxidative phosphorylation
PAI-1	plasminogen activator inhibitor type I
PCR	polymerase chain reaction
PDE3B	phosphodiesterase 3 B
PGC1-alpha	proliferator-activated receptor coactivator 1alpha
PKA	protein kinase A
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real time PCR
QTL	quantitative trait loci
RMR	resting metabolic rate
SAM	significance analysis of microarrays
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
SVF	stromal vascular fraction
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TAG	triacylglycerol
TCA	tricarboxylic acid cycle
TNF-alpha	tumour necrosis factor alpha
TZD	thiazolidinedione
UCP	uncoupling protein
VCO ₂	volume carbon dioxide production
VLDL	very low density lipoprotein
VO ₂	volume oxygen consumption
WAT	white adipose tissue
WHO	World health organization
WHR	waist- to-hip ratio

1 INTRODUCTION

1.1 OBESITY

Obesity, the excess of fat mass, has evolved to become an increasing problem worldwide (1). Obesity is a result of an imbalance between food intake and energy expenditure which results in a storage of excess energy as fat, primarily in the adipose tissue. There are several complications following obesity, for example high levels of serum free fatty acids and dyslipidemia (2), cardiovascular disease (CVD) as well as insulin resistance which eventually can develop into type 2 diabetes mellitus (T2DM). The prevalence of T2DM is increasing in the footsteps of obesity worldwide (3; 4). In Sweden obesity and T2DM are also becoming increasing health problems (5-7).

Body Mass Index (BMI) is the most common method to assess obesity. BMI is calculated as total body weight in kg divided by square of length in meter. According to the International Obesity Task Force (IOTF) the definition of obesity is when BMI equals or exceeds 30.00 kg/m² and overweight is defined as BMI equalling or exceeding 25.00 kg/ m². It should be noted that healthy individuals can have a BMI in the range of 25-30 kg/ m² without being overweight. Therefore it is just as important to also have additional measures when defining obesity in the clinic; examples are waist circumference and waist-to-hip ratio (WHR). These parameters can give a more correct estimation of the body composition. Waist circumference has recently been shown to strongly predict insulin resistance in a retrospective study (8). One drawback of using waist circumference as an assessment of obesity is the bad reproducibility.

Table 1. BMI classification according to the World Health Organization, (WHO) (1).

Classification	BMI kg/m²
Normal range	18.50-24.99
Overweight	≥ 25.00
<i>Pre obese</i>	25.00 – 29.99
Obese	≥ 30.00
<i>Obese class I</i>	30.00- 34.99
<i>Obese class II</i>	35.00 – 39.99
<i>Obese class III</i>	≥ 40.00

Regarding waist circumference there are gender differences and the thresholds for increased risk of developing metabolic complications are:

Women: waist circumference exceeding 80 cm and a WHR over 0.8

Men: waist circumference exceeding 94 cm and a WHR over 1.0

These thresholds are used for defining abdominal adiposity, which is an independent strong risk factor of insulin resistance and CVD in both women and men (8; 9). Thus abdominal adiposity is a stronger predictor of insulin resistance and CVD than an increased BMI alone

There are additional gender differences to consider when it comes to adipose tissue distribution. Men have a tendency to accumulate excess adipose tissue in an apple like fashion around the abdominal region while women have a more pear like shape when excess adipose tissue is stored over the thighs and hips. These different types of adiposity are called android and gynoid obesity, respectively. The gynoid obesity is preferred over the android given the increased metabolic complications with visceral adiposity (8). It is even suggested that gynoid adiposity has a protective effect (10). The best way to assess risk of metabolic complications and disease following obesity is most probably a combination of BMI and waist circumference.

1.2 DIABETES

Diabetes is defined as a fasting blood glucose level equalling or exceeding 7.0 mmol/l or a blood glucose level after a glucose tolerance test equalling or exceeding 11.1 mmol/l. The reasons for diabetes can be either lack of insulin secretion, which is type I diabetes mellitus (T1DM), or resistance to insulin in liver, muscle and adipose tissue combined with an insufficient production from the pancreas, which is T2DM. The definitions of glucose intolerance are fasting venous blood glucose level below 7.0 mmol/l and blood glucose level after a glucose tolerance test ≥ 7.8 and < 11.1 mmol/l.

In the early stages of T2DM development the major sign is insulin resistance of muscle, liver and adipose tissue. Insulin resistance means a reduced sensitivity in the tissues to the action of insulin. In the beginning this peripheral insulin resistance is balanced out by an increased secretion of insulin from the pancreas, and thus the plasma glucose

level is kept normal. With time this compensating insulin secretion becomes insufficient and leads to hyperglycaemia and T2DM (11; 12).

It is believed that an increased level of circulating free fatty acids (FFA) in obesity contributes to insulin resistance in liver and muscle, as well as decreased insulin secretion from pancreas. It has been shown that plasma free fatty acids associate to the risk of developing T2DM (13). One of the first theories on the involvement of free fatty acids in the development of insulin resistance was the Randle hypothesis, postulating that there is a competition between free fatty acids and glucose for oxidative phosphorylation in the cells. This would lead to a decrease in glucose utilization when the lipid concentration is increased in turn leading to an increase in systemic glucose concentration (14). The molecular pathways underlying the development of T2DM and the role of FFA are incompletely understood (15). In muscle, it is suggested that the increased intramyocellular lipid metabolites interfere with the insulin receptor substrate 1 and thus disturb the normal insulin signalling (16).

1.3 ADIPOSE TISSUE

Humans have two main depots of white adipose tissue; the subcutaneous, found under the skin around the abdomen and in the periphery, and the abdominal visceral fat depot found in the peritoneal cavity. Different regions of human adipose tissue have different properties (17; 18). The human adipose tissue is a tissue comprised of various cell types. Besides adipocytes there are the cells of the stromal vascular fraction (SVF) for example endothelial cells, fibroblasts and macrophages (19).

1.3.1 Lipolysis

Adipocytes are cells well equipped for storing triacylglycerol (TAG) to be released as free fatty acids and glycerol for energy needs in the rest of the body. Lipolysis is the breakdown of TAG into free fatty acids and glycerol (reviewed in (20-22)). One molecule of TAG is cleaved into one molecule of glycerol and three free fatty acids. There is a basal lipolysis in adipocytes, a continuous breakdown of TAG, at approximately $2 \mu\text{mol glycerol}/10^7$ cells. Stimulated lipolysis is highly regulated by the two catecholamines; adrenalin secreted from the adrenal gland and noradrenalin secreted from the adrenal gland and central nervous system. The major inhibitor of lipolysis is insulin secreted from the pancreas.

In lipolysis, the catecholamines bind to the stimulatory beta (subtypes 1, 2 and 3) and inhibitory alpha 2-adrenoceptors (α_2 -AR) on the adipocyte cell membrane. The adrenoceptors are coupled to either stimulatory or inhibitory G-proteins (Gs or Gi), see figure 1. The G-proteins either activate or inhibit adenylate cyclase, which in turn synthesises cyclic AMP (cAMP) from ATP. cAMP is a very potent second messenger involved in many cellular processes. cAMP binds and activates protein kinase A (PKA) which in turn phosphorylates hormone sensitive lipase (HSL), the rate-limiting enzyme in lipolysis. HSL catalyses the first step of releasing a free fatty acid from the TAG.

The inhibition of lipolysis is mediated either through the alpha 2-adrenoceptor coupled to Gi-proteins or through the insulin receptor. Insulin binds to the insulin receptor which by autophosphorylation and downstream signalling eventually causes an activation of the enzyme phosphodiesterase 3 B (PDE3B). PDE3B breaks down the cAMP thus inhibiting the activation of PKA.

The TAG is further broken down stepwise into diacylglycerol (DAG) and monoacylglycerol (MAG). The last step of lipolysis, the hydrolysis of MAG into FFA and glycerol, is made by a non-specific monoacylglycerol lipase. Glycerol is released from the adipocytes by a channel forming membrane protein, aquaporin. The transport of fatty acids from the cell is still not clearly known, either they passively diffuse or they are actively transported by a specific fatty acid binding protein (FABP). The FFAs that are released are used as fuel for other organs, but at higher levels in the blood system they may also play an important role in the development of insulin resistance and T2DM.

Different regions of human adipose tissue have different lipolytic properties. The abdominal subcutaneous adipose depot is more sensitive to insulin and is not as lipolytically active as the visceral abdominal adipose depot. This is associated with clear differences in expression of the different receptors regulating lipolysis in adipocytes. The beta adrenoceptors 1, 2 and 3 are more active in the visceral adipocytes while the inhibitory alpha 2-adrenoceptors and the insulin receptors are more active in subcutaneous adipocytes. In addition, these differences in lipolytic activity are even more pronounced in obesity (23).

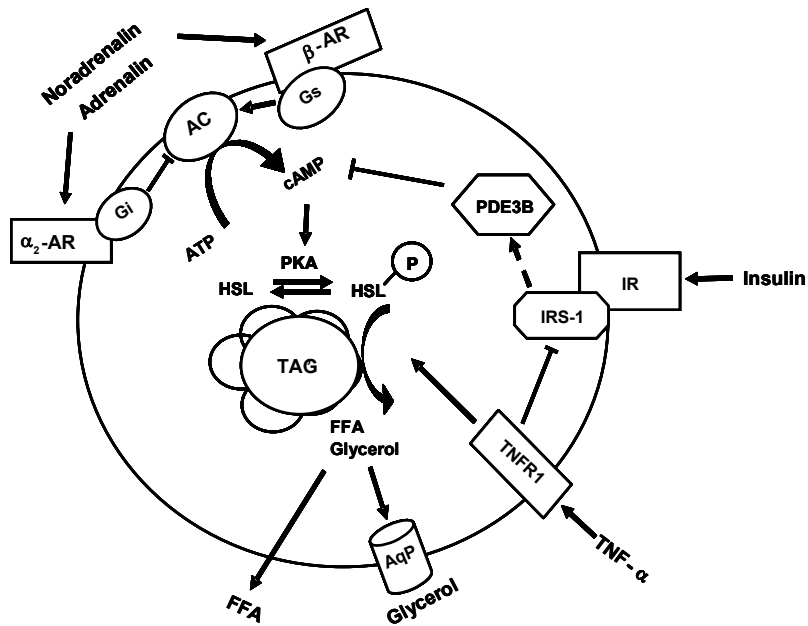


Figure 1. Simplified figure of adipocyte with some of the different pathways regulating lipolysis.

AR adrenoceptor, AC adenylate cyclase, Gs, Gi stimulatory and inhibitory G-proteins, ATP adenosine trisphosphate, cAMP cyclic adenosine monophosphate, PKA protein kinase A, HSL hormone sensitive lipase (inactive), HSL-P phosphorylated HSL (active), TAG triacylglycerol, FFA free fatty acids, IR insulin receptor, IRS-1 insulin receptor substrate 1, PDE 3B phosphodiesterase 3 B, TNFR1 TNF-alpha receptor 1, AqP aquaporin.

→ activating pathway —| inhibiting pathway

1.3.2 Lipogenesis

Lipogenesis is the process of building up lipids as TAG in the adipocyte. It starts with the uptake of glucose from the blood through the stimulation of the glucose transporter 4 (GLUT4) by insulin. In lipogenesis the backbone carbons of the glucose molecule are used in synthesising fatty acids, for example palmitate, or the glycerol backbone of the TAG. The fatty acids either come from the adipocyte itself, as described above, or they are taken up from the blood stream by fatty acid transporters on the cell membrane (FATP). The TAG formed in lipogenesis form a lipid droplet in the adipocyte. From here they are either stored or metabolised in lipolysis (22).

1.4 ADIPOSE TISSUE AS AN ENDOCRINE ORGAN

Adipose tissue has for long been considered as a passive storage organ for excess energy, in the form of TAG, for meeting the energy needs of the body. This idea has since then been reformed and now the adipose tissue is seen as a highly active endocrine organ secreting factors acting both locally in the adipose tissue and systemically in other organs. Through adipokines the adipose tissue regulates and interplays with the brain and other peripheral organs in weight maintenance and metabolic regulation (24).

One of the most important adipokines discovered is leptin (25). Leptin works as a messenger signalling from the fat depot, giving information about the size of the depot to the hypothalamus in the brain. The most important satiety centre in the CNS is the ventromedial nucleus of the hypothalamus (26).

One more example of an adipokine is adiponectin (27). Adiponectin is secreted systemically from the adipose tissue and has been shown to have effect on several peripheral tissues such as liver and skeletal muscle. It improves insulin sensitivity and is anti-inflammatory (28).

An important cytokine secreted by adipose tissue is TNF-alpha (29). Its secretion is shown to be increased in obesity and to directly interfere with insulin signalling in adipocytes (30; 31). TNF-alpha has local effects in the adipose tissue, and is shown to exert somewhat different effects in mice and humans. mRNA expression of TNF-alpha in adipose tissue is associated with obesity and insulin resistance in both rodents and humans (29). Both obese humans and rodents have an increased secretion of TNF-alpha from the adipose tissue and display an increased lipolysis in response to TNF-alpha (32). An important species difference is that the adipose tissue derived TNF-alpha in rodents is released to the circulation which is not the case in humans (33). The increased lipolysis in adipocytes mediated by TNF-alpha is shown to go through the mitogen-activated protein kinase (MAPK) pathway, and also through inhibition of the insulin receptor. The inhibition of the normal signalling of insulin receptor is shown to go through the inhibition of the autophosphorylation of tyrosine residues directly on the receptor and by induction of the serine phosphorylation of the insulin receptor substrate 1 (IRS-1) (34).

Subsequently, several additional inflammatory mediators have been discovered to be secreted from the adipose tissue. Interleukin 6 (IL6) is released to the general circulation from the adipose tissue. IL6 secretion increases with adiposity, and IL6 reduces lipoprotein lipase (LPL) activity and stimulates thermogenesis (33).

Another mediator shown to be secreted by adipocytes is plasminogen activator inhibitor type I (PAI-1) (35). PAI-1 takes part in the coagulation of blood, and thereby is a pathogenic link between obesity and CVD.

Macrophage migration inhibitory factor (MIF) is very potent in inhibiting macrophage migration. The secretion of MIF from adipocytes has a positive correlation with BMI. It is suggested that MIF controls the macrophage infiltration into adipose tissue in obesity (36).

A potent inflammatory chemokine is monocyte chemoattractant protein 1 (MCP1) also known as C-C motif chemokine ligand-2 (CCL2). MCP1 is secreted by *in vitro* differentiated primary cultures of human adipocytes and its receptor CCR2 is expressed in these cells (37). TNF-alpha induces MCP1 mRNA expression in human primary cultured adipocytes and secretion from adipose tissue cultures (37; 38). Circulating MCP1 positively correlates to BMI (39). Like TNF-alpha, MCP1 seems to interfere with insulin stimulated glucose uptake in murine cell cultures (40). MCP1 and its role in inflammation and insulin resistance will be further discussed under the next section.

Secretion of adipokines can also differ between different regions of WAT (18; 41). For example TNF-alpha shows a higher secretion from the visceral depot compared to the abdominal subcutaneous adipose tissue adding on to the concept of the dangerous visceral fat depot.

1.5 ADIPOSE TISSUE AND INFLAMMATION IN OBESITY

It is established that there is a low-grade inflammation present in obese subjects indicated by an increased level of circulating C reactive protein (CRP). Inflammation is implicated in obesity complications such as insulin resistance and also ischemic heart

disease. CRP has been shown to be directly related to adiposity and BMI. An increased level of circulating CRP has also been associated to development of insulin resistance and T2DM. CRP is a non-specific marker of inflammation secreted by the liver (42; 43).

In addition to the systemic low-grade inflammation in obesity, there is also a local inflammation present in the adipose tissue. It has been shown in both humans and in animal models that there is an infiltration of macrophages into the adipose tissue, which in turn gives a local insulin resistance (44-46). There is a correlation found between obesity and the amount of infiltrating macrophages in the adipose tissue. In addition, the amount of macrophages correlates with the size of the adipocytes in several adipose tissue depots in mice and the abdominal subcutaneous adipose tissue in humans (45). It is shown that there are twice as many macrophages in the visceral adipose tissue compared to the subcutaneous adipose depot even though the adipocytes in the visceral depot are significantly smaller than in the subcutaneous depot. The amount of macrophages in the visceral depot in morbidly obese humans is also associated with two markers for hepatic inflammatory lesions, and with measures of overall insulin sensitivity (47).

A characterization of the adipose tissue macrophages shows that they are a unique type of macrophages. They display an ability of endocytosis and also show ability of secreting proinflammatory molecules such as MCP1. MCP1 secretion by these adipose tissue macrophages is also stimulated by TNF-alpha (48). The regulation of the recruitment of macrophages into adipose tissue is incompletely understood. Factors derived from human adipocytes attract blood monocytes to migrate through capillary endothelial cells, most probably by inducing the expression of adhesion molecules on the adipose tissue endothelial cells (49).

One of the inflammatory chemokines secreted by the adipose tissue, MCP1, has been studied in further detail in its role in local inflammation in adipose tissue and insulin resistance. Overexpression of MCP1 in adipose tissue in mice contributes to macrophage infiltration and gives an insulin resistant phenotype. In addition pre-treatment of skeletal muscle cells with MCP1 blunts insulin stimulated glucose uptake (50; 51). When the MCP1 receptor CCR2 is knocked out in mice made obese on a high fat diet, there is an improvement of insulin sensitivity in obesity (52). In addition to

these findings implicating MCP1 in the development of inflammation and insulin resistance in obesity, treatment with the insulin sensitizers thiazolidinediones (TZD) decreases the *in vitro* adipose tissue secretion of MCP1 (38). In contrast to these findings, there is one study showing no reduction of macrophage infiltration in adipose tissue in MCP1 knockout mice (53). In another study there is no weight gain on a high fat diet nor is there any change in glucose or insulin levels compared to control mice when the MCP1 receptor is knocked out in adipose tissue (54).

One gene that was firstly implicated in ischemic heart disease is the proinflammatory arachidonate 5-lipoxygenase activating protein (ALOX5AP). The protein of ALOX5AP, FLAP, is located at the nuclear membrane and donates arachidonic acid to its head enzyme 5-lipoxygenase (5-LO), which catalyzes one step in the synthesis of leukotrienes. Common haplotypes of the ALOX5AP gene is associated with increased leukotriene production in neutrophils and confer risk of myocardial infarction and stroke in men (55). The gene of 5-LO is implicated in obesity in mice (56). We investigated the ALOX5AP gene in human adipose tissue.

The inflammatory profile in the adipose tissue in obesity can be decreased by marked weight loss with a substantial change of fat mass. Both the gene expression of inflammation related genes of the SVF as well as the number of adipose tissue macrophages are decreased with weight loss (57; 58). One example is the mRNA expression of MCP1 in subcutaneous adipose tissue pieces that is significantly downregulated three months after weight loss.

1.6 MITOCHONDRIA

Mitochondria are cell organelles which are key players in energy turnover. Here the beta oxidation of fatty acids and the tricarboxylic acid cycle (TCA) take place. The beta oxidation and glycolysis, the breakdown of glucose, converge onto the TCA as acetyl CoA. Through the TCA the electron donors NADH and FADH₂ are formed. These work as electron donors in the electron transport chain, also called the oxidative phosphorylation pathway (OXPHOS). The electrons pass through the complexes to O₂ as the end electron acceptor, building up a proton gradient which in the end results in the generation of energy rich ATP from ADP and phosphate, see figure 2.

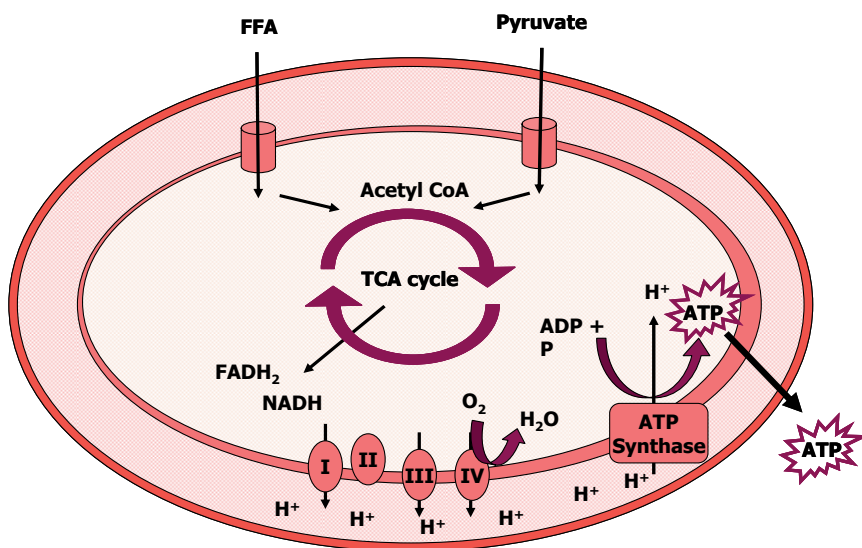


Figure 2. Schematic picture of a mitochondrion and oxidative phosphorylation (adapted from(59).

FFA free fatty acids, TCA tricarboxylic acid cycle, FADH₂ flavin adenine dinucleotide, reduced form, NADH nicotinamide adenine dinucleotide, reduced form, complex I-IV electron transport chain proteins

It is established with both microarray studies (60; 61) and with *in vivo* studies (62; 63) that mitochondrial function in skeletal muscle is impaired in human type 2 diabetic individuals. Recently the mitochondria and their function have been studied in adipose tissue as well. Several groups show that it is possible to increase mitochondria number and fatty acid oxidation capacity in murine and human adipocytes by treatment with TZD both *in vitro* (64; 65) and *in vivo* (66; 67).

The mitochondrial uncoupling proteins (UCPs) 1, 2 and 3 are expressed in the inner membrane of the mitochondria. They all work as uncouplers of the oxidative phosphorylation pathway, uncoupling oxidation from the synthesis of ATP. Instead of ATP, the energy is released as heat (68). This makes the UCPs candidates for obesity regulation. The different uncoupling protein subtypes are expressed in different tissues in mammals. UCP1 is most abundant in brown adipose tissue, which is present in humans at birth and only in a limited amount in adults. UCP2 is ubiquitously

expressed. UCP3 is expressed in skeletal muscle and adipose tissue. Single nucleotide polymorphisms (SNP's) found in these genes have been coupled to obesity and energy expenditure (69).

An important transcriptional regulator of mitochondrial biogenesis and enzymes of the respiratory chain is the peroxisome proliferator-activated receptor coactivator 1-alpha (PGC1-alpha). Tiraby C. and colleagues have successfully overexpressed PGC1-alpha with adenotransfection in differentiated primary human adipocytes (70). The overexpression of PGC1-alpha induced the mRNA expression of UCP1 and other mitochondrial proteins involved in the oxidative phosphorylation and fatty acid oxidation. In addition the fatty acid oxidation capacity increased in these cells. These results together imply that mitochondria have a potential role in obesity and insulin resistance.

1.7 GENETICS OF OBESITY

Many studies have been performed to prove that obesity clearly displays a genetic predisposition. First, there are monogenic forms of obesity. The most common cause of monogenic obesity is a mutation in the melanocortin receptor 4 gene (MC4R). MC4R mediates the anorexigenic response to leptin in the brain. There are also more rare forms of monogenic obesity, examples of this are mutations in the leptin gene and the leptin receptor gene (71).

Common obesity, also called polygenic obesity, depends upon mutations or alterations in several genes giving the obese phenotype (71). Quantitative trait loci (QTL) are determined by investigating the inheritance behind a quantitative trait, for example BMI or waist circumference. The QTLs are regions of DNA that are associated to a trait. There are numerous human QTLs shown to be associated with obesity (reviewed in (72)). QTLs help us find candidate genes for obesity. Underlying susceptibility genes for obesity are likely to act both in the central nervous system (CNS) on food intake, and on metabolic pathways in peripheral organs. Recently, the first susceptibility genes for common obesity identified by genome wide approaches have been identified, INSIG2 (73) and FTO (74). Their mode of action is unknown.

Obesity genes in the adipose tissue affecting the metabolism of adipocytes and adipose tissue are reviewed by Dahlman I (69). Examples of obesity genes in adipose tissue are the beta adrenoceptors 2 and 3. Polymorphisms in the beta 2 adrenoceptor are shown to give functional alterations of this receptor *in vitro* and are associated with lipolysis rate in adipocytes, levels of FFAs and obesity (75). Also a polymorphism in the promoter of TNF-alpha influencing the gene expression is associated with obesity (76). Another example of a gene implicated in common obesity is leptin. There are several polymorphisms in this gene that are shown to be associated with common obesity (77).

I believe that obesity is a consequence of an interaction between gene and environment. There are forms of monogenic obesity but the common obesity is explained by interactions between inheritance and environment. An individual is predisposed to obesity by carrying a certain set up of genes and in the “right” environment this interaction comes into play.

2 OVERALL AIMS

The description above of human adipose tissue gives support to the idea that human white adipose tissue (WAT) is an active tissue in the regulation and origin of obesity and insulin resistance. The mechanisms thereof are so far incompletely characterized and need to be investigated further.

The overall aims of this thesis were to identify new genes in subcutaneous and visceral adipose tissue regulating obesity and insulin resistance. The other aim was to characterize the function of these genes in human WAT and the association between SNP's and obesity.

2.1 AIMS STUDY I AND II

- Characterize inflammatory mediators in human adipose tissue in obesity.

2.2 AIMS STUDY III AND IV

- Determine regulation of mitochondrial OXPHOS genes in T2DM in human WAT.
- Determine the impact of mitochondria number in human white adipose tissue and adipocytes in relation to adipocyte and clinical variables that are linked to insulin sensitivity.

3 METHODOLOGICAL CONSIDERATIONS

All studies were approved by appropriate ethical committees at Karolinska Hospital with informed consent from all study individuals.

All details of the methods used can be found in each paper. This section will be a discussion of the choice of methods.

3.1 DESCRIPTION OF STUDY COHORTS

All studies were performed in humans, this in order to investigate how obesity and insulin resistance is regulated specifically in humans. There are many animal studies performed in this field of research but there are many differences in the metabolism and fat depots between humans and animals. In all four studies the definition of obesity was defined as a BMI greater than 30.00 kg/m². Both women and men were included in the studies but it is easier to recruit women in obesity studies.

3.1.1 Study I

Cohort 1 comprised of healthy nonobese and obese women studied for gene profiling of subcutaneous abdominal adipose tissue pieces.

Cohort 2 comprised of women undergoing cosmetic breast surgery and were used for the protein secretion study from paired samples of adipose tissue pieces and isolated adipocytes.

Cohort 3 comprised of healthy lean and obese women for the paired quantification of *in vitro* protein secretion and of mRNA with quantitative real time PCR (qRT-PCR) in adipose tissue pieces.

Cohort 4 comprised of two groups of both men and women with varying BMI. In these groups the *in vivo* secretion of MCP1 was quantified comparing the levels between blood from the abdominal vein and arterialized blood.

3.1.2 Study II

Cohort 1 comprised of nonobese and obese men for the genetic analysis. Here we chose only men since the association between ALOX5AP SNP's and CVD was much stronger in men than in women (55). In this cohort we included subjects treated for hypertension, dyslipidemia and type 2 diabetes with diet, sulfonurea or metformin,

respectively. These phenotypes are usually complications of obesity and are unlikely in themselves to cause an obese phenotype.

Cohort 2 included lean and obese women and men for mRNA quantification with qRT-PCR.

Cohort 3 was a group of obese women undergoing gastric banding surgery for weight loss. The mRNA expression was assessed before and 2-4 years after surgery when their body weight had reached a steady-state level.

In addition mRNA expression was quantified in different cell types of adipose tissue from subcutaneous abdominal adipose tissue samples from a group of individuals undergoing cosmetic liposuction.

3.1.3 Study III

Cohort 1 was used for Affymetrix gene profiling and comprised of three groups with eight subjects in each; nonobese, obese healthy and obese with a newly diagnosed T2DM. Adipose tissue samples from abdominal subcutaneous and visceral fat depot were obtained. No subject had any sign of general inflammation, measured by serum CRP before operation. The healthy obese subjects had a normal glucose tolerance while the obese T2DM individuals had a pathological glucose tolerance.

Cohort 2 was used for confirmation. Since it was only possible to measure a limited number of individual mRNA's in cohort 1, a subset of mRNA s were quantified in a separate group. In this group ten nonobese and ten obese with T2DM of various duration were included. The T2DM subjects in cohort 2 were on antidiabetic treatment. Cohort 3 comprised of healthy women who were not selected in the basis of BMI or age. They were all undergoing cosmetic liposuction or gastric banding surgery because of obesity. Subcutaneous and visceral adipose tissue samples were taken for experimental studies.

3.1.4 Study IV

Cohort 1 included obese and nonobese healthy women and men who all were free from continuous medication. A subgroup of the subjects was studied before and after a 10 week energy restricted diet.

Cohort 2 comprised of a small group of obese women studied before and after gastric banding operation or behavioural modification after reaching steady state in body weight.

Indirect calorimetry was performed on all study subjects and an insulin tolerance test on a subset of individuals.

3.2 CLINICAL MEASUREMENTS

3.2.1 Assessment of body composition and metabolic rate

BMI and waist circumference are measurements to make a primary assessment of obesity. Waist circumference has also recently been proven to be a good predictor of insulin resistance in a retrospective study (8).

To assess the resting metabolic rate (RMR) and energy expenditure (EE), indirect calorimetry was performed using an open ventilated hood system during 25 minutes (Deltatrac II; Datex-Ohmeda, Helsinki, Finland). This system measures caloric needs and estimates substrate oxidation by continuously measuring oxygen uptake, VO_2 , and CO_2 production, VCO_2 . Assessment of body composition such as percentage fat free mass, total body fat, lean body mass and total body weight was made using bioimpedance with Body Stat (Quad Scan, Isle of Man, UK) used in study IV. These methods are easy and non-invasive and give a good assessment of the metabolic profile of the study individual.

3.3 ASSESSMENT OF INSULIN RESISTANCE

3.3.1 Homeostasis model assessment index (HOMA index)

The HOMA index is an indirect measure of insulin resistance, using fasting plasma insulin and plasma glucose.

The formula being:

$$\text{Fasting plasma glucose (mmol/l)} \times \text{Fasting serum insulin } (\mu\text{U/ml}) / 22.5$$

A drawback is that the HOMA index is an indirect measurement of insulin resistance, but it gives a good reflection of insulin sensitivity without being too time consuming and invasive for the test-person. The HOMA index was used in all four studies.

According to Bonora E. and colleagues the HOMA index is a good predictor of insulin resistance, also this index has been shown to be a good independent predictor of CVD in type 2 diabetic individuals (78; 79). The HOMA index based on the formula above

was used in study I-III. In study IV an updated version of the HOMA index to assess insulin sensitivity was used, HOMA 2%S (80).

3.3.2 Insulin tolerance test

As a complement to the indirect method of HOMA index, an intravenous insulin tolerance test was performed during 16 min, measuring the percentage fall in glucose/min after insulin injection (81). The intravenous insulin test was used in study IV.

3.4 ADIPOSE TISSUE SAMPLING

To obtain subcutaneous abdominal adipose tissue samples a needle aspiration biopsy was obtained under local anaesthesia giving a sample size between 1-2 g. A fasting venous blood sample was taken before the biopsy in all the four studies for the quantification of proteins, insulin, glucose, total and high density lipoprotein (HDL) cholesterol, and TAG. In addition a glucose tolerance test was performed. All study individuals came to the examination in the morning after an overnight fast. For the adipose tissue studies we usually aimed at using individuals that were healthy except obesity or T2DM and that do not take medication, since disease and medication can have impact on adipose tissue function.

3.5 QUANTIFICATION OF RNA

3.5.1 Isolation of DNA and RNA

In the case of quantification of RNA and DNA it is very important to treat the samples correctly in order to avoid degradation. To keep the integrity of the nucleic acids, the adipose tissue biopsy samples were frozen directly in liquid nitrogen and kept at -70°C until isolation of RNA and DNA was performed. The quality of the RNA was evaluated using the Agilent 2100 bioanalyzer where the dominant RNA population of the cell, the ribosomal 18S and 28S which make up 85% of the total RNA, are evaluated for integrity. The cDNA synthesis was made with random primers.

Previously, we used the poly A-tail of the mRNA as the start of cDNA synthesis but the drawback with this method is that the extension of the cDNA is limited and it is easy to miss important sequences in the mRNAs.

3.5.2 Microarray

Microarray based gene expression profiling, with parallel quantification of thousands of genes, was used to screen for new genes with obesity and T2DM associated expression in adipose tissue. There are two main types of microarrays; those with thousands of different cDNAs spotted onto different cells on slides and those with oligos representing different genes synthesized *in situ* on the slides. In both cases, labelled cDNA or cRNA samples are hybridized to the microarrays, followed by washing to get rid of non-hybridized material and quantification of the signal emitted from each cell on the microarray, which is a measure of the amount of specific mRNA in the original samples. For the spotted arrays, two different samples labelled with different fluorophores, usually rhodamine and fluorescein are hybridized to the same array. These two fluorophores give two different fluorescent colours (red and green respectively). The relative intensities of the two fluorophores are used to find up- and downregulated genes. Thus, this is a paired comparison between two samples.

We have used microarrays with *in situ* synthesized oligos from Affymetrix. To evaluate sample integrity, cRNA samples were first hybridized to Test-3 arrays and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 3' to 5' probeset hybridization ratios compared. Unlike spotted cDNA arrays, only one labelled cRNA sample is hybridized to each Affymetrix microarray. To allow comparison of gene expression between samples, we scaled the average intensity on each array to a common target signal. To evaluate the specificity of hybridizations, each gene on the Affymetrix arrays is represented by several matched as well as miss-matched oligos. Based on the calls from the cRNA hybridized to the different oligos, an algorithm is used to evaluate whether specific gene expression is detected on the arrays; that is the transcript is scored as present or absent. Another algorithm is used to estimate amount of specific mRNA, which is reported as an absolute call in arbitrary units. Despite these quality controls, microarray results always need to be confirmed by another independent expression assay. For this purpose we used the sensitive technique of qRT PCR.

There are several drawbacks with microarray gene profiling. If probesets are poor, genes will be scored as absent in the tissue on the microarrays, although they may be expressed in the tissue. Furthermore, with the microarray technique it is difficult to accurately measure and detect differences in mRNA levels for genes with low

expression levels. In addition, the microarrays we have used cannot distinguish between different mRNA splice variants of a gene.

3.5.3 Quantitative real time PCR (qRT-PCR)

A common method to measure specific mRNA levels is quantitative real time PCR. This is a good independent method used often to verify the results given from microarrays.

We used two different approaches to the qRT-PCR technique; the intercalation dye technique with SYBR Green and the TaqMan probe approach. The basis of both techniques is that a fluorophore binds to the PCR product and that the intensity of the fluorescence increases as the PCR product accumulates. This is done in different ways in the two techniques. The SYBR Green technique is a non-specific reporter of product amount in the PCR reaction. In the TaqMan approach you use a labelled hybridization probe, usually 20-30 nucleotides long, for the gene of interest. The fluorescent probe is labelled at the 5' end with a fluorescent reporter molecule (ex. fluorescein) and on the other end 3' with a quencher – usually a tetramethylrhodamine derivative. The probe capitalizes on the 5' exonuclease activity of Taq polymerase to cleave a labelled probe during the extension phase of the PCR. When the fluorophore is at close proximity to the quencher the fluorophore does not emit any light when excited by an outside light source. When the Taq polymerase encounters the probe during extension from one of the primers, it digests the probe freeing the reporter from the quencher and the reporter fluorescence can be detected and measured.

In early cycles of the reaction the change in amount of reporter fluorescence is usually undetectable, but at a point of amplification the accumulation of product results in a measurable change in the total fluorescence of the reaction mixture. This point in the reaction when the fluorescence reaches above the background is called the threshold cycle (Ct). There is a linear relationship between the log of the starting amount of different samples and their corresponding Ct during qRT-PCR.

The assessments of mRNA expression in the samples are calculated by either a standard curve or the 2 Delta Ct ratio formula:

$$2^{(Ct \text{ target gene calibrator} - Ct \text{ target gene sample})}$$

$$2^{(Ct \text{ reference gene calibrator} - Ct \text{ reference gene sample})}$$

The calibrator sample is randomly picked for each PCR run. Thus the quantification is relative to a calibrator. That is why arbitrary units are used. In the qRT-PCR you can use a plasmid standard curve of known copy number for absolute quantification of starting amount of mRNA in each sample.

One of the main differences between using the SYBR Green and the TaqMan approach is that the intercalating dye technique can have a lower specificity. The SYBR Green intercalating dye binds into all double stranded PCR products and thus can give a false positive signal. It is therefore very important to make a melt curve to see that the product is specific to one melting point. Also to be on the safe side one should visualise the product on a gel by electrophoresis. The TaqMan probes are in comparison much more specific. The one drawback with this technique is that it is more expensive than SYBR Green.

3.5.3.1 Use of reference genes

The mRNA expression assessed by qRT-PCR needs to be normalized to a gene which is stably expressed, a so called reference gene. A single perfect reference gene is hard to select. The crucial factor with the reference gene is that it should be stably expressed and not be regulated by the complication or treatment being studied. At our laboratory we have chosen to use both beta 2 microglobulin (B2MG) and the ribosomal 18S. In our hands, these two reference genes appear to be the best to use, so far. There is still a continuing discussion about the best reference gene to be used in qRT-PCR and many studies use several reference genes.

3.6 QUANTIFICATION OF MITOCHONDRIAL DNA

The mitochondrial DNA (mtDNA) assay is based on qRT-PCR. In this assay the quantification is based on a standard curve of plasmids in known copy numbers. The plasmids either contain the mitochondrial gene NADH dehydrogenase subunit 1 (ND1)

or the nuclear gene lipoprotein lipase (LPL). We obtained total DNA from adipose tissue pieces, isolated adipocytes and SVF and diluted to the proper concentrations. The qRT-PCR is based on the TaqMan probe method. The ratio of ND1 to LPL reflects the amount of mtDNA/cell in each sample. It is important to keep in mind that this is only a reflection of the mtDNA amount of the cells, not a direct measure of mitochondria mass or function. To complement this assay it is recommended to also assess the mRNA expression of electron transport chain genes and the genes involved in beta oxidation of fatty acids to get a picture of the function of the mitochondria in the sample.

3.7 QUANTIFICATION OF PROTEIN

3.7.1 Western blot

Western blot is used for most protein quantifications. First the total protein is isolated from adipose tissue pieces and isolated adipocytes using a method previously described (82). After isolation the protein concentration is measured spectrophotometrically.

Western blot is based on the separation of proteins according to size on a gel with electrophoresis. It is therefore possible to distinguish between different isoforms of the same protein if they are of different sizes. The protein samples are denatured by heating and adding sodium dodecyl sulphate (SDS). In study II where SDS PAGE was used, we first immunoprecipitated the proteins with a polyclonal antibody overnight and then added protein A sepharose in order to enrich the protein before the gel electrophoresis. This is a way to obtain reliable results at low protein concentration. In addition to this we added dithiothreitol (DDT), a strong reducing agent, in order to optimize the reduction of the FLAP protein which is found as tri- di- and monomers (83). After the gel electrophoresis the proteins are blotted to a polyvinylidene fluoride (PVDF) membrane. On this membrane the protein of interest is detected with specific antibodies. The quantification of the protein is made with chemiluminescence. In the western blot there should always be a positive control together with the samples as well as a size marker. These are both usually commercially available today.

3.7.2 Enzyme linked immunosorbent assay (ELISA)

ELISA is another antibody based method primarily used to measure circulating or secreted proteins. The antibodies directed to the protein of interest are bound to a test plate. The samples together with an appropriately diluted standard curve are added to

the assay. After protein binding, a second specific antibody is added to the assay. The protein amount is quantified by chemiluminescence. We used ELISA for quantifying protein from *in vitro* secretion studies. In the adipocyte incubations it is important to make sure that the cell fraction is clean from any contaminating cells, i.e. endothelial cells, macrophages etc. In order to validate the purity of the cell fractions we use the microscope.

One drawback with these two protein quantification methods is that they do not reflect any functional effect of the eventual difference in protein levels. In order to investigate whether the protein of interest is giving a functional effect in the cell one should always do complementary experiments for elucidating the function of the protein.

3.8 CELL CULTURE

There are several types of cell cultures used in obesity research and I will briefly describe the cell culture used in study III.

3.8.1 Human primary adipocyte cultures

The method of isolating human adipocyte precursor cells (19) were further developed in our laboratory (84). Briefly, the SVF is isolated from adipose tissue pieces from the subcutaneous or visceral depot and the adipocyte precursor cells of the SVF are isolated, proliferated and then differentiated on cell culture plates for approximately 16 days. These cells are easy to work with in the laboratory since they are attached to the plate. A big advantage is also that they are primary cells whose phenotype could reflect primary factors that are genetically induced (84). A disadvantage is that the cells are affected by the *in vitro* culturing in itself. These cells are differentiated from the SVF and are different from the mature adipocytes isolated from adipose tissue pieces. In study I the protein secretion was quantified from isolated mature adipocytes.

3.9 ASSESSMENT OF LIPID TURNOVER

To estimate the lipid turnover in human mature adipocytes we performed lipolysis, lipogenesis and fatty acid oxidation experiments as follows.

3.9.1 Lipolysis

The lipolysis experiments are made on isolated mature adipocytes from adipose tissue pieces. In this method glycerol release from the adipocytes are used as an index of lipolysis.

Briefly, diluted cell suspensions, 2% vol/vol, were incubated in duplicates for 2 hours with air as the gas phase at 37°C in Krebs-Ringer phosphate buffer at pH 7.4. The buffer was supplemented with glucose, ascorbic acid and bovine serum albumin without, basal, or with increasing concentrations of either noradrenaline or insulin and glycerol release was determined. In the lipolysis experiments with insulin, the standard medium was also supplemented with the PDE3B sensitive cAMP homologue, 8 bromo cyclic AMP and adenosine deaminase. Glycerol release was measured based on a bioluminometric assay (85). The amount of lipolysis is expressed as the amount of glycerol released per 2h per 10^7 fat cells.

3.9.2 Lipogenesis

The lipogenesis experiments to assess the incorporation of tritiated glucose into TAG in the adipocytes were performed on isolated mature adipocytes (86). In the lipogenesis experiments, the standard medium was supplemented with a low concentration of unlabeled and ^3H -labelled glucose and insulin whereafter radioactive incorporation into total lipids was determined. Results are expressed as amount of glucose incorporated into lipids per 2h per 10^7 fat cells. Half maximum effective hormone concentration, EC_{50} , is determined and turned into a pD_2 value ($-\log_{10} \text{EC}_{50}$). Maximum effect was calculated as lipolysis or lipogenesis at maximum effective concentration.

3.9.3 Fatty acid oxidation

The assay of quantification of fatty acid oxidation is foremost used in assessing fatty acid oxidation rates in human skeletal muscle cells. The method has been set up on differentiated primary human adipocytes, described by Tiraby C. and colleagues (70). Briefly, the differentiated adipocytes were incubated with ^{14}C -palmitate substituted medium. Afterwards, the retrieval of fatty acid oxidation metabolites was made. There are two fractions of fatty acid oxidation metabolites, the $^{14}\text{CO}_2$ - fraction and the acid soluble products (ASP). The two ^{14}C -labelled fractions of palmitate oxidation metabolites reflect the rate of oxidation in the cells. This method was used in study III on TNF-alpha treated human preadipocytes. In the ^{14}C palmitate oxidation method it is

somewhat hard to get reliable results since the level of fatty acid metabolites in differentiated primary human adipocytes is quite low.

3.10 DATA ANALYSIS

Microarray gene expression profiles were analyzed both for differences in expression of single genes, as well as gene sets or pathways, between nonobese, obese and or type 2 diabetic patients. We had to consider a number of factors in these analyses:

- (1) The simultaneous analysis of thousands of genes on the microarrays represents a multiple testing problem, i.e. with a nominal P value of 5% there will be many false positive significant results.
- (2) For multifactorial traits like obesity and type 2 diabetes, the change in expression of specific genes may be small and non-significant. However, small differences in expression of several genes may cause important up- or downregulation of biological pathways or gene sets.
- (3) One last question that is partially related to the previous ones; what threshold for fold changes in expression can be reliably measured and validated, and is biologically relevant?

In paper I we used Student's *t* test when we analyzed specific immune response genes, as opposed to all transcripts on the microarrays, for differences between groups. Student's *t* test does not consider multiple comparisons. However, the results were highly significant, i.e. allowed for adjustment for multiple comparisons, and were confirmed by qRT-PCR. In paper III, the microarrays were screened for differences in expression of individual genes between groups by Significance analysis of microarrays (SAM) (87), which uses permutations to adjust for multiple tests caused by the presence of thousands of genes on the microarrays. The results are based on a false discovery rate rather than a significance level. We chose a false discovery rate of 5%. We accepted that this threshold would produce some false positive genes. This could not be avoided as we wanted to detect genes with small differences in expression between groups for subsequent pathway analysis.

We used computational methods that determine whether an *a priori* defined set of genes shows statistically significant, concordant differences between two biological states to screen for differences in expression of pathways or genes sets. We used the

MAPPFinder program to rank the magnitude in gene expression differences of the GenMAPP pathways and Gene Ontology terms between nonobese and obese or type 2 diabetic patients (88-90). The analysis in MAPPFinder is based on the percentage of genes in each pathway that meets a user-defined criterion for change in expression. We used a relaxed criterion, since it has been shown in pathway analysis to be relevant to include information about genes that are regulated by type 2 diabetes, although the regulation may not be significant at the single-gene level (60). In paper I, criteria for change in expression were nominal significant *t test* for change in expression of individual genes and mean signal fold change > 25%. In paper III, criterion for change was significant difference in expression according to SAM. To obtain additional support for the MAPPFinder results, we also used, in paper III, Gene Set Enrichment Analysis (GSEA), which does not require significant change in expression of individual genes (91).

Values reported in the papers are means and standard deviations as measure of dispersion. If possible, non-normally distributed phenotypes were log-transformed to become normally distributed. We used parametric tests with appropriate covariates for normally distributed phenotypes. Otherwise non-parametric tests were used. In analyses, except microarrays, a nominal p-value below 0.05 was considered significant

4 DISCUSSION OF RESULTS

4.1 STUDY I

In study I, we wanted to investigate which inflammatory markers were most upregulated at gene level in subcutaneous abdominal adipose tissue in obesity. In addition we wanted to investigate how this overexpression related to the corresponding protein secretion.

In initial analysis, we observed that among the obesity-associated gene ontology terms, immune response genes were most upregulated, so we further investigated these genes. Of these immune response genes, 53 were upregulated in obesity and three were downregulated. Among the 11 genes with a fold change in expression greater than 2 between obese and nonobese, seven were chemokines. This group also was the largest subgroup of immune response genes affected by obesity. No chemokine gene was downregulated in obesity. We also had chemokine receptors on the same microarray and we confirmed the expression of chemokine receptors in adipose tissue but there was no difference between the two groups.

For six investigated upregulated chemokine genes, the microarray results were confirmed by qRT-PCR in the same subjects. Next, we investigated the corresponding protein secretion of the chemokines with ELISA. Monocyte chemoattractant protein 1 (MCP1) was the only chemokine to show a time dependent secretion into the incubation medium for two hours from adipose tissue pieces and isolated adipocytes. The rate of release was about three times more rapid from adipose tissue pieces than from isolated mature adipocytes, see fig 3. The secretion of MCP1 from adipose tissue was six-fold increased in obese when compared with nonobese subjects. There was on the other hand no difference in the serum concentration of MCP1 between the two groups.

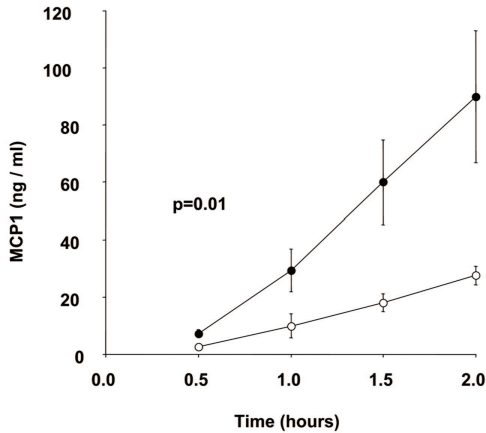


Figure 3.
Secretion of MCP1 from adipose tissue pieces (filled circles) and isolated mature fat cells (unfilled circles) derived from human subcutaneous adipose tissue.

To further elucidate whether the MCP1 secreted from the subcutaneous adipose tissue contributed to the systemic level of MCP1, MCP1 and leptin were quantified in a separate samples, in blood from the abdominal vein and arterialized blood. A similar experiment was performed in a cohort of obese and nonobese individuals. A higher level of adipokine in the abdominal vein would support that adipose tissue contributed so the systemic levels. However, there was no difference in the levels of MCP1 between the two blood compartments. There was no difference in MCP1 levels between obese and nonobese subjects, neither in the abdominal vein nor in arterialized blood.

We confirm hereby what other groups have found that obesity is characterized by activation of a number of immune and defence response genes in adipose tissue (45; 46). Our data are in line with a recent study showing MCP1 secretion from human adipose tissue with an increased level secreted from obese compared to lean individuals (38). In contrast to the study made by Christiansen T and colleagues (39) we could not report a difference in circulating MCP1 between nonobese and obese individuals. The novel finding in our study is that MCP1 has a time dependent secretion from adipose tissue pieces and more important from isolated mature adipocytes. MCP1 is supposedly a local mediator in adipose tissue since there was no difference in MCP1 levels between the abdominal and arterialized blood of nonobese and obese subjects.

When the MCP1 receptor CCR2 is knocked out in mice there is a significant decrease in diet induced obesity as well as adipose tissue inflammation measured as amount of macrophages (52). By overexpressing MCP1 specifically in adipose tissue in mice the amount of infiltrating macrophages increased into the tissue (50; 51). At low concentrations, MCP1 has earlier been shown to blunt insulin stimulated glucose uptake in murine 3T3-L1 adipocytes (40). Another interesting finding made by Bruun JM. and colleagues was that incubation with TZD decreased the secretion of MCP1 from pieces of human subcutaneous adipose tissue (38). This suggests that TZD increase insulin sensitivity by anti-inflammatory action.

These findings together give MCP1 a potential important role in promoting local inflammation in abdominal adipose tissue in human obesity. We suggest that MCP1 secreted from mature adipocytes and cells of the stromal vascular fraction attract macrophages and by itself induce insulin resistance (40; 50). Also TNF-alpha has been shown to stimulate the expression of MCP1 in differentiated primary human adipocytes suggesting that TNF-alpha is an inducer of MCP1 secretion from adipocytes (37).

4.2 STUDY II

In study II, we investigated the role of the gene ALOX5AP in human obesity. SNPs in the ALOX5AP gene were in an Icelandic study implicated in myocardial infarction and stroke in men (55). We wanted to elucidate whether ALOX5AP could contribute to adipose tissue insulin resistance or obesity.

We found no association between the CVD-risk ALOX5AP SNPs and obesity or insulin resistance measured by HOMA index. In contrast, the mRNA expression in adipose tissue pieces showed to be associated with both obesity and HOMA index. High adipose tissue mRNA expression of ALOX5AP was significantly associated with a high HOMA index, i.e. insulin resistance, independent of obesity. ALOX5AP mRNA was found to be expressed by several cells found in adipose tissue. We also found that the protein FLAP encoded by the ALOX5AP gene, as well as the enzyme 5-lipoxygenase, were expressed in subcutaneous adipocytes. We conclude that ALOX5AP may play a role in the development of insulin resistance in human adipose tissue in obesity.

We believe that the increased expression of ALOX5AP in obesity gives increased levels of the protein FLAP which, together with 5-LO results in an increased adipose tissue production of leukotriene B4 (LTB4). LTB4 has been shown to induce the secretion of MCP1 from human monocytes (92) connecting these two inflammatory markers in the development of the local inflammation found in the obese adipose tissue. Also MCP1 and LTB4 have been shown to recruit leukocytes to inflammatory sites (93). We hypothesise that these two inflammatory genes lead to an increased infiltration of macrophages, leading in turn to an increased secretion of other inflammatory markers and local insulin resistance in the adipose tissue of obese individuals.

Study I and II completes the inflammatory profile in obesity with two more factors possible contributing to the local inflammation found in obesity and suggested to induce insulin resistance.

4.3 STUDY III

In study III, we wanted to detect new pathways regulated by T2DM in adipose tissue. Based on preliminary results we subsequently focused on the impairment of mitochondrial oxidative phosphorylation.

Gene expression profiling was performed in adipose tissue pieces from the subcutaneous and visceral depot in healthy obese, obese T2DM and nonobese individuals. Using SAM we found several genes which were differentially expressed between the three groups. The biggest difference in gene expression was found between T2DM obese individuals and nonobese, especially in the visceral adipose tissue samples. This led us to further investigate the genes differentially expressed between obese T2DM and nonobese subjects by pathway analysis using MAPPFinder and GSEA. Two pathways regulated by T2DM in our samples were common to these analyses, electron transport and Krebs TCA cycle. This led us to further study the regulation of electron transport chain genes in visceral fat of the T2DM individuals. 28 out of 91 electron transport chain genes were shown to be downregulated in the type 2 diabetic individuals. No gene was upregulated, and no electron transport chain gene was downregulated in visceral fat of healthy obese individuals.

We found an overlap between our findings and the findings by Mootha VK. et al. and Patti ME. et al. (60; 61). Six downregulated genes in our study overlapped with the genes in OXPHOS found in Moothas study, and 11 genes overlapped with the OXPHOS genes shown to be downregulated in skeletal muscle in insulin resistant individuals by Patti ME. and colleagues. We next verified our findings with qRT-PCR on five randomly selected electron transport chain genes that were downregulated and five that were unchanged between T2DM and controls. Our data on the ten genes from qRT-PCR were consistent with the microarray data.

Next, our aim was to understand how this downregulation of electron transport chain genes was mediated. We hypothesised that TNF-alpha was the factor behind the downregulation of OXPHOS genes. We incubated differentiated primary human adipocytes with TNF-alpha during 48 h. This resulted in a 20-40% decrease in gene expression of the electron transport genes we studied; in addition there was a significant decrease in fatty acid oxidation of about 30 % in the TNF-alpha treated cells. Even though no more than 0.5 % of the fatty acids produced during lipolysis are oxidized in the adipocytes this process is highly regulated (94). T2D's are known to increase the expression of fatty acid oxidation genes as well as fatty acid oxidation itself (64; 66). Since T2D increase insulin sensitivity this implies that reduced fatty acid oxidation is a factor behind insulin resistance in adipose tissue.

Our finding of downregulation of electron transport chain genes are in line with a study performed in mice where weight gain has been associated with downregulation of OXPHOS genes in visceral fat (95). What is novel with our study is that the downregulation in gene expression is depot specific and associated with T2DM independent of obesity. We have also found a new role for TNF-alpha in visceral fat, to disturb adipocyte metabolism through mediating the downregulation of OXPHOS genes and fatty acid oxidation.

4.4 STUDY IV

Study IV was performed with the aim to further investigate the role of adipose tissue mitochondria in relation to clinical and experimental parameters of insulin resistance. We here assessed the mitochondrial DNA (mtDNA) amount/cell in adipose tissue as a measure of mitochondria amount. Firstly, we found a minor decrease of adipose tissue

mtDNA/cell with increased BMI and age, independent of each other. MtDNA/cell was not affected by either moderate or marked weight reduction. Also the mitochondrial DNA in adipose tissue was suggested to be found mainly in adipocytes, since the mtDNA/cell was higher in this celltype compared to SVF cells. We found no association between adipose tissue mtDNA/cell and either HOMA index or intravenous insulin tolerance test.

The striking finding we made was that there was a strong positive association between mtDNA/cell in adipose tissue and both basal and insulin stimulated lipogenesis. This finding suggests a link between adipocyte mitochondria and lipid metabolism. As a complement to the mtDNA measurements we also measured mRNA expression of several electron transport chain genes, as well as genes regulating fatty acid oxidation, in order to see if there was a correlation between mtDNA/cell and these parameters. The association between the mRNA expressions in the various genes was all significantly stronger than the association between mtDNA/cell and the individual gene expressions. This suggests that mtDNA/cell is one, but not the only, factor regulating mitochondrial energy expenditure. We also found association between the expression of the electron transport chain genes cytochrome c oxidase 1 (COX1), ATP synthase 6 (ATP6) and NADH dehydrogenase 1 β subcomplex 4 (NDUFB4) and basal lipogenesis. Responsiveness of lipogenesis to insulin was found to associate with the expression of COX1, ATP6, medium-chain specific acyl-coenzyme A dehydrogenase (MCAD) and PGC1 alpha. We conclude that the amount of mitochondrial DNA/cell is fairly stable in human white adipose tissue in healthy adults and may play a role as a local regulator of adipocyte lipogenic capacity.

It has been reported that in human skeletal muscle insulin increases oxidative capacity and increases gene expression of mitochondrial and nuclear proteins involved in oxidative phosphorylation (96). In T2DM individuals this effect was not seen. Giving our results linking mtDNA/cell in adipose tissue with insulin induced lipogenesis in isolated mature adipocytes. We propose that there is a link between insulin and mitochondria also in adipocytes which we not yet know of.

This study adds to the new findings that put the adipose tissue mitochondria into focus in the study of obesity and insulin resistance. Previous studies have shown that mitochondria are impaired in white adipose tissue in obese mice, and can be

normalized with TZD treatment (64). Two independent studies in humans have shown that TZDs induce mitochondrial biogenesis in WAT *in vivo* in T2DM patients (66; 67). Whether the impairment of mitochondria is a cause or a consequence of obesity and insulin resistance still remains to be investigated.

5 GENERAL DISCUSSION

The four studies in this thesis contribute to enhance the picture on how adipose tissue, in particular through inflammatory mediators and mitochondria, can play a role in development of insulin resistance in obesity in humans.

The chemokine MCP1 was the only upregulated chemokine to be secreted in a time-dependent manner from adipose tissue pieces as well as from isolated mature adipocytes giving this chemokine a potential role as a contributor of local inflammation, macrophage recruitment and insulin resistance in adipose tissue (50). The MCP1 secreted from the abdominal adipose tissue does not contribute to the systemic levels of MCP1. Thus MCP1 seems to act in a local manner to induce insulin resistance. Of note, there are studies that do not support this theory where the knockout of both MCP1 and its receptor CCR2 in adipose tissue in mice caused no increase of macrophage infiltration in the adipose tissue and no change in insulin or glucose levels (53; 54). However it is well known that genetic manipulation such as gene knockout can give paradoxical results as well as different effects in different genetic backgrounds. This may be due to compensatory mechanisms. We therefore interpret these animal studies with caution.

We found that adipose tissue mRNA of the gene ALOX5AP was strongly associated to a high HOMA index independent of obesity. Its protein FLAP is expressed by human mature adipocytes. The expression level of ALOX5AP mRNA was normalized after a marked weight reduction suggesting that this gene is not a cause of obesity but a regulator of insulin resistance in human WAT. Thereby this gene is not only implicated in CVD risk in men, but also in the regulation of insulin resistance in human adipose tissue.

Our finding of downregulated expression of several electron transport chain genes selectively in the visceral adipose tissue of obese T2DM individuals was intriguing. We showed that the cytokine TNF-alpha, which is upregulated in obesity and involved in insulin resistance in adipose tissue (30), downregulated the electron transport chain genes in differentiated primary human adipocytes as well as decreased fatty acid oxidation; an important pathway for generating electron donors for the OXPHOS chain.

This gives TNF-alpha yet another pathway in which it interferes with adipocyte metabolism and leads to disturbed energy turnover.

To complement the results in study III we undertook study IV in which we assessed mtDNA copy number in subcutaneous adipose tissue in relation to parameters of insulin resistance in healthy obese and nonobese women and men. A somewhat surprising finding was that mtDNA copy number/cell displayed a significant and positive correlation to basal lipogenesis and especially with insulin induced lipogenesis, independent of BMI and age. As much as around 14% of the variance in lipogenesis could be explained by the variance in mtDNA/cell. The downregulation of PGC1 alpha mRNA in adipose tissue in obesity is in agreement with previous results in skeletal muscle of T2DM individuals (60; 61). It should be noted that this study was made in subcutaneous adipose tissue and there could be even stronger associations between mtDNA/cell, mitochondrial gene expression and adipocyte metabolism in visceral adipose tissue.

The study made by Bogacka I and colleagues (67) showed a clear decrease of adipose tissue mtDNA copy number in T2DM individuals compared to healthy controls which was normalized by TZD treatment. This, together with our finding, gives WAT mitochondria a possible role in development of insulin resistance and impaired lipogenesis. If the impaired mitochondria are the cause or the result of T2DM and impaired lipogenesis remains to be elucidated, but it is an attractive theory that the impaired mitochondria with their important role as the power plants of the cells play a role in the development of insulin resistance.

Our finding that TNF-alpha downregulates electron transport chain genes supports the theory that adipose tissue mitochondria play a role in obesity and insulin resistance. We know that TNF-alpha secretion is upregulated in obesity and acts as a local inducer of insulin resistance in adipocytes (29). If our *in vitro* experiment hold true for the *in vivo* situation, this reveals a new route for TNF-alpha in inducing insulin resistance in visceral adipose tissue. It also implicates reduced fatty acid oxidation in adipocytes in the development of insulin resistance in obesity. The downregulated fatty acid oxidation indirectly leads to a higher systemic FFA level and these FFAs are known to lead to ectopic fat disposition in skeletal muscle and liver and thus induce insulin resistance in these organs.

The fact that the electron transport chain genes were downregulated markedly in visceral fat in T2DM gives this depot another important role in the generation of insulin resistance in obesity. The adipocytes in this depot have a higher basal lipolysis together with lower insulin sensitivity, which together ends up in a higher secretion of FFA. The visceral depot is drained by the portal vein straight to the liver. So the FFAs released from the adipocytes go directly through the portal vein into the liver (97). The intrahepatic lipids lead to increased gluconeogenesis and increased synthesis of the lipoproteins VLDL and LDL. We hypothesize that reduced fatty acid oxidation in WAT contribute to increased release of fatty acids from visceral fat, hereby contributing to insulin resistance.

We suggest after these studies an additional connection between the inflammatory profile found in human obesity, with an increased secretion of TNF-alpha, MCP1 and 5-LO products in the WAT, and insulin resistance. The increased TNF-alpha induces an impairment of the mitochondrial oxidative phosphorylation. The reduced fatty acid oxidation indirectly leads to a higher level of circulation free fatty acids, known to induce peripheral insulin resistance.

It is becoming clear that the human white adipose tissue is highly active in the development of insulin resistance in obesity. In figure 4, I suggest a summary of the hypothetical interconnections between the findings in this thesis.

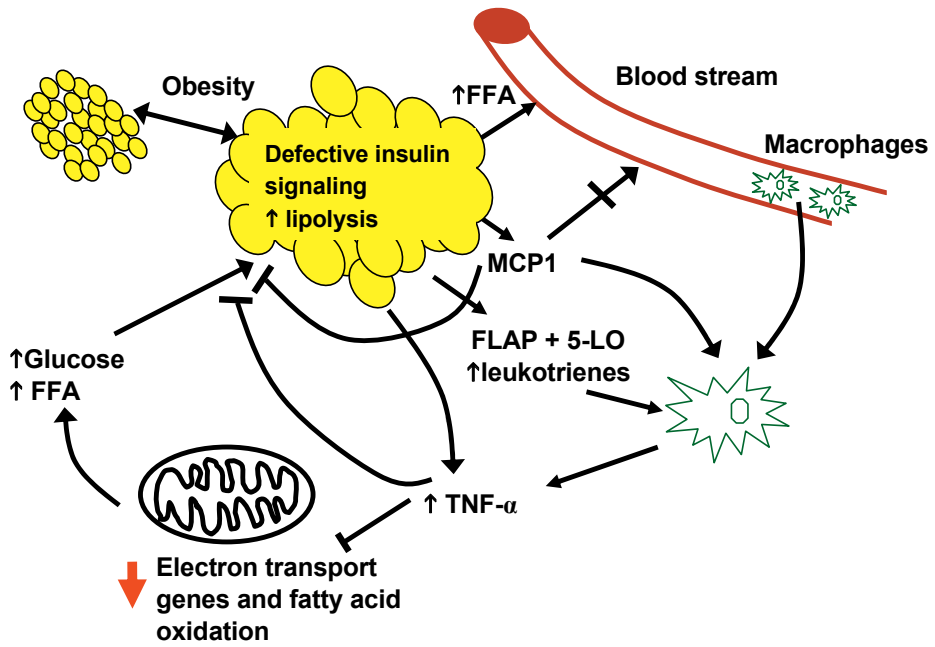


Figure 4. Overview of the factors studied in this thesis and there hypothesized interconnections and mediation of insulin resistance in human adipose tissue in obesity.

6 FUTURE PERSPECTIVES

The findings in this thesis add on to the importance of investigating human adipose tissue metabolism and its role in development of insulin resistance in obesity.

To continue the characterization of the inflammation present in adipose tissue in obesity one would like to measure other inflammatory genes and products in adipose tissue, such as 5-LO products like LTB₄.

The findings of the downregulation of mitochondrial genes in visceral fat in T2DM open for many studies to investigate the function of white adipocyte mitochondria. It would be interesting to investigate how cytokines like TNF- α could mediate the downregulation of electron transport chain genes and the fatty acid oxidation.

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