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PATHOGENESIS AND TREATMENT OF LIPODYSTROPHY IN HIV-INFECTED PATIENTS RECEIVING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

Jussi Sutinen

ACADEMIC DISSERTATION

To be presented with the permission of the Medical Faculty of the University of Helsinki for public examination in Auditorium 2, Biomedicum, Haartmaninkatu 8,
on December 19th, 2003, at 1 p.m.

Helsinki 2003

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ISBN 952-91-6730-X (paperback)
ISBN 952-10-1568-3 (pdf)
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2003

ABSTRACT

Background and aims. Highly active antiretroviral therapy (HAART) has improved the prognosis of HIV-infected patients, but is also associated with adverse events, such as lipodystrophy and insulin resistance caused by unknown mechanisms. Glitazones appear promising drugs to treat HAART-associated lipodystrophy (HAL), since they both improve insulin sensitivity and increase the amount of subcutaneous adipose tissue (SAT) in patients with type 2 diabetes. Present studies were undertaken to gain insight into the pathogenesis of HAL, and to evaluate whether rosiglitazone could increase the amount of SAT in these patients.

Subjects and methods. Three groups were included in the study: HIV-infected, HAART-treated patients with (HAART+LD+, n=25-30) and without lipodystrophy (HAART+LD-, n=9–13), and HIV negative subjects (HIV-, n= 15–35). Effects of rosiglitazone (8 mg/d for 24 weeks) were studied in a randomized, double-blind, placebo-controlled trial in the HAART+LD+ group. Body composition was measured using magnetic resonance imaging, liver fat by proton spectroscopy, and gene expression in SAT by real-time PCR.

Results. Liver fat content was increased in the HAART+LD+ compared to the HAART+LD- and the HIV-group, and correlated with fasting serum insulin concentrations. Serum adiponectin and its expression in SAT were decreased in the HAART+LD+ compared to the HAART+LD- group, and correlated inversely with features of insulin resistance. The expression of peroxisome proliferator-activated receptor (PPAR) γ and δ, sterol regulatory element-binding protein 1c, PPARγ coactivator-1 (PGC-1), lipoprotein lipase, acyl CoA synthase and glucose transport protein 4 were decreased, whereas the expression of CD45 and interleukin 6 were increased in the HAART+LD+ compared to the HAART+LD- group. Rosiglitazone treatment did not increase the amount of SAT. Rosiglitazone decreased serum insulin concentration and liver fat content, but worsened dyslipidemia. Rosiglitazone increased the expression of adiponectin, PPARγ and PGC-1, and decreased the expression of IL-6. PAI-1 concentration in plasma and its expression in SAT were increased in the HAART+LD+ compared to the HAART+LD- and the HIV- group. Rosiglitazone did not change the expression of PAI-1 in SAT, but caused a decrease in plasma PAI-1 concentration, which correlated with the decrease in the liver fat content.

Conclusions. Increased liver fat content may contribute to insulin resistance and to plasma PAI-1 concentrations in patients with HAL. Multiple alterations in gene expression in SAT imply decreased adipocyte maturation, increased inflammation and decreased adiponectin production, which all may contribute to insulin resistance. The present data do not support use of rosiglitazone in patients with HAL, although it decreased liver fat content and fasting serum insulin concentrations. The insulin-sensitizing effects of rosiglitazone may have been mediated by the increased expression of adiponectin.

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ORIGINAL PUBLICATIONS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I. Sutinen J, Häkkinen AM, Westerbacka J, Seppälä-Lindroos A, Vehkavaara S, Halavaara J, Järvinen A, Ristola M, Yki-Järvinen H. Increased fat accumulation in the liver in HIV-infected patients with antiretroviral therapy-associated lipodystrophy. *AIDS* 2002;16:2183-93.

II. Sutinen J, Korsheninnikova E, Funahashi T, Matsuzawa Y, Nyman T, Yki-Järvinen H. Circulating concentration of adiponectin and its expression in subcutaneous adipose tissue in patients with highly active antiretroviral therapy-associated lipodystrophy. *J Clin Endocrinol Metab* 2003;88:1907-1910.

III. Kannisto K, Sutinen J, Korsheninnikova E, Fisher RM, Ehrenborg E, Gertow K, Virkamäki A, Nyman T, Vidal H, Hamsten A, Yki-Järvinen H. Expression of adipogenic transcription factors, peroxisome proliferator-activated receptor gamma co-activator 1, IL-6 and CD45 in subcutaneous adipose tissue in lipodystrophy associated with highly active antiretroviral therapy. *AIDS* 2003;17:1753-62.

IV. Sutinen J, Häkkinen AM, Westerbacka J, Seppälä-Lindroos A, Vehkavaara S, Halavaara J, Järvinen A, Ristola M, Yki-Järvinen H. Rosiglitazone in the treatment of HAART-associated lipodystrophy – a randomized double-blind placebo-controlled study. *Antivir Ther* 2003;8:199-207.

V. Sutinen J, Kannisto K, Korsheninnikova E, Fisher RM, Ehrenborg E, Nyman T, Virkamäki A, Funahashi T, Matsuzawa Y, Vidal H, Hamsten A, Yki-Järvinen H. Effects of rosiglitazone on gene expression in subcutaneous adipose tissue in highly active antiretroviral therapy - associated lipodystrophy. Submitted.

VI. Yki-Järvinen H, Sutinen J, Silveira A, Korsheninnikova E, Fisher RM, Kannisto K, Ehrenborg E, Eriksson P, Hamsten A. Regulation of plasma PAI-1 concentrations in HAART-associated lipodystrophy during rosiglitazone therapy. *Arterioscler Thromb Vasc Biol* 2003;23:688-694.

The publications II and III have also been included in the doctoral thesis by Elena Korsheninnikova entitled ”Molecular mechanisms of insulin resistance in human skeletal muscle and lipodystrophic adipose tissue” (University of Helsinki, 2003).

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ABBREVIATIONS

11β-HSD	11β-hydroxysteroid dehydrogenase
ACC	acetyl coenzyme A carboxylase
ACS	acyl coenzyme A synthase
AIDS	acquired immunodeficiency syndrome
ALBP	adipocyte lipid binding protein
ALT	alanine aminotransferase
AMPK	adenosine monophosphate-activated protein kinase
ASP	acylation stimulating protein
BIA	bioelectrical impedance analysis
BMI	body mass index
cAMP	cyclic adenosine monophosphate
C/EBP	CCAAT/enhancer-binding protein
CETP	cholesteryl ester transfer protein
cIAP	cellular inhibitor of apoptosis protein
CoA	coenzyme A
CRP	C-reactive protein
CT	computed tomography
DEXA	dual-energy x-ray absorptiometry
DNA	deoxyribonucleic acid
FABP	fatty acid binding protein
FABPpm	plasma membrane-associated fatty acid binding protein
FAS	fatty acid synthase
FAT	fatty acid translocase
FATP	fatty acid transport protein
FFA	free fatty acids
GLUT	glucose transport protein
HAART	highly active antiretroviral therapy
HAART+LD+ group	HAART-treated patients with lipodystrophy
HAART+LD- group	HAART-treated patients without lipodystrophy
HAL	HAART-associated lipodystrophy
HDL	high density lipoprotein
HIV	human immunodeficiency virus
HIV- group	HIV-negative subjects
HSL	hormone sensitive lipase

IL	interleukin
IMCL	intramyocellular lipid
IRS	insulin receptor substrate
KLBP	keratinocyte lipid binding protein
LDL	low density lipoprotein
LPL	lipoprotein lipase
MAP kinase	mitogen-activated protein kinase
MRI	magnetic resonance imaging
mRNA	messanger RNA
mtDNA	mitochondrial DNA
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
ND	not done
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NS	non significant
OGTT	oral glucose tolerance test
PAI-1	plasminogen activator inhibitor-1
PBMC	peripheral blood mononuclear cell
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1	PPARγ coactivator –1
PI	protease inhibitor
PI 3-kinase	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator-activated receptor
SAT	subcutaneous adipose tissue
SEM	standard error of mean
SREBP	sterol regulatory element-binding protein
TG	triglycerides
TNF	tumor necrosis factor
UCP	uncoupling protein
VAT	visceral adipose tissue
VLDL	very low density lipoprotein
WHR	waist to hip ratio

1. INTRODUCTION

The prognosis of human immunodeficiency virus (HIV) -infected people has dramatically improved after the introduction of highly active antiretroviral therapy (HAART) in 1996 (1). However, eradication of the virus is not possible with current regimens (2), and therefore patients need to use HAART permanently. HAART is also associated with adverse events, such as lipodystrophy, i.e. loss of subcutaneous fat (lipoatrophy) and accumulation of intra-abdominal fat, and insulin resistance (3). During the last few years, HAART-associated lipodystrophy (HAL) has become the most common form of human lipodystrophy. Severe lipodystrophy, especially facial lipoatrophy can be stigmatizing and reduce adherence to otherwise effective HAART (4,5). Long term consequences of the adverse events still remain unknown, but preliminary data suggest that HAART is associated with increased cardiovascular morbidity (6).

The pathogenesis of HAL remains unknown. It is not known whether lipoatrophy results from decreased differentiation of adipocytes, increased loss of adipocytes, or both. The inability to store fat in adipose tissue in patients with non-HIV lipodystrophies and in lipodystrophic mouse models results in fat accumulation in the liver and skeletal muscle, which is associated with development of insulin resistance (7,8). Whether this occurs also in HAL is not known. Adipose tissue is an active endocrine organ, which produces several proteins that regulate whole body metabolism (9). Data are sparse regarding the possible contribution of altered secretory function of the adipose tissue to the pathogenesis of HAL.

Currently there is no pharmacological treatment available for HAL. Thiazolidinediones are novel insulin-sensitizing agents, which increase subcutaneous fat mass in patients with type 2 diabetes (10). The latter is an undesirable side effect in patients with type 2 diabetes. However, in patients with HAL, both the adipose tissue-increasing and insulin-sensitizing effects of thiazolidinediones would be beneficial. Thiazolidinediones therefore appear promising drugs for the treatment of HAL, but have not been tested in a controlled trial.

The present studies were undertaken to gain insight into the pathogenesis and treatment of HAL. We examined whether the adipocyte differentiation is abnormal in lipodystrophic adipose tissue by measuring the expression of several transcription factors and other genes necessary for normal maturation of adipocytes. We also evaluated physiologic function of lipodystrophic adipose tissue by quantifying the expression of several adipocytokines, e.g. adiponectin, leptin and interleukin (IL) -6 in adipose tissue and their circulating concentrations. We studied whether liver fat content measured using proton spectroscopy is increased in HAL, and whether liver fat content is associated with features of insulin resistance. We also studied the possibility that liver fat content could be a significant correlate of the concentration of plasminogen activator inhibitor-1 (PAI-1) in plasma. Finally, we conducted a randomized, placebo-controlled, double-blind trial to

evaluate whether rosiglitazone could increase the amount of subcutaneous adipose tissue (SAT) in patients with HAL. Currently there are no human *in vivo* data available on the effects of rosiglitazone on gene expression in adipose tissue. We therefore quantified the expression of multiple genes, which could possibly be involved in the insulin-sensitizing action of rosiglitazone in subcutaneous fat of patients with HAL.

2. REVIEW OF THE LITERATURE

2.1. INSULIN RESISTANCE AND ADIPOSE TISSUE METABOLISM

2.1.1. PHYSIOLOGIC ACTIONS OF INSULIN

GLUCOSE METABOLISM

Maintenance of plasma glucose concentration within narrow limits is of vital importance to humans. Insufficient glucose availability would be deleterious especially to the brain and other neuronal tissues, which cannot use alternative energy sources. At any given moment, plasma glucose concentration represents the balance between glucose absorption from the intestine, endogenous glucose production and glucose utilization. Insulin serves as the main regulator of blood glucose concentration by inhibiting hepatic glucose production and by increasing glucose uptake primarily in skeletal muscle (11).

Endogenous glucose production

In the fasting (postabsorptive) state, an equal amount of glucose is produced and utilized. The liver produces most of the circulating glucose in the fasting state. Also the kidneys can synthesize glucose, but it is considered important only following prolonged fasting (11). The liver can produce glucose by breaking down glycogen (glycogenolysis) or by *de novo* glucose synthesis mainly from lactate, alanine, pyruvate and glycerol (gluconeogenesis) (11). The early studies suggested that glycogenolysis accounted for ~75% of glucose production after an overnight fast (11). However, novel *in vivo* measurements using ^{13}C magnetic resonance imaging (MRI) spectroscopy have shown that gluconeogenesis accounts for up to 50% of the hepatic glucose production even during early hours of fasting (12). Total depletion of hepatic glycogen (70 to 150 g) occurs within 24-64 hours depending on the method used for quantification of glycogen stores (13).

Insulin inhibits both gluconeogenesis and glycogenolysis. In normal subjects, serum insulin concentration of ~30 mU/l halve hepatic glucose production and complete suppression is achieved at insulin concentrations of 50-60 mU/l in studies employing [3-^{3}H] glucose under non-steady state conditions (14). Insulin induces the transcription of sterol regulatory element binding protein 1c (SREBP-1c) by a phosphatidylinositol 3 (PI 3)-kinase dependent mechanism (*vide infra*) (15). After the proteolytic cleavage of the precursor SREBP-1c, the truncated, mature form of SREBP-1c translocates into the nucleus, where it activates transcription of glucose kinase, an enzyme that increases glucose phophorylation and glycogen repletion (15). Mature form of SREBP-1c also inhibits the transcription of phosphoenolpyruvate carboxykinase (PEPCK), an important enzyme in gluconeogenesis (15). Insulin also decreases the activity of the enzyme glycogen phosphorylase, which stimulates breakdown of glycogen to glucose (11,13). Furthermore, insulin indirectly decreases gluconeogenesis by suppressing lipolysis and proteolysis, thus reducing peripheral release of gluconeogenic precursors (11). An increase in plasma glucose concentration regulates hepatic glucose production by

inhibiting both gluconeogenesis and glycogenolysis, independent of changes in glucoregulatory hormones (11).

Glucagon rapidly increases both glycogenolysis and gluconeogenesis (16). Catecholamines also rapidly stimulate glycogenolysis and gluconeogenesis. However, their role for preventing hypoglycemia is considered significant only as a compensatory mechanism if glucagon secretion is deficient (17). Also glucocorticoids enhance hepatic glucose production, but in contrast to the acute stimulatory effects of glucagon and catecholamines, the effects of corticosteroids take several hours to occur. Corticosteroids activate gluconeogenic enzymes and augment the transfer of free fatty acids (FFA) to the liver (11). Growth hormone impairs the ability of insulin to suppress hepatic glucose production (18). In addition, a complex paracrine signaling system operates between Kuppfer cells, hepatocytes and endothelial cells and may, at least judging from animal data, regulate glucose production (13).

Other factors involved in hepatic glucose production include fat accumulation in the liver (Chapter 2.1.4), which is associated with hepatic insulin sensitivity in several animal models (Chapter 2.1.5.) and in humans (19). Adiponectin is an adipocyte-derived protein (Chapter 2.1.3.), which *in vitro* and in animal models has been shown to increase the ability of insulin to suppress glucose production and to downregulate the expression of enzymes involved in gluconeogenesis (20,21). Interestingly, adiponectin infusion in animals increases insulin sensitivity and decreases liver fat content (22).

Glucose utilization

Insulin regulates glucose utilization mainly by increasing glucose uptake in skeletal muscle. Under fasting conditions, when circulating concentration of insulin is low, glucose utilization occurs mainly in insulin-independent tissues, such as the brain, renal medulla and erythrocytes, which cannot use alternative energy sources. According to various studies, it has been estimated that the brain accounts for ~50%, splanchnic area (the liver and gut) ~25%, skeletal muscle and fat ~10%, kidneys ~6% and heart ~5% of the basal glucose disposal (14). Under fasting conditions, insulin-dependent tissues, such as skeletal muscle and splanchnic tissues use FFA as the main source of energy (11).

After oral glucose administration, insulin-dependent tissues switch their energy supply from FFA to glucose. Consequently, one third of glucose is taken up by skeletal muscle, one third by the splanchnic tissues and one third by other tissues, especially the brain (14,23).

Under intravenously maintained normoglycemic hyperinsulinemia, e.g. during hyperinsulinemic euglycemic clamp, glucose utilization can increase up to 6-fold compared to glucose utilization rate after an overnight fast (14). Under these experimental conditions, skeletal muscle by far accounts for most of glucose

utilization (~70%), the brain for 14%, heart 6%, splanchnic area 6%, kidneys 2%, and adipose tissue for 1% (14).

In order to exert its effect on cells, insulin must first bind to an extracellular α-subunit of its cell membrane-associated receptor. This binding leads to autophosphorylation of the intracellular β-subunit of the receptor, which consequently results in activation of the tyrosine kinase activity of the receptor (24). Tyrosine kinase catalyzes phosphorylation of several insulin receptor substrate (IRS) proteins. IRS-1 is the main IRS in skeletal muscle (25). Intracellular insulin signaling involves two major pathways: the mitogen-activated protein (MAP) kinase and the PI 3-kinase pathway. The MAP kinase pathway mediates growth-promoting effects of insulin and PI 3-kinase most of the metabolic responses to insulin, such as translocation of intracellular glucose transport protein 4 (GLUT4) (*vide infra*) to the cell membrane, and glycogen and protein synthesis (24).

Specific glucose transport proteins are needed for glucose entry into the cells. Seven functional isomers of glucose transport proteins are known today (11). GLUT4 is the main insulin-dependent glucose transport protein expressed in skeletal muscle and adipose tissue (26,27). Insulin-induced intracellular signaling results in translocation of the intracellular GLUT4 to the cell membrane and also enhances GLUT4 activity (16). GLUT1 is the main insulin-independent glucose transporter. It is expressed ubiquitously and is present on the cell surface (16). GLUT2 is present on the plasma membrane and mediates glucose entry into the hepatocytes (15). GLUT2 also mediates the export of glucose out of hepatocytes during gluconeogenesis (11).

LIPID METABOLISM

Lipoproteins are particles that transport hydrophobic lipids in the blood and mediate their delivery to various tissues. Dietary fat enters circulation in chylomicrons, which are triglyceride-rich lipoproteins synthesized by enterocytes in the small intestine (28). On the vascular endothelium, lipoprotein lipase (LPL) releases fatty acids from chylomicrons (28). FFA can then be taken up by tissues, such as skeletal muscle and adipose tissue. The resulting chylomicron remnant particles are cleared from the circulation by the liver (28).

The liver synthesizes both triglyceride and cholesterol, which are released into the circulation as very low density lipoproteins (VLDL) (28). Following the release of fatty acids from VLDL by endothelial LPL, VLDL are converted into VLDL remnants, intermediate density lipoproteins and finally into low density lipoproteins (LDL) (28). High density lipoprotein (HDL) particles can originate from the liver and the gut, and hydrolysis of chylomicrons and VLDL yield components which can form HDL particles (28).

Insulin suppresses VLDL secretion by directly inhibiting the assembly and production of VLDL particles (29). In addition, insulin suppresses VLDL production indirectly by decreasing FFA availability for VLDL

assembly by inhibiting lipolysis in adipose tissue (29). Insulin acutely increases the activity of LPL in adipose tissue (30), but normally decreases the activity of LPL in skeletal muscle (29,31). Lipolysis in adipose tissue is a very insulin sensitive process. Insulin inhibits lipolysis primarily through inhibiting hormone sensitive lipase (HSL), the rate-limiting enzyme of intracellular triglyceride hydrolysis in adipose tissue (32,33). These combined effects of insulin tend to "keep fat where it belongs", i.e. in adipose tissue (29).

FIBRINOLYSIS AND OTHER EFFECTS

PAI-1 is an inhibitor of fibrinolysis. Plasma PAI-1 concentrations are increased in insulin resistant subjects (34). *In vitro*, insulin increases the synthesis of PAI-1 in human vascular endothelial and smooth-muscle cells, and in hepatoma HepG2 cells (34,35). Insulin also increases PAI-1 expression in human subcutaneous adipocytes *in vitro* (36). The relative contributions of these tissues *in vivo* to PAI-1 production in different physiological and pathological situations are unknown.

Physiologic concentrations of insulin acutely decrease the stiffness of large arteries measured using pulse wave analysis (37). Insulin has also been shown to cause vasodilatation in peripheral resistance vessels, but this effect requires prolonged or high doses of insulin and its physiologic relevance has therefore been questioned (29). In hypothalamus, insulin stimulates sympathetic nervous system resulting in e.g. increases in sympathetic nervous activity in muscle (29). Insulin also regulates the autonomic control of heart rate by decreasing vagal and increasing sympathetic tone (29).

2.1.2. INSULIN RESISTANCE

Insulin resistance is defined as the inability of insulin to produce its usual biological actions at circulating concentrations that are effective in normal subjects (29). Insulin resistance can develop to any of the metabolic actions of insulin.

CAUSES OF INSULIN RESISTANCE

Obesity

Obesity is associated with an impaired action of insulin to inhibit glucose production and to increase glucose uptake (29). Body mass index (BMI), however, accounts only for a part of the variance in insulin sensitivity in the normal population, and the mechanisms by which obesity induces insulin resistance are poorly understood (29). Recent data would suggest that the amount of fat stored within the liver and skeletal muscle is the most proximal correlate of insulin resistance in obesity (38). In fact, fat may also accumulate in the liver and skeletal muscle in the absence of subcutaneous fat, in lipodystrophic conditions in humans and animals, as will be discussed later.

Physical inactivity

Several prospective epidemiological studies have shown an inverse correlation between physical activity and the incidence of type 2 diabetes (29,39). Physical inactivity increases the risk of diabetes, even after adjusting for age, smoking, alcohol consumption, family history of diabetes, BMI, HDL-cholesterol, triglycerides and hypertension (29). Studies on the effects of physical exercise training in diabetic and non-diabetic subjects suggest a preferential loss of visceral fat over total fat and a decrease in inflammatory markers, such as C-reactive protein (CRP) and tumor necrosis factor (TNF) α (40,41).

Insulin and contractions of muscle fibers stimulate glucose uptake in skeletal myocytes through independent mechanisms (42). Contractions of the myocytes increase glucose uptake by stimulating the adenosine monophosphate-activated protein kinase (AMPK) (43). AMPK is an energy-sensing enzyme, which is activated in response to cellular fuel depletion, hypoxia and contraction (44). AMPK activation leads to increased glucose uptake, enhanced insulin sensitivity and increased oxidation of fatty acids in skeletal muscle, and to an increase in hepatic fatty acid oxidation and inhibition of glucose production in the liver (45,46).

Gender

The glucose uptake is 45% higher in women than in men when expressed per kilogram of muscle tissue after controlling for age and maximal oxygen uptake (47). Female sex steroids are unlikely to be responsible for this gender difference, since estradiol does not improve insulin sensitivity in postmenopausal women (48).

Age

Several factors, such as increasing adiposity, a reduction in muscle mass, physical inactivity, medications and coexisting illnesses may contribute to age-related insulin resistance (49). In a recent report, increased insulin resistance in healthy elderly people was associated with increased fat accumulation in skeletal muscle and the liver, and with a ~40% reduction in mitochondrial oxidative and phosphorylative activity in the muscle when compared to healthy young people matched for body composition and physical activity (50). These data would support the hypothesis that an inability of skeletal muscle and the liver to metabolize fatty acids, possibly because of mitochondrial dysfunction, may lead to intracellular accumulation of fatty acid metabolites and defects in insulin signaling and action in these tissues (51).

2.1.3. ADIPOSE TISSUE

Traditionally, adipose tissue was regarded merely as a passive energy reserve capable of storing lipids in the form of triacylglycerol at times of energy surplus, and releasing FFA and glycerol at times when energy expenditure exceeds energy intake. A grown-up person has usually 10 - 25 kg of fat, which stores 90 000 to 225 000 kcal energy in the form of triglyceride (11). However, it is now recognized that adipose tissue has a wide range of endocrine and paracrine functions, and participates in the regulation of metabolism in other tissues. It is also important to bear in mind that adipose tissue does not consist of adipocytes only but also of

a variety of other functionally active cells such as preadipocytes, vascular endothelial and smooth muscle cells, fibroblasts, mast cells and macrophages (52).

DIFFERENTIATION AND FUNCTION OF ADIPOCYTES

Adipocytes originate from pluripotent mesenchymal stem cells, which can differentiate to adipocytes, myocytes, chondrocytes or osteoblasts (**Fig. 1**) (52,53). Stem cells first develop into preadipocytes and so become committed to the adipocyte lineage (54). The regulation of this first step is poorly known (54). Interestingly, it has recently been shown that under experimental conditions in mice preadipocytes can be converted also into macrophages (55).

After being committed to the adipocyte lineage, the preadipocytes have an exponential growth phase, which leads to cell confluence and subsequently to a cell cycle arrest usually achieved through contact inhibition (54). Thereafter contact-inhibited preadipocytes re-enter the cell cycle due to hormonal induction and undergo a limited number of cell divisions known as the clonal expansion of preadipocytes (52).

In the final step of differentiation, fibroblast-like preadipocytes accumulate intracellular lipids and become typical round adipocytes. The main regulators of the terminal differentiation are three classes of transcription factors: CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptor (PPAR) γ and SREBP-1c (**Fig. 1**) (52). These transcription factors act in a sequential cascade. First, C/EBPβ and δ are transiently induced and seem to have a direct transcriptional effect through C/EBP binding sites in the PPARγ promoter. PPARγ is then responsible for inducing C/EBPα. PPARγ and C/EBPα reinforce the expression of each other, thus ensuring sufficient expression of the two major stimulators of adipocyte differentiation (53). PPARγ and C/EBPα synergistically activate differentiation-linked gene expression. Many of these genes are known to have binding sites for both C/EBP proteins and PPARγ (53). In addition to C/EBPβ- and δ-dependent induction, PPARγ expression can also be induced by SREBP-1c, which may additionally be involved in the production of an endogenous PPARγ ligand and consequently increase PPARγ activity (53). SREBP-1c stimulates adipogenesis not only via inducing PPARγ, but also by directly activating expression of adipogenic genes (56). Eventually, the activation of the transcription factors results in *de novo* or enhanced expression of genes that characterize the mature adipocyte phenotype along with massive triglyceride accumulation. The products of these genes include e.g. fatty acid synthase (FAS), GLUT4, insulin receptor and adipocyte lipid binding protein (ALBP) (53).

Factors stimulating adipogenesis

The combination of insulin, dexamethasone and cyclic adenosine monophosphate (cAMP) is conventionally used to stimulate adipocyte differentiation *in vitro* (57). Insulin increases the percentage of preadipocytes

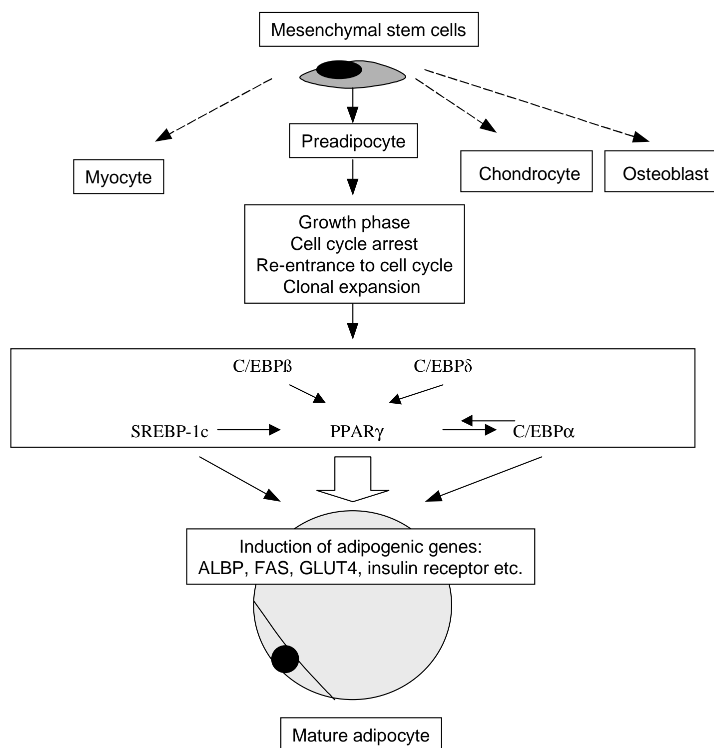


Figure 1. *Differentiation of an adipocyte from a multipotent mesenchymal stem cell. After the clonal expansion of preadipocytes a cascade of several transcription factors gets activated. PPARγ is the major transcription factor for the activation of adipogenic genes, which results in lipid accumulation and final maturation of the adipocyte.*

that differentiate, adipocyte lipogenesis and it also has antiapoptotic activity (53). Glucocorticoids are believed to stimulate adipogenesis through binding to glucocorticoid receptor. Glucocorticoid-induced transcriptional effects in adipocyte differentiation may include induction of C/EBPδ expression and reduction of the expression of preadipocyte factor-1, which is a negative regulator of adipogenesis (53). Increase in cellular cAMP concentration promotes adipocyte differentiation at least in part, by inducing C/EBPβ, but may also act through the cAMP response element binding protein (CREB) (53).

Factors inhibiting adipocyte differentiation

Inflammatory cytokines, such as TNFα, IL-1, IL-6, IL-11 and interferon γ inhibit adipocyte differentiation *in vitro*, and may contribute to atrophy of adipose tissue in cancer cachexia, inflammatory and chronic infectious diseases (56). Exposure of preadipocytes to TNFα or to other inflammatory cytokines inhibits adipogenesis by blocking induction of PPARγ and C/EBPα (56). Growth hormone has been shown to

decrease adiposity *in vivo* through activation of lipolysis (56). However, *in vitro* growth hormone can both promote and inhibit adipocyte differentiation (58).

Physiology of mature adipocytes

Surplus energy is stored in adipocyte lipid droplets as triglycerides. Depending on the size of the lipid droplet, the mature adipocyte can change its diameter by 20-fold and the volume by several thousand-fold (9).

Adipocytes synthesize triglycerides from fatty acids. In order to enter the adipocytes, fatty acids must first be released from circulating triglyceride-rich lipoproteins, chylomicrons and VLDL (**Fig. 2**). The release of fatty acids from circulating lipoproteins is catalyzed by LPL, which is located on the adipose tissue capillary endothelium (11). The activity of LPL is regulated mainly by insulin, but is also controlled by the removal rate of liberated fatty acids from the capillary, i.e. if fatty acids are not taken up by adipocytes, LPL activity decreases (59). The less fatty acids are taken up by the adipocytes, the more fatty acids enter the general circulation and reach the liver and skeletal muscle (59).

Entry of fatty acids into the adipocyte is likely to occur both by passive diffusion and active transport (**Fig. 2**) (60). Three groups of proteins have been implicated in the transport process: fatty acid transport proteins (FATPs), CD36 also known as fatty acid translocase (FAT), and plasma membrane-associated fatty acid binding protein (FABPpm). Their expression is upregulated during adipocyte differentiation (60). Acylation stimulating protein (ASP) is another protein regulating the uptake of fatty acids by the adipocyte. ASP is formed via posttranslational interactions of three proteins secreted by adipocytes: factor B, adipsin (factor D) and the third component of complement C3 (61).

Once inside the adipocyte, fatty acids are bound to cytoplasmic fatty acid-binding proteins (FABP). Two FABPs are expressed in human white adipose tissue, ALBP (the human homologue of the mouse aP2) and keratinocyte lipid binding protein (KLBP) (**Fig. 2**) (62). Acyl coenzyme A synthase (ACS) in turn catalyzes the conversion of long-chain fatty acids to their acyl CoA esters than can then be used either for the synthesis of triglycerides or for oxidation in mitochondria (63).

The breakdown of adipocyte intracellular triglycerides, lipolysis, is catalyzed by HSL (**Fig. 2**). Insulin and ASP decrease lipolysis by increasing re-esterification of fatty acids and inhibiting HSL activity (61,64). Other regulators of lipolysis include TNFα, which increases lipolysis (65), and autonomous nervous system, which increases lipolysis via β1- and β2- receptors, or decreases lipolysis via α2-receptors (66).

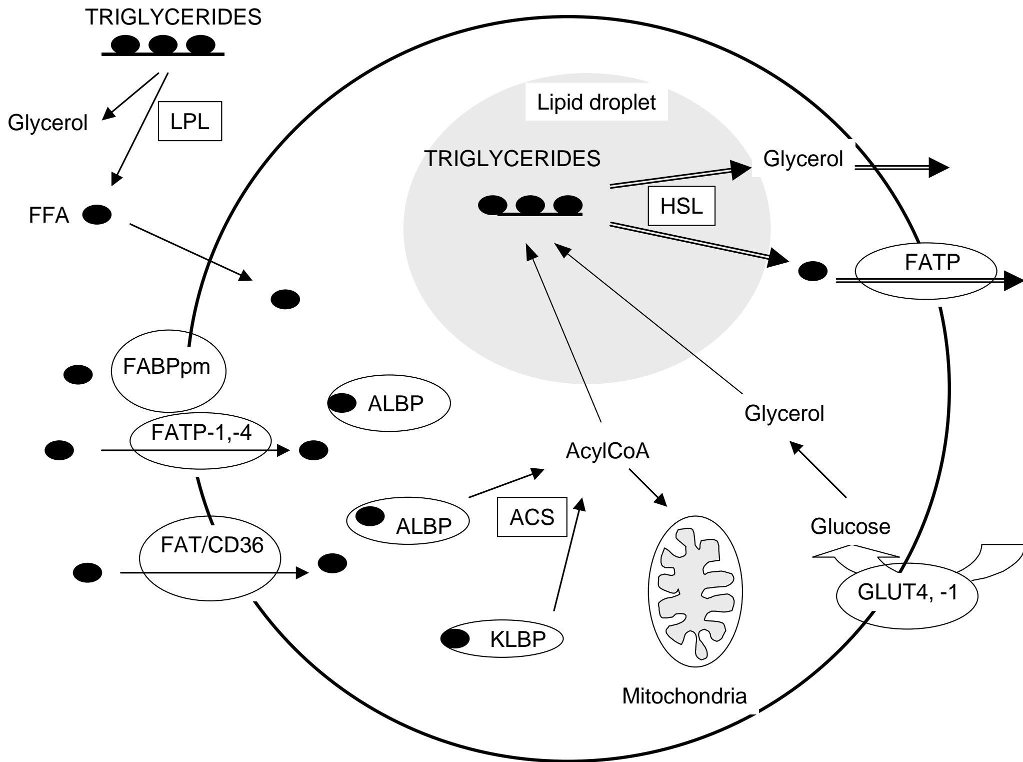


Figure 2. *Schematic picture of FFA trafficking in the adipocyte. FFA (•) are released from triglyceride-rich lipoproteins (VLDL, chylomicrons) on capillary endothelium by LPL. FFA can then enter the adipocyte via passive diffusion or by using transport proteins (FATP-1 and -4, FAT/CD36, FABPpm). Intracellular FFA are bound to ALBP or KLBP. ACS catalyzes the formation of AcylCoA, which can either be oxidized in mitochondria or used for triglyceride synthesis. Glucose uptake via GLUT4 and GLUT1 transporters is needed for glycerol formation. HSL catalyzes breakdown of intracellular triglycerides.*

In addition to storing and releasing fatty acids, adipose tissue is capable of producing a large number of proteins such as adiponectin, leptin, TNFα, IL-6, LPL, PAI-1, tissue factor, angiotensinogen, adipsin, ASP, some of which are important in the regulation of whole body metabolism (Chapter 2.1.3.). Of note, some of these proteins do not exclusively originate from adipocytes, but also from other cells such as macrophages and endothelial cells present in adipose tissue.

Brown adipose tissue

The primary function of brown adipose tissue is not to store energy but to produce heat. Brown adipocytes differ from white adipocytes morphologically: brown adipocytes are rich in mitochondria and store lipids in small droplets instead of one large droplet as seen in white adipocytes (67). In rodents, brown and white adipocytes have specific tissue distribution; inguinal, epididymal and retroperitoneal depots contain mainly white adipocytes, whereas interscapular and perirenal depots contain mainly brown adipocytes (68).

Abundant brown adipose tissue is present in human newborns, primarily in the thoracic cavity surrounding the great vessels (53). In healthy human adults, there are no specific brown fat depots, but occasional brown adipocytes can be detected within normal white adipose tissue (68). Expression of uncoupling protein-1 (UCP-1), which confirms the presence of brown adipocytes, has been found to be significantly increased in omental vs. subcutaneous fat both in lean and obese subjects (69). It has been estimated that in omental fat approximately 1 in 100-200 adipocytes is brown (69).

The PPARγ coactivator –1 (PGC-1) is expressed in brown fat, skeletal muscle, heart, kidney and brain, but not in white fat in mice (53). PGC-1 may preferentially direct preadipocytes to a brown adipocyte phenotype, since overexpression of PGC-1 in human and mouse white adipocytes in culture induces endogenous UCP-1 expression and mitochondrial biogenesis (53,70).

PGC-1 expression has not been measured in patients with HAL, but is an interesting protein in this context for multiple reasons. As a co-activator of PPARγ (71), it may affect adipogenesis via PPARγ activation. In addition, PGC-1 has been shown to regulate mitochondrial biogenesis (72), which may have impact in the pathogenesis of the mitochondrial alterations observed in HAL (Chapter 2.3.4.). Furthermore, in muscle cells *in vitro*, adenovirus-mediated PGC-1 expression results in increased GLUT4 expression (73). PGC-1 expression in transgenic mice has been shown to convert type II muscle fibers into type I which are rich in mitochondria, express more GLUT4 and are more dependent in oxidative metabolism than type II fibers (74).

ADIPOSE TISSUE AS A REGULATOR OF WHOLE BODY INSULIN RESISTANCE

Mechanisms of insulin resistance in adipose tissue

The mechanisms underlying insulin resistance in adipocytes are not fully understood. Subcutaneous adipocytes from patients with type 2 diabetes have reduced IRS-1 protein expression and reduced PI 3-kinase activity when compared to adipocytes from non-diabetic subjects (75). Low messanger RNA (mRNA) and protein levels of IRS-1 in subcutaneous adipocytes have also been found in healthy individuals with an increased risk of type 2 diabetes, i.e. in massively obese subjects and subjects with first-degree relatives with type 2 diabetes (76). Those healthy adults, who had low IRS-1 expression in subcutaneous adipocytes had also impaired downstream insulin signaling, reduced PI 3-kinase activation, GLUT4 expression and insulin-stimulated glucose transport in adipocytes (77). Low IRS-1 expression in subcutaneous adipocytes of insulin resistant subjects was associated with decreased expression of genes related to fat cell differentiation, such as adiponectin, ALBP, PPARγ and LPL (77,78). Women with gestational diabetes have been reported to have a decreased cellular content of GLUT4, but normal content of GLUT1 in isolated omental adipocytes (79). Similarly, GLUT4 expression in SAT has been reported to be reduced both in obese patients with type 2 diabetes and in obese non-diabetic subjects when compared to lean controls (80).

Free fatty acids

FFA have emerged as a major link between obesity and insulin resistance (81). In normal subjects, an acute elevation of FFA by a lipid infusion decreases insulin-stimulated glucose uptake (82,83). It has been demonstrated using MRI spectroscopy that FFA infusion into healthy humans causes a decrease in intracellular glucose-6-phosphate concentration in skeletal muscle (84). This decrease was a consequence of reduced insulin-stimulated glucose transport and insulin-stimulated induction of PI 3-kinase activity (84).

Acute elevation of FFA in the plasma inhibits the ability of insulin to suppress glucose production in the liver (82,85). Although data are somewhat contradictory, increased plasma FFA may reduce hepatic insulin clearance (86). Because approximately half of the insulin secreted by β-cells is removed on first pass by the liver, this reduction in clearance may contribute to peripheral hyperinsulinemia in insulin resistance (86).

Adipocytokines

The term adipocytokine is used to describe a wide range of proteins produced by adipose tissue. Adipocytokines include both classical cytokines such as TNFα and IL-6, and other proteins, such as adiponectin and leptin (87). Adipocytokines may act locally as autocrine or paracrine factors, or have remote-acting endocrine functions.

Adiponectin

In 1995, a novel 30-kDa secretory protein, which was later named adiponectin, was described in 3T3-L1 adipocytes (88). The protein was expressed exclusively in adipocytes and its mRNA was induced 100-fold during adipocyte differentiation (88). Adiponectin was originally named Acrp30 (adipocyte complement-related protein of 30 kDa) and later was also called AdipoQ, apM1, GBP28 (89).

Since adiponectin is exclusively expressed in adipocytes, it was surprising that the plasma concentrations in humans were inversely rather than directly correlated with BMI both in women and men, although women had higher plasma concentrations than men (90). Adiponectin concentrations have also been shown to increase after weight loss (91). Adiponectin seems to act as a metabolically protective adipocytokine, since age- and BMI-matched diabetic patients have lower serum adiponectin concentrations than non-diabetic subjects (91). Furthermore, diabetic and non-diabetic patients with coronary artery disease have lower adiponectin concentrations than diabetic or non-diabetic subjects without coronary artery disease, respectively (91,92). Adiponectin mRNA levels were significantly reduced in omental adipose tissue of obese patients with type 2 diabetes compared with lean and obese normoglycemic subjects (93). Although less pronounced, adiponectin mRNA levels were reduced also in SAT of type 2 diabetic patients (93).

In addition to the cross-sectional studies listed above, the role for adiponectin in the development of insulin resistance has been evaluated in some longitudinal animal and human studies. In a prospective study with

rhesus monkeys, decrease in plasma adiponectin concentration paralleled with the development of insulin resistance and this decrease preceded overt hyperglycemia (94). In apparently healthy humans, high concentrations of adiponectin seem to be associated with a substantially reduced relative risk of developing type 2 diabetes even after adjusting for age, sex, waist to hip ratio (WHR), BMI, smoking, exercise, alcohol consumption, education and HbA1c concentration at baseline (95). Similarly, baseline plasma adiponectin concentration was lower in those Pima Indians who after a mean follow-up of 6.7 years developed diabetes than in those who did not develop diabetes matched for age, sex, BMI (96).

Further evidence for an antidiabetic and cardioprotective role of adiponectin has been obtained in animal and *in vitro* studies. Infusion of adiponectin reverses insulin resistance both in obese and lipoatrophic mouse models (22). *In vitro* adiponectin has been shown to inhibit the TNFα-induced expression of endothelial adhesion molecules (97). Furthermore, adiponectin suppresses the *in vitro* transformation of human monocyte-derived macrophages into foam cells (98).

Regulation of adiponectin expression has recently been evaluated in several studies. Known inhibitory regulators of adiponectin expression in 3T3-L1 adipocytes include TNFα and dexamethasone (99), IL-6 (100) and ghrelin (101). TNFα also decreases adiponectin expression in differentiating primary human adipocytes (102). Both TNFα and IL-6 decrease adiponectin mRNA levels also in cultured human SAT (103). In non-diabetic subjects, adiponectin expression in SAT has been shown to have an inverse correlation with the expression of TNFα, but not with the expression or plasma levels of IL-6 concentration (104). The effect of insulin on adiponectin expression remains controversial even in 3T3-L1 cell line; one study showed an insulin-induced inhibition of adiponectin expression (99), whereas in another study insulin enhanced the secretion of adiponectin (105). In humans, insulin appears to decrease circulating levels of adiponectin (106). β-Adrenergic stimulation inhibits adiponectin expression in human visceral adipose tissue (VAT) explants (107) and 3T3-L1 adipocytes (108). In mice, castration increases plasma adiponectin concentrations and improves insulin sensitivity (109). Treatment with thiazolidinediones increases adiponectin plasma concentrations in humans (110) and adiponectin mRNA concentrations in adipose tissue of obese mice (111). A functional PPAR-responsive element was recently identified in the human adiponectin promoter (112).

The molecular mechanisms by which adiponectin enhances insulin sensitivity are still incompletely understood. Infusion of adiponectin decreases insulin resistance and triglyceride content in skeletal muscle and in the liver both in obese and lipoatrophic mice (22). Adiponectin increases fatty acid oxidation in isolated muscle in mice (113). Both globular and full-length adiponectin stimulate phosphorylation and activation of AMPK in skeletal muscle (21). In parallel with the activation of AMPK, adiponectin stimulates phosphorylation and thereby inhibition of acetyl coenzyme A carboxylase (ACC) activity (21). Lower ACC activity leads to a fall in malonyl-CoA content and relieves the inhibitory effect of malonyl-CoA on carnitine

palmitoyl transferase 1, which results in enhanced entry of fatty acids into mitochondria for oxidation (21,114). In isolated rat hepatocytes, adiponectin increases the ability of insulin to suppress glucose production (20). Full-length adiponectin, but not the globular domain was capable of activating AMPK in the mouse liver and subsequently reduced expression of molecules involved in gluconeogenesis, such as PEPCK and glucose-6 phosphatase (21).

Very recently, two adiponectin receptors (AdipoR) have been cloned (115). Human and mouse AdipoR1 share 96.8% and AdipoR2 95.2% identity (115). In mice, AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver (115).

Leptin

Leptin is the protein product of the obese (ob) mouse gene cloned in 1994 (116). Leptin is expressed mainly, but not exclusively in white adipocytes (117). Originally leptin was thought to act merely as a satiety hormone (118) and reduce food intake via central mechanisms. Today, however, it is clear that leptin has multiple other functions, such as regulation of the hypothalamic-pituitary-endocrine axes, hematopoiesis, angiogenesis, immune functions, osteogenesis, and wound healing (117).

Since leptin expression in adipose tissue is increased in obese humans (119), and serum leptin concentration and mRNA in adipocytes are positively correlated with total body fat, it has been suggested that obese people are resistant to the effects of leptin (120). Since leptin treatment induces weight loss in leptin-deficient (*ob/ob*) obese mice (121), exogenous leptin therapy has also been tested in human obesity. However, leptin treatment in normal obese humans with high leptin concentrations, induced only modest weight loss in a few subjects and had no effect on glycemic control (122). However, in patients with different forms of non-HIV lipodystrophy with low baseline leptin levels, leptin treatment induced a marked improvement in glycemic control (123). In this study, the improvements in hepatic and skeletal muscle insulin sensitivity were associated with a decrease in hepatic and muscle triglyceride content (124).

Pro-inflammatory cytokines

TNFα has been suggested to contribute to obesity-induced insulin resistance. TNFα is overexpressed in adipose tissue of obese humans and its expression is decreased by weight loss (125). Although TNFα appears to be secreted into conditioned media of human adipose tissue explants (126), the release of TNFα from adipose tissue to the circulation has not been shown *in vivo* (127). TNFα mRNA levels in human adipose tissue have been shown to correlate closely with the level of hyperinsulinemia (126). However, correlations between TNFα expression in SAT and BMI or insulin sensitivity have not been found in all studies (128).

The cellular actions of TNFα are mediated through two receptors, TNFα receptor 1 (TNFR1 or p60 in humans, and p55 in rodents), and TNFR2 (p80 in humans and p75 in rodents) (129). The suggested mechanisms of TNFα to cause insulin resistance involve increased lipolysis and a consequent increase in circulating FFA levels, decrease in GLUT4, insulin receptor and IRS-1 synthesis, inhibition of PPARγ synthesis and/or function, and serine phosphorylation of IRS-1 (130). However, the contribution of TNFα to insulin resistance in humans still remains to be defined; the first trial using TNFα-neutralizing antibodies failed to change insulin sensitivity in patients with type 2 diabetes (131).

IL-6 is secreted from SAT to the circulation and adipose tissue-derived IL-6 is estimated to account for 15-35% of its total circulating concentration in humans (127). Serum concentrations of IL-6 are increased in obesity (132) and in type 2 diabetes (133), and correlate with the degree of insulin resistance in non-diabetic subjects (134,135). IL-6 protein content in adipose tissue has been found to be inversely correlated with *in vivo* insulin-stimulated glucose uptake, and *in vitro* glucose uptake in human subcutaneous adipocytes (136). Weight loss enhances insulin sensitivity and is associated with a decrease in IL-6 protein levels in both SAT and serum (137). Furthermore, the change in circulating IL-6 level has been found to correlate with the improvement in insulin sensitivity after weight loss (138).

The mechanisms linking IL-6 to insulin resistance are not fully understood. In the human hepatocarcinoma cell line, HepG2, IL-6 decreases tyrosine phosphorylation of IRS-1 and the association of the p85 subunit of PI 3-kinase with IRS-1, and inhibits insulin-dependent activation of protein kinase B (139). IL-6 does not cause an acute lipolytic effect in human adipocytes (140). In 3T3-L1 adipocytes, IL-6 decreases transcription of IRS-1, GLUT4 and PPARγ genes, and insulin-stimulated glucose transport (140).

Resistin

Resistin is a peptide hormone, which has been shown to impair glucose tolerance and insulin action in normal mice (141). Administration of anti-resistin antibody has been shown to improve glycemia and insulin action in mice with diet-induced obesity (141). However, several studies have later reported an association between decreased rather than increased resistin expression and insulin resistance in various rodent models (142). Resistin mRNA (143) and protein (144) concentrations were significantly increased in abdominal subcutaneous and omental fat when compared with breast and thigh subcutaneous fat in non-diabetic subjects. However, the role of resistin in human insulin resistance remains elusive, since several studies have not been able to detect resistin mRNA in human adipocytes (142).

METABOLIC CHARACTERISTICS OF DIFFERENT ADIPOSE TISSUE DEPOTS

Already in the 1950s, the association between android, i.e. upper body obesity and type 2 diabetes was recognized (145). In 1985, Ashwell et al. studied fat distribution using computed tomography (CT) and

suggested that the metabolic complications of obesity may relate specifically to the amount of intra-abdominal fat (146). Intra-abdominal fat can be further divided into an intraperitoneal depot (omental [0.5-3 kg] and mesenteric [0.5-2 kg]) and retroperitoneal, i.e. perirenal fat (0.5-2 kg) (147). Omental and mesenteric fat depots are also referred to as visceral fat since their venous drainage is mainly through the portal vein (147). However, subcutaneous fat is the largest abdominal fat depot with an estimated weight of 1-20 kg (147). Of the whole body adipose tissue mass, subcutaneous fat constitutes at least 80% in both lean and obese subjects (148).

Intra-abdominal fat

VAT constitutes less than 20% of the whole body adipose tissue. Thus, for VAT to be more important than SAT in the pathogenesis of insulin resistance, there should be significant differences in the metabolic activity of VAT vs. SAT. Indirect evidence in favor of major functional differences between fat depots comes from a study, in which obesity was treated surgically with adjustable gastric binding (AGB) only, or with AGB and removal of the greater omentum fat which represented less than 1% of total fat mass (149). After 24 months, improvements in insulin sensitivity, and decreases in fasting plasma glucose and insulin concentrations were 2-3 times greater in omentectomized subjects as compared to those treated with AGB only (149).

The anatomic location of VAT may make it more important than SAT in the development of insulin resistance. Due to the portal venous drainage of visceral fat, the liver may get exposed to high concentrations of FFA and adipocytokines released from VAT, which could then stimulate hepatic glucose production and triglyceride synthesis, and decrease insulin clearance by the liver (147,150).

Gene expression in VAT vs. SAT in humans has been evaluated in several studies (**Table 1**). Omental fat secretes more IL-6 than subcutaneous fat, although IL-6 secreted from the isolated adipocytes is estimated to account only for ~10% of the total adipose tissue release (151). By contrast, leptin expression is higher in SAT than VAT (152,153). Some other adipocytokines, such as TNFα are similarly expressed in both SAT and VAT (148). It is impossible to draw firm conclusions regarding the expression of most genes, since they have only been evaluated in a single study or because the results from diverse studies show conflicting results. Furthermore, since many of these studies have included morbidly obese subjects, the results may not be generalizable to people with normal or moderately increased body weight.

Table 1. *Comparisons of gene expression in human subcutaneous (SAT) vs. visceral adipose tissue (VAT).*

	Number of subjects (F/M)	BMI (kg/m^2)	Measurement	Result	Ref.
Adiponectin	6	28-29	Protein from adipose tissue sample.	VAT < SAT	(154)
Adiponectin	9	41	Secretion of protein from isolated adipocytes.	VAT = SAT	(155)
Adipsin	31 (19/12)	28 (Female) 24 (Male)	mRNA from isolated adipocytes (n=12).	VAT = SAT	(156)
ALBP	29 (20/9) total 20 (13/7) obese 9 (7/2) lean	44 (Obese) 23 (Lean)	mRNA from adipose tissue samples in obese only. Protein from adipose tissue samples in all subjects.	VAT < SAT (both mRNA and protein, but in obese only)	(62)
Angiotensinogen	20 (8/12)	41	mRNA from adipose tissue sample (n=16).	VAT > SAT	(157)
Angiotensinogen	9 (5/4)	34	mRNA from adipose tissue sample.	VAT > SAT	(158)
ASP / C3a	9 (5/4)	34	mRNA from adipose tissue sample.	VAT > SAT	(158)
CETP	9 (5/4)	34	mRNA from adipose tissue sample.	VAT < SAT	(158)
cIAP2	31 (19/12)	28 (Female) 24 (Male)	mRNA from isolated adipocytes (n=8).	VAT > SAT	(156)
cIAP2	11 (9/2)	24	mRNA from preadipocytes.	VAT > SAT	(159)
Complement components:C2, C3,C4,C7,Factor B	10 (0/10)	42	mRNA from adipose tissue sample.	VAT > SAT	(160)
Glucocorticoid receptor	14 (14/0)		mRNA from adipose tissue sample.	VAT > SAT	(161)
GLUT4	12 (7/5)	20-53	mRNA from adipose tissue.	VAT < SAT	(162)
GLUT4	9 (9/0)	45	mRNA (n=6-8) and protein from isolated adipocytes from 3 sites: round ligament,. greater omentum, subcutaneous fat.	Round ligament > omentum or subcutaneous fat	(163)
Glycerol-3-phosphate dehydrogenase	24 (15/9)	20-34	mRNA from isolated adipocytes (n=20).	VAT = SAT	(152)
Glycogen synthase	12 (7/5)	20-53	mRNA from adipose tissue.	VAT < SAT	(162)
11β-HSD-1	16 (7/9)	Weight 78kg	mRNA and enzyme activity in isolated preadipocytes.	VAT > SAT in enzyme activity	(164)
HSL	31 (19/12)	28 (Female) 24 (Male)	mRNA from isolated adipocytes (n=12).	VAT = SAT	(156)
HSL	21 (12/9)	32	mRNA and HSL activity in isolated adipocytes.	VAT < SAT	(165)
HSL	12 (7/5)	20-53	mRNA from adipose tissue.	VAT = SAT	(162)

IL-6	10 (6/4)	52	Protein release into media from adipose tissue fragments (n=6) or isolated adipocytes (n=3).	VAT > SAT	(151)
Insulin receptor	12 (7/5)	20-53	mRNA from adipose tissue. Most mRNA from insulin receptor lacking exon 11.	VAT > SAT	(162)
Insulin receptor	55 (28/27)	19-37	mRNA (n=20) and protein (n=9) from isolated adipocytes.	VAT = SAT	(166)
IRS-1	12 (7/5)	20-53	mRNA from adipose tissue.	VAT = SAT	(162)
IRS-1	55 (28/27)	19-37	Protein (n=5) from isolated adipocytes.	VAT < SAT	(166)
KLBP	29 (20/9) total 20 (13/7) obese 9 (7/2) lean	44 (Obese) 23 (Lean)	mRNA from adipose tissue samples in obese only. Protein from adipose tissue samples in all subjects.	VAT > SAT (protein level in lean only)	(62)
Leptin	31 (19/12)	28 (Female) 24 (Male)	mRNA from isolated adipocytes (n=12).	VAT < SAT	(156)
Leptin	24 (15/9)	20-34	mRNA from isolated adipocytes.	VAT < SAT	(152)
Leptin	9 (5/4)	34	mRNA from adipose tissue sample.	VAT < SAT	(158)
Leptin	12 (7/5)	20-53	mRNA from adipose tissue.	VAT < SAT	(162)
Leptin	23 (23/0) 15 obese 8 lean	28-60 (obese) 20-27 (lean)	Protein secretion from adipose tissue samples (in all subjects) and mRNA in obese women.	VAT < SAT	(153)
LPL	31 (19/12)	28 (Female) 24 (Male)	mRNA from isolated adipocytes (n=11).	VAT = SAT	(156)
LPL	63 (45/18)	46 (Female) 50 (Male)	mRNA from adipose tissue samples (n=12 women and 5 men).	VAT < SAT in women VAT = SAT in men	(167)
LPL	12 (7/5)	20-53	mRNA from adipose tissue.	VAT = SAT	(162)
PAI-1	22 (14/8)	43	mRNA from adipose tissue and protein secretion from adipose tissue samples.	VAT < SAT	(168)
PAI-1	28 (28/0)	28	mRNA from adipose tissue samples.	VAT = SAT	(169)
PAI-1	26 (22/4)	41	mRNA from adipose tissue samples.	VAT > SAT	(170)
PAI-1	7 (3/4)	18-28	Protein secretion from adipose tissue explant.	VAT > SAT	(171)
PAI-1	40	28	Protein (n=7) and mRNA release from adipose tissue samples.	VAT > SAT	(172)
PAI-1	18 (10/8) total 11 (8/3) obese 7 (2/5) non-obese	45 (obese) 24 (non-obese)	Protein release from adipose tissue culture.	VAT > SAT	(173)
6-Phosphofructo-1-kinase	12 (7/5)	20-53	mRNA from adipose tissue.	VAT = SAT	(162)
PI 3-kinase	12 (7/5)	20-53	mRNA from adipose tissue.	VAT = SAT	(162)

p85-subunit					
PPARα	12 (7/5)	20-53	mRNA from adipose tissue (n=6).	VAT = SAT	(162)
PPARδ	12 (7/5)	20-53	mRNA from adipose tissue (n=6).	VAT = SAT	(162)
PPARγ	12 (7/5)	20-53	mRNA from adipose tissue.	VAT < SAT in those with BMI < 30 only	(162)
PPARγ	31 (19/12)	28 (Female) 24 (Male)	mRNA from isolated adipocytes (n=11).	VAT = SAT	(156)
Resistin	10	26	Protein from adipose tissue sample.	VAT > thigh or breast SAT VAT = abdominal SAT	(144)
SREBP-1c	20 (14/6)	51	mRNA from adipose tissue.	VAT < SAT	(174)
TNFα			Reviewed by Arner (148).	VAT = SAT	(148)
TNF receptors p60 and p80	40 (40/0) 28 obese 12 non-obese	48 (obese) 26 (lean)	mRNA from adipose tissue samples.	VAT < SAT only obese subjects included.	(175)

Omental and mesenteric adipocytes have higher rate of lipolysis, i.e. FFA release, than subcutaneous adipocytes, and their lipolysis is more readily stimulated by catecholamines and less readily suppressed by insulin (147,150,176). Unexpectedly, in one study the mRNA expression and the activity of HSL, which is the major determinant of the maximum lipolytic capacity of human fat cells (177), was found to be higher in SAT than in VAT (165). In two other studies available, HSL expression was not different between VAT and SAT (152,162).

In the view of stable or increasing amount of VAT in obese subjects, increased lipolysis in VAT should be compensated by increased lipogenesis. This has been shown in a study, where the uptake of orally administered fatty acids was ~50% higher in VAT than in SAT (178). However, *in vitro* triacylglycerol synthesis was greater in human SAT fragments and subcutaneous preadipocytes than in omental adipose tissue and preadipocytes (179). LPL regulates hydrolysis of plasma triglycerides and consequently FFA availability for deposition in adipose tissue. LPL mRNA expression has either been reduced in VAT compared to SAT (167) or it has been similar in both fat depots (152,162).

Subcutaneous fat

The origin and concentration of FFA in the human portal vein are poorly known due to the difficult anatomic accessibility of the portal vein. According to catheterization studies, only ~10% of the FFA reaching the liver originate from VAT (147,180). Postprandial FFA delivery to the liver is greater in women with upper than lower body obesity (181). The excess FFA, however, seem to originate from the non-splanchnic adipose tissues rather than from VAT (181).

Paucity rather than excess of lower body subcutaneous fat may independently contribute to the development of insulin resistance and diabetes. In a cross-sectional study, a narrow hip circumference adjusted for age, BMI and waist circumference was associated with features of insulin resistance (182). Conversely, the protective role of abundant lower body subcutaneous fat was demonstrated in a recent prospective study where large hip and thigh circumferences at baseline were associated with a lower risk of development of type 2 diabetes, independently of BMI, age and waist circumference (183).

Taken together, there are differences in the metabolic activity of VAT vs. SAT. However, the exact mechanisms of these different fat depots to contribute to insulin resistance in humans *in vivo* are currently not fully understood.

2.1.4. FAT IN INSULIN SENSITIVE TISSUES OTHER THAN ADIPOSE TISSUE

Patients with excess (the obese) or too little (the lipoatrophic) adipose tissue are insulin resistant and at increased risk of developing type 2 diabetes. A common denominator for both groups appears to be excessive deposition of lipids in the liver and skeletal muscle (38).

THE LIVER

The term “nonalcoholic fatty liver disease” (NAFLD) is used to describe a spectrum of abnormalities ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) (184). The term “nonalcoholic steatohepatitis” was originally used to describe liver disease that histologically mimicked alcoholic hepatitis and that could progress to cirrhosis (185). Steatosis without inflammation seems to be a benign condition (186). It has been suggested that the development of NASH requires two pathogenic steps: hepatic fat accumulation and thereafter oxidative stress capable of initiating significant lipid peroxidation and cytokine induction (187,188).

Both steatosis and NASH are associated with obesity and diabetes (189). Subjects with normal glucose tolerance, who had biopsy-proven NAFLD with or without steatohepatitis had central fat accumulation, increased triglycerides and uric acid, and a low HDL cholesterol irrespective of BMI (190). In the same study, patients with NAFLD had impaired insulin-induced suppression of hepatic glucose production, and reduced glucose disposal rate when compared to healthy subjects even after adjusting for age, BMI and WHR (190).

Liver fat content measured using spectroscopy has been found to be more closely correlated with insulin-induced suppression of hepatic glucose production in type 2 diabetic patients than any other measure of body composition (191). In healthy non-diabetic men, liver fat content was associated with several features of insulin resistance, including hyperinsulinemia, hypertriglyceridemia, a low HDL cholesterol concentration and high 24-h systolic blood pressure, and impaired insulin-induced suppression of hepatic glucose production and of serum FFA concentration (19). Similarly in obese non-diabetic women, those with higher liver fat content had an increased serum triglyceride and insulin concentrations, a lower HDL cholesterol concentration, higher 24-h systolic and diastolic blood pressure, and lower glucose uptake during hyperinsulinemic euglycemic clamp than women with lower liver fat content but similar BMI (192).

SKELETAL MUSCLE

With MRI proton spectroscopy it is possible to non-invasively differentiate intramyocellular lipid (IMCL) from extramyocellular lipid (193,194). Using spectroscopy, a group of healthy men with higher IMCL have been shown to have reduced glucose uptake when compared to healthy men with lower IMCL independent of BMI and physical fitness (195). In obese subjects with unaltered insulin sensitivity, the preservation of insulin sensitivity has been associated with unaltered IMCL content, but increased fat oxidation when compared to lean subjects (196).

Aerobic fitness and recent strenuous exercise are important confounding factors when interpreting the relationship between IMCL and insulin resistance. A 2-week training program has been shown to

significantly increase IMCL whereas insulin sensitivity tended to improve (197). On the other hand, a 3-hour cycling exercise has been found to acutely decrease IMCL content (197).

MECHANISMS OF INSULIN RESISTANCE WITH FAT ACCUMULATION IN THE LIVER AND SKELETAL MUSCLE

Since triglycerides themselves are inert, increased intracellular triglyceride content is likely to be merely a surrogate marker of some other fatty acid-derived factor(s), such as long chain acyl-CoA, which can induce insulin resistance by multiple mechanisms, including diacylglycerol formation, activation of protein kinase C-theta, and ultimately serine, instead of tyrosine, phosphorylation of IRS-1, and reduction in PI 3-kinase activity (84,198,199). Other potential mechanisms of long chain acyl-CoA to induce insulin resistance include inhibition of insulin signaling via *de novo* ceramide synthesis and direct inhibition of hexokinase and glycogen synthase (200).

2.1.5. MOUSE MODELS OF LIPODYSTROPHY

Several mouse models of lipodystrophy with varying severity of fat loss have been reported in recent years. These models have been crucial in understanding the deleterious effects of the accumulation of lipids in insulin sensitive tissues, such as the liver and skeletal muscle, when adipose tissue cannot normally store fat.

Adipocyte specific-expression of diphtheria toxin A chain virtually eliminates white and brown fat in mice (8). Histologic examination of fat revealed atrophy, necrosis and monocytic infiltration, livers were enlarged and filled with fat, and mice developed diabetes (8). Troglitazone significantly decreased serum glucose, insulin, triglyceride, FFA and cholesterol concentrations, but did not change serum leptin concentration, or liver or muscle fat content in these mice (8).

Another model of fatless mouse was developed by expressing a truncated nuclear version of SREBP-1c in adipocytes (201,202). Surprisingly, overexpression of truncated SREBP-1c, a known adipogenic transcription factor, reduced adipose tissue mass and induced hypoleptinemia, hepatic steatosis and type 2 diabetes (201,202). Continuous leptin infusion decreased liver fat content and corrected insulin resistance (202).

A fatless mouse has also been developed by expressing a dominant-negative protein, termed A-ZIP/F (203). These A-ZIP/F-1 transgenic mice have no white fat, dramatically reduced brown fat, severe hepatosteatosis, diabetes, reduced serum leptin and increased glucose, insulin, triglyceride and FFA concentrations (203). Transplantation of wild-type fat tissue to A-ZIP/F-1 fatless mice reversed hyperglycemia, lowered insulin concentrations, improved muscle insulin sensitivity, corrected insulin signaling defects and normalized fat content in the liver and muscle (204,205).

2.1.6. NON-HIV HUMAN LIPODYSTROPHIES

Human lipodystrophies are rare genetic or acquired disorders characterized by total or partial loss of adipose tissue (lipoatrophy) and occasionally by local accumulation of fat (lipohypertrophy). The prevalences for the congenital forms have been estimated to be in the range of 1:10 million people (7). Of note, lipodystrophy must be differentiated from paucity of adipose tissue due to negative energy balance, e.g. due to starvation or extreme physical training. In contrast to lipodystrophies, in the latter condition the remaining adipocytes are functionally normal and retain the ability to store lipids during periods of energy surplus.

Human lipodystrophies can be classified according to the presumed etiopathogenesis, i.e. genetic, presumed inflammatory or autoimmune, and acquired lipodystrophies; or according to the severity of fat loss, i.e. generalized or partial (206). Various forms of congenital lipodystrophies have been described, such as congenital generalized (Berardinelli-Seip syndrome) and familial partial (Dunnigan and Köbberling variety) lipodystrophies (7,206). Presumed inflammatory or autoimmune lipodystrophies include acquired generalized (Lawrence syndrome), acquired partial (Barraquer-Simons syndrome) and injection site - associated lipodystrophy e.g. due to insulin injections before the availability of purified or human insulin (7,206).

Mutations causing Dunnigan-type partial lipodystrophy have recently been described (207). These mutations are found in the LMNA gene, which encodes type A lamins (207). Nuclear lamins are filament-type proteins that are the major building blocks of the nuclear lamina, a fibrous proteinaceous meshwork underlying the inner nuclear membrane thereby giving the cell nucleus its shape and interacting with proteins that regulate gene expression (206,208). The binding of lamin A to SREBP-1 is reduced by mutations causing lipodystrophy (209). Since SREBP-1 is a transcription factor, which promotes adipocyte differentiation, this defective interaction has been suggested to, at least partly, cause loss of fat in Dunnigan lipodystrophy (209).

Clinical and laboratory findings of lipodystrophic patients vary considerably, but they usually include insulin resistance and type 2 diabetes, hypertriglyceridemia, low HDL-cholesterol concentration, hypertension and a fatty liver (7,206). Women may suffer from oligo-amenorrhea and polycystic ovaries. Severe hypertriglyceridemia may cause pancreatitis, and fatty liver may lead to cirrhosis (7). Early onset diabetes and dyslipidemia are likely to accelerate atherosclerosis (7,210).

There are only limited data regarding treatment of lipodystrophy and its complications. Metformin may work in lipoatrophic diabetic patients, but the efficacy has been described in a single case report only (206,211). Insulin at very large doses may correct metabolic abnormalities in lipodystrophic patients with diabetes, whereas sulfonylureas have not been useful (206). Thiazolidinediones would seem ideal to treat lipodystrophy since they both improve insulin sensitivity and increase adipose tissue mass (212). In an open-label, uncontrolled study including 20 patients with various forms of lipodystrophy, use of troglitazone for

six months was shown to significantly decrease HbA1c levels, triglyceride and FFA concentrations (213). The respiratory quotient decreased significantly suggesting increased fat oxidation. Body fat measured by dual-energy x-ray absorptiometry (DEXA) increased significantly and MRI showed an increase in subcutaneous but not visceral fat. The size of the liver measured using MRI decreased (213). The effect of leptin has been studied in a small open-label, uncontrolled study in nine lipodystrophic patients with low serum leptin concentrations at baseline (123). Treatment with subcutaneous recombinant leptin injections for four months decreased HbA1c, triglyceride and FFA concentrations. Liver size decreased on the average by 28%. Body weight decreased in all but one patient. The effects on body composition were not reported (123). Leptin treatment in three of these patients significantly reduced hepatic and intramyocellular lipid content, and enhanced the insulin-induced suppression of hepatic glucose production and the insulin-stimulated peripheral glucose disposal (124).

2.2. HIV INFECTION AND ANTIRETROVIRAL THERAPY

2.2.1 HISTORY OF THE HIV EPIDEMIC

On June 5, 1981 Centers for Disease Control published a report of five cases of Pneumocystis carinii pneumonia among previously healthy young men in Los Angeles (214); this report is often referred to as the beginning of general awareness of acquired immunodeficiency syndrome (AIDS). It was soon realized that a new acquired disease of cellular immunodeficiency that manifested as Pneumocystis carinii pneumonia, Kaposi’s sarcoma, mucosal candidiasis and other opportunistic infections had emerged among gay men, intravenous drug users and hemophiliacs (215). By 1982, the new disease was being referred to by its new name “AIDS” (216). In May 1983, Dr Montagnier’s group from France reported an isolation of a new virus, which they believed was the cause of AIDS (217). In 1984, Dr Gallo’s group reported isolation of a new human retrovirus from blood samples of patients with AIDS and at risk for AIDS (218). It was later shown that LAV (lymphadenopathy-associated virus) described by Dr Montagnier and HTLV-III (human T-cell leukemia virus III) described by Dr Gallo were the same virus, and in 1986 the virus was renamed HIV (Human Immunodeficiency Virus) (219).

By 1999, according to World Health Organization, HIV infection had become the fourth leading cause of death worldwide after ischemic heart disease, cerebrovascular disease and acute lower respiratory infections (220). Among infectious disease pathogens, HIV causes more deaths than any other single agent (220). In 2002, 42 million people were estimated to live with HIV, the vast majority of them in sub-Saharan Africa, and in 2002 alone, 3.1 million people died from HIV/AIDS (221).

2.2.2. PATHOGENESIS

CD4+ T LYMPHOCYTES

Loss of immune system competence, and in particular, loss of cellular immunity is the major cause of clinical symptoms of HIV infection. T-lymphocytes expressing a CD4-receptor (CD4+ cells) are a critical component of intact cellular immunity. Loss of CD4+ cells occurs throughout HIV disease with an approximate decline of 80-90 cells/mm^3 per year (222). Since the beginning of the HIV epidemic, the blood CD4+ count has been used to indicate disease stage. CD4+ counts of healthy subjects are in the range of 500 to 1300 cells/mm^3 (223). A patient with a CD4+ cell count below 200 cells/mm^3 is at substantial risk of developing opportunistic infections and malignancies, and according to classification by Centers for Disease Control, is categorized as having AIDS even in the absence of opportunistic infections (224).

VIRAL LIFE CYCLE (**Fig. 3**)

Binding of the virus to the CD4 molecule on the host cell surface initiates a complex mechanism of viral entry, which leads to fusion of the viral lipid envelope with the cell membrane. The core of the virus penetrates into the cytoplasm and liberates viral genomic RNA. Viral reverse transcriptase enzyme converts RNA into double-stranded DNA, which is transported to the nucleus. In the nucleus, a viral enzyme called integrase mediates the integration of the viral DNA into the host chromosomal DNA. Thereafter viral DNA functions as a mammalian gene resulting in production of viral RNA and proteins. The viral structural proteins assemble around viral genomic RNA at the plasma membrane to produce new viruses. The final stage of the viral life cycle consists of proteolytic cleavage of precursor molecules by the viral protease enzyme (225).

During its clinical latency, HIV infection was earlier considered to be a relatively static infection. However, it is currently known that as many as 10^{10} virion particles are produced and cleared daily in an untreated individual (226). Rapid rate of virus production and high mutation rate create genetically diverse virus population within an individual patient. It is estimated that every possible point mutation will occur at least once daily in an HIV-infected non-treated person (227). The diversity of the viral population places high requirements for the potency of antiretroviral treatment.

2.2.3. CLINICAL COURSE

HIV is transmitted either through unprotected sexual contact, contaminated blood products, contaminated needles or injection equipment, or through mother-to-child transmission as transplacental or intra-partum infection or during breast-feeding (228). Symptomatic primary infection develops in ~50% of infected persons, usually 2-8 weeks after contracting the virus, and is characterized by fever, lymphadenopathy, pharyngitis and morbilliform skin eruption (222). The natural course of HIV infection thereafter is variable, but chronic HIV infection can remain clinically latent for years with minimal or no symptoms (229). With

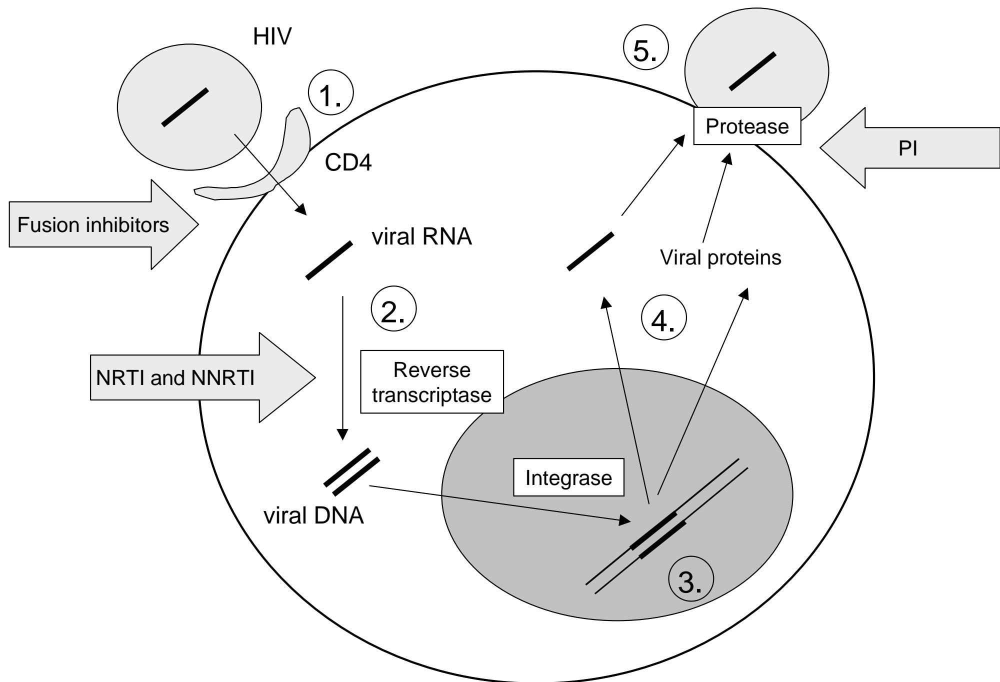


Figure 3. *Schematic picture of the life cycle of HIV and the site of action of antiretroviral agents. HIV enters the human host cell by using the CD4- and co-receptors (1). The released viral single-stranded RNA is converted into double-stranded DNA by the viral reverse transcriptase (2). Double-stranded viral DNA enters the cell nucleus and is incorporated into the cell DNA in a reaction catalyzed by the viral integrase enzyme (3). Viral proteins and RNA are synthesized (4) and new viruses assembled (5). Viral proteins are modified by the viral protease. Fusion inhibitors block the viral entry into the cell, NRTIs and NNRTIs inhibit the reverse transcriptase and PIs the protease enzyme.*

advancing immunodeficiency, patients often develop nonspecific symptoms such as fever, night sweats, mucosal and dermatological manifestations (229). The final stage of the infection is named AIDS and is defined by the occurrence of an opportunistic infection or tumor considered indicative of advanced infection with HIV. In untreated individuals, the median time for progression from contracting HIV to the development of AIDS is ~10 years (223).

2.2.4. TREATMENT

HISTORY OF ANTIRETROVIRAL THERAPY

Until late 1980s, the only therapeutic interventions available for HIV-infected persons were prophylaxis and treatment of opportunistic diseases. Since the treatment of opportunistic diseases does not affect the gradual loss of CD4+ T cells, patients remained at constant risk for further opportunistic diseases and the overall prognosis remained poor. The first antiretroviral agent to decrease mortality among patients with AIDS was

zidovudine (230). The superiority of a dual nucleoside combination over zidovudine monotherapy on survival and disease progression was shown later (231).

After mid 1990s it became possible to monitor the progression of HIV infection not only by measuring the CD4+ count but also by quantifying the amount of HI-virus in plasma (viral load). Viral load was soon shown to independently predict disease progression (232) and, even more importantly, the measurement of viral load made it possible to monitor the virologic efficacy of antiretroviral treatment. In 1995-96, it was understood that mono and dual therapies could only transiently suppress viral replication, whereas the introduction of triple-drug regimens resulted in durable virologic suppression and lead to enormous positive impact on the prognosis of HIV-infected people (233,234). The acronym HAART, originally referring to triple combination therapy, was widely used already in 1996. After the introduction of HAART, the mortality rates declined by ~60-80% (**Fig. 4**) (1). Improved prognosis was based on the recovery of immune system after the viral replication was controlled for by the combination therapy. Successful HAART is not only able to stop the gradual loss of CD4+ cells, but it also allows the regeneration of these cells. Prophylaxis for opportunistic infections can be successfully discontinued after sustained increase in CD4+ count (235). However, since it is impossible to eradicate the virus with present antiretroviral agents (236), HAART most likely needs to be continued permanently.

ANTIRETROVIRAL AGENTS

Nucleoside reverse transcriptase inhibitors (NRTI)

NRTIs are structurally similar to the natural building blocks of nucleic acids, but are unable to form phosphodiester linkage essential for deoxyribonucleic acid (DNA) elongation. They compete with the natural substrates of reverse transcriptase enzyme and when incorporated into the viral DNA, they terminate further elongation of DNA (**Fig. 3**) (237). NRTI-associated adverse events include e.g. polyneuropathy, gastrointestinal side effects, myositis, lipodystrophy, and in rare cases lactic acidosis (237,238). As of summer 2003, there are six NRTIs available in Finland: abacavir, didanosine, lamivudine, stavudine, zalcitabine, zidovudine (**Table 2**). These agents require intracellular activation through incorporation of three phosphate groups into the molecule before they can function as NRTIs. A novel agent, tenofovir, has one phosphate group bound to it, and hence it only requires two additional intracellular phosphorylations (239). Tenofovir is therefore regarded a nucleotide analogue instead of a nucleoside analogue, albeit eventually the mechanism of action is identical to that of other NRTIs.

Non-nucleoside reverse transcriptase inhibitors (NNRTI)

NNRTIs bind reversibly at a non-substrate binding site in the reverse transcriptase enzyme (**Fig. 3**). They inhibit non-competitively the function of reverse transcriptase by causing a change in the shape of the enzyme or blocking the polymerase active site (240). Rash is the most common side effect of NNRTIs, in

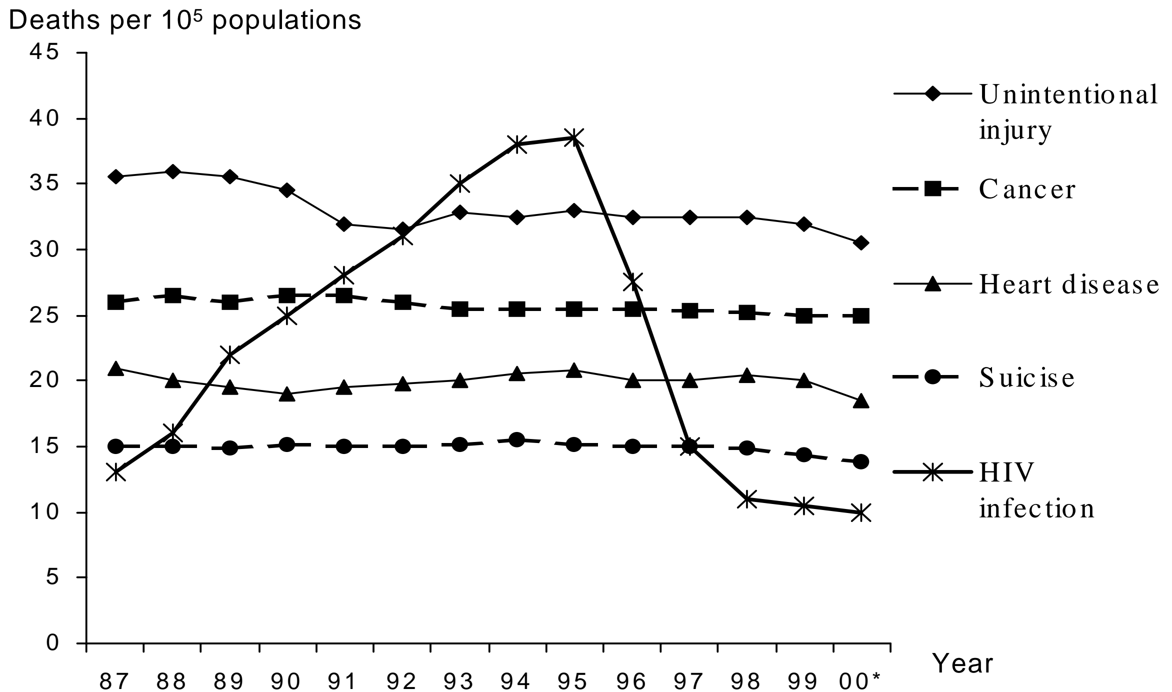


Figure 4. *Trends in annual rates of death per 100 000 population due to the leading causes of death among persons 25-44 years of age in the United States 1987-2000. HAART became widely available in 1996. Modified from http://www.cdc.gov/hiv/graphics/images/l285/l285-10.htm.*

addition, nevirapine is associated with liver toxicity and efavirenz with central nervous system symptoms (237). Currently there are three NNRTIs available in Finland: delavirdine, efavirenz and nevirapine (**Table 2**).

Protease inhibitors (PI)

The HIV protease, the target molecule of the PIs, is a 99 –amino-acid protein with aspartyl protease activity (241). PIs hinder the cleavage of the viral precursor proteins and therefore the released viral particles remain non-infectious to uninfected cells (**Fig. 3**). Mammalian cells also contain aspartyl proteases, but they do not efficiently cleave the viral polyproteins. PIs used for the treatment of HIV infection are inactive or only weakly active against human aspartyl proteases (241). All PIs may cause gastrointestinal side effects, other adverse events include elevations in liver function tests, hyperlipidemia (with the exception of atazanavir), glucose intolerance and fat redistribution (241,242). There are currently 8 PIs available in Finland: amprenavir, atazanavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir (**Table 2**).

Table 2. *Antiretroviral agents available in Finland as of autumn 2003.*

Nucleoside reverse transcriptase inhibitors NRTI	Non-nucleoside reverse transcriptase inhibitors NNRTI	Protease inhibitors PI	Fusion inhibitor
Zidovudine AZT/ ZDV	Nevirapine NVP	Saquinavir SQV	Enfuvirtide T-20
Lamivudine 3TC	Efavirenz EFV	Ritonavir RTV	
Didanosine ddI	Delavirdine DLV	Indinavir IDV	
Stavudine d4T		Nelfinavir NFV	
Zalcitabine ddC		Amprenavir APV	
Abacavir ABC		Lopinavir LPV	
Tenofovir TDF		Fosamprenavir fAPV	
		Atazanavir ATZ	

Fusion inhibitors

Fusion inhibitors are the newest class of antiretroviral agents. They block the viral entry into the host cell by preventing fusion of the viral envelope with the cell membrane (**Fig. 3**) (243). The only fusion inhibitor available today for clinical use, enfuvirtide, is a synthetic peptide and must be administered by subcutaneous injections. The most common adverse events include injection site complications and gastrointestinal disturbances (244,245). Due to its parenteral administration and high cost, enfuvirtide is currently used in salvage regimens of patients who harbor viruses resistant to other antiretroviral agents (246).

INITIATION OF THERAPY

HAART is recommended for HIV-infected patients with severe symptoms and also for asymptomatic patients with CD4+ cell counts less than 200/mm^3 (247,248). Treatment should be considered in patients with CD4+ counts between 200-350/mm^3, but is rarely indicated in patients with CD4+ counts above 350/mm^3 (248). The first-line alternatives are the combinations of two NRTIs with either a NNRTI or a PI (248). The success of the initial treatment is mainly determined by the patient's adherence to the medication. The combination of drugs must be individualized, since different combinations have different dosing patterns, dietary restrictions, and antiretroviral agents may have interactions with concurrent illnesses and medications. It is estimated that at least 90-95% of the doses must be taken at correct times with drug-specific eating or fasting requirements to maintain optimal drug concentrations in plasma and complete virologic suppression (247). Since the eradication of the virus is not possible with current regimens (2), high level of adherence must be maintained permanently.

2.3. HAART-ASSOCIATED LIPODYSTROPHY

2.3.1. METABOLIC ALTERATIONS OF HIV INFECTION BEFORE HAART

BODY COMPOSITION

Before HAART was available, the progression of HIV infection was often accompanied by loss of body weight. In its most severe form, loss of body weight results in HIV wasting syndrome, which is recognized as one of the AIDS-defining conditions (224). The wasting syndrome is characterized by loss of both lean body mass and fat mass (249). Loss of lean body mass makes the wasting syndrome distinctly different from lipodystrophy, which affects fat tissue only.

FEATURES OF INSULIN RESISTANCE

Before the HAART era, diabetes was rare in HIV-infected patients (250). The observed disturbances in glucose metabolism were often considered to be induced by medications, such as pentamidine (251), corticosteroids or megestrol acetate (250). Prior to HAART, patients with advanced HIV infection typically had increased serum concentrations of triglycerides, but decreased concentrations of total, LDL and HDL cholesterol (252).

2.3.2. CLINICAL AND METABOLIC CHARACTERISTICS OF HAL

HAART became widely available in 1996. The first case reports describing HAART-associated buffalo humps (253) and thinning of the buttocks and thighs together with hypertrophy of breasts (254) were published as early as 1997. Soon thereafter, accumulation of intra-abdominal fat (255) and facial lipoatrophy (256) were reported in patients using HAART. Today, these features are recognized as symptoms of HAL, which has also been named fat redistribution or fat maldistribution syndrome. Patients with HAL often have also hyperlipidemia and insulin resistance (257).

2.3.3. DEFINITION OF HAL

The main clinical features of lipodystrophy are subcutaneous fat loss and fat accumulation intra-abdominally, within breasts or over the dorsocervical spine (**Fig. 5**) (3,258,259). However, as of today there is no uniformly approved definition for lipodystrophy. Recently, an objective case definition of lipodystrophy has been suggested (260). However, despite its relatively complex composition (the definition requires DEXA scan, abdominal CT scan, measurement of HDL cholesterol and anion gap in blood) the model reaches only 79% sensitivity and 80% specificity in diagnosing lipodystrophy.

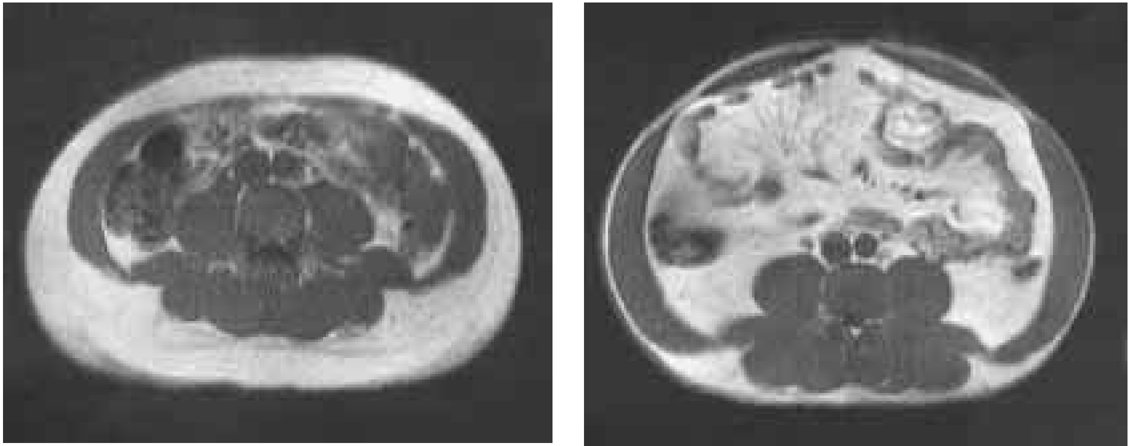


Figure 5. *Abdominal MRI scan of a person with normal fat distribution characterized by abundant subcutaneous and little intra-abdominal fat (left), and of a person with HAL (right) with almost complete loss of subcutaneous and severe accumulation of intra-abdominal fat. Fat is shown in white in these MRI scans.*

2.3.4. PATHOGENESIS AND ETIOLOGY OF HAL

LIPODYSTROPHIC HUMAN ADIPOSE TISSUE

There are limited *in vivo* human adipose tissue data describing changes in lipodystrophic adipose tissue. Morphology of lipoatrophic tissue is characterized by a greater variation in adipocyte size with an increased number of small adipocytes when compared to HIV-infected treatment naive patients or HIV negative controls (261-263). Adipocytes have been found to contain clusters of small lipid droplets instead of a single large droplet (264). Lipogranulomata with lipid-laden macrophages and vascular proliferation have been reported (261,263). Also apoptosis has been present in lipoatrophic adipose tissue (263).

Gene expression in SAT in patients with HAL has only been evaluated in a single study (262). Patients with HAL had decreased mRNA concentrations of PPARγ, SREBP-1c, C/EBPα, C/EBPβ, HSL, GLUT4, leptin, but increased mRNA concentration of TNFα (262). At the protein level, SREBP-1 was increased, although SREBP-1c mRNA was decreased. Protein levels of the β-subunit of insulin receptor and the insulin-signaling kinase protein kinase B were decreased. Protein concentration of PPARγ was also decreased in keeping with its decreased mRNA concentration. SREBP-1c mRNA concentration correlated negatively with insulin resistance, and weaker inverse correlations were found between insulin resistance and PPARγ, leptin and GLUT4 mRNA concentrations. The mRNA concentrations of TNFα correlated positively with insulin resistance (262). In this study patients with HAL are compared to a group of healthy HIV negative subjects. It therefore remains uncertain to what extent the observed alterations are due to lipodystrophy, HAART *per se* or HIV-infection (262).

NRTI-induced mitochondrial toxicity due to inhibition of mitochondrial DNA polymerase γ has been suggested to cause lipodystrophy, especially lipoatrophy (265). In keeping with this hypothesis, mitochondrial DNA (mtDNA) has been found to be reduced in SAT of patients with HAL when compared to HAART-treated patients without lipodystrophy, HIV-infected treatment-naive patients or HIV negative controls (264,266). The number of mtDNA copies per cell has been shown to be decreased in adipocytes from patients with HAL when compared to treatment naive HIV-infected patients or healthy controls (261). Loss of mtDNA was more severe in stavudine- than zidovudine-treated patients (261). Electron microscopy has revealed abnormal cristae and inclusions in mitochondria in lipoatrophic adipose tissue (264). No large mitochondrial deletions, insertions or point mutations have been found in these studies (264,266).

There are no tissue data on the hypertrophic intra-abdominal fat in patients with HAL. In a single case report of a patient with HAART-associated buffalo hump, gross pathological examination revealed unremarkable adipose tissue with a few septae and the microscopic appearance was consistent with non-encapsulated mature fat tissue (267).

INSULIN RESISTANCE IN PATIENTS WITH HAL

In one of the most comprehensive studies on insulin resistance in patients with HAL, multiple alterations in glucose metabolism were detected: lipodystrophic patients had increased post-absorptive glucose production, decreased insulin-induced suppression of endogenous glucose production and lipolysis, and decreased insulin-stimulated glucose disposal when compared to healthy subjects (268). In a longitudinal study, 12 weeks of PI-containing treatment reduced insulin-stimulated glucose disposal implying insulin resistance in skeletal muscle, but did not affect the ability of insulin to suppress hepatic glucose production (269). Insulin resistance was also suggested to affect adipose tissue, since insulin-induced suppression of lipolysis was impaired after 12 weeks of treatment (269). In keeping with insulin resistance in skeletal muscle, IMCL content is increased in patients with HAL and correlates with insulin resistance (270,271). Increased circulating FFA concentrations may also contribute to HAART-associated insulin resistance. Two doses of acipimox were used as an inhibitor of lipolysis in a pilot study of seven men with HAL (272). Acipimox decreased FFA concentrations significantly and resulted in a significant increase in insulin sensitivity compared to placebo (272).

ETIOLOGY OF HAL

Since HAART usually consists of at least three drugs, it is difficult, if not impossible to assess the effect of an individual antiretroviral agent on lipodystrophy in HIV-infected patients. PIs were primarily suspected to cause lipodystrophy and other metabolic abnormalities. However, since basically all PI-treated patients receive also two or more NRTIs, their potential contribution to the development of adverse events must be taken into account. Furthermore, HIV infection itself, nadir CD4+ count, degree of the HAART-induced

viral suppression, older age, gender and ethnicity may play a role in the development of lipodystrophy and metabolic abnormalities (273).

PROTEASE INHIBITORS

Effects of PIs in vitro

Several PIs have been shown to inhibit differentiation of various adipocyte cell lines (**Table 3**). The proposed mechanisms include both PPARγ-dependent (274,275) and –independent (276,277) mechanisms. The earlier events of differentiation, such as mitotic clonal phase of preadipocytes does not seem to be affected (274,275). PIs have also been shown to increase lipolysis (278) and decrease LPL expression (279) and activity (280). Nelfinavir has been associated with increased apoptosis in mature adipocytes (274). Pre- or co-treatment of cells with rosiglitazone or troglitazone has been shown to reverse the PI-induced inhibition of adipocyte differentiation and the increased basal lipolysis (275,281,282), whereas concomitant treatment with TNFα has been shown to exacerbate the suppressive effects of PIs on adipogenesis (281). Although patients with HAL often present with both subcutaneous lipoatrophy and hypertrophy of visceral fat, there are hardly any data suggesting mechanism(s) for fat hypertrophy. Stimulation of differentiation of 3T3-L1 adipocytes by a PI has been reported in a single study, which showed increased expression of the active, mature SREBP-1 protein, but decreased expression of PPARγ and C/EBPα (283).

PIs have been shown to decrease insulin-stimulated glucose uptake in several models, but there is no consensus regarding the mechanisms of insulin resistance. In some (282), but not all models (284), PIs seem to inhibit GLUT4 translocation. PIs inhibit the action of insulin also in HepG2 hepatoma cells, which express no or almost no GLUT4 (285). PIs have caused inhibition of early insulin signaling in HepG2 hepatoma cells (286), but not in 3T3-L1 adipocytes (284). It has also been shown that different PIs and different exposure times result in different effects on glucose transport (287). Troglitazone pre- and co-treatment with nelfinavir had no effect on the impairment in insulin-stimulated glucose uptake induced by nelfinavir (282).

Effects of PIs in animal models

There are limited data from animal models evaluating metabolic adverse events of PIs. In Wistar rats, a single intravenous dose of indinavir decreased glucose uptake in the muscle but did not alter the suppression of hepatic glucose output under hyperinsulinemic conditions (288). Ritonavir has been shown to increase plasma triglyceride and cholesterol levels, and liver fat content (289). These abnormalities were due to accumulation of the active SREBP-1 protein, but not mRNA of SREBP in the liver and white adipose tissue (289). The increase of the active form of SREBP-1 was suggested to be due to its decreased degradation (289). However, in another model ritonavir treatment in rats decreased serum triglyceride concentration, increased body fat and caused no change in insulin sensitivity (290).

Table 3. *In vitro effects of PIs on cell and tissue models.*

Protease inhibitor	Cell / tissue	Effect	Ref.
APV, IDV, NFV, RTV	3T3-L1	Inhibition of differentiation, but not via inhibition of PPARγ-mediated gene transcription.	(276)
IDV, SQV	Cultured primary human preadipocytes	Inhibition of differentiation via PPARγ-independent mechanism.	(277)
IDV	3T3-F442A	Decrease in protein expression of SREBP-1, PPARγ and insulin receptor. Defective maturation and altered localization of SREBP-1.	(275)
IDV	Human embryonic kidney and hepatoma cells	Inhibition of SREBP-1c –dependent genes, such as LPL and FAS, but no effect on SREBP-1c independent gene encoding LDL receptor.	(279)
IDV	3T3-L1	Inhibition of insulin-stimulated glucose uptake, but no effect on early insulin signaling or translocation of GLUT4 or GLUT1.	(284)
IDV	Xenopus oocytes	Inhibition of the activity of glucose transporter isoforms in decreasing order GLUT4 >> GLUT2 > GLUT3 > GLUT1 ~ GLUT8.	(291)
IDV	L6 skeletal muscle cells	Stronger inhibition of GLUT4 over GLUT1.	(292)
IDV	Rat skeletal muscle	Decrease in both insulin- and contraction-stimulated glucose transport, decrease in cell surface GLUT4. No effect on insulin stimulation of PI 3-kinase and phosphorylation of protein kinase B.	(293)
IDV	HepG2 hepatoma cells	Reduced insulin effect on IRS-1 tyrosine phosphorylation, on the association of PI 3-kinase with IRS-1 and phosphorylation of protein kinase B. Impaired insulin-stimulated glycogen synthesis.	(285,286)
APV, IDV, NFV, SQV, RTV	C3H10T1/2 stem cells	Inhibition of lipogenesis and increased acute lipolysis (APV and IDV had only little effect). Only SQV inhibited ligand-binding to PPARγ.	(278)
NFV, IDV, SQV	3T3-L1, L6 myotubes	Acute exposure to NFV, IDV or SQV: decreased insulin-stimulated glucose uptake through direct interaction with GLUT4. Prolonged exposure to NFV (but not IDV or SQV): increased basal lipolysis and glucose uptake with elevated GLUT1 expression and decreased insulin-stimulated	(287)

		glucose transport and protein kinase B phosphorylation.	
NFV	3T3-L1	Decrease in the expression of C/EBPα, PPARγ. Reduced level of active, but not the precursor protein of SREBP-1c. Increased apoptosis in differentiated adipocytes.	(274)
NFV	3T3-L1	Decreased insulin-stimulated glucose uptake and impaired GLUT4 translocation and increased basal lipolysis.	(282)
RTV	3T3-L1	Enhancement in adipocyte differentiation. Increase in active SREBP-1 protein, blunted upregulation of PPARγ and C/EBPα during differentiation.	(283)
SQV	3T3-F442A	Inhibition of synthesis of lipids, increase in lipolysis. Inhibition of LPL activity, but no effect on LPL mRNA. Increase in basal glucose transport, but decrease in insulin-stimulated glucose transport.	(280)

For abbreviations of antiretroviral agents, see Table 2.

These discrepant *in vitro* and animal data regarding the metabolic effects of HAART underscore the importance of defining *in vivo* effects of antiretroviral agents on gene expression in humans.

Effects of PIs in humans

Lipodystrophy was originally described as an adverse event of PIs (257,294). Although lipodystrophy has been described in patients not receiving PIs (295), the prevalence of lipodystrophy is greater in patients receiving PIs than in PI-naive or antiretroviral therapy-naive HIV-infected patients (296). In a study with 655 HIV-infected patients receiving first-line antiretroviral therapy and followed for a median of 86 weeks, indinavir exposure significantly increased the risk of developing combined form of both lipoatrophy and fat accumulation (297). In another report with 366 patients receiving their first antiretroviral therapy, lipoatrophy and lipohypertrophy were independently associated with the initiation of a PI-containing regimen (298). One study found an association between high nelfinavir trough concentrations in plasma and overall lipodystrophy and peripheral fat wasting scores (299), and another study found an increased risk of lipodystrophy with increasing duration of PI therapy (300).

In cross-sectional studies, PI-treated patients have higher prevalence of insulin resistance than non-PI-treated (301,302), treatment-naive HIV-infected patients (302) or healthy subjects (257). In longitudinal studies, treatment with a PI-containing HAART for 3 months increased fasting serum insulin concentration by 96% (303), and diabetes was diagnosed in 6% of 117 patients after a 12-month treatment with a PI-containing regimen (304). In a cohort of 1785 women, the use of PIs was associated with a threefold increase in the risk of developing diabetes (305).

Most of the studies evaluating the effects of PIs on insulin resistance have compared PI-treated patients with either HIV-infected drug naive or healthy subjects. Therefore it remains unknown to what extent PI treatment *per se* and to what extent PI-associated lipodystrophy contribute to insulin resistance. There are some data demonstrating the development of insulin resistance in PI-treated patients in the absence of changes in body composition. The effects of a single antiretroviral drug can be evaluated in HIV negative subjects, since there is no risk for development of viral resistance due to suboptimal HIV therapy. In these studies, a 4-week treatment with indinavir or even a single dose of it decreased insulin sensitivity without changes in circulating lipoproteins or FFA concentrations, or in the amount of intra-abdominal or subcutaneous fat (306,307). However, several studies have shown that PI-treated patients with lipodystrophy are more insulin resistant than PI-treated patients without lipodystrophy (257,308,309). Patients with both subcutaneous lipoatrophy and intra-abdominal fat accumulation have been found to be more insulin resistant than those with atrophy only or those with no lipodystrophy (302,309). Taken together, it seems likely that both PI treatment *per se* and lipodystrophy may contribute to the development of insulin resistance in HIV-infected patients.

NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

Effects of NRTIs in vitro and in animal models

There are substantially less *in vitro* data on the effects of NRTIs than of PIs on adipocyte differentiation. When 3T3-F442A cells were treated either with zidovudine, stavudine, didanosine or lamivudine, only zidovudine exerted a significant antiadipogenic effect (310). NRTI-associated lipoatrophy has been suggested to result from NRTI-induced inhibition of mitochondrial DNA polymerase γ (265). *In vitro* studies of the NRTIs demonstrate the following hierarchy of mitochondrial DNA polymerase γ inhibition: zalcitabine > didanosine > stavudine > lamivudine > zidovudine > abacavir (311). In lean mice, stavudine decreased hepatic and muscle mtDNA, but only in obese (*ob/ob*) mice stavudine decreased mtDNA in white adipose tissue (312).

Effects of NRTIs in humans

Lipodystrophy, especially lipoatrophy, has been described in HIV-infected patients who have been treated with NRTIs only (295,313,314). Trunk-to-extremity fat ratio was increased and extremity-to-total fat ratio decreased in NRTI-treated, PI-naive patients when compared with BMI-matched HIV-infected, treatment naive controls (315). In addition, lipodystrophy was significantly more common among patients randomized to receive two PIs and stavudine than among patients who received dual PI therapy without stavudine (316). Of the various NRTIs, stavudine has most commonly been associated with lipodystrophy (295,297,317-319). The use of lamivudine has been associated with the development of lipodystrophy in one study (319).

There are only limited and contradictory data regarding insulin resistance and the use of NRTIs. In a cross-sectional study with 45 patients, NRTI treated patients were not more insulin resistant than HIV-infected therapy-naive patients (302). In a small longitudinal study with 9 subjects, dual NRTI therapy for 5 months did not change serum glucose, insulin or lipid concentrations (303). However, the duration of NRTI treatment has independently predicted fasting hyperinsulinemia (309), and the use of stavudine and the rate of lipolysis have been strong independent predictors of insulin resistance (320). The use of stavudine has also been associated with an increased risk of developing diabetes in a large retrospective study with 1011 patients (321). However, when therapy naive patients were compared with PI-naive, but either zidovudine or stavudine treated patients, those on stavudine had lower total body fat, markedly reduced subcutaneous-to-visceral fat ratio and higher serum triglycerides, but plasma glucose, insulin and C-peptide concentrations were not different between the groups (295).

NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

There are limited human and no *in vitro* data on the effects of NNRTIs on HAL and metabolic adverse effects. In a longitudinal study using DEXA scans, the use of nevirapine has been associated with a reduced rate of loss of subcutaneous fat when compared with PI-containing regimens (322). Favorable effects of

nevirapine on blood lipids have been reported in a study comparing three different first-line antiretroviral combinations: in addition to 2 NRTIs, patients were randomized to receive nevirapine, indinavir or lamivudine. The nevirapine group had a 49% increase from baseline in HDL-cholesterol concentration after 24 weeks of treatment as opposed to 16% increase in the lamivudine and indinavir groups (323).

HOST-ASSOCIATED FACTORS

Patients with older age (317,324) and female gender appear to have an increased risk for developing lipodystrophy (297,298,324). Co-infection with hepatitis C (325), male gender (326) and white race (322) have been associated with an increased risk of developing lipoatrophy.

Preliminary data suggest that genetic susceptibility may influence the development of HAART-associated metabolic adverse events. TNFα polymorphism has been evaluated in two studies. Data from a genetic case-control study suggest that a G to A substitution in the –238 position in the promoter region of the TNFα gene predisposes to HAL (327). Similarly, the TNFα -238G/A promoter polymorphism has been found to independently enhance the progression of lipodystrophy in a cohort of HAART-recipients (328). A single-nucleotide polymorphism (3'322C/G) in the SREBP-1c gene was found to be predictive of HAART-related dyslipidemia in a group of 67 patients, although it did not alter the amino acid sequence of the protein (329). A larger study with 355 HAART-treated patients, however, could not confirm the association of this SREBP-1c polymorphism and dyslipidemia (330).

2.3.5. PREVALENCE OF HAL

LIPODYSTROPHY

The reported prevalences of lipodystrophy vary from as low as 2% among 272 PI-treated patients (331) up to 83% among 113 PI-treated patients (332) depending on the age and sex of the patients, type and duration of HAART, and the different definitions of lipodystrophy. Estimates from large surveys indicate a 50% prevalence of at least one physical abnormality after 12-18 months of therapy (3,273).

ABNORMALITIES IN GLUCOSE METABOLISM

It is difficult to compare the results from different studies examining glucose metabolism in patients with HAL due to differences in the methods used. In oral glucose tolerance test (OGTT), 35% of patients with HAL had impaired glucose tolerance (IGT), and an additional 7% had diabetes (309). These prevalences of IGT and diabetes were 7-fold higher than those in age- and BMI-matched controls (309). Other studies have reported a 17 - 46% prevalence of IGT and a 7 - 13% prevalence of diabetes measured using the OGTT (301,332), and up to a 61% prevalence of peripheral insulin resistance measured using the intravenous insulin tolerance test (333) among patients using PIs. In a retrospective study of 1011 patients followed for a median of 289 days, 16 new cases of diabetes were diagnosed; the risk for developing diabetes was significantly increased in patients receiving indinavir or stavudine (321). A large cohort study with 17 852

HIV-infected subjects with or without antiretroviral therapy has found only a 2.5% -prevalence of diabetes (334). NNRTI and a combination of NNRTI and PI therapy were independently associated with the presence of diabetes in this large cohort (334).

LIPID ABNORMALITIES

The prevalence of hyperlipidemia among HIV-infected patients using PIs is in the range of 30-75% (301,332,334). Among patients receiving non-PI containing HAART, 11% had cholesterol levels >6.5 mmol/l and 26% triglycerides >2.2 mmol/l (335). In a longitudinal study, treatment with HAART including a PI for a mean of 3.4 months increased serum total cholesterol by 23% and triglyceride concentration by 48% (303).

IMPAIRED FIBRINOLYSIS

In addition to having altered glucose and lipid metabolism, patients with HAL have also been found to have increased plasma PAI-1 and tPA antigen levels when compared to HIV negative controls (336). PAI-1 and fibrinogen levels were also significantly higher in 266 patients (42% with lipodystrophy) receiving PI-containing HAART when compared to 97 treatment naive HIV-infected subjects (337). In this study, the plasma PAI-1 concentration was independently correlated with the use of PI-containing HAART, serum triglyceride and insulin concentrations, and BMI (337).

2.3.6. SIGNIFICANCE OF HAL

Lipodystrophy has a variety of physical and psychological effects, ranging from bodily discomfort to low self-esteem and depression (338,339). Especially facial lipoatrophy may be a visible marker of HIV infection (340) and may lead to social isolation (338). In addition, self-perceived lipodystrophy is an independent cause of nonadherence to HAART (5).

Due to the short history of HAART, long-term effects of HAART-associated insulin resistance and dyslipidemia on cardiovascular morbidity still remain unknown. Dyslipidemias have been associated with an increased intima-media thickness of the carotid artery suggesting an increased risk of atherosclerosis in HIV-infected patients (341,342), but in a multivariate analysis HAART or lipodystrophy were not independent risk factors for the increased intima-media thickness (342). HIV-infected patients may have a high prevalence of conventional cardiovascular risk factors, such as cigarette smoking (343). Also dietary habits may be inappropriate especially among individuals with long history of HIV infection, since dietary advice given to HIV-infected persons prior to mid 1990s was targeted to prevent wasting by increasing caloric, especially fat intake.

Data from large cohort studies are inconsistent regarding the risk of cardiovascular events in HAART-treated patients. In the Veterans Affairs Cohort with follow-up of 36 766 HIV-infected persons, the introduction of

HAART was associated with a reduction, and not an increase, in the rate of hospital admission for cardio- or cerebrovascular disease (344). However, these patients had been exposed to combination antiretroviral therapy for relatively short duration: the median exposure time for NRTIs was 17 months, for PIs 16 months and for NNRTIs 9 months (344). In the HOPS cohort (HIV Outpatient Study) with 5672 HIV-infected patients, the frequency of myocardial infarction increased significantly after the introduction of PIs, and the use of PIs was strongly associated with the likelihood of having a myocardial infarction (345). In the DAD cohort (Data collection on Adverse events of anti-HIV Drugs) with 23 468 HIV-infected people, each year of exposure to antiretroviral therapy increased the risk of myocardial infraction by 26% even after adjusting for known cardiovascular risk factors such as age, gender and smoking (6).

2.3.7. TREATMENT OF HAL

Attempts to treat HAL are based either on the modification of HA ART, or on the addition of another agent to treat the metabolic complication. Simple removal of an agent from HAART has been shown to lead to an unacceptably high risk of virologic failure (346). Therefore, in the so called "switch-studies" the suspected toxic antiretroviral agent is usually replaced by another agent, which is believed to be metabolically less toxic .

If adverse events are severe, discontinuation of all antiretroviral agents may be considered. Development of viral resistance is unlikely to occur, if all agents are discontinued simultaneously (347). However, due to the inevitable rebound in viral replication and the consequent decrease in the CD4+ cell count, it is usually not possible to discontinue HAART for a long time. In an uncontrolled study, 7-week interruption of all antiretroviral agents caused a significant decrease in triglyceride, total and LDL cholesterol concentration, but there were no significant changes in glucose or insulin levels or anthropometric measurements(348).

SWITCHING ANTIRETROVIRAL AGENTS

It is difficult to draw firm conclusions regarding the switch studies due to differences in study population, study design and methods applied (**Table 4**). Most of the published studies have evaluated the effect of replacing a PI by a NNRTI. There are no constant beneficial effects on body composition in these studies. However, blood lipids have improved in most (349-353), but not all (354-356) studies after commencing a NNRTI. Although the effects on glucose metabolism are less pronounced, some studies report an improvement (349,350,356). Another approach is to replace a PI by a NRTI, mainly by abacavir. This switch does not appear to correct body composition, but is associated with improvements in blood lipids (357-359). Data regarding effects on glucose metabolism are very limited.

A couple of studies have evaluated the effect of replacing a thymidine analogue NRTI, i.e. stavudine (or zidovudine), by abacavir. This switch seems to result in an increase in the amount of limb fat measured using

DEXA. However, the absolute change in fat mass has been very modest, e.g. limb fat increased by 310 g during 24 weeks of abacavir therapy compared to the control group (360), and in another study leg fat increased by 9 g and arm fat by 14 g per month after switching to abacavir (361). Although statistically significant, the increase in leg fat was not noticed either by the patients themselves or the treating physicians (360). Blood lipid and glucose concentrations have remained unchanged in these studies (360,361).

Switching of antiretroviral agents has been shown to be virologically safe in several studies in patients with no previous virologic failure (362). Virologic failure has been reported to be less or at most equally common in patients who were randomized to switch their PI to abacavir (358), nevirapine (352,353,355) or efavirenz (352) as compared to those who were randomized to continue with the PI. Switching of stavudine or zidovudine to abacavir had no effect on viral load when compared to those who continued with stavudine or zidovudine treatment (360). However, some studies have reported an increased risk of virologic failure among patients who switched their PI to abacavir (363), efavirenz or nevirapine (359), especially among patients who had received prior partially suppressive NRTI mono- or dual therapy.

MODIFICATIONS OF LIFE STYLE

The amount of aerobic, or combined aerobic and resistant training has been independently inversely associated with fasting triglyceride concentration and almost significantly associated with insulin resistance in a group of 120 HIV-infected patients and also in the subgroup of 69 patients with HAL (364). In small intervention trials involving HAART-treated patients with and without lipodystrophy, physical exercise has been shown to increase lean body mass, either to cause no change or decrease fat mass, and to decrease blood lipids, especially serum triglycerides (365-368). Taken together, physical exercise appears to improve blood lipids, and might be helpful in patients with abdominal lipohypertrophy but not with subcutaneous lipoatrophy.

The effect of diet on HAL and associated metabolic adverse events is unclear. In a study involving 100 HIV-infected subjects, there was no significant difference in the intake of total or saturated fat between patients with or without lipodystrophy (369). However, total energy intake was higher in lipodystrophic patients compared to non-lipodystrophic patients (369). Lipid-lowering diet for 6 months decreased serum cholesterol by 10% and triglycerides by 23% in those patients who self-reported good compliance with dietary advice, whereas there were no changes in blood lipids in patients who reported poor compliance (370).

Table 4. *The effects of changing antiretroviral agents on HAL.*

Intervention Group A	Control Group B	N	Baseline characteristics	Follow-up time	Results: lipodystrophy	Results: lipids	Results: glucose	Ref.
Switch PI to ABC+NVP+ADF +hydroxyurea	Continue PI	81	All with HAL	24 weeks	A > B loss of total fat and SAT, and VAT in those with moderate-severe fat accumulation at baseline	A: total chol ↓, TG ↓, HDL chol ↑ B: HDL chol ↑	No change, no change in OGTT	**(371)**
Switch PI to EFV	No control group	41	All with HAL	1 year	No change in VAT; SAT ↓ (CT); no change in WHR	No change	No change, no change in OGTT	**(354)**
Switch PI to EFV	No control group	20	All with HAL	6 months	WHR ↓, no change in SAT (ultrasonography)	TG ↓	Insulin resistance index ↓	**(349)**
Switch PI to NVP	No control group	23	All with HAL	6 months	WHR ↓	Total chol ↓, TG ↓	Glucose ↓, insulin resistance index ↓	**(350)**
Switch PI to NVP	Continue with PI	138	Simplification trial, ~72% had HAL	6 months	A: improvement in 50% as reported by the patient and doctor (no objective measurements)	No change	ND	**(355)**
Switch PI to EFV or to NVP	Continue PI	77	Simplification trial, ~75% had HAL	12 months	No change in those with HAL at baseline (DEXA, anthropometry)	NVP: total and LDL cholesterol ↓, TG ↓	No change	**(352)**
Switch PI to NVP	Continue PI	106	All with HAL	48 weeks	No change (DEXA)	A: Total chol ↓ and TG ↓	ND	**(353)**
Switch PI to NVP	Continue PI	34	All with HAL	24 weeks	No change (DEXA, anthropometry)	A: total and LDL chol ↓, VLDL-1 TG ↓, HDL chol ↑, HDL size ↑	No change	**(351)**
Switch PI to NVP	Continue PI	14	All with HAL	12 months	No change in WHR. Apoptosis in SAT did not change.	No change	A: insulin ↓	**(356)**
Switch PI to ABC (A), EFV (E) or NVP (N)		460	Simplification trial, ~30% lipoaccumulation, ~42% lipoatrophy	12 months	No change (no objective measurements)	A: total cholesterol ↓, proportion with TG>4.5 mmol/l ↓	glucose ↑ more in E than in A or N	**(359)**
Switch PI to ABC	Continue PI	211	Simplification trial, lipodystrophy not reported	48 weeks	ND	A: total chol ↓, TG ↓	ND	**(358)**

Switch PI to ABC	Continue PI	31		12 months		A: total chol ↓, TG ↓	A: insulin sensitivity ↑	**(357)**
Switch PI-containing regimen to AZT+3TC+ABC	Continue PI	163	Simplification trial, lipodystrophy not reported	84 weeks	No change in WHR	A: total chol↓, TG↓	ND	**(363)**
Switch AZT or d4T to ABC	Continue AZT or d4T	111	Moderate-severe lipoatrophy	24 week	A: Limb fat ↑ (DEXA), SAT ↑ (CT)	No change	No change	**(360)**
Switch d4T to AZT, and PI to ABC	Continue d4T and PI	37	LD not reported	48 weeks	A: limb fat ↑ (DEXA), no change in VAT (CT)	No change	No change	**(361)**
Switch d4T to ABC (A); PI or NNRTI to ABC (B); d4T+PI or d4T+NNRTI to ABC+AZT (C)		27	Patients had hypercholesterolemia and/or lipoatrophy	48 weeks	A: Total and limb fat ↑ (DEXA) No change in VAT (CT)	B: total chol ↓, LDL chol ↓, TG ↓ C: total chol ↓, LDL chol ↓	No change, no change in OGTT	**(362)**

For abbreviations of antiretroviral agents, see Table 2.

LIPID-LOWERING AGENTS

Overall, lipid-lowering agents often fail to reduce lipid concentrations to target levels in HAART-associated hyperlipidemia (372). There are several small studies reporting effects of pravastatin, atorvastatin and fluvastatin on HAART-associated hyperlipidemia. The mean decrease in total cholesterol has been in the range of 17-27%, while that in serum triglycerides has varied from no effect to a 37% decrease (372). Gemfibrozil and fenofibrate have decreased serum triglyceride concentrations by 18-54% in small trials (372).

PIs, NNRTIs and statins are all either metabolized by or affect the activity of various cytochrome P450 isoforms; therefore their concomitant use may lead to adverse pharmacological interactions (372). In healthy volunteers, the combination of ritonavir and saquinavir increased the median estimated area under the curve of simvastatin by 3059%, atorvastatin by 79% and decreased that of pravastatin by 50% (373). Pravastatin is considered safe, fluvastatin may be a safe alternative and atorvastatin can be used with caution with low initial doses in patients using PIs (372). Any statin can probably be used safely in persons using efavirenz or nevirapine, although more data are needed (372). Drug-drug interactions with fibrates and antiretroviral agents are unlikely to occur (372).

METFORMIN

The effect of metformin on HAART-associated insulin resistance and lipodystrophy has been evaluated in two studies. In a randomized, open-label study with 29 non-diabetic patients with HAART-associated insulin resistance, 2-month treatment with metformin significantly decreased plasma glucose, insulin, C-peptide and triglyceride concentrations when compared to placebo. VAT and VAT-to-total fat ratio also decreased in the metformin group (374). In a randomized, double-blind, placebo-controlled trial with 26 patients, metformin demonstrated significant reductions in insulin area under the curve during OGTT in patients with HAL with abnormal OGTT or hyperinsulinemia at baseline (375). The metformin group lost weight, and the amount of both VAT and SAT decreased (375). Metformin also decreased plasma tPA and PAI-1 concentrations (336).

THIAZOLIDINEDIONES

Thiazolidinediones (glitazones) are novel insulin-sensitizing anti-diabetic agents, two of which, rosiglitazone and pioglitazone, are available for treatment of type 2 diabetes both in Europe and the U.S. The first agent in this group, troglitazone was withdrawn due to hepatotoxicity (376). Thiazolidinediones are ligands for the transcription factor PPARγ, activation of which is critical for adipocyte differentiation (Chapter 2.1.3.) (377,378). In patients with type 2 diabetes, treatment with rosiglitazone improves insulin sensitivity despite increasing body weight and fat mass (10,379-382). The increase in fat mass amounts to 3.5-4.0 kg in 12 weeks (379,382) and appears to occur almost exclusively in SAT (380,381,383), an effect which would be desirable in patients with lipodystrophy. The expression of PPARγ in SAT has been shown to be decreased

in HIV-infected patients with lipodystrophy (262). *In vitro*, rosiglitazone has been shown to increase the expression of PPARγ (377) and to prevent the block in adipocyte differentiation induced by PIs (275).

In an uncontrolled study of patients with various forms of non-HIV lipodystrophy, troglitazone treatment improved insulin sensitivity, increased body fat % and the amount of SAT, but not that of VAT (213). Thiazolidinediones have also been used in two small, uncontrolled studies in patients with HAL. Six diabetic patients with HAL were treated with troglitazone for 3 months (384). Due to the small sample size, statistical significances were not reported, but a potential improvement in insulin sensitivity, an increase in total, LDL and HDL cholesterol concentrations, and a decrease in triglyceride concentration were reported (384). Troglitazone also appeared to decrease the amount of VAT and increase the amount of SAT (384). In another uncontrolled study involving 8 patients with HAL, rosiglitazone treatment for 6 to 12 weeks was reported to significantly improve insulin sensitivity, decrease the amount of VAT and increase the amount of SAT (385). There are, however, no controlled studies evaluating the effects of thiazolidinediones in HAL or in non-HIV lipodystrophies.

GROWTH HORMONE

Growth hormone with doses ranging from 6 mg/day to 4 mg every other day has been shown to decrease % body fat and the amount of VAT in patients with HAL (386-388). However, body composition rebounds to or near baseline after a wash out period of 12 weeks (387). Furthermore, insulin sensitivity decreased, and four out of 30 patients developed diabetes and three developed cancer of unknown relationship to treatment (387). In another study, insulin sensitivity decreased after one month of therapy, but returned to almost baseline after 6 months of treatment (388).

OTHER INTERVENTIONS

In small series of patients, topical hyaluronic acid injections for severe facial lipoatrophy have been reported to give good results (389,390). Liposuction has been used for the treatment of buffalo hump (391).

3. AIMS OF THE STUDY

The present studies were undertaken to answer the following questions:

1) Is hepatic fat content increased, and does the amount of hepatic fat correlate with features of insulin resistance in HIV-infected patients with HAL?

2) Are the circulating concentration of adiponectin and its expression in SAT decreased, and does adiponectin concentration correlate with features of insulin resistance in HIV-infected patients with HAL?

3) Is expression of genes involved in adipogenesis, fatty acid metabolism and inflammation altered in SAT in HIV-infected patients with HAL compared to HAART-treated patients without lipodystrophy?

4) Does treatment with rosiglitazone increase the amount of subcutaneous fat and improve features of insulin resistance in HIV-infected patients with HAL?

5) Does rosiglitazone treatment affect gene expression in SAT in HIV-infected patients with HAL?

6) Is plasma PAI-1 concentration increased, and does it correlate with hepatic fat content before and after rosiglitazone treatment in HIV-infected patients with HAL?

4. SUBJECTS AND STUDY DESIGNS

SUBJECTS

All HIV-infected patients were enrolled from the outpatient clinic of the Helsinki University Central Hospital. Both male and female subjects were included in the study. They had to be older than 18 years, have been treated with HAART for at least 18 months with no changes in the treatment regimen during eight weeks prior to enrollment, and did not have signs or symptoms of current opportunistic infections. Patients with HAART-associated lipodystrophy (HAART+LD+) had self-reported symptoms of loss of subcutaneous fat with or without increased abdominal girth, breast size or development of a buffalo hump. HIV-infected patients without lipodystrophy (HAART+LD-) had received HAART without developing symptoms of lipodystrophy. Both the presence and absence of the signs of lipodystrophy were confirmed by the single investigator (J.S.) before enrollment. Exclusion criteria for the rosiglitazone vs. placebo treatment study (Studies IV-VI) included serum transaminase concentrations greater than three times the upper limit of normal, heart failure, severe hypertriglyceridemia (serum triglycerides > 10 mmol/l), diabetes and pregnancy. HIV negative normal subjects (HIV-) were recruited from occupational health services in Helsinki. They were healthy as judged by history and physical examination and standard laboratory tests, and did not use any regular medication. None of the study subjects was a carrier of hepatitis B or C. Baseline characteristics of the study subjects are given in **Tables 5 and 6**.

The purpose, nature and potential risks of the study were explained to the study subjects before their written informed consent was obtained. Treatment study with rosiglitazone was investigator-initiated and not supported by the manufacturer of rosiglitazone. The study protocols were approved by the ethics committee of Helsinki University Central Hospital.

STUDY I: Hepatic fat in HAL

In this cross-sectional study, liver fat content was measured using proton spectroscopy in three age- and weight-matched groups of men: HIV-infected men using HAART who had developed lipodystrophy (HAART+LD+), HIV-infected men using HAART but without lipodystrophy (HAART+LD-) and HIV negative normal subjects (HIV-). Interrelationships between liver fat content and volumes of intra-abdominal and subcutaneous fat, and various laboratory parameters associated with insulin resistance were studied.

STUDY II: Adiponectin and HAL

Serum adiponectin and adiponectin mRNA concentration in SAT were measured in two gender-, age- and BMI-matched groups of HIV-infected, HAART-treated patients either with (HAART+LD+) or without lipodystrophy (HAART+LD-). Correlations of serum adiponectin concentration and its expression in SAT were examined with features of insulin resistance and liver fat content.

STUDY III: Gene expression in SAT in HAL

Expressions of multiple genes in SAT were compared between two gender-, age- and BMI-matched groups: HIV-infected, HAART-treated patients with lipodystrophy (HAART+LD+) and HIV-infected, HAART-treated patients without lipodystrophy (HAART+LD-). The mRNA concentrations measured using real-time PCR were determined for transcription factors (PPARγ, SREBP-1c, PPARδ) and PGC-1, for genes involved in lipogenesis and fatty acid metabolism (LPL, ACS, ALBP, KLBP, FATP-1, FATP-4), glucose transport (GLUT4, GLUT1) and inflammation (IL-6 and CD45).

STUDY IV: Treatment of HAL with rosiglitazone

Thirty HIV-infected patients with HAL participated in this randomized, double-blind, placebo-controlled study that consisted of treatment with either rosiglitazone 8 mg/d (n=15) or an identical-looking placebo (n=15) in a parallel fashion for 24 weeks. The following characteristics were considered during randomization: age, sex, BMI, fasting serum triglyceride and cholesterol concentrations, and use of PIs. The primary aim was to determine whether rosiglitazone increases the amount of subcutaneous fat in these patients. Measurements of body composition were performed at baseline and 24 weeks, and included quantification of intra-abdominal and subcutaneous fat using MRI, total body fat by bioelectrical impedance analysis (BIA), liver fat by proton spectroscopy, serum leptin concentration as a marker of adipose tissue mass and anthropometric measurements. Secondary aims included evaluation of the effects of rosiglitazone on features of insulin resistance and safety parameters. For the secondary aims of the study, blood samples were taken at outpatient visits at baseline, 2, 6, 12, 18 and 24 weeks.

STUDY V: Effects of rosiglitazone on gene expression in SAT in HAL

Thirty HIV-infected, HAART-treated patients with lipodystrophy who participated in Study IV had a subcutaneous fat biopsy performed at baseline and after 24 weeks of treatment. Effects of rosiglitazone vs. placebo on the expression of multiple genes were analyzed and these changes were correlated with the changes in features of insulin resistance and body composition.

STUDY VI: PAI-1 in HAL

In the cross-sectional part of the study, three gender- and BMI-matched groups were included: HIV-infected patients using HAART who had developed lipodystrophy (HAART+LD+), HIV-infected patients using HAART but without lipodystrophy (HAART+LD-) and HIV negative normal subjects (HIV-). In addition to measuring plasma PAI-1 and tPA concentrations, and mRNA of PAI-1 in SAT, body composition was measured using MRI and liver fat by proton spectroscopy. In the treatment part of the study, the HAART+LD+ group was treated with rosiglitazone or placebo for 24 weeks. Correlations of plasma PAI-1 concentration and its expression in SAT with features of insulin resistance were studied both before and after rosiglitazone vs. placebo treatment.

Table 5. *The number, age, body composition and metabolic characteristics of the study subjects.*

Variable	STUDY I HAART+LD+	HAART+LD-	HIV-	STUDY II-VI HAART+LD+	STUDY II,III,V,VI HAART+LD-	STUDY IV HIV-	STUDY VI HIV-
Number of subjects (f/m)	25 (0/25)	9 (0/9)	35 (0/35)	30 (5/25)	13 (4/9)	30 (5/25)	15 (3/12)
Age (years)	44 ± 2	40 ± 3	42 ± 1	43 ± 2	39 ± 2	43 ± 2	33 ± 3 ##
Body weight (kg)	75 ± 2	73 ± 4	76 ± 1	73 ± 2	69 ± 4	73 ± 2	71 ± 2
BMI (kg/m^2)	23.9 ± 0.6	23.3 ± 1.4	23.7 ± 0.3	23.6 ± 0.5	22.4 ± 1.1	23.6 ± 0.4	23.2 ± 0.6
Waist to hip ratio	1.00 ± 0.01 *	0.93 ± 0.03	0.94 ± 0.1 ##	0.99 ± 0.01 ***	0.89 ± 0.03	0.92 ± 0.01 ###	0.87 ± 0.02 ###
Total abdominal fat (cm^3)	230 ± 20	200 ± 50	220 ± 90	3100 ± 300	2700 ± 500	3200 ± 200	2700 ± 500
Subcutaneous fat (cm^3)	80 ± 10	110 ± 30	140 ± 10 ###	1100 ± 200 *	1800 ± 300	2100 ± 100 ###	1900 ± 300 #
Intra-abdominal fat (cm^3)	150 ± 20 *	90 ± 30	80 ± 20 ###	1900 ± 200 **	900 ± 300	1100 ± 100 ###	800 ± 200 ##
Liver fat (%)	8.1 ± 2.0 **	2.9 ± 1.6	5.3 ± 1.1 #	7.6 ± 1.7 ***	2.1 ± 1.1	3.2 ± 0.9 ###	3.6 ± 1.2 #
fP-glucose (mmol/l)	5.6 ± 0.3	5.2 ± 0.1	5.4 ± 0.1	5.5 ± 0.3	5.0 ± 0.1	5.3 ± 0.1	5.2 ± 0.1
fS-insulin (mU/l)	12.1 ± 1.7 *	7.1 ± 1.5	6.1 ± 0.5 ###	11.1 ± 1.2 **	6.5 ± 1.1	5.3 ± 0.5 ###	5.5 ± 0.7 ##
fS-triglycerides (mmol/l)	3.6 ± 0.4 ***	1.2 ± 0.2	1.1 ± 0.1 ###	3.4 ± 0.4 ***	1.2 ± 0.1	1.0 ± 0.1 ###	0.9 ± 0.1 ###
fS-HDL cholesterol (mmol/l)	1.1 ± 0.1 ***	1.5 ± 0.1	1.4 ± 0.1 ###	1.1 ± 0.1 ***	1.6 ± 0.1	1.5 ± 0.1 ###	1.5 ± 0.1
fS-cholesterol (mmol/l)	5.9 ± 0.2 **	4.8 ± 0.3	5.1 ± 0.2 ##	5.9 ± 0.2 **	4.9 ± 0.2	5.3 ± 0.2 #	4.6 ± 0.3 ###
S-ALT (U/l)	50 ± 5 **	28 ± 3	29 ± 2 ###	46 ± 5 **	28 ± 3	26 ± 2 ###	27 ± 3 ##

Data are shown as mean ± SEM. *<0.05, **<0.01, ***<0.001 for the comparison between HAART+LD+ vs. HAART+LD-; #<0.05, ##<0.01, ###<0.001 for the comparison between HAART+LD+ vs. HIV-. The reference intervals of the laboratory tests are: fP-glucose 4.0-6.1 mmol/l, fS-insulin 2-20 mU/l, fS-triglycerides 0.4-1.7 mmol/l, fS-HDL cholesterol >0.9 mmol/l, fS-cholesterol <5.0 mmol/l, S-ALT 10-50 U/l for men and 10-40 U/l for women.

Table 6. *HIV- and HAART-related characteristics of the study subjects.*

Variable	STUDY I HAART+LD+	STUDY I HAART+LD-	STUDY II-VI HAART+LD+	STUDY II-VI HAART+LD-
Number of subjects	25	9	30	13
Time since diagnosis of HIV (years)	8.3 ± 0.7	8.6 ± 1.9	8.4 ± 0.6	8.7 ± 1.3
Currently using PI	76%	56%	73%	69%
Currently using NNRTI	32%	44%	33%	31%
Currently using NRTI	100%	100%	100%	100%
Duration of PI therapy (months)	39 ± 4	29 ± 8	39 ± 4	39 ± 7
Duration of NNRTI therapy (months)	6 ± 2	9 ± 4	6 ± 2	9 ± 3
Duration of NRTI therapy (months)	69 ± 5 *	45 ± 11	68 ± 5	57 ± 10
Most recent CD4+ cell count (x 10^6/l)	561 ± 64	504 ± 102	572 ± 54	516 ± 70
Most recent HIV-1 RNA log 10 (copies/ml)	1.9 ± 0.2	1.8 ± 0.4	1.9 ± 0.1	1.6 ± 0.2

Data are shown as mean ± SEM. *<0.05 for the comparison between the HAART+LD+ vs. HAART+LD-.

5. METHODS

5.1. BODY COMPOSITION

The volumes of intra-abdominal and subcutaneous fat were measured using MRI. A total of 16 T1-weighted trans-axial scans extending from 8 cm above to 8 cm below the 4th and 5th lumbar interspace (a single scan at the level of the 4th and 5th lumbar interspace in Study I) were analyzed for the determination of intra-abdominal and subcutaneous fat (field of view 375 x 500 mm^2, slice thickness 10 mm, breath-hold repetition time 138.9 msec, echo time 4.1 msec). Intra-abdominal and subcutaneous fat volumes were measured using an image analysis software (Alice 3.0, Parexel, Waltham, MA). A histogram of pixel intensity of each MRI scan was displayed, and the intensity corresponding to the nadir between the lean and fat peaks was used as a cut point. First, total abdominal adipose tissue was defined as the area of pixels above this cut point. Intra-abdominal adipose tissue was thereafter manually erased and the area of subcutaneous fat was calculated. Intra-abdominal fat was finally determined by subtracting the SAT area from the total abdominal adipose tissue area. MRI analyses were performed by a single investigator (J.S.) blinded for treatment randomization (Study IV-VI). The reproducibility of intra-abdominal and subcutaneous fat measurements performed on two separate occasions in non-diabetic subjects (n=10) is 5 and 3% (coefficient of variation) at our institute.

Percentage of total body fat and body fat mass were determined using BIA (BioElectrical Impedance Analyzer System model #BIA-101A; RJL Systems, Detroit, MI). Waist circumference was measured midway between the lower rib margin and the iliac crest, and hip circumference over the great trochanters. Skinfold thicknesses (sum of mean values of triplicate measurements) were determined at 6 sites (triceps, biceps, subscapular, iliac crest, thigh and cheek). Bioelectrical impedance analyses and all anthropometric measurements were performed by a single investigator (J.S.) blinded for treatment randomization.

5.2. LIVER FAT

Liver fat content was measured using MRI proton spectroscopy. Localized single voxel (2 x 2 x 2 cm^3) proton spectra were recorded using a 1.5 T whole body system (Siemens Magnetom Vision, Erlangen, Germany), which consisted of the combination of whole body and loop surface coils for radiofrequency transmitting and signal receiving. T1-weighed high resolution MRI images were used for localization of the voxel within the right lobe of the liver. Vascular structures and subcutaneous fat were avoided in localization of the voxel. Subjects were lying on their stomach on the surface coil, which was embedded in a mattress in order to ensure a firm contact between the chest wall and the surface coil, and to minimize movement artefact caused by breathing.

The single voxel spectra were recorded using the stimulated –echo acquisition mode sequence with an echo time of 20 msec, a repetition time of 3000 msec, a mixing time of 30 msec, 1024 data points over 1000 kHz spectral width with 32 averages. Water-suppressed spectra with 128 averages were also recorded to detect weak lipid signals. The short echo time and long repetition time were chosen to ensure fully relaxed water signal, which was used as an internal standard. Chemical shifts were measured relative to water at 4.80 ppm. The methylene signal, which represents intracellular triglyceride, was measured at 1.4 ppm (**Fig. 6**) (392). Signal intensities were quantified using an analysis program VAPRO-MRUI (393). Spectroscopic intracellular triglyceride content (in percent) was expressed as a ratio of the area under the methylene peak to that under the sum of the methylene and the water peaks x 100. All spectra were analyzed by a single physicist who was unaware of any of the clinical data and treatment randomization. The reproducibility of repeated measurements of liver fat in non-diabetic subjects studied on two occasions at our institute is 11% (coefficient of variation).

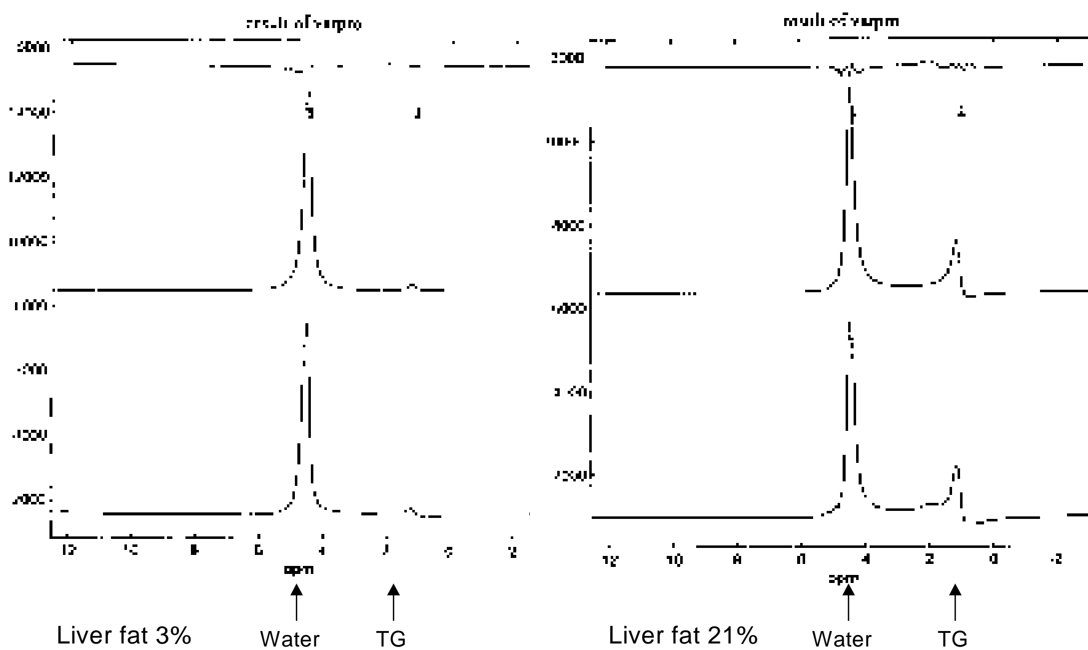


Figure 6. *Spectra from the liver of a person with a 3% (left) and 21% (right) hepatic fat content measured using MRI proton spectroscopy. TG = triglyceride.*

5.3. GENE EXPRESSION IN SAT

A needle aspiration biopsy of abdominal SAT was taken under local anesthesia. The fat sample was immediately frozen and stored in liquid nitrogen until analysis. A part of the biopsy was immediately treated with collagenase for 30 min at 37 °C. From this sample, the diameter of 200 adipocytes was determined using a microscope. In order to measure blood contamination in the samples, 100 µl of supernatant was quantified for hemoglobin concentration after homogenization. Supernatant hemoglobin was measured by absorbance at 560 nm, compared to the hemoglobin standard curve and the hemoglobin reading was divided by the individual blood hemoglobin concentration.

Total RNA and cDNA preparation

Frozen fat tissue (50-150 mg) was homogenized in 2 ml of RNA STAT-60 (Tel-Test, Friendswood, TX) and total RNA was isolated according to the manufacturer's instructions. After DNase treatment (RNase-free DNase set, Qiagen, Hilden, Germany), RNA was purified using the RNeasy mini kit (Qiagen). RNA concentrations were measured using the RiboGreen fluorescent nucleic acid stain (RNA quantification kit, Molecular Probes, Eugene, OR). The quality of RNA was checked by agarose gel electrophoresis. Average yields of total RNA were 3.3 ± 0.4 µg per 100 mg of adipose tissue wet weight, and did not differ between the groups. Isolated RNA was stored at –80 ^{o}C until quantification of the target mRNAs. A total of 0.1 µg of RNA was transcribed into cDNA using M-MLV reverse transcriptase (Life Technologies, Paisley, UK) and oligo $(dT)_{12\text{-}18}$ primer.

Quantification of mRNA concentration of β-actin, adiponectin, PPARγ, LPL and SREBP-1c

Quantification of the mRNAs was performed by real-time PCR using LightCycler technology (Roche Diagnostics GmbH, Mannheim, Germany). 2 µl of 1:10 diluted cDNA was brought to a final volume of 20 µl, which contained 3 mM $MgCl_2$, 2 µl of LightCycler-FastStart DNA SYBR Green I Mix (Roche Diagnostics), and 0.5 µM of primers. After initial activation of the DNA polymerase at 95 ^{o}C for 10 min, the amplification conditions were as follows: 40 cycles consisting of denaturation at 95 ^{o}C for 15 sec, annealing for 5 sec at 57 ^{o}C (β-actin), 58 ^{o}C (adiponectin), 56 ^{o}C (PPARγ), 58 ^{o}C (LPL), or for 10 sec at 60 ^{o}C (SREBP-1c) and extension at 72 ^{o}C. The extension times (sec) were calculated from the amplicon size (base pairs/25). Fluorescent data were acquired at the end of each extension phase. After amplification, a melting curve analysis from 65 ^{o}C to 95 ^{o}C with a heating rate of 0.1 ^{o}C/sec with a continuous fluorescence acquisition was made. The primers for β-actin, adiponectin, PPARγ, LPL and SREBP-1c are listed in **Table 7**. For β-actin, adiponectin, LPL and SREBP-1c expression, standard curves were created from a specific PCR product. A standard curve for PPARγ was created using purified cloned plasmid cDNA (QIAquick PCR purification kit, Qiagen, Hilden, Germany). To account for differences in RNA loading, adiponectin, PPARγ, SREBP-1c and LPL were expressed relative to β-actin.

Quantification of mRNA concentration of β2-microglobulin, PAI-1, leptin, GLUT1, GLUT4, PGC-1, PPARδ, ALBP, KLBP, FATP-1, FATP-4, ACS, CD45 and IL-6

TaqMan real-time semiquantitative PCR was performed according to the manufacturer's protocol using ABI PRISM 7000 Sequence Detection System instrument and software (PE Applied Biosystem, Foster City, CA). Primer and probe sets were designed using the manufacturer's software and sequences available in GeneBank (**Table 7**). IL-6 was measured using Pre-Developed TaqMan Assay Reagents (PE Applied Biosystem, Foster City, CA). The GLUT4 primer set has been published (394). Expression levels were quantified (arbitrary units) by generating a six-point serial standard curve (395). The mRNA concentrations of PAI-1, leptin, GLUT1, GLUT4, PGC-1, PPARδ, ALBP, KLBP, FATP-1, FATP-4, ACS, CD45 and IL-6 were given relative to β2-microglobulin mRNA concentration.

5.4. LABORATORY ANALYSES

Serum free insulin concentrations were determined with radioimmunoassay (Phadeseph Insulin RIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) after precipitation with polyethylene glycol (396). Serum C-peptide concentrations were determined by a time-resolved fluoroimmunoassay (AUTOdelfia^{TM} C-peptide, Wallac, Turku, Finland). HbA_{1c} was measured by a high pressure liquid chromatography using a fully automated Glycosylated Hemoglobin Analyzer System (BioRad, Richmond, CA). Plasma glucose concentrations were measured by a hexokinase method. Serum total and HDL cholesterol, and triglyceride concentrations were measured by respective enzymatic kits from Roche Diagnostics using an autoanalyzer

Table 7. *Primers and probes used for mRNA analyses.*

Gene Bank Number	Sense primers	Antisense primers	Probe
PPARγ U79012	5’-CTCATATCCGAGGGCCAA	5’-TGCCAAGTCGCTGTCATC	*
β-actin M10277	5’-CACACTGTGCCCATCTACGA	5’-CCATCTCTGCTCGAAGTCC	*
SREBP-1c NM004176	5’-GCGGAGCCATGGATTGCAC	5’- CTCTTCCTTGATACCAGGCCC	*
LPL NM000237	5’-GGTCGAAGCATTGGAATCCAG	5’-TAGGGCATCTGAGAACGAGTC	*
Adiponectin XM 003191	5’-CAGAGATGGCACCCCTGGTG	5’-TTCACCGATGTCTCCCTTAG	*
β2-microglobulin AH002619	5’-GCCTGCCGTGTGAACCAT	5’-TTACATGTCTCGATCCCACTTAACTAT	5’-TGACTTTGTCACAGCCCA
GLUT1 NM006516	5’-CCTGTGGGAGCCTGCAAA	5’-TCTATACACAACAGGGCAGGAGTCT	5’-CACTGCTCAAGAAGAC
GLUT4 14758189	5’-GCTACCTCTACATCATCCAGAATCTC	5’-CCAGAAACATCGGCCCA	5’-CTGCCAGAAAGAGTCTGAAGCGCCT
PGC-1 AH008808	5’-AGAGACAAATGCACCTCCAAAAA	5’-AAAGTTGTTGGTTTGGCTTGTAAGT	5’-AAGTCCCACACACAGTCGCAGTCACAA
PPARδ NM006238	5’-CACACGGCGCCCTTTG	5’-CCTTCTCTGCCTGCCACAA	5’-ATCCACGACATCGAGAC
CD45 NT004612	5’-TCTTGGCATTTGGCTTTGC	5’-GGAGACTGACTGCGTGTGTGA	5’-CTGGACACAGAAGTATTT
ALBP NM001442	5’-TGATAAACTGGTGGTGGAATGC	5’-CCCTTGGCTTATGCTCTCTCA	5’-TCATGAAAGGCGTCACTTCCACGAGA
KLBP NM001444	5’-GGGAAGGAAAGCACAATAACAAGA	5’-CGAGTACAGGTGACATTGTTCATG	5’CACACTCCACCACTAATTTCCCATCTTTCAA TT
FATP-1 AX015323	5’-AGATGCCCGCTCACTTCG	5’-GCTAAGGCCCTGATCTTTGGA	5’-CCACCGCCGCCACCATTTCTC
FATP-4 AF055899	5’-CAAGACCATCAGGCGCG	5’-CGCACCTTTGCCTTCACCT	5’-TATCTTTGGCGGCCTGGTCCTCCT
ACS L09229	5’-CAAAGACGGCTGGTTACACACA	5’-TCCGGTCGATAATTTTCAAGGT	5’-CCATTTGGTAACCATTTTCCGATGTCCC
PAI-1	5´-CGCCAGAGCAGGACGAA	5´-GGAGACATCTGCATCCTGAAGTT	5´-CGCCAATCGCAAGGCACCTCTG
Leptin	5’-CCAAAACCCTCATCAAGACAATT	5’-GGAGACTGACTGCGTGTGTGA	5’-CACCAGGATCAATGACADD

(Roche Diagnostics Hitachi 917, Hitachi Ltd, Tokyo, Japan). Serum aspartate aminotransferase, alanine aminotransferase (ALT), and gamma glutamyltransferase activities were determined according to recommendations of the European Committee for Clinical Laboratory Standards using the Roche Diagnostics Hitachi 917 autoanalyzer. Venous blood gas analysis was performed using specific electrodes with a blood gas analyzer (Ciba Corning 850, Medfield, MA). Blood lactate was determined using an enzymatic method (Dade Behring ACA Analytical Test Packs, Dade Behring, Deerfield, IL).

The concentration of leptin in serum was measured by radioimmunoassay using a commercial kit (Human leptin RIA kit, Linco Research, St. Charles, MO). Serum concentration of adiponectin was measured using a commercial enzyme-linked immunosorbent assay (Human Adiponectin ELISA kit, B-Bridge International, San Jose, CA). Serum concentrations of IL-6 and TNFα were measured using commercial enzyme-linked immunoassays (Quantikine, R&D Systems, Minneapolis, MN). Serum CRP was analyzed using a commercial kit (Ultrasensitive CRP Kit, Orion Diagnostica, Espoo, Finland). Serum FFA were measured by a fluorometric assay (397). Plasma PAI-1 and tPA concentrations were measured by enzyme immunoassays TintElize PAI-1 and TintElize tPA, respectively (Biopool International, Umeå, Sweden).

HIV viral load was measured using the HIV-1 Monitor Test (Roche Diagnostics, Branchburg, NJ) with a detection limit of 50 copies/ml. Serum PI trough concentrations were determined using liquid chromatography; the assay was available for indinavir, nelfinavir, ritonavir and saquinavir.

All blood samples were drawn after an overnight fast and either analyzed immediately, or stored at –20 ºC or - 80 ºC until analyses.

5.5. STATISTICAL METHODS

The unpaired t-test or analysis of variance followed by pairwise comparison using Fisher's Least-Significant-Difference test was used to compare differences between the groups. Correlations were calculated using Spearman's rank correlation coefficient. Categorical variables were compared using Fisher's exact test. Effects of rosiglitazone and placebo treatment were calculated by comparing changes between the groups during 24 weeks using the unpaired t-test. Changes within rosiglitazone and placebo groups were calculated by the paired t-test. Repeated measurements over time were compared using analysis of variance followed by Fisher's Least-Significant-Difference test. Logarithmic transformation was performed on skewed data. All data are given as mean ± standard error of mean (SEM). Sample size for study IV was calculated based on the effects of troglitazone on the amount of subcutaneous fat measured by MRI in patients with non-HIV lipodystrophy (213). In this study, subcutaneous fat in the abdominal region increased by 837 ml after 6 months of troglitazone treatment. In the present study, a sample size of 15 in each group has 95 % power to detect a difference in means of abdominal subcutaneous fat of 450 ml assuming that the common standard

deviation is 300 ml using a two group t-test with a significance level of 0.05. All calculations were performed using the Systat statistical package, version 10.0 (Systat, Evanston, IL) or GraphPad Prism version 2.01 (GraphPad Inc, San Diego, CA). A p-value less than 0.05 was considered statistically significant.

6. RESULTS

6.1. HEPATIC FAT IN HAL (Study I)

The HAART+LD+, HAART+LD- and HIV- groups were comparable with respect to age and BMI (**Table 5**). Alcohol consumption was comparable between the groups (107 ± 27 vs. 153 ± 57 vs. 78 ± 11 grams/week in the HAART+LD+ vs. HAART+LD- vs. HIV-, non-significant [NS]). None of the subjects was a carrier of hepatitis B or hepatitis C. All HIV-infected patients had contracted HIV through sexual contact. HIV-related characteristics were comparable between the HAART+LD+ and the HAART+LD- groups with the exception of the HAART+LD+ group having had a longer duration of NRTI therapy (**Table 6**).

Body composition

The total amount of fat in the abdominal region was comparable between the groups, but its distribution was different (**Table 5**). The HAART+LD+ group had significantly more intra-abdominal fat than the HAART+LD- or the HIV- group, and significantly less subcutaneous fat than the HIV- group (**Table 5**). The ratio of intra-abdominal to subcutaneous fat was 4.4-fold higher in the HAART+LD+ group (3.1 ± 0.6) than in the HAART+LD- group (0.7 ± 0.1, $p<0.001$) and 6.2-fold higher than in the HIV- group (0.5 ± 0.1, $p<0.001$). The WHR was significantly higher in the HAART+LD+ group than in the HAART+LD- or the HIV- group (0.94 ± 0.01, $p<0.01$).

Biochemical characteristics

Serum insulin concentrations were significantly higher in the HAART+LD+ group than either in the HAART+LD- or the HIV- group (**Table 5**). Serum insulin concentrations did not correlate with the amount of intra-abdominal fat within the HAART+LD+ group ($r=0.26$, NS). The HAART+LD+ group had significantly lower serum HDL cholesterol concentration, and higher concentrations of triglyceride and total cholesterol than the HAART+LD- or the HIV- groups (**Table 5**). Serum ALT concentrations were significantly higher in the HAART+LD+ group than in the HAART+LD- or the HIV- groups (**Table 5**). Blood lactate concentrations were similar in both HIV-infected groups (1.3 ± 0.1 vs. 1.1 ± 0.2 mmol/l in the HAART+LD+ vs. HAART+LD- group, NS) and none of the patients had acidosis.

Liver fat

Liver fat content in the HAART+LD+ group was 53% higher than in the HIV- group and 179% higher than in the HAART+LD- group (**Table 5).** Liver fat content was not significantly different between the HAART+LD- and HIV- groups. Liver fat content correlated significantly with fasting serum insulin concentration in the HAART+LD+ and the HIV- group (**Fig. 7**). Similar significant relationships were observed between liver fat content and serum C-peptide concentrations (**Fig. 7**). The slopes of the regression lines relating liver fat and fasting insulin concentration were similar in the HAART+LD+ and the HIV-

groups. The intercepts of the regression lines were, however, significantly different between the HAART+LD+ and the HIV- group (p<0.001) implying that for a given percentage of liver fat, serum fasting insulin concentrations were significantly higher in the HAART+LD+ than in the HIV- group (**Fig. 7**). Liver fat did not correlate with the amount of intra-abdominal fat or WHR in the HAART+LD+, the HIV- (**Fig. 7**) or the HAART+LD- group (data not shown).

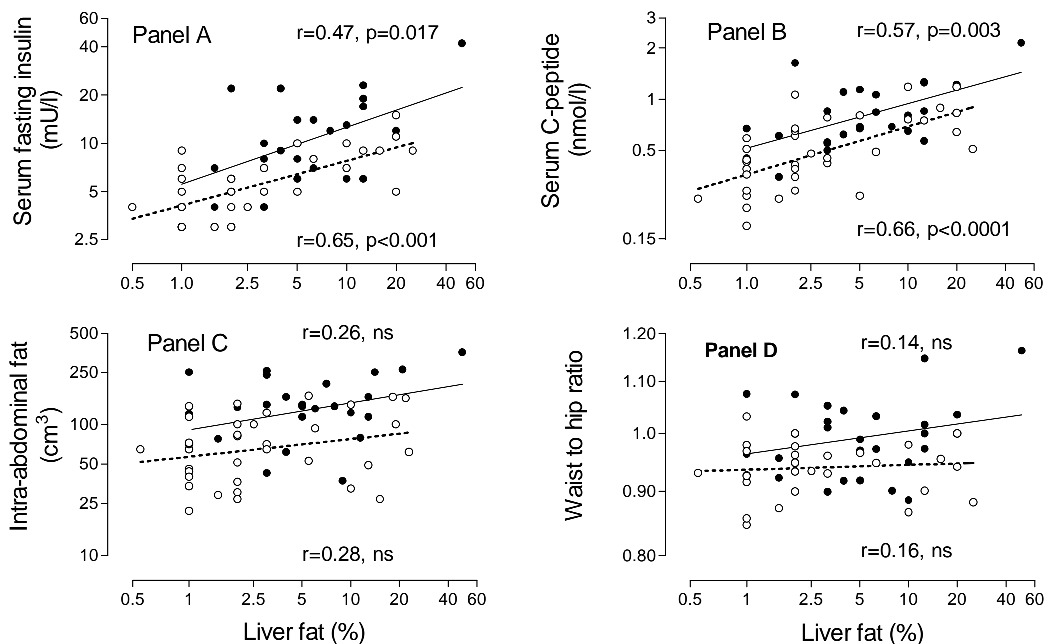


Figure 7. *Relationships between liver fat and serum fasting insulin concentration (panel A), serum C-peptide concentration (panel B), the amount of intra-abdominal fat (panel C), and the waist-to-hip ratio (panel D) in patients with HAL (•, solid line) and in HIV negative subjects (o, dotted line). r = correlation coefficient.*

Leptin

The HAART+LD+ group had significantly lower leptin concentrations than the two other groups (2.9 ± 0.3 vs. 4.0 ± 0.6 ng/ml in the HAART+LD+ vs. the two other groups, p<0.05). Serum leptin concentrations were closely correlated with the amount of SAT in both the HAART+LD+ and the HIV- groups (**Fig. 8**). Within the HAART+LD+ and the HIV- groups, serum leptin correlated with BMI, but the slopes of these relationships were different (**Fig. 8**). For the same BMI above approximately 20 kg/m^2, the HAART+LD+ group had a lower leptin concentration than the HIV- group.

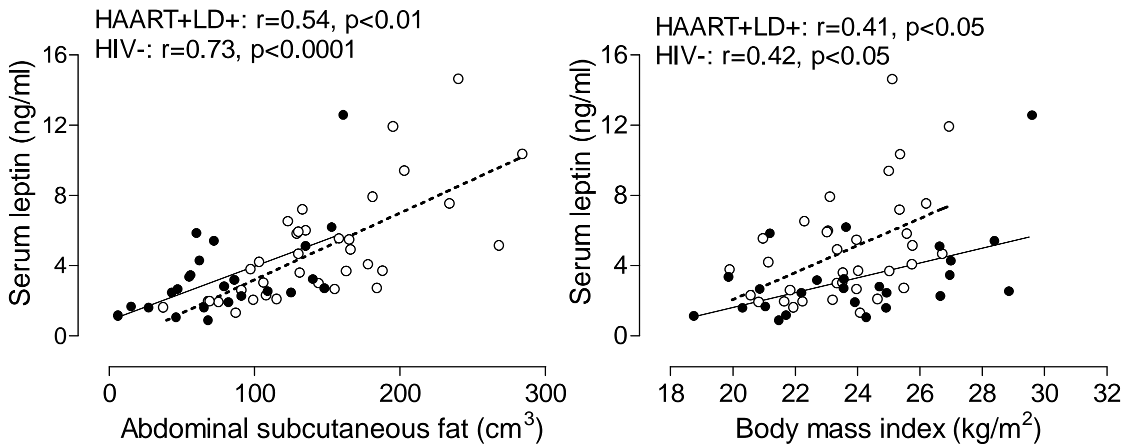


Figure 8. *Relationship between the amount of abdominal subcutaneous fat and serum leptin concentration (left), and between body mass index and serum leptin concentration (right) in patients with HAL (•, solid line) and in HIV-negative subjects (o, dotted line). r = correlation coefficient.*

6.2. ADIPONECTIN IN HAL (Study II)

Body composition and biochemical characteristics of the study groups

Age and BMIs were comparable between the HAART+LD+ and the HAART+LD- groups (**Table 5**). The amount of total abdominal fat measured using MRI was similar between the groups, but the HAART+LD+ group had significantly less subcutaneous and more intra-abdominal fat than the HAART+LD- group (**Table 5**). The HAART+LD+ group had significantly higher fasting serum insulin and triglyceride, and lower HDL cholesterol concentrations than the HAART+LD- group (**Table 5**). Liver fat content was significantly higher in the HAART+LD+ group than the HAART+LD- group (**Table 5**). Among all HAART-treated patients, liver fat content correlated closely with serum fasting insulin concentration (r= 0.60, p<0.001).

Adiponectin

The mRNA concentration of adiponectin in SAT was significantly decreased in the HAART+LD+ patients when compared with the HAART+LD- patients (**Fig. 9**). Serum adiponectin concentration was significantly lower in the HAART+LD+ than in the HAART+LD- group (**Fig. 9**).

In all HAART-treated patients, adiponectin expression in SAT correlated significantly with serum concentration of adiponectin (r=0.52, p<0.001). Serum adiponectin concentrations correlated with features of insulin resistance: serum triglyceride (r=-0.52, p<0.001), HDL cholesterol (r=0.39, p<0.01), insulin (r=-0.36, p<0.05) and C-peptide (r=-0.38, p<0.05) concentrations. In addition, serum adiponectin concentrations correlated inversely with liver fat content (r=-0.50, p<0.001, **Fig. 9**) and with the amount of intra-abdominal fat (r=-0.54, p<0.001), but not with the amount of subcutaneous (r=0.20, NS) fat. Serum adiponectin concentrations also correlated inversely with those of ALT (r=-0.51, p<0.001). Similar correlations were

found between the mRNA concentration of adiponectin in SAT and features of insulin resistance: fasting serum triglycerides (r=-0.65, p<0.001), HDL cholesterol (r=0.44, p<0.01), insulin (r=-0.43, p<0.01), C-peptide (r=-0.44, p<0.01), liver fat (r=-0.55, p<0.001, **Fig.9**) and intra-abdominal fat content (r=-0.68, p<0.001), but not with the amount of subcutaneous fat (r=0.27, NS).

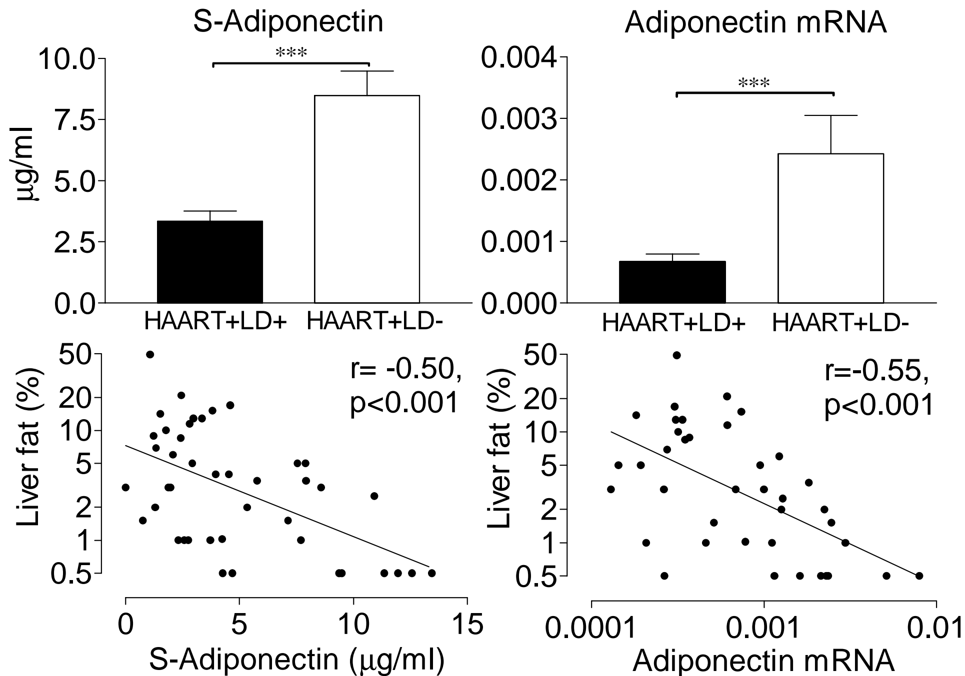


Figure 9. *Serum adiponectin and adiponectin mRNA concentration in SAT (upper panels) and their relationships with liver fat content (lower panels). Error bar = SEM, r = correlation coefficient. ***p<0.001.*

6.3. GENE EXPRESSION IN SAT IN HAL (Study III)

Body composition and biochemical characteristics of the study groups

The study subjects were the same as in Study 2 and their clinical and biochemical characteristics are given in **Table 5**. HIV- and HAART-related characteristics did not differ between the groups (**Table 6**).

Adipose tissue gene expression

The mRNA concentrations of control genes were not different between the groups (β-actin 217 ± 44 vs. 180 ± 40 in the HAART+LD+ vs. HAART+LD-, NS; β2-microglobulin 606 ± 71 vs. 645 ± 73, respectively, NS). The blood contamination percentage did not differ significantly between the groups (17.2 ± 2.2 vs. 14.3 ± 2.1% volume/weight, HAART+LD+ vs. HAART+LD-, NS).

Transcription factors and coactivator: PPARγ, SREBP-1c, PPARδ and PGC-1

The mRNA concentrations of PPARγ, SREBP-1c, PPARδ and PGC-1 are shown in **Table 8**. Expressions of all these genes were significantly lower in the HAART+LD+ than in the HAART+LD- group. PPARγ and SREBP-1c expressions were significantly interrelated within the HAART+LD+ group ($r = 0.68$, $p<0.0001$).

Table 8. *The expression of the defined genes in SAT in the HAART+LD+ and the HAART+LD- groups.*

Variable	HAART+LD+	HAART+LD-
PPARγ	$13 \pm 2 \times 10^{-3}$ **	$28 \pm 6 \times 10^{-3}$
SREBP-1c	1.8 ± 0.2 **	3.1 ± 0.5
PGC-1	$19 \pm 4 \times 10^{-3}$ *	$27 \pm 7 \times 10^{-3}$
PPARδ	$15 \pm 3 \times 10^{-3}$ *	$20 \pm 3 \times 10^{-3}$
LPL	$7 \pm 1 \times 10^{-3}$ ***	$19 \pm 3 \times 10^{-3}$
ACS	$10 \pm 2 \times 10^{-4}$ *	$15 \pm 3 \times 10^{-4}$
FATP-1	$16 \pm 3 \times 10^{-4}$	$15 \pm 2 \times 10^{-4}$
FATP-4	$13 \pm 2 \times 10^{-4}$	$12 \pm 1 \times 10^{-4}$
KLBP	$13 \pm 1 \times 10^{-3}$	$10 \pm 2 \times 10^{-3}$
ALBP	0.26 ± 0.03	0.27 ± 0.02
GLUT4	0.8 ± 0.2 *	1.6 ± 0.3
GLUT1	$10 \pm 1 \times 10^{-3}$	$13 \pm 2 \times 10^{-3}$
IL-6	0.8 ± 0.3 *	0.2 ± 0.1
CD45	$16 \pm 3 \times 10^{-4}$ *	$9 \pm 1 \times 10^{-4}$
Adiponectin	$68 \pm 10 \times 10^{-5}$ ***	$242 \pm 62 \times 10^{-4}$

Data are shown as mean ± SEM.
*p<0.05, **p<0.01, ***p<0.001 for comparisons between HAART+LD+ and HAART+LD-.

Lipogenesis and fatty acid metabolism: LPL, ACS, ALBP, KLBP, FATP-1 and FATP-4

Expression of LPL and ACS were significantly decreased in the HAART+LD+ group compared to the HAART+LD- group (**Table 8**). The mRNA concentrations of the fatty acid transport proteins FATP-1 and FATP-4, or the fatty acid intracellular binding proteins ALBP and KLBP did not differ between the groups (**Table 8**).

Glucose transport proteins: GLUT4 and GLUT1

The mRNA concentration of GLUT4 was significantly decreased in patients with lipodystrophy (**Table 8**). Expression of PGC-1 correlated closely with that of GLUT4 within the HAART+LD+ group (**Fig. 10**). The mRNA concentration of GLUT1 was not different between the groups (**Table 8**).

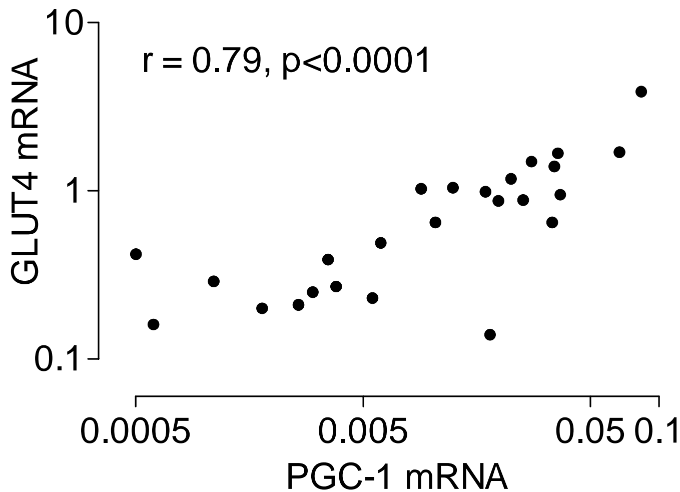


Figure 10. *Relationship between mRNA concentration of PGC-1 and GLUT4 in SAT in patients with HAL. r = correlation coefficient.*

Markers of inflammation: CD45 and IL-6

The mRNA concentrations of CD45 and IL-6 were significantly higher in the HAART+LD+ than the HAART+LD- group (**Table 8**). Serum IL-6 concentration (2.2 ± 0.3 vs. 1.9 ± 0.6 pg/ml, HAART+LD+ vs. HAART+LD-, NS) did not differ between the groups. There was no correlation between serum IL-6 and adipose tissue mRNA concentration of IL-6 (r=0.24, p=0.14).

6.4. TREATMENT OF HAL WITH ROSIGLITAZONE (Study IV and V)

Body composition and biochemical characteristics at baseline

At baseline, the placebo and rosiglitazone groups were similar with respect to age, gender, body weight and composition (**Table 9**). The HIV-related characteristics of the subjects are given in **Table 6**. There were no significant differences between the rosiglitazone and the placebo group with respect to HIV-related characteristics at baseline (data not shown). None of the patients changed any of the antiretroviral agents during the study.

Compared to the age- and weight-matched HIV- group, the HAART+LD+ group had significantly less subcutaneous and more intra-abdominal fat, and higher WHR (**Table 5**). Fasting serum insulin and triglyceride concentrations were significantly higher and HDL cholesterol concentrations lower in the HAART+LD+ than in the HIV- group (**Table 5**). Plasma glucose (**Table 5**) or HbA_{1c} (5.2 ± 0.2 vs. 5.5 ± 0.1 %, HAART+LD+ vs. HIV-, NS) concentrations were not different between the groups.

Table 9. *The number, age, body composition and laboratory characteristics of the subjects in the rosiglitazone and the placebo groups at baseline and at 24 weeks.*

Variable	Rosiglitazone		Placebo	
	0 weeks	**24 weeks**	**0 weeks**	**24 weeks**
Number of patients	15	15	15	15
Males / females	12 / 3		13 / 2	
Age (years)	44 ± 3		42 ± 2	
Body mass index (kg/m^2)	23.7 ± 0.7	23.9 ± 0.6	23.6 ± 0.8	23.9 ± 0.9
Body weight (kg)	73.0 ± 2.5	73.7 ± 2.5	73.3 ± 3.4	74.4 ± 3.6
Subcutaneous fat (cm^3)	980 ± 200	1040 ± 230	1300 ± 250	1330 ±250
Intra-abdominal fat (cm^3)	2000 ± 250	2030 ± 260	1830 ± 330	1780 ± 320
Total body fat (%)	17.6 ± 1.6	18.0 ± 1.6	17.4 ± 1.9	18.0 ± 1.9
Skinfolds (mm)	38 ± 4	36 ± 4	38 ± 4	38 ± 3
Serum leptin (ng/ml)	4.2 ± 1.0	3.8 ± 0.9	3.8 ± 0.8	3.9 ± 0.9
Serum insulin (mU/l)	12.6 ± 1.5	9.3 ± 0.6 * †	9.6 ± 1.8	16.3 ± 5.7
Serum HDL cholesterol (mmol/l)	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Liver fat content (%)	7.3 ± 1.6	6.2 ± 1.3 †	8.0 ± 3.1	10.1 ± 3.3
Serum CRP (mg/l)	1.5 ± 0.3	1.0 ± 0.3 *	1.6 ± 0.4	1.6 ± 0.3
Serum IL-6 (pg/ml)	2.0 ± 0.3	1.8 ± 0.3	2.4 ± 0.4	2.1 ± 0.4
B-leukocytes (x 10^9/l)	5.6 ± 0.3	5.0 ± 0.3 * †	5.9 ± 0.6	6.1 ± 0.6
Serum FFA (µmol/l)	550 ± 35	422 ± 36 *	572 ± 64	516 ± 55
Serum TNFα (pg/ml)	1.7 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	1.8 ± 0.3
Serum adiponectin (ug/ml)	3.6 ± 0.5	6.2 ± 1.1 * †	3.1 ± 0.6	3.6 ± 0.6

Data are shown as mean ± SEM. *$p<0.05$ for the change between 0 and 24 weeks within rosiglitazone or placebo group. † $p<0.05$ for the comparisons of changes between the rosiglitazone and placebo group.

Clinical effects of rosiglitazone

After 24 weeks of treatment, there were no significant changes in body weight or in the amount of intra-abdominal or subcutaneous fat as determined by MRI, serum leptin concentrations, the sum of skinfold thicknesses or other measures of adiposity in either placebo or rosiglitazone group (**Table 9**).

Serum insulin concentrations and liver fat content decreased in the rosiglitazone group, but increased in the placebo group (**Table 9**); the changes between the groups were statistically significant. In the rosiglitazone group, the change in serum insulin concentration correlated with the change in the liver fat content ($r=0.52$, $p<0.05$), but not with any other measure of body composition. Serum triglyceride concentrations increased significantly during rosiglitazone treatment (**Fig. 11**). Serum total cholesterol concentrations also increased

significantly (**Fig. 11**). Serum HDL cholesterol did not change significantly in either group (**Table 9**). Plasma glucose concentration decreased non-significantly in both groups: by 0.01 ± 0.2 in the placebo and 0.15 ± 0.1 mmol/l in the rosiglitazone group. Serum CRP and FFA concentrations, and total white blood cell count decreased significantly in the rosiglitazone group (**Table 9**). Serum adiponectin concentration increased significantly in the rosiglitazone group, but remained unchanged in the placebo group (**Table 9**).

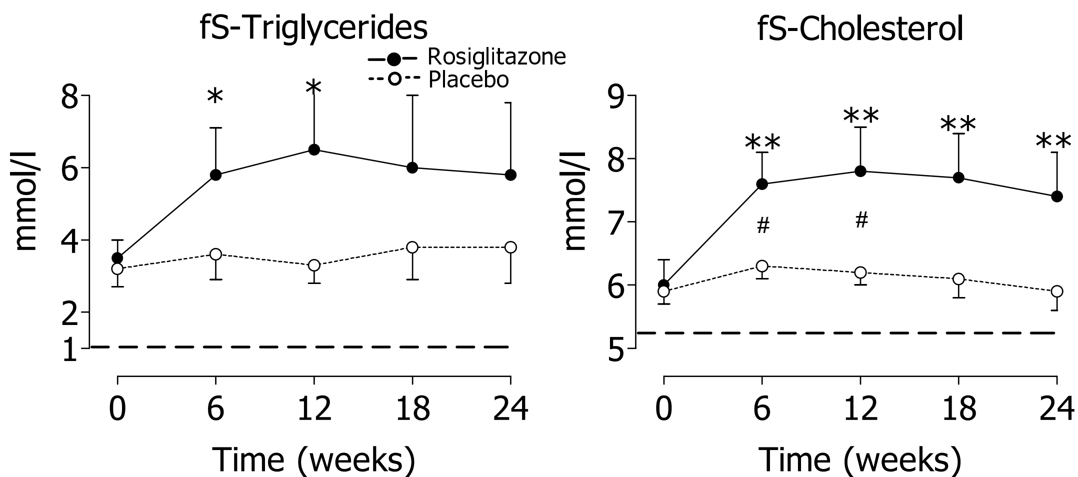


Figure 11. *The effect of rosiglitazone vs. placebo on serum triglyceride and total cholesterol concentration. Error bars = SEM. Dashed line = mean value of HIV negative normal subjects. *p<0.05, **p<0.01 for comparisons vs. baseline. #p<0.05 for comparisons between the rosiglitazone and the placebo groups.*

Serum ALT and hemoglobin concentrations decreased significantly in the rosiglitazone group and remained stable in the placebo group (**Fig. 12**). One patient in the rosiglitazone group discontinued the study after 12 weeks of treatment due to increased triglyceride concentration (32.5 mmol/l). None of the patients developed hypoglycemia or clinically detectable edema. Venous blood pH and bicarbonate concentrations did not change significantly in either group (data not shown). CD4+ cell counts and serum PI concentrations did not change significantly in either group (data not shown). None of the patients lost virologic control during the study.

Effects of rosiglitazone on gene expression in SAT

There were no significant differences in the mRNA concentrations for any of the genes at baseline between the placebo and rosiglitazone group (**Table 10**). Rosiglitazone induced a significant increase in the expression of adiponectin and PGC-1, and a significant decrease in the expression of IL-6. In addition, the expression of PPARγ was increased in the rosiglitazone group when compared with the placebo group. Expression of other genes involved in lipogenesis, fatty acid metabolism or glucose transport remained unchanged in both groups.

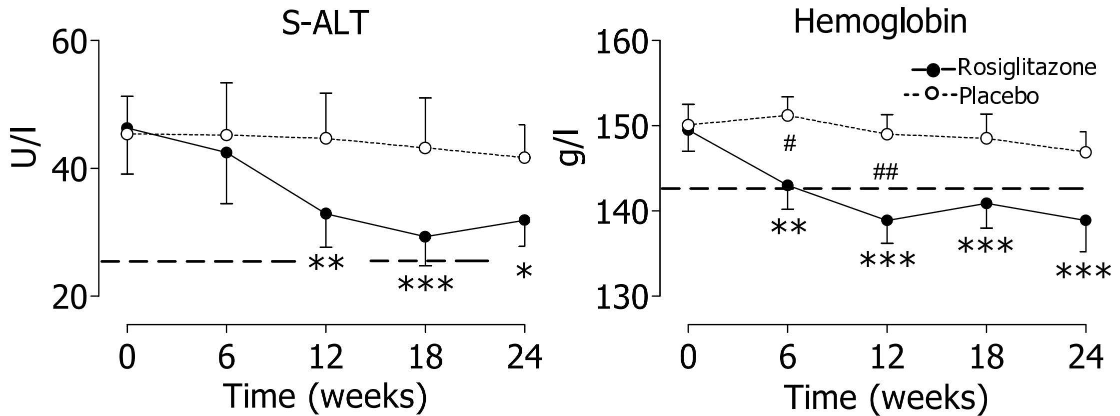


Figure 12. *The effect of rosiglitazone vs. placebo on serum ALT and hemoglobin concentration. Error bars = SEM. Dashed line = mean value of HIV negative normal subjects. *p<0.05, **p<0.01, ***p<0.001 for comparisons vs. baseline. $^{\#}$p<0.05, $^{\#\#}$p<0.01 for comparisons between the rosiglitazone and the placebo groups.*

Interrelationships between gene expression and features of insulin resistance

Among lipodystrophic patients at baseline, the mRNA concentration of adiponectin in SAT correlated with that of SREBP-1c (r=0.69, p<0.0001) and LPL (r=0.95, p<0.0001), and with liver fat content (r=-0.39, p<0.05). Similar correlations were also found after 24 weeks between adiponectin expression and the liver fat content (r=-0.34, p=0.07), and between adiponectin and SREBP1-c (r=0.79, r<0.0001) and LPL (r=0.94, p<0.0001) expression in SAT. Serum adiponectin concentration at 24 weeks but not at baseline correlated significantly with the liver fat content (r=-0.36, p<0.05) and the fasting serum insulin concentration (r=-0.38, p<0.05).

In all patients with HAL, the change in the concentration of adiponectin mRNA in SAT correlated closely with the corresponding changes in the concentrations of LPL mRNA (r=0.89, p<0.0001) and SREBP-1c mRNA (r=0.47, p<0.05). The change in serum adiponectin concentration was inversely correlated with the change in fasting serum insulin concentration (r=-0.49, p<0.01), the liver fat content (r=-0.45, p<0.05) (**Fig. 13**) and serum ALT concentration (r=-0.38, p<0.05). The change in fasting serum insulin concentration and the change in liver fat content correlated significantly (r=0.45, p<0.05).

The change in the mRNA concentration of IL-6 in adipose tissue correlated positively with the changes in fasting serum FFA (r=0.52, p<0.01) and serum CRP (r=0.40, p<0.05) concentrations. The change in the serum concentration of CRP also correlated inversely with the changes in mRNA concentrations of SREBP-1c (r=-0.45, p<0.05) and LPL (r=-0.45, p<0.05) in SAT.

Table 10. *The expression of the defined genes in the rosiglitazone and the placebo group at baseline and at 24 weeks.*

	Rosiglitazone		Placebo	
	0 weeks	**24 weeks**	**0 weeks**	**24 weeks**
PPARγ	12.5 ± 3.3 x 10^{-3}	13.3 ± 2.4 x 10^{-3} †	13.9 ± 2.6 x 10^{-3}	12.0 ± 2.4 x 10^{-3}
SREBP-1c	1.7 ± 0.4	1.7 ± 0.2	1.8 ± 0.3	1.7 ± 0.3
PGC-1	15 ± 4 x 10^{-3}	20 ± 4 x 10^{-3} *	23 ± 7 x 10^{-3}	23 ± 6 x 10^{-3}
PPARδ	14 ± 4 x 10^{-3}	14 ± 3 x 10^{-3}	16 ± 4 x 10^{-3}	12 ± 3 x 10^{-3}
Adiponectin	62 ± 16 x 10^{-5}	72 ± 14 x 10^{-5}*	73 ± 18 x 10^{-5}	74 ± 16 x 10^{-5}
GLUT1	9 ± 2 x 10^{-3}	12 ± 2 x 10^{-3}	10 ± 2 x 10^{-3}	10 ± 1 x 10^{-3}
GLUT4	0.64 ± 0.15	0.66 ± 0.16	0.96 ± 0.26	0.67 ± 0.12
IL-6	1.05 ± 0.60	0.33 ± 0.08 ** †	0.56 ±0.33	0.43 ± 0.12
CD45	19 ± 6 x 10^{-4}	19 ± 5 x 10^{-4}	14 ± 2 x 10^{-4}	15 ± 2 x 10^{-4}
LPL	7 ± 2 x 10^{-3}	7 ± 1 x 10^{-3}	7 ± 2 x 10^{-3}	7 ± 2 x 10^{-3}
ACS	7 ± 1 x 10^{-4}	7 ± 1 x 10^{-4}	13 ± 3 x 10^{-4}	10 ± 3 x 10^{-4}
FATP-1	14 ± 4 x 10^{-4}	13 ± 3 x 10^{-4}	18 ± 4 x 10^{-4}	13 ± 2 x 10^{-4}
FATP-4	12 ± 3 x 10^{-4}	10 ± 2 x 10^{-4}	15 ± 3 x 10^{-4}	10 ± 2 x 10^{-4}
KLBP	11 ± 2 x 10^{-3}	9 ± 1 x 10^{-3}	14 ± 2 x 10^{-3}	11 ± 1 x 10^{-3}
ALBP	0.24 ± 0.04	0.25 ± 0.03	0.27 ± 0.05	0.22 ± 0.03
Leptin	0.078 ± 0.025	0.061 ± 0.023	0.052 ± 0.020	0.046 ± 0.019

Data are shown as mean ± SEM. *p<0.05 and **p<0.01 for the change between 0 and 24 weeks within the rosiglitazone or placebo group. † p<0.05 for the comparisons of changes between the rosiglitazone and placebo group.

6.5. PAI-1 IN HAL (Study VI)

Body composition and biochemical characteristics of the groups

Clinical and biochemical characteristics of the study groups are given in **Table 5.**

Circulating PAI-1, tPA and cytokine concentrations

Plasma PAI-1 antigen concentrations were significantly increased in the HAART+LD+ group (28.4 ± 2.4 ng/ml) compared with the HAART+LD- (17.8 ± 2.6, p<0.05) and the HIV- groups (10.4 ± 2.7, p<0.001) as were plasma tPA concentrations (10.7 ± 0.6 vs. 7.3 ± 0.6 vs. 6.1 ± 0.8 ng/ml, p<0.01 for HAART+LD+ vs. HAART+LD-, p<0.001 for HAART+LD+ vs. HIV-). These differences persisted even after age was included as a covariate in the analysis of variance.

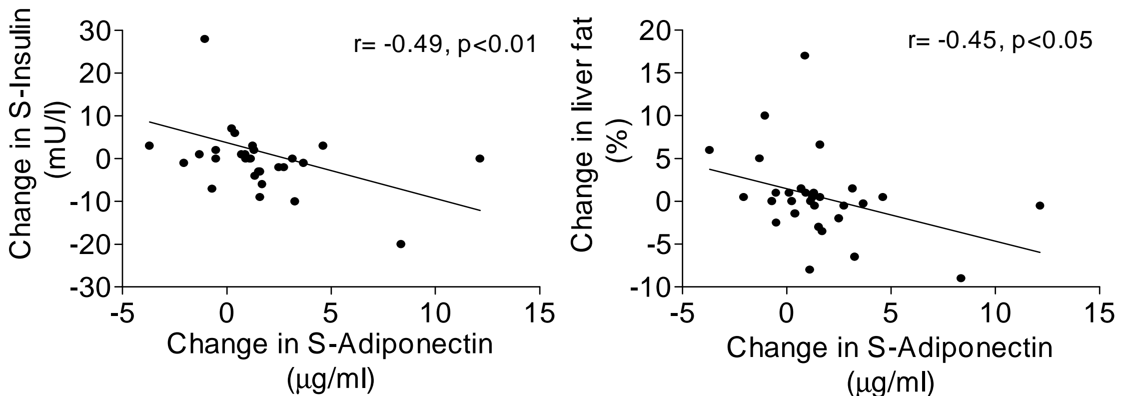


Figure 13. *Relationship between the change in serum adiponectin concentration and the change in serum insulin concentration (left), and between the change in serum adiponectin concentration and the change in liver fat content (right) in patients with HAL. r = correlation coefficient..*

Serum TNFα concentrations were not significantly different between the groups (1.6 ± 0.1 vs. 1.5 ± 0.2 vs. 2.0 ± 0.8 pg/ml, HAART+LD+ vs. HAART+LD- vs. HIV-). Serum IL-6 concentrations, on the other hand, were increased in both HIV-infected groups relative to the HIV- group (2.2 ± 0.2 vs. 1.9 ± 0.6 vs. 0.7 ± 0.2 pg/ml, p<0.05 for HIV-infected groups vs. HIV-).

PAI-1 and leptin expression in SAT

In SAT, PAI-1 mRNA concentration averaged 0.019 ± 0.003 in the HAART+LD+ group, which was significantly higher than in the HAART+LD- (0.007 ± 0.002, p<0.005) and the HIV- (0.006 ± 0.001, p<0.005) groups (**Fig. 14**). Fat cell diameter was greater in the HIV- (94 ± 3 µm) than in the HAART+LD+ (74 ± 3 µm, p<0.01) or in the HAART+LD- group (80 ± 4 µm, p<0.05). Leptin mRNA concentration in SAT was significantly lower in the HAART+LD+ group (0.06 ± 0.02) when compared to the HAART+LD- (0.17 ± 0.03, p<0.005) or the HIV- (0.28 ± 0.06, p<0.001) group.

Relationships between PAI-1 and physical and biochemical characteristics before rosiglitazone therapy

Fig. 15 depicts the relations of plasma PAI-1, and **Fig. 16**, those of plasma tPA antigen concentrations to liver fat content, and to the amount of subcutaneous and intra-abdominal fat in all study groups. Within the HAART+LD+ group, plasma PAI-1 antigen concentration correlated with liver fat content before rosiglitazone therapy (r=0.49, p<0.01, **Fig. 15**) but not with serum TNFα (r=-0.03, NS) or IL-6 (r=0.18, NS) concentrations, the amount of subcutaneous (r=-0.05, NS**, Fig. 15**) or intra-abdominal fat (r=0.33, NS, **Fig. 15**), serum C-peptide (r=0.33, NS) or insulin (r=0.26, NS) concentrations, or body weight (r=0.26, NS). Within the HAART+LD+ group, the correlation coefficient between PAI-1 mRNA concentrations in SAT and plasma PAI-1 concentrations before rosiglitazone therapy was 0.39 (p<0.05).

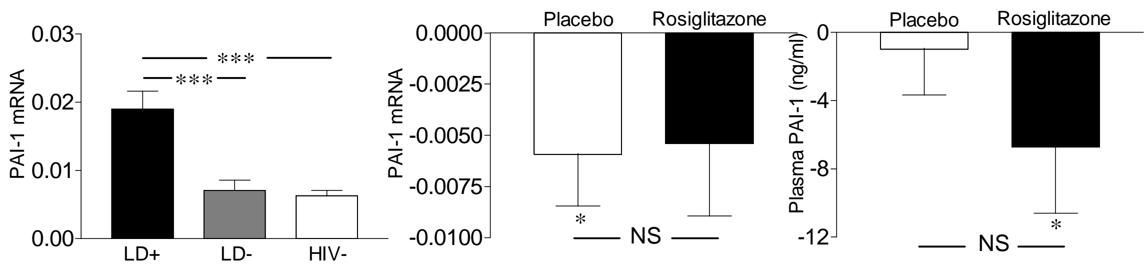


Figure 14. *The mRNA concentration of PAI-1 in SAT in the HAART+LD+, HAART+LD- and HIV- groups (left); the change by rosiglitazone vs. placebo treatment in PAI-1 mRNA concentration in SAT (middle) and plasma PAI-1 antigen concentration (right). Error bars = SEM. *p<0.05, ***p<0.005.*

Effects of rosiglitazone treatment

Compared with baseline, PAI-1 mRNA concentration in SAT decreased slightly in the placebo group (p<0.05 for before vs. after) but not in the rosiglitazone group (p>0.1); the changes between the groups were not statistically significant (**Fig. 14**). Rosiglitazone treatment was associated with a decrease in plasma PAI-1 concentration from 30 ± 4 to 23 ± 2 ng/ml (p<0.05; **Fig. 14**). Plasma PAI-1 remained unchanged in the placebo group (27 ± 3 vs. 26 ± 3 ng/ml, before vs. after, NS; **Fig. 14**). The mean size of the adipocytes or the distribution of cell sizes were not changed by placebo or rosiglitazone treatment (data not shown). Plasma tPA decreased from 11.3 ± 0.8 to 10.5 ± 0.8 ng/ml (p<0.05) in the rosiglitazone group but remained unchanged in the placebo group (10.1 ± 0.7 vs. 9.8 ± 0.7 ng/ml, NS). Serum IL-6 concentration did not change with rosiglitazone treatment when compared to placebo (**Table 9**). Serum TNFα concentration increased almost significantly in the placebo group (p=0.06), but remained unchanged in the rosiglitazone group (**Table 9**). Leptin expression remained unchanged in both rosiglitazone and placebo groups (**Table 10**).

The change in plasma PAI-1 concentration within the HAART+LD+ group was significantly correlated with the change in serum insulin concentration (r=0.42, p<0.05), which in turn was significantly correlated with the change in liver fat content (r=0.49, p<0.01). The correlation coefficient between the change in plasma PAI-1 concentration and the change in liver fat content was 0.37 (p<0.05). Except for the latter correlation, there were no significant correlations between the change in plasma PAI-1 concentration and changes in body composition, nor were there any significant correlations between the change in plasma PAI-1 concentration and the changes in serum TNFα or IL-6 concentrations (data not shown). The change in plasma PAI-1 concentration did not correlate with the change in PAI-1 mRNA concentration in SAT (r=-0.04, NS).

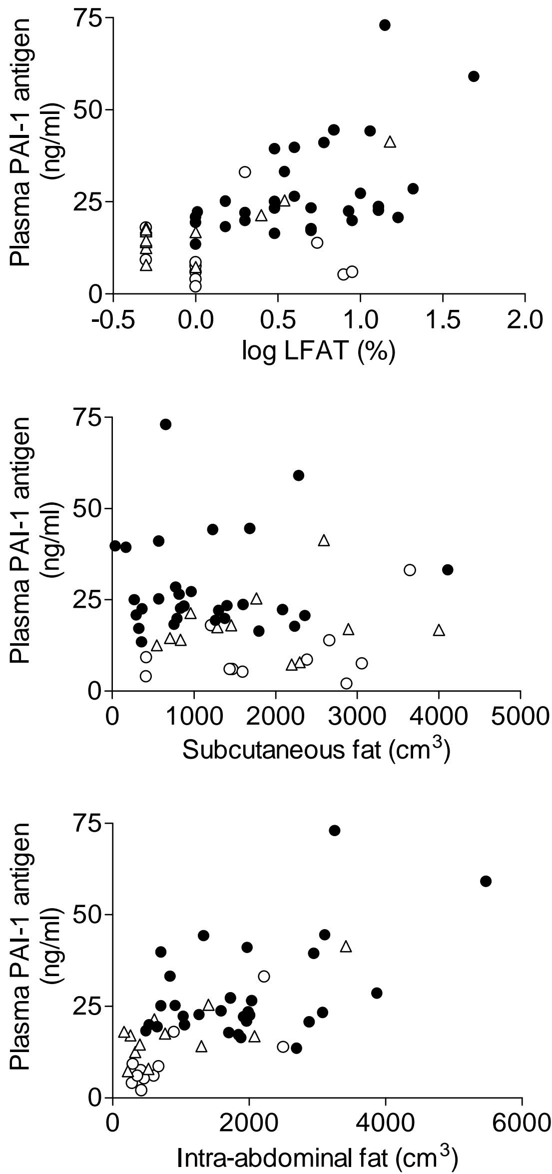


Figure 15. *Relationships between liver fat (LFAT), subcutaneous and intra-abdominal fat, and plasma PAI-1 antigen concentrations in HAART-treated patients with (closed circles, HAART+LD+) and without (open triangles, HAART+LD-) lipodystrophy, and in HIV-negative normal subjects (open circles, HIV-). The correlation coefficients and the corresponding p-values were for LFAT: r = 0.49, p<0.01 in the HAART+LD+ group, r = 0.54, p<0.05 in the HAART+LD- group, r = -0.17, NS in the HIV- group; for subcutaneous fat: r = -0.05, NS in the HAART+LD+ group, r = 0.17, NS in the HAART+LD- group, r = 0.19, NS in the HIV- group; for intra-abdominal fat: r = 0.33, NS in the HAART+LD+ group, r = 0.45, NS in the HAART+LD- group, r = 0.63, p<0.05 in the HIV- group.*

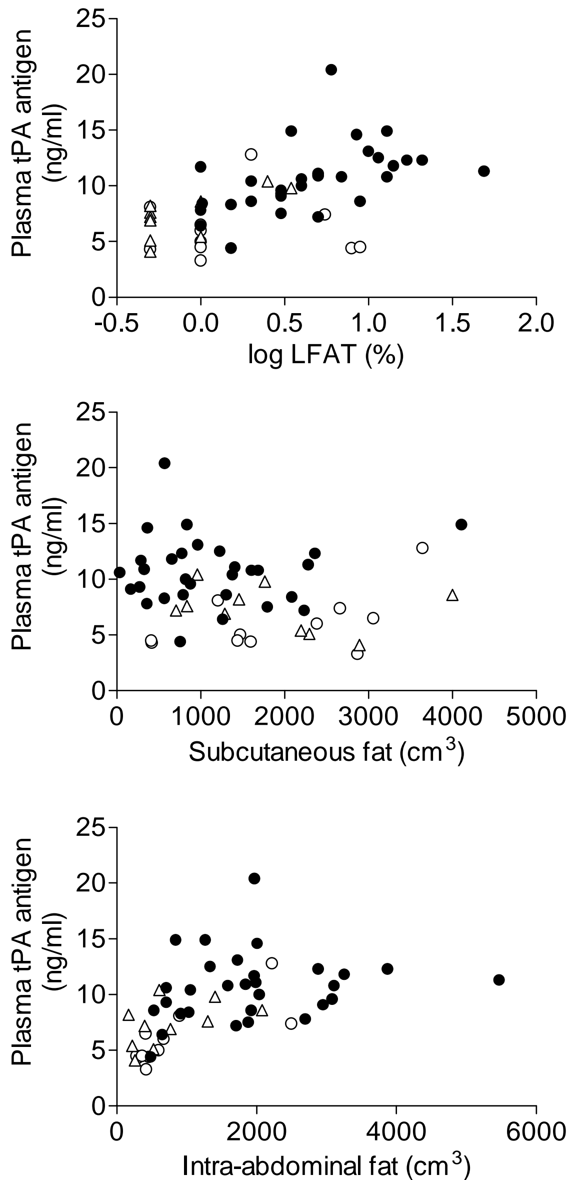


Figure 16. *Relationships between liver fat (LFAT), subcutaneous and intra-abdominal fat, and plasma tPA antigen concentrations in HAART-treated patients with (closed circles, HAART+LD+) and without (open triangles, HAART+LD-) lipodystrophy, and in HIV-negative normal subjects (open circles, HIV-). The correlation coefficients and the corresponding p-values were for LFAT: $r = 0.65$, $p<0.0001$ in the HAART+LD+ group, $r = 0.65$, $p<0.05$ in the HAART+LD- group, $r = 0.03$, NS in the HIV- group; for subcutaneous fat: $r = 0.02$, NS in the HAART+LD+ group, $r = -0.30$, NS in the HAART+LD- group, $r = 0.36$, NS in the HIV- group; for intra-abdominal fat: $r = 0.33$, NS in the HAART+LD+ group, $r = 0.49$, NS in the HAART+LD- group, $r = 0.71$, $p<0.05$ in the HIV- group.*

7. DISCUSSION

7.1. SUBJECTS AND METHODS

7.1.1. SUBJECTS

In the current study, three groups of subjects were included. Most of the comparisons were made between the HAART+LD+ and the HAART+LD- groups in order to distinguish findings associated with lipodystrophy from those associated with HIV and HAART. This is important, since a chronic HIV infection *per se* induces expression of inflammatory cytokines (398,399). Furthermore, it has recently been shown that human preadipocytes express CD4, CXCR4 and CCR5 receptors, which are necessary for entry of HIV into inflammatory host cells (400). At least *in vitro*, adipocytes can actually express viral proteins (400). Therefore the mere presence of HIV may influence gene expression in adipocytes. When two HIV-infected, HAART-treated groups were compared, these potential interferences could be avoided.

There is no international consensus regarding the diagnosis of lipodystrophy. Therefore clinical criteria, i.e. presence of symptoms (HAART+LD+) or lack thereof (HAART+LD-) reported by the patients and the findings confirmed by the single investigator (J.S.), were used for grouping of the HIV-infected patients. The clinical criteria classified the patients correctly as was later shown by objective measurements of body composition: regardless the similar body weight and total body fat, the HAART+LD+ group had significantly less subcutaneous fat and more intra-abdominal fat than the HAART+LD- group (**Table 5**). The HAART+LD+, HAART+LD- and HIV- groups were matched for gender and BMI, and also for age with the exception of Study VI, in which the HIV- group was somewhat younger than the HAART+LD+ group. However, the results of Study VI did not change when age was included as a covariate in the analyses.

At the time of the initiation of Study IV, there were no data available regarding the use of rosiglitazone in HAL. Patients with serum transaminase concentrations higher than three times the upper limit of normal, patients with heart failure and with severe hypertriglyceridemia were excluded from the study in order to minimize the risk of potential side effects due to rosiglitazone. These exclusion criteria must be taken into account before generalizing the results. Although there were no interaction studies available evaluating concomitant use of thiazolidinediones and antiretroviral agents, harmful interactions were considered unlikely, since rosiglitazone does not interact with other cytochrome P450 3A4 substrates (401,402). As expected, there were no significant changes in PI concentrations and none of patients who had undetectable amount of the virus in plasma at baseline lost virologic control during the study.

7.1.2. LIVER FAT

MRI proton spectroscopy is a novel method that allows non-invasive quantification of liver fat without radiation exposure. Other common non-invasive methods for evaluating liver fat content, such as ultrasound are at best only semi-quantitative (403). The recorded methylene signal from the liver specifically represents intrahepatocellular triglyceride, since there is no extrahepatocellular fat in the liver. Quantification of hepatic fat by proton spectroscopy correlates closely with that determined histologically from liver biopsies and with liver density measurements calculated by CT (191,404,405), although the units of measurement are different. In our hands, the spectroscopic fat percentage is about half of the histologic fat percentage (Vehkavaara S et al, unpublished data). Spectroscopy has also the advantage that a larger volume of liver (8 cm^3) can be analyzed than by performing a liver biopsy (406). An important limitation of all non-invasive methods is their inability to differentiate benign fat accumulation (hepatosteatosis) from steatohepatitis, which has a significantly worse prognosis as a liver disease (407).

7.1.3. BODY COMPOSITION

Body composition was measured using MRI, BIA and anthropometric measurements. MRI and CT imaging allow depot-specific quantification of abdominal subcutaneous vs. intra-abdominal fat in contrast to DEXA, which can measure total truncal fat, but not separate the two depots (408). This limitation of DEXA is of great significance in patients with lipodystrophy (409) since, as in the present study, the total amount of abdominal fat does not necessarily differ between lipodystrophic and non-lipodystrophic subjects (**Table 5**). Abdominal subcutaneous and intra-abdominal fat depots are most commonly measured using a single CT or MRI scan at the level of L4-L5. Even though a good correlation has been shown between the fat mass measured by MRI covering total abdominal cavity and the fat mass from a single MRI scan (410), we preferred to quantify adipose tissue mass by using a total of 16 slices 1 cm apart to increase the accuracy of the measurement.

BIA is a non-invasive, inexpensive method for determining total, but not compartment-specific body composition, and has been validated in HIV-infected patients before the era of HAART (411). However, the method may not be reliable in patients with HAL and the predictive equations should be developed specifically for patients with lipodystrophy (412). Reliability of anthropometric measurements could perhaps also be questioned. However, in the current study anthropometry was considered useful, since measurements are known to be more reliable in lean subjects with thin subcutaneous fat layer than in obese subjects (408). The inter-observer error was eliminated by having a single investigator to perform all measurements. The coefficient of variation of repeated skinfold measurements by the same trained observer is approximately only 5%, but it rises to 10-20% between different observers (408).

Lack of DEXA imaging should be considered a limitation of the current study, since DEXA would have provided an additional measure of total limb fat mass. This would have been useful when evaluating the

effects of rosiglitazone on the amount of SAT. However, since rosiglitazone is not known to affect lean body mass, an increase in the amount of subcutaneous fat should either have caused an increase in total body weight, or alternatively it should have been compensated by a reciprocal decrease in the amount of intra-abdominal fat if the body weight remained unchanged. The lack of change in body weight or in the amount of intra-abdominal fat measured by covering almost all abdominal cavity (16 MRI scans), makes it unlikely that such a significant increase in the amount of subcutaneous fat remained unnoticed.

7.1.4. GENE EXPRESSION

Gene expression was quantified using real-time polymerase chain reaction (PCR). Because RNA cannot serve as a template for PCR, mRNA must first be converted into cDNA. DNA is thereafter amplified by a PCR reaction. Real-time PCR is currently the most sensitive method for quantification of mRNA (414).

PCR reaction generates copies of the DNA template in an exponential fashion. Due to various limiting factors, the reaction eventually does not remain exponential, but reaches a ”plateau phase”; some reactions reach a higher plateau than others (415). The variability of the plateau level reached makes the end-point quantification of PCR products unreliable (415). With the ability to follow the concentration of the PCR product as they are accumulating, i.e. in ”real time”, it is possible to measure the amount of the PCR product at a point in which the reaction is still in the exponential range. It is only during this exponential phase of the PCR reaction that it is possible to extrapolate back to determine the starting amount of the template (415).

Measurements of gene expression in adipose tissue in humans are often limited by the size of the sample and by the high lipid content of adipose tissue samples (416). The extreme paucity of subcutaneous fat in patients with severe lipodystrophy limited the size of fat samples also in the current study. Protein expression could not be evaluated in these small samples, which is a limitation of the current study. One must also keep in mind, when interpreting results of gene expression in adipose tissue samples that adipose tissue is not composed of adipocytes only, but also of other cells, such as endothelial cells and macrophages (52), which contribute to the total mRNA and protein concentrations of the adipose tissue samples.

7.2. GENE EXPRESSION IN SAT AND ADIPOCYTOKINE PRODUCTION

Lipodystrophy in its most typical form is characterized not only by a loss of subcutaneous fat but also by an accumulation of intra-abdominal fat and occasionally by a development of a buffalo hump or an increase in breast size. There are, however, no human or animal data regarding the effects of antiretroviral agents on gene expression in the hypertrophic adipose tissue. Although also non-HIV human data are limited, there is some evidence that differences in gene expression exist between different fat depots even in non-lipodystrophic conditions (**Table 1**).

Prior to the current study, gene expression in SAT of patients with HAL had been evaluated in one study (262). Since the subjects in the control group of this study were not HIV-infected and did not use HAART, it remained unclear as to whether the differences between the groups were due to the HIV infection, HAART or lipodystrophy.

7.2.1. TRANSSCRIPTION FACTORS AND CO-ACTIVATOR **(Table 8)**

The mRNA concentrations of PPARγ, SREBP-1c, PPARδ and PGC-1 in SAT were decreased in patients with HAL when compared to HIV-infected, HAART-treated patients without lipodystrophy. Since PPARγ is considered the main adipogenic transcription factor, and its expression is decreased also when patients with HAL are compared to HIV negative subjects (262), it seems likely that defective PPARγ expression plays a role in the development of lipodystrophy. Upstream regulators of PPARγ in the cascade of adipocyte differentiation include C/EBPβ, δ and α (53). These transcription factors were not measured in the current study, but C/EBPβ and α expressions were reported to be decreased in patients with HAL in the study of Bastard et al (262). PPARγ can also be activated by SREBP-1c, the expression of which was decreased in patients with HAL compared to the HAART+LD- group in the current study and compared to healthy controls in the study of Bastard et al (262). We also found a close correlation between PPARγ and SREBP-1c mRNA concentrations. The expression of PPARδ, which may play a role in the earlier events of adipocyte differentiation, such as proliferation of adipocyte precursor cells (417), was found to be decreased in patients with HAL and may contribute to the reduction in adipocyte differentiation.

The expression of PGC-1 has not previously been evaluated in patients with HAL. PGC-1 is an interesting protein in this context, since it was originally identified as a co-activator of PPARγ (71), but has also been found to be involved in the biogenesis of mitochondria (72). In the present study, PGC-1 expression in SAT was decreased in patients with HAL. Low expression of PGC-1 may therefore have contributed both to the low transcriptional activity of PPARγ and to the mitochondrial alterations in SAT described earlier in patients with HAL (264,266). As mitochondrial dysfunction may lead to apoptosis (418), mitochondrial damage in adipose tissue may, at least partly, explain the apoptotic findings in SAT of patients with HAL (263). PGC-1 expression in adipose tissue was closely correlated with that of GLUT4 in patients with HAL (**Figure 10**). This is in keeping with a recent report, which found PGC-1 expression in SAT to be reduced in the insulin-resistant subjects and its expression to correlate with the expression of GLUT4, IRS-1, UCP-1 and insulin sensitivity (419). In the same study, a correlation between PGC-1 and GLUT4 expression was found also in skeletal muscle (419). Currently there are no data regarding PGC-1 expression in the liver in man, but hepatic expression of PGC-1 in mice somewhat surprisingly has resulted in increased gluconeogenesis (420).

7.2.2. GENES OF LIPOGENESIS AND FATTY ACID METABOLISM **(Table 8)**

The expression of fatty acid transport proteins (FATP-1, FATP-4) and intracellular binding proteins (ALBP, KLBP) were similarly expressed in the HAART+LD+ and the HAART+LD- groups. The expression of most fatty acid transport proteins are known to be upregulated by PPARγ in mice *in vivo* and *in vitro* (60,421). However, the lower expression of PPARγ in patients with HAL in the current study was not associated with reduced levels of expression of fatty acid transport or intracellular binding proteins. Whether this is due to different regulation of the expression of these genes in man when compared to mice is unclear.

Both ALBP and KLBP, and at least CD36 of the fatty acid transport proteins, are also expressed in human macrophages (422,423). It is thus possible that the unchanged expression of these genes in SAT of patients with HAL could reflect a decrease in the expression in adipocytes and an increase in macrophages. A recent mouse model, in which macrophages were deficient in aP2 (corresponding human ALBP), expressed low levels of pro-inflammatory cytokines (TNFα, IL-1β, IL-6) (422). If ALBP expression was increased in macrophages in the current study, this might have contributed to the increased expression of IL-6. Interestingly, incubation of human peripheral blood mononuclear cells (PBMCs) with PIs increased the expression of CD36, which may increase the rate of lipid accumulation in macrophages and development of atherosclerosis (424). Human data in this regard are, however, as yet inconclusive. Seven to 30 day treatment of healthy volunteers and HIV-infected subjects with HAART has been shown to decrease rather than increase CD36 expression in PBMCs (425).

Expression of ACS and LPL genes were reduced in the HAART+LD+ group compared with the HAART+LD- group. PPARγ is known to increase the expression of ACS both in preadipocytes *in vitro* and in adipose tissue in the rat *in vivo* (426). Pro-inflammatory cytokines decrease the expression of ACS in the liver and adipose tissue of hamsters (427). The low ACS expression in patients with HAL may therefore be a consequence of downregulation of PPARγ and upregulation of inflammatory cytokines, i.e. IL-6 and TNFα (262). Regulation of LPL expression resembles that of ACS in adipose tissue. Both SREBP-1 and PPARγ increase LPL expression *in vitro* and in rat adipose tissue (428,429), whereas pro-inflammatory cytokines decrease LPL expression (9). Thus, both the decreases in PPARγ and SREBP-1, and the increases in IL-6 and TNFα (262) expressions could have contributed to the low LPL expression.

7.2.3. MARKERS OF INFLAMMATION **(Table 8)**

In the present study, the expression of IL-6 in SAT was increased in the HAART+LD+ group when compared to the HAART+LD- group. IL-6 is a multifunctional cytokine produced by several different cell types including immune cells, adipocytes, fibroblasts, stromal-vascular cells, endothelial cells, myocytes, and a variety of endocrine cells (9). As much as a third of the total circulating concentration of IL-6 has been estimated to originate from adipose tissue (9), but only ~10% of the IL-6 secreted from adipose tissue

originates from adipocytes (9). The HAART+LD+ group had a higher number of white blood cells capable of IL-6 production in SAT as judged from the significantly higher concentration of the mRNA coding for CD45. CD45 is a tyrosine phosphatase, which is expressed exclusively in white blood cell lineage (430). The expression of a specific macrophage marker, CD68 (431), is of great interest in these patients regarding the source of inflammatory cytokines. The expression of CD68 appears to be increased in patients with HAL (Sutinen J et al., unpublished). TNFα has considerable catabolic effects in adipose tissue, including inhibition of CEBPα, PPARγ and LPL expression, and induction of apoptosis (9). TNFα and IL-6 may also inhibit the expression of adiponectin (Chapter 7.2.4.). Increased inflammation in lipodystrophic adipose tissue may therefore have multiple direct and indirect effects in the pathogenesis of lipodystrophy and the associated insulin resistance.

Because of the cross-sectional design of the current study, the results are limited to show associations between altered gene expression and lipodystrophy in the pathogenesis of HAL. Only prospective studies can prove the cause and effect. In addition, the role of a specific drug or even a drug class cannot be evaluated due to the small number of subjects and the large variety of HAART-combinations used by the patients. Taken together, multiple alterations have been found to characterize gene expression in SAT of patients with HAL compared with HIV-infected, HAART-treated patients without lipodystrophy implying that the changes were attributable to or responsible for lipodystrophy.

7.2.4. ADIPONECTIN

In the present study, adiponectin expression in SAT and its serum concentration (**Fig. 9**) were significantly decreased in the HAART+LD+ when compared to the HAART+LD- group. Both serum concentration and adipose tissue mRNA concentration of adiponectin correlated closely with features of insulin resistance including liver fat content, suggesting that adiponectin deficiency may contribute to hepatic insulin resistance in these patients.

The expression of adiponectin in SAT of patients with HAL was measured for the first time in the current study. Low expression of adiponectin in lipodystrophic adipose tissue has thereafter been reported by Lihn et al (432). The low serum adiponectin concentration in HAL in the current study is in keeping with findings of other recent reports comparing patients with HAL with HIV-infected subjects without HAL or with HIV negative healthy controls (432-435). All of these studies have also found an inverse correlation between circulating adiponectin concentrations and features of insulin resistance (432-436). In one study, adiponectin concentration was inversely correlated with the use of NRTI, and was suggested to mediate the worsening of insulin resistance during NRTI therapy (435). Serum adiponectin concentrations are decreased also in the generalized vs. the partial forms of non-HIV lipodystrophies and correlate with features of insulin resistance (437). Taken all data together, there is striking consistency, in contrast to data with leptin (Chapter 7.2.5.),

demonstrating adiponectin deficiency in human lipodystrophy. Even though cross-sectional studies cannot define the cause and effect, adiponectin is a candidate link between adipose tissue dysfunction and disturbances in whole body metabolism.

The serum concentrations of adiponectin correlate inversely with BMI in persons with normal body weight and in obese individuals (90). However, body weight did not explain low adiponectin levels in the current study, since the HAART+LD+ and the HAART+LD- groups had comparable BMIs. Because in general population lean subjects have increased adiponectin, the paucity of subcutaneous fat *per se* in the HAART+LD+ group is not sufficient to explain low expression of adiponectin. Since adiponectin expression is induced 100-fold during adipocyte differentiation (88), the decrease in the expression of PPARγ and other transcription factors may have contributed to the low adiponectin expression.

Current data are controversial regarding the expression of adiponectin in SAT vs. VAT (**Table 1**). Visceral fat has also been suggested to produce an as-yet unidentified substance, which may destabilize adiponectin mRNA (438). The significantly decreased serum adiponectin concentration together with significantly increased intra-abdominal fat mass in the HAART+LD+ group and the strong inverse correlation between the serum adiponectin concentration and the intra-abdominal fat mass, would suggest an inhibitory rather than stimulatory effect of intra-abdominal fat on adiponectin production.

In the present study, serum adiponectin concentrations correlated closely with liver fat content, and liver fat content correlated with serum insulin concentrations. Currently there are no other human data available relating liver fat content and adiponectin concentrations. However, adiponectin has been found to be negatively correlated with IMCL in the oxidative soleus muscle, but not in the non-oxidative tibialis anterior muscle in healthy volunteers (439,440). Serum adiponectin concentration has also been shown to correlate positively with the increase in insulin-stimulated insulin receptor tyrosine phosphorylation in human skeletal muscle (441).

In mice, adiponectin infusion enhances insulin sensitivity, fat oxidation in muscle and decreases liver and muscle fat content (22). In another mouse model, adiponectin inhibits the expression of hepatic gluconeogenic enzymes PEPCK and glucose 6 phosphatase and the rate of endogenous glucose production (442). In isolated primary rat hepatocytes, physiologic doses of adiponectin have been shown to enhance insulin-induced suppression of glucose production (20). Adiponectin treatment of mice with alcohol- or obesity-induced fatty livers has been shown to alleviate hepatomegaly, steatosis and decrease ALT concentrations (443). These therapeutic effects resulted partly from an increase in the carnitine palmitoyltransferase-1 activity and hepatic fatty acid oxidation, and from a decrease in the activities of enzymes involved in fatty acid synthesis in the liver, including ACC and FAS (443). These data strongly suggest that adiponectin regulate hepatic lipid accumulation and endogenous glucose production.

Adiponectin has also anti-inflammatory properties antagonizing the effects of TNFα (444), which may contribute to the insulin-sensitizing effects of adiponectin. Disruption of the adiponectin gene in mice is associated with high levels of TNFα in adipose tissue and plasma (445). Adenovirus-induced adiponectin expression in these mice normalized the increased TNFα expression (445). In mice with a fatty liver, adiponectin treatment suppresses hepatic production of TNFα and its plasma concentrations (443). On the other hand, TNFα has been shown to decrease the expression of adiponectin in 3T3-L1 adipocytes (99), and both TNFα and IL-6 have been found to decrease the expression of adiponectin in human adipose tissue culture *in vitro* (103). Plasma TNFα and its expression in SAT have been shown to be increased, and to correlate negatively with plasma adiponectin concentration in patients with HAL (432). IL-6 expression is also increased in SAT in patients with HAL as shown in the current study and by Lihn et al (432). It is therefore possible that increased TNFα and/or IL-6 contribute to the decreased adiponectin expression in HAL or *vice versa*. Very recently, the expression of CRP has been demonstrated in human SAT (446). The expression of CRP in SAT correlated inversely with that of adiponectin (446).

Adiponectin deficiency may also enhance atherosclerosis by increasing the rate of foam cell formation, since *in vitro* adiponectin has been shown to reduce human macrophage-to-foam cell transformation (98). In this study, adiponectin suppressed the expression of the class A macrophage scavenger receptor without affecting the expression of CD36 (98). *In vitro* data therefore suggest that HAART may increase macrophage lipid accumulation by two mechanisms, i.e. by a direct PI-induced increase in the expression of CD36 in human PBMCs (424) and by an adiponectin deficiency-mediated increase in the expression of macrophage scavenger receptor (98).

7.2.5. LEPTIN

The HAART+LD+ group had a significantly lower serum leptin concentration than the HAART+LD- or the HIV- group (Study I). These data are consistent with data comparing patients with HAL to HIV negative controls (447). In some studies, however, serum leptin concentrations have not been different between lipodystrophic and non-lipodystrophic HIV-infected patients (270,433,448), and HAL has also been associated with an increased leptin concentration when compared to non-lipodystrophic HIV-infected patients (449). The reasons for the contrasting results between the current study and some previous studies are not clear, but may include lack of statistical power due to a smaller sample size (270), or less severe lipoatrophy of study subjects as judged from BMI (433) than in the current study. The latter hypothesis is supported by the findings of a study, in which only patients with pure lipoatrophy, as opposed to patients with pure lipohypertrophy or mixed form of lipodystrophy, had decreased circulating leptin concentrations when compared to non-lipodystrophic subjects (450). Large variation in the results regarding leptin in these studies also raises the question whether altered leptin expression is at all a characteristic feature of HAL.

In the current study, serum leptin concentration correlated with BMI and especially with the amount of subcutaneous fat both in the HAART+LD+ and the HIV- group (**Fig. 8**). The slope of the relationship between BMI and serum leptin concentration was significantly different between the HAART+LD+ and the HIV- groups; for a given BMI above 20 kg/m^2 the lipodystrophic patients had a lower leptin concentration than the HIV negative subjects. This could be explained by increased leptin expression in SAT vs. VAT (**Table 1**), since for the same BMI lipodystrophic patients have less SAT and more VAT than non-lipodystrophic subjects. When the relationship between subcutaneous fat mass and serum leptin concentration was evaluated in the lipodystrophic and HIV negative subjects, the relationships had similar slopes in both groups (**Fig. 8**). But again, for the same amount of SAT, the lipodystrophic patients have additionally more VAT than the non-lipodystrophic patients. The contribution of the additional VAT to serum leptin concentration in the HAART+LD+ group should be compensated by a decreased leptin expression in SAT when compared to HIV-group. This was later directly shown when leptin expression was measured by quantifying mRNA concentration in SAT: patients with HAL had lower leptin expression than HAART-treated patients without lipodystrophy or HIV negative subjects (Study VI) (262).

Data from lipodystrophic mouse models suggest that leptin can reverse insulin resistance when normal adipose tissue is absent (202). Leptin treatment has also been shown to decrease serum cholesterol and triglyceride concentrations and interscapular fat mass, and to improve liver steatosis in a ritonavir-induced lipodystrophic mouse model (451). In non-HIV lipodystrophic humans with a low baseline leptin concentration, leptin-replacement therapy for four months has been shown to improve glycemic control and to decrease triglyceride concentrations and liver volume (123). As of today there are no data regarding the effect of leptin therapy in HAL.

7.2.6. PAI-1

The increased plasma PAI-1 and tPA concentrations in the HAART+LD+ group when compared to the HAART+LD- or the HIV- group (Study VI) are in keeping with a previous study comparing patients with HAL to HIV negative subjects (336). PAI-1 expression in adipose tissue of patients with HAL has not been previously reported.

PAI-1 mRNA concentrations in SAT are approximately 2-fold increased in obese as compared to lean subjects (452,453). Given that the obese subjects in the latter studies had mean BMIs of 42.6 kg/m^2 (452) and 35.6 kg/m^2 (453), adipose tissue mass was markedly increased and thus SAT probably contributed substantially to the increase in plasma PAI-1 concentrations. We found a 2-3 -fold increase in PAI-1 gene expression in SAT in the HAART+LD+ group compared with the HAART+LD- and the HIV- groups (**Fig. 14**). However, SAT was almost 2-fold reduced in the abdominal region (**Table 5**). This implies that even though the concentration of PAI-1 mRNA was 2-3 -fold increased in subcutaneous fat, this increase, in the

face of a 2-fold reduction in total SAT mass, is unlikely to explain the greater than 2-fold increase in plasma PAI-1 antigen concentration. Another factor worth considering is that PAI-1 has been suggested to originate from stromal cells rather than adipocytes in adipose tissue (170). There are more inflammatory cells in the lipodystrophic than in the non-lipodystrophic adipose tissue as judged from the increased CD45 expression (**Table 8**). The lack of a correlation between the size of the SAT depot and plasma PAI-1 (**Fig. 15**) could therefore be due to altered composition of adipose tissue. Again, even here opinions are divided as some authors have suggested that SAT does not contribute to circulating plasma PAI-1 concentrations (454).

Significant associations between plasma PAI-1 concentrations and the amount of VAT rather than SAT have been found (455). Visceral fat may produce more PAI-1 than subcutaneous fat (170,455). In the present study within the HAART+LD+ group, which had a 2-fold increase in intra-abdominal fat (**Table 5**), there was no correlation between the amount of intra-abdominal fat and plasma PAI-1 concentration (**Fig. 15**) suggesting that other factors are important in the regulation of plasma PAI-1 concentration (Chapter 7.4.3).

7.3. LIVER FAT

In the current study, the liver fat content was significantly higher in the HAART+LD+ group when compared to the HAART+LD- and the HIV- group (**Table 5**). Liver fat has not been previously quantified in patients with HAL.

The increased hepatic fat content of the patients with HAL could not be explained by alcohol consumption, which was comparable in all groups (Study I). Hepatitis C has recently been shown to increase the risk for severe liver damage (456) and insulin resistance (325) during HAART. This association, however, cannot explain the findings in the current study, since none of the subjects had serologic evidence of hepatitis C or B. The HAART+LD+ and the HAART+LD- groups were comparable with respect to the HIV-related characteristics with the exception of the HAART+LD+ group having a longer exposure to NRTIs (**Table 6**). Even though this is unlikely to alone explain lipodystrophy and insulin resistance, we cannot exclude the possibility that NRTI-specific effects, such as mitochondrial toxicity (265) could have contributed to these side effects. NRTI-induced mitochondrial toxicity has been suggested to cause lactic acidosis and hepatic steatosis in some patients using HAART (457,458). None of the subjects in the current study had symptoms of lactic acidosis and serum lactate concentrations were similar in the HAART+LD+ and the HAART+LD- groups (Study I). However, in the absence of liver biopsies, we cannot exclude the possibility that mitochondrial abnormalities, such as those described in insulin resistant patients with non-alcoholic steatohepatitis (459), characterized also the present participants. Very recently, mild to diffuse steatosis and ultrastructural mitochondrial abnormalities have been found in liver biopsies of HIV-infected patients who had been treated with NRTI-based regimens and had only mildly elevated transaminases (460) suggesting that mitochondrial abnormalities may contribute to liver steatosis also in the absence of lactic acidosis.

Data are limited regarding the direct effects of HAART on the liver. Treatment of monkeys with stavudine has been shown to deplete mtDNA and disturb oxidative phosphorylation in the liver (461). The effects of stavudine in the human liver are not known, but the use of stavudine has been associated with the loss of mtDNA in subcutaneous adipocytes in patients with HAL (261). In mice, ritonavir caused enlargement of the liver and hepatic lipid accumulation especially when fed with a high fat diet (289). The proposed mechanism was an excessive accumulation of SREBP-1 and –2 in the nucleus of hepatocytes. These data in mice would suggest that features of insulin resistance in patients with HAL were a consequence of a primary effect of PIs in the liver. This is, however, controversial. At least in adipocytes, PIs have been shown both to decrease and to increase the expression of SREBP (**Table 3**). In human SAT, SREBP-1 mRNA concentration is decreased in patients with HAL when compared to HAART-treated patients without lipodystrophy (**Table 8**) and to HIV- negative subjects (262).

It is also possible that steatosis is not due to a direct effect of the drugs in the liver, but is a consequence of the inability of the adipose tissue to store triglycerides (38). Furthermore, the dysfunction of the remaining lipoatrophic subcutaneous fat and hypertrophic intra-abdominal fat may result in upregulation of insulin resistance-inducing cytokines, such as TNFα (432), and downregulation of beneficial adipocytokines, such as adiponectin (**Table 8**).

In the current study, liver fat content correlated with fasting serum insulin and C-peptide concentrations, but not with the amount of intra-abdominal fat or WHR (**Figure 7**). Furthermore, the amount of intra-abdominal fat did not correlate with serum insulin concentrations (Study I). The lack of this correlation challenges the idea that accumulation of fat intra-abdominally is, at least alone, responsible for insulin resistance in HAL. For a given amount of liver fat, serum insulin concentrations were higher in the lipodystrophic patients than the HIV negative subjects (**Fig. 7**) implying that liver fat alone was insufficient to explain all the variation in serum insulin concentrations. Fat may not be deposited only in the liver but also in skeletal muscle cells. The amount of IMCL has been shown to correlate negatively with insulin sensitivity in HIV negative subjects (195,462-465). IMCL is also increased in patients with HAL when compared to HIV negative controls (271) or to HIV-infected non-lipodystrophic patients (270), and correlates inversely with insulin sensitivity (270,271). In humans, HAART-associated insulin resistance is most likely caused by a combination of direct and indirect mechanisms, e.g. indinavir has been shown to induce an acute decrease in glucose disposal even after a single dose in healthy subjects (307).

Accumulation of liver fat may not only affect patients' health via contributing to insulin resistance, but may also lead to steatohepatitis and eventually to cirrhosis (407). The presence of steatohepatitis can only be confirmed by performing liver biopsies. Since this is an invasive procedure and places the subjects at risk for

bleeding complications, biopsies were not performed in the current study. Fatty liver disease is a significant contributor to morbidity in patients with non-HIV lipodystrophy (206) and NAFLD is the most common cause of cryptogenic cirrhosis, which is the third leading cause of liver transplantation in the United States (466). It has also recently been shown that patients who have hepatitis C and NAFLD have an increased risk of advanced fibrosis when compared to hepatitis C patients without NAFLD (467). This additive effect may be especially important in patients with HAL, since co-infections with HIV and hepatitis C are common.

7.3.1. LIVER FAT AND PAI-1

A striking finding in the present study was the close correlation of plasma PAI-1 concentrations with liver fat content in the HAART+LD+ and the HAART+LD- groups (**Fig. 15**). It can be concluded that plasma PAI-1 concentration is likely to be regulated directly via changes in production or uptake of PAI-1 by the liver, or indirectly via alterations in serum insulin concentrations due to changes in hepatic insulin sensitivity (19,191). This interpretation is also supported by the intervention study (Chapter 7.4.3.).

In the normal human liver, PAI-1 mRNA has been localized to endothelial cells but not to hepatocytes (468). On the other hand, PAI-1 synthesis in hepatocytes is induced by specific mediators and under certain pathological conditions. Mediators of the acute phase response (IL-1 alone and in combination with IL-6) stimulate PAI-1 gene transcription in HepG2 hepatoma cells (469,470). Insulin can increase PAI-1 production in cultured hepatocytes and HepG2 cells (471,472). Hepatosteatosis is associated with increased synthesis of a variety of proteins including hepatic enzymes and coagulation factors (188). The latter include PAI-1 and tPA, which are increased in men with steatosis, independently of obesity (473). Liver enzymes are also correlated with plasma PAI-1 concentrations, independently of serum triglycerides in hypertriglyceridemic subjects (474), and independently of triglycerides, insulin and obesity in asymptomatic hyperlipidemic men (475). Recently, increases in PAI-1 mRNA concentrations were found in hepatocytes of rabbits with a fatty liver after high fat feeding (476). These studies support the notion that the liver may be a source of circulating PAI-1 in subjects with a fatty liver. Whether it is the simultaneous increase in serum triglycerides or insulin, or perhaps changes in circulating FFA or some other factor, which increases PAI-1 under such conditions remains unclear. Since PAI-1 is cleared by the liver (477), fat accumulation in the liver could increase PAI-1 concentration in plasma by impairing its clearance.

7.4. TREATMENT OF HAL WITH ROSIGLITAZONE

7.4.1. CLINICAL EFFECTS

This is the first controlled study evaluating the efficacy and safety of a thiazolidinedione in patients with HAL. In contrast to results in type 2 diabetic patients (10,379-382), in non-HIV lipodystrophic patients (213) and *in vitro* data (275), treatment with rosiglitazone did not increase any of the several measures of adiposity. The results of the current study contrast also those of an uncontrolled study evaluating the effects of

rosiglitazone in HAL. In the latter study, treatment of 8 patients with 8 mg of rosiglitazone for 6-12 weeks significantly increased the amount of SAT and decreased the amount of VAT measured using a single CT scan (385). Peripheral SAT was measured at baseline using DEXA. Body weight or DEXA results after rosiglitazone treatment were not reported (385). Possible explanations for the different results may arise from the differences in study design, i.e. open-label uncontrolled vs. double-blind placebo-controlled, or possibly from differences in the background HAART regimens.

The lack of effect of a 24-week treatment with rosiglitazone on body fat in the present study demonstrates that either rosiglitazone is unable to increase adipose mass in patients with HAL, or these patients require much longer treatment than HIV negative patients. It can also be hypothesized that rosiglitazone caused a stimulatory effect on adipocyte differentiation, but this beneficial effect was neutralized by the unaltered use of HAART. It therefore remains to be studied, whether thiazolidinediones could increase fat mass under the circumstances that the concomitant HAART could be simultaneously modified to exert a less deleterious effect on the differentiating adipocytes, or if thiazolidinediones were given prophylactically.

In HIV negative subjects, rosiglitazone lowers or has neutral effects on serum triglyceride concentrations (10,379-382,478). Both LDL and HDL cholesterol concentrations generally increase by rosiglitazone (380,478). In the study by Gelato et al., rosiglitazone increased serum triglycerides non-significantly by 1.5 mmol/l in patients with HAL (385). In the current study, serum triglycerides increased markedly in the rosiglitazone group but remained unchanged in the placebo group (**Fig. 11**). At baseline, serum triglycerides exceeded 5 mmol/L in 20% of the patients both in the rosiglitazone and the placebo groups. After 6 and 12 weeks of treatment with rosiglitazone these percentages had increased to 40% and 53%, respectively. These data imply, given the risk of pancreatitis and the need of lipid-lowering drugs when triglycerides exceed 10 mmol/l (479) that triglycerides need to be monitored closely in future trials using rosiglitazone in patients with HAL. The cause of the increase in serum triglycerides remains speculative as effects of rosiglitazone on VLDL kinetics are unknown even in HIV negative individuals. Possibly, rosiglitazone mobilized triglycerides from the liver, but was unable to sufficiently enhance their clearance by adipose tissue.

Despite the lack of effect on adipose tissue mass or distribution, rosiglitazone decreased liver fat content and fasting serum insulin concentrations in the current study (**Table 9**). Rosiglitazone improved insulin sensitivity measured using the clamp technique also in the study by Gelato et al. in patients with HAL (385). In the present study, liver function tests continuously improved in the rosiglitazone group, possibly as a consequence of the decrease in the liver fat content (**Fig. 12**). The decrease in the liver fat content by rosiglitazone is similar to that reported in HIV negative subjects in an uncontrolled study (382).

Regarding treatment of HAL, the effects of rosiglitazone in the current study should be compared with those of metformin in HAART-treated patients (375). Although inclusion criteria were somewhat different, both

metformin and rosiglitazone improved insulin sensitivity and reduced PAI-1 concentrations (**Fig. 14**) (336,375). Metformin decreased serum triglycerides, whereas rosiglitazone at least temporarily worsened dyslipidemia. Rosiglitazone, however, decreased liver fat content and transaminase concentrations, which remained unchanged after metformin treatment. Liver fat content was not assessed in the metformin study. Although neither rosiglitazone nor metformin reversed lipodystrophy, metformin might be considered at the moment the drug of choice to treat insulin resistance in these patients in view of the significant increases in blood lipids by rosiglitazone. On the other hand, the two drugs have not yet been compared in the same study in patients with HAL.

In vitro, both PIs (**Table 3**) and NRTIs (310) can inhibit adipocyte differentiation. The suggested mechanisms include both SREBP-1 / PPARγ -dependent and -independent mechanisms (**Table 3**). *In vitro*, the PI-induced block in adipocyte differentiation can be prevented by pre- or co-incubation of preadipocytes with rosiglitazone (**Table 3**). However, it is unclear to what extent PIs vs. NRTIs are responsible for the loss of subcutaneous fat in humans (Chapter 2.3.4.). The present data imply that the reversal of the PI-induced block in adipocyte differentiation observed *in vitro* does not appear to happen *in vivo*. Because glitazones promote preadipocyte differentiation into mature adipocytes through activation of PPARγ (378), the low baseline expression of PPARγ in the lipodystrophic adipose tissue may contribute to the poor effect (**Table 8**) (262). Another possibility is that the loss of adipocytes could perhaps be prevented if patients were treated with glitazones before rather than after the development of lipodystrophy simulating the *in vitro* experiments of pre- or co-incubation with rosiglitazone (**Table 3**).

7.4.2. EFFECTS ON GENE EXPRESSION IN SAT

Effects of thiazolidinediones on gene expression in human adipose tissue *in vivo* have not been previously reported. In human adipocytes *in vitro*, rosiglitazone has been shown to increase the expression of UCP-2 and the p85α-subunit of PI 3 kinase, decrease leptin expression, and have no effect on the expression of insulin receptor, IRS-1, GLUT4, LPL, HSL, ASP, FATP-1, angiotensinogen, PAI-1 and PPARγ (480).

Despite the lack of effect on the amount of subcutaneous and intra-abdominal fat (**Table 9**), rosiglitazone induced changes in gene expression in SAT of patients with HAL. These included significant increases in adiponectin and PGC-1 expression, and a decrease in the expression of IL-6. Rosiglitazone also increased PPARγ expression. However, the increase in PPARγ expression was of limited magnitude and significant only when compared with the decrease in the placebo group. Rosiglitazone also caused a significant increase in the serum concentration of adiponectin, which correlated significantly with the decrease in serum insulin concentration and liver fat content. These data demonstrate that rosiglitazone can have insulin-sensitizing effects without increasing the amount of SAT.

A 23% decrease in FFA concentration in the rosiglitazone group in the present study is in keeping with previous findings of 20-30% decreases in FFA concentrations in patients with type 2 diabetes treated with rosiglitazone (379,478). The decrease in serum FFA concentration can be due to decreased production or increased clearance of FFA. In patients with type 2 diabetes, rosiglitazone seems to lower fasting FFA concentrations by decreasing lipolysis (379). In patients with HAL, rates of lipolysis have been suggested to be increased (320). Since the sizes of adipose tissue depots remained unchanged, it is not possible to explain the decrease in serum FFA concentration by a decrease in lipolysis in adipose tissue. FFA originating from intravascular lipolysis is unlikely to be decreased, since serum triglycerides increased and the expression of LPL remained unchanged. Other possibilities, which cannot be resolved based on the present study, include increased FFA utilization in skeletal muscle as has been found in the rat (481), possibly mediated by an increase in adiponectin production (113). In mice, thiazolidinediones induce expression of fatty acid transport proteins (FATP-1, CD36), intracellular fatty acid binding protein (aP2) and acyl CoA synthase in white adipose tissue (482). Such changes could increase the clearance of FFA. Human data are limited regarding effects of rosiglitazone on the expression of genes involved in FFA utilization. In isolated human adipocytes, rosiglitazone has been reported to have no effect on the expression of FATP-1 (480). The lack of induction of these genes and of other genes involved in lipogenesis (SREBP-1c, ACS, PPARδ, LPL) may have contributed to the lack of increase in adipose tissue mass in the current study. Rosiglitazone did not increase the expression of LPL as has been described in human SAT *in vitro* (483). The lack of increase in LPL expression could have contributed to the increase in serum triglyceride concentration but cannot explain why triglycerides increased in the first place. Possibly, rosiglitazone mobilized triglycerides from the liver, the fat content of which significantly decreased compared to placebo treatment.

There are conflicting *in vitro* data regarding the effects of thiazolidinediones on glucose transport proteins (480,484,485). The mRNA concentrations of GLUT1 and GLUT4 in SAT remained unchanged in the current study and thus cannot explain the improved insulin sensitivity. These data do not exclude the possibility that rosiglitazone increased GLUT4 expression or translocation in muscle (486,487). On the other hand, the major physiological function of fasting insulin is to control hepatic glucose production (14). Changes in liver fat content have been found to be closely correlated with changes in the ability of insulin to suppress hepatic glucose production (19). Consistent with these data and the idea that the decrease in serum fasting insulin was due, at least in part, to enhanced hepatic insulin sensitivity, the decrease in serum fasting insulin and liver fat content were significantly correlated in the present study.

In the current study, treatment with rosiglitazone decreased serum CRP concentration and total white blood cell count, but did not change serum IL-6 concentration (**Table 9**). Similarly, in patients with type 2 diabetes, rosiglitazone has been found to decrease serum CRP and matrix metalloproteinase-9 concentrations and total white blood cell count, but did not change serum IL-6 concentrations (488). Thiazolidinediones have anti-inflammatory effects both in animals and in humans. In mice, troglitazone decreases the expression of TNFα

and IL-6 in white adipose tissue and in the liver (489). IL-6 is a key regulator of CRP production in hepatocytes (490). The unchanged serum IL-6 concentration in the present study cannot explain the decrease in serum CRP concentration. In mice, thiazolidinediones dowregulate pro-inflammatory cytokines in Kupffer cells in the liver (491). Whether thiazolidinediones have similar local anti-inflammatory properties in the liver of humans is not known. In the present study, despite having no effect on the total circulating IL-6 concentration (**Table 9**), rosiglitazone markedly decreased the expression of IL-6 in SAT (**Table 10**). Very recently, expression of CRP has been demonstrated also in human adipose tissue (446). If IL-6 regulates the expression of CRP in adipose tissue, it is possible that the decreased IL-6 expression in SAT may have decreased the expression of CRP in SAT. However, the contribution of adipose tissue-derived CRP to serum CRP concentration is unknown.

Rosiglitazone significantly increased adiponectin expression in SAT (**Table 10**) and almost doubled its circulating concentration (**Table 9**). Thiazolidinediones appear to have a direct effect on adiponectin expression via a recently identified functional PPAR-responsive element in the promoter region of the human adiponectin gene (112). The change in serum adiponectin concentration correlated inversely with the change in serum insulin concentration and liver fat content (**Fig. 13**). In rats, pioglitazone treatment increases plasma adiponectin, which is inversely correlated with hepatic glucose output (481). *In vivo* expression of adiponectin during thiazolidinedione treatment has previously not been reported in humans. *In vitro* incubation of isolated human adipocytes from omental but not from subcutaneous depots with rosiglitazone increases the secretion of adiponectin (155). In keeping with the results of the current study, an increase in the serum adiponectin concentration by rosiglitazone treatment has also been reported in patients with type 2 diabetes (110), glucose intolerant (111) and normal (492) subjects.

Based on animal data, one can hypothesize that adiponectin may have mediated most of the favorable effects of rosiglitazone treatment, such as the decrease in liver fat content (22), in serum insulin and FFA concentrations (113), and in inflammatory markers (445). However, other effects of rosiglitazone, such as a decrease in the expression of 11β-hydroxysteroid dehydrogenase type 1 could also have contributed (493).

7.4.3. EFFECTS ON PAI-1

In the current study, the mRNA concentrations of PAI-1 in SAT did not change in the rosiglitazone group, but plasma PAI-1 concentrations decreased significantly (**Fig. 14**). The decrease in plasma PAI-1 concentrations is similar to that reported with troglitazone in studies with type 2 diabetic patients (494-496). We did observe a small decrease in the PAI-1 expression in SAT in the placebo group, the cause of which is unclear. However, the changes in the mRNA concentrations of PAI-1 in SAT did not differ between the groups, and could therefore not explain the decrease of plasma PAI-1 concentration in the rosiglitazone group (**Fig. 14**). Of all clinical and biochemical parameters, the only significant correlates of the decrease in

plasma PAI-1 concentration were the decreases in serum insulin concentration and liver fat content (Study VI). The data thus suggest that the fatty liver may significantly contribute to plasma PAI-1 concentrations via affecting either the synthesis or the clearance of PAI-1. This hypothesis is supported by a recent study, which showed a correlation between the degree of steatosis in liver biopsies and plasma PAI-1 concentrations in obese humans (497). In the same report, a significant correlation was found between PAI-1 expression in the liver and its plasma concentration, thus further supporting a role of the liver in the regulation of plasma PAI-1 concentration (497).

7.5. FUTURE PROSPECTS

Life-long treatment with HAART will most likely increase the risk of severe adverse events, such as enhanced cardiovascular morbidity and stigmatizing lipodystrophy. Large prospective studies are needed to determine the incidence and risk factors of HAL, and clinical significance of long-term toxicity of HAART. It is important to increasingly combine the expertise in infectious diseases and metabolism in the routine care of HIV-infected individuals in order to assure the optimal virologic response to HAART and to minimize the adverse consequences of long-term HAART. In order to prevent future complications, blood lipid and glucose levels must be regularly monitored in patients receiving HAART. Emphasis should also be placed on the reduction of classical risk factors of cardiovascular diseases, such as smoking. Fully developed lipodystrophic, especially lipoatrophic changes appear to be resistant to reversal. Therefore it is increasingly important to try to prevent the loss of subcutaneous fat in patients receiving HAART.

Understanding of the pathogenesis of HAL is ultimately needed for its successful prevention and treatment. Since HIV-infected patients are treated with a combination of antiretroviral agents, it is difficult to determine the contribution of each individual antiretroviral agent to the metabolic adverse events. Single antiretroviral agents could be tested as monotherapy in HIV negative subjects, since in the absence of the virus there is no risk of the development of viral drug resistance. Quantifying gene expression in SAT instead of waiting for the appearance of visible changes in body composition may considerably shorten the exposure time needed for these studies.

The finding of an increased liver fat content in patients with HAL warrants further studies on its pathogenesis and clinical impact. The potential role of NRTI-induced mitochondrial toxicity in the pathogenesis of hepatosteatosis warrants to be evaluated. Hepatosteatosis in patients with HAL may not only contribute to insulin resistance, but also increase the risk of steatohepatitis and cirrhosis. Monitoring of the potential consequences of the fatty liver in patients with HAL should be included in future studies.

Although rosiglitazone did not alleviate lipoatrophy in the current study, it decreased liver fat content and serum insulin levels. It remains to be studied, whether treatment with rosiglitazone could increase SAT in patients whose HAART regimen would be simultaneously modified by replacing the more toxic drug(s) with

metabolically less toxic antiretroviral agent(s). Under these circumstances, the adipogenic effects of rosiglitazone could possibly exceed the diminished anti-adipogenic effects of the modified HAART, and eventually increase the amount of SAT. It would also be of interest to study whether rosiglitazone could prevent HAL if started before lipoatrophy has fully developed. Pioglitazone, which may act as a partial PPARα agonist (498), would also be an interesting agent, since pioglitazone appears to have more favorable effects on blood lipids than rosiglitazone at least in patients with type 2 diabetes (499). In the future, agonists which are active for both PPARγ and α may offer a dual effect by improving insulin sensitivity and correcting dyslipidemia (500). However, the effects of these drugs on lipoatrophy are probably not different from those of thiazolidinediones, since their action in adipose tissue is dependent on the activation of PPARγ as is that of rosiglitazone.

Currently there are no human data, and very limited *in vitro* or animal data regarding the mechanisms underlying the intra-abdominal fat accumulation in HAL. Since VAT is anatomically difficult to access, studies on the hypertrophic fat in buffalo humps might provide information regarding the pathogenic differences between the hypertrophic and atrophic adipose tissue in HAL.

8. SUMMARY

The results of the Studies I-VI can be summarized as follows:

I. Liver fat content was significantly increased in HIV-infected patients with HAL when compared to HIV-infected, HAART-treated patients without lipodystrophy and healthy controls. Fasting serum insulin concentrations correlated more closely with liver fat content than with other measures of body composition.

II. The expression of adiponectin in SAT and its circulating concentrations were decreased in HIV-infected patients with HAL when compared to HIV-infected, HAART-treated patients without lipodystrophy. Both adiponectin concentration in serum and its expression in SAT correlated with features of insulin resistance including liver fat content.

III. The expression of several transcription factors (PPARγ, SREBP-1c, PPARδ, PGC-1) important for the normal maturation of adipocytes were decreased in SAT of patients with HAL when compared to HIV-infected, HAART-treated patients without lipodystrophy. Also the expression of other genes involved in adipogenesis (LPL, ACS) and in glucose transport (GLUT4, GLUT1) were decreased in patients with HAL. The markers of inflammation, i.e. the expression of IL-6 and CD45 were increased in SAT of patients with HAL compared to HIV-infected, HAART-treated patients without lipodystrophy.

IV. Rosiglitazone did not increase the amount of subcutaneous fat in patients with HAL after 24 weeks of treatment. Rosiglitazone appeared to ameliorate insulin resistance as judged by the decrease in fasting serum insulin concentration and in liver fat content. Rosiglitazone markedly increased serum triglyceride and cholesterol concentrations in most patients.

V. Rosiglitazone upregulated the expression of adiponectin, PPARγ and PGC-1, and downregulated the expression of IL-6 in SAT of patients with HAL. Rosiglitazone also increased serum adiponectin concentration. The change in serum adiponectin concentration correlated with the change in fasting serum insulin concentration and the change in liver fat content.

VI. PAI-1 concentration in plasma and its expression in SAT were increased in HIV-infected patients with HAL when compared to HIV-infected, HAART-treated patients without lipodystrophy. Plasma PAI-1 concentration correlated with the amount of hepatic fat. Rosiglitazone did not change the expression of PAI-1 in SAT, but decreased PAI-1 concentration in plasma. The decrease in plasma PAI-1 concentration correlated with the decrease in liver fat content.

9. CONCLUSIONS

The results of the current studies imply that the pathogenesis of HAL involves multiple mechanisms. Downregulation of several transcription factors and other genes involved in adipogenesis in SAT may result in reduced adipocyte maturation and lipid accumulation in fat cells. Increased inflammation in SAT may inhibit adipocyte differentiation and increase lipolysis. Increased inflammation and mitochondrial alterations may also increase apoptosis of adipocytes.

Increased liver fat content in patients with HAL appears to contribute to hepatic insulin resistance. Liver fat also seems to regulate plasma PAI-1 concentrations in these patients. Increased hepatic fat accumulation may be a consequence of a direct effect of antiretroviral agents on the liver, or alternatively the inability to store fat in SAT may cause shifting of lipids into the liver and skeletal muscle. Decreased expression of adiponectin and increased inflammation in SAT may also contribute to the increased liver fat content and insulin resistance.

These data do not support the use of rosiglitazone for the treatment of subcutaneous lipoatrophy in HAART-treated patients, although rosiglitazone appeared to ameliorate insulin resistance and hepatosteatosis. Because of the worsened dyslipidemia by rosiglitazone, blood lipid concentrations should be monitored carefully in future trials using thiazolidinediones in patients with HAL. Increased expression of adiponectin may contribute to the insulin-sensitizing effects of rosiglitazone.

Finally, regardless the potential long-term toxicity of HAART, it is important to emphasize the dramatically decreased total HIV-associated mortality and morbidity after the introduction of HAART. Therefore, HAL and other metabolic adverse events must not prevent the use of HAART in HIV-infected patients when treatment is clinically indicated.

10. ACKNOWLEDGEMENTS

This study was carried out at the Department of Medicine, Divisions of Diabetes and Infectious Diseases at the Helsinki University Central Hospital during the years 2000-2003. I want to express my gratitude to Professor Hannele Yki-Järvinen and Professor Ville Valtonen for providing me with excellent research facilities.

I am most grateful to my supervisor Professor Hannele Yki-Järvinen for her ever-lasting energy, devotion to science, her attitude not only to look for challenges but also to accomplish them.

I want to express my gratitude to Docent Petri Kovanen and Docent Esa Rintala for their valuable advice and constructive criticism during the review of the thesis.

This work would not have been possible without our collaborators in Finland, Sweden, France and Japan. I am especially grateful to MD, PhD Matti Ristola for his unfailing support and guidance in HIV medicine, Docent Anna-Maija Häkkinen for her expert spectroscopy analyses and Professor Anders Hamsten for the fruitful Finnish-Swedish collaboration.

The help and support from my coworkers in Yki-team have been fundamental for the completion of this work. I want to thank Docent Antti Virkamäki for being the first "metabolic person" interested in HIV-lipodystrophy. I am deeply indebted to Elena Korsheninnikova and Katja Kannisto for their invaluable work in the lab. Jukka Westerbacka and Mirja Tiikkainen have not only been great coworkers, but also good friends already prior to this research project. I feel very fortunate to have shared so much work and so much fun with Robert Bergholm, Marjo Tamminen, Satu Vehkavaara, Anja Corner and Kirsi Pietiläinen. A special thank you goes to Katja Tuominen for her energetic spirit, superb organizing skills and good laughs. Maarit Toivonen and Pentti Pölönen are gratefully acknowledged for skilled technical assistance. I also want to express my greatest appreciation for the welcoming and helpful attitude that I have always experienced from Professor Marja-Riitta Taskinen and her team, and from all the staff, especially Tuulikki Nyman and Mia Urjansson, at Minerva Foundation.

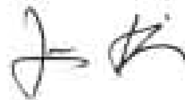
I am deeply grateful to my original home base, Infectious Disease Clinic. The help and support from Docent Asko Järvinen, other colleagues and staff, particularly the skilled nurses at the HIV Clinic, are highly acknowledged. Their encouraging attitude to my shuttling between the worlds of metabolism and infectious diseases has made it a pleasant adventure.

Without volunteers there would not be any clinical research. The time and effort of all the volunteers participating in these studies are greatly appreciated.

My gratitude for persistently keeping me aware of the non-scientific world during the last three years goes to my true friends: Helena, Hörps, Juha & Sönke, Korppu, La Profa & Los Estudiantes, Mappe & Hepa, Mersu, Miia, Pekka & Tita, Petteri, Risto & Eija, Satu, Terhi, Timo & Maarit, Tuija and others. I am very privileged to have parents with genuine wisdom reaching far beyond the rational thinking required in science. Thank you, Kaisa and Matti, for teaching me some of your tolerant and humorous attitude in life. I also want thank my sister Maikki, brother Asko and their families for their continuous support. My ever-growing appreciation goes to my little brother Köpi with Sanni and Kaisla for always remaining calm and having time to help. The final thank you goes to Aatu for sharing all the ups and downs with never-failing humor and patience. Kiitos. :-)

This work has been supported by grants from the Finnish Medical Foundation and Research Foundation of Orion Corporation. I also want to express my sincere appreciation to Thanks to Scandinavia for generously financing my stay at Beth Israel Medical Center, New York 1997-1998 where Dr Usha Mathur-Wagh and Dr Donna Mildvan originally introduced me to the metabolic complications of HAART.

Helsinki, December 2003



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