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Combination antiretroviral therapy -associated lipodystrophy: insights into pathogenesis and treatment

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ACADEMIC DISSERTATION

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*"Causa latet, vis est notissima."
("The cause is hidden. The effect is visible to all.")*

Ovid (Publius Ovidius Naso)
Roman poet (43 BC - 17 AD)

ABSTRACT

Introduction: Combination antiretroviral therapy (cART) has decreased morbidity and mortality rates of individuals infected with human immunodeficiency virus type 1 (HIV-1). Its use, however, is associated with severe metabolic adverse effects which increase the patients' risk of conditions such as diabetes and coronary heart disease. Perhaps the most stigmatizing side effect of cART is lipodystrophy, i.e., the loss of subcutaneous adipose tissue (SAT) in the face, limbs and abdomen with concurrent accumulation of adipose tissue in the intra-abdominal and dorsocervical regions. The pathogenesis of cART-associated lipodystrophy remains unclear with a number of antiretroviral agents associated with its development, and a variety of mechanisms proposed to date. For example, nucleoside reverse transcriptase inhibitors (NRTI) have been suggested to induce lipoatrophy through mitochondrial toxicity. At present, there is no known effective treatment for cART-associated lipodystrophy during unchanged cART in humans. Promising *in vitro* data have, however, been published showing that uridine can abrogate NRTI-induced toxicity in adipocytes.

Aims: The present studies were undertaken to elucidate whether i) cART as such or lipodystrophy associated with its use affect arterial stiffness; ii) lipoatrophic abdominal SAT is inflamed as compared to non-lipoatrophic SAT; iii) abdominal SAT from patients with as compared to those without cART-associated lipoatrophy differs with respect to mitochondrial DNA (mtDNA) content, adipose tissue inflammation and gene expression, and if the NRTIs stavudine and zidovudine are associated with different degrees of lipoatrophic change; iv) abdominal SAT (lipoatrophic in lipodystrophy) differs from dorsocervical SAT (preserved in lipodystrophy) with respect to mtDNA content, adipose tissue

inflammation and gene expression in patients with and without cART-associated lipodystrophy and v) oral uridine supplementation can revert lipoatrophy and the associated metabolic disturbances during ongoing stavudine or zidovudine-containing cART.

Subjects and methods: A total of 64 HIV-1-infected cART-treated patients with (n=45) and without lipodystrophy/-atrophy (n=19) of similar age, gender and body mass index were compared in a series of cross-sectional studies. A surrogate marker of arterial stiffness, heart rate-corrected augmentation index (AgI_{HR}), was measured by pulse wave analysis. Body composition was measured by magnetic resonance imaging and dual-energy X-ray absorptiometry, and liver fat content by proton magnetic resonance spectroscopy. Gene expression and copy numbers of mtDNA in SAT were assessed by real-time reverse transcriptase polymerase chain reaction and/or microarray technique. Adipose tissue composition as well as signs of inflammation were assessed by histology and immunohistochemistry. Both dorsocervical and abdominal SAT were studied. The efficacy and safety of uridine for the treatment of cART-associated lipoatrophy were evaluated in a randomized, double-blind, placebo-controlled 3-month trial in 20 lipoatrophic cART-treated patients.

Results: Duration of antiretroviral therapy and the cumulative exposure to NRTIs and protease inhibitors predicted AgI_{HR} independent of age and blood pressure. Time since HIV-1 diagnosis, severity of immunodeficiency or the presence of cART-associated lipodystrophy did not affect AgI_{HR} . Gene expression of macrophage markers and inflammatory cytokines was increased in the abdominal SAT of lipodystrophic as compared to that of non-lipodystrophic patients, and was seen to correlate with liver fat content. Expression of genes involved in adipogenesis, triglyceride synthesis and glucose disposal was significantly lower and of those

involved in mitochondrial biogenesis, apoptosis, inflammation and oxidative stress significantly higher in the abdominal SAT of patients with as compared to those without cART-associated lipoatrophy. Most changes were more pronounced in stavudine-treated than in zidovudine-treated patients. Lipoatrophic abdominal SAT had significantly lower mtDNA content than the abdominal SAT of non-lipoatrophic cART-treated patients. Expression of inflammatory genes was significantly lower in dorsocervical as compared to abdominal SAT. Neither adipose tissue depot had characteristics of brown adipose tissue. Despite being spared from lipoatrophy, the dorsocervical SAT of lipodystrophic cART-treated patients had lower mtDNA content than the phenotypically similar corresponding depot of non-lipodystrophic patients. The greatest difference in gene expression between dorsocervical and abdominal SAT, irrespective of lipodystrophy status, was in expression of homeobox genes that regulate transcription and cranio-caudal localization of organs during embryonal development. With regards to the clinical trial, uridine supplementation significantly

increased the absolute amount of limb fat as well as its proportion of total fat. Lean body mass, liver fat content and markers of insulin resistance remained unchanged in both uridine and placebo groups.

Conclusions: Long-term cART is associated with increased arterial stiffness and, consequently, with higher cardiovascular risk. Lipoatrophic abdominal SAT is characterized by inflammation, apoptosis and mtDNA depletion. However, as mtDNA is depleted even in the non-lipoatrophic dorsocervical SAT, lipoatrophy is unlikely to be caused directly by a decrease in cellular mtDNA content. Dorsocervical SAT of patients with cART-associated lipodystrophy is less inflamed than their lipoatrophic abdominal SAT, and does not resemble brown adipose tissue. The greatest difference in gene expression between dorsocervical and abdominal SAT is in expression of transcriptional regulators, homeobox genes, which might explain the differential susceptibility of these adipose tissue depots to cART-induced toxicity. Uridine supplementation is able to increase peripheral SAT in lipoatrophic patients during unchanged cART.

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LIST OF ORIGINAL PUBLICATIONS

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

- I. **Sevastianova K**, Sutinen J, Westerbacka J, Ristola M, Yki-Järvinen H. Arterial stiffness in HIV-infected patients receiving highly active antiretroviral therapy. *Antiviral Therapy* 2005; 10:925-35.
- II. **Sevastianova K**, Sutinen J, Kannisto K, Hamsten A, Ristola M, Yki-Järvinen H. Adipose tissue inflammation and liver fat in patients with highly active antiretroviral therapy –associated lipodystrophy. *American Journal of Physiology: Endocrinology and Metabolism* 2008; 295:E85-91.
- III. Sievers M*, Walker UA*, **Sevastianova K**, Setzer B, Wågsäter D, Eriksson P, Yki-Järvinen H, Sutinen J. Gene expression and immunohistochemistry in adipose tissue of HIV-1-infected patients with NRTI-associated lipodystrophy. *Journal of Infectious Diseases* 2009; 200:252-62.
- IV. **Sevastianova K**, Sutinen J, Greco D, Sievers M, Salmenkivi K, Perttilä J, Olkkonen VM, Wågsäter D, Lidell EM, Enerbäck S, Eriksson P, Walker UA, Auvinen P, Ristola M, Yki-Järvinen H. Comparison of dorsocervical to abdominal subcutaneous adipose tissue in patients with and without antiretroviral therapy –associated lipodystrophy. *Diabetes* 2011; 60:1894-900.
- V. Sutinen J, Walker UA, **Sevastianova K**, Häkkinen A-M, Ristola M, Yki-Järvinen H. Uridine supplementation for the treatment of HAART-associated lipodystrophy – a randomized, placebo-controlled trial. *Antiviral Therapy* 2007; 12:97-105.

* These authors have contributed equally to this work.

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ABBREVIATIONS

¹ H-MRS	proton magnetic resonance spectroscopy
16SRNA	16S ribosomal RNA
36B4	acidic ribosomal phosphoprotein P0
ACS	acyl-coenzyme A synthase
ACTB	actin β
ADAM8	a disintegrin and metalloproteinase domain 8
ADP	aortic diastolic pressure
AgI _{HR}	augmentation index corrected for heart rate
AGPAT2	1-acylglycerol-3-phosphate O-acyltransferase 2
AIDS	acquired immunodeficiency syndrome
AKT2	RAC- β serine/threonine protein kinase
ALT	alanine aminotransferase
aRNA	amplified RNA
ART	antiretroviral therapy
ASP	aortic systolic pressure
ATP	adenosine triphosphate
AZT	zidovudine
AZT+LA+	lipotrophic patients on zidovudine-containing antiretroviral regimen
B2M	β 2-microglobulin
BAT	brown adipose tissue
BIA	bioelectric impedance analysis
BMI	body mass index
BMP2	bone morphogenetic protein 2
BMP4	bone morphogenetic protein 4
BMP7	bone morphogenetic protein 7
BSCL2	Berardinelli-Seip congenital lipodystrophy 2
cART	combination antiretroviral therapy
cART+LD+	cART-treated patients with lipodystrophy
cART+LD-	cART-treated patients without lipodystrophy
CCL2	chemokine (C-C motif) ligand 2
CCL3	chemokine (C-C motif) ligand 3
CCR5	chemokine (C-C motif) receptor 5
cDNA	complementary DNA
CEBPA	CCAAT-enhancer-binding protein α
CEBPB	CCAAT-enhancer-binding protein β
CEBPD	CCAAT-enhancer-binding protein δ
CEBPG	CCAAT-enhancer-binding protein γ
CHOP	transcription factor homologous to CCAAT-enhancer-binding protein
CLS	crown-like structure
COX3	cytochrome c oxidase subunit III
COX4	cytochrome c oxidase subunit IV
CT	computed tomography
CXCR4	chemokine (C-X-C motif) receptor 4
d4T	stavudine
d4T+LA+	lipotrophic patients on stavudine-containing antiretroviral regimen
DAD	Data Collection on Adverse Events of Anti-HIV Drugs
DAG	diacylglycerol

DEXA	dual-energy X-ray absorptiometry
DNA	deoxyribonucleic acid
ED ₅₀	half-maximal effective dose
EMR1	epidermal growth factor –like module-containing, mucin-like, hormone receptor –like 1
FABP	fatty acid binding protein
FAS	factor of apoptotic stimulus
FASN	fatty acid synthase
FATP	fatty acid transport protein
FDG PET	fluorodeoxyglucose positron emission tomography
FFA	free fatty acid
GADPH	glyceraldehyde-3-phosphate dehydrogenase
GATA2	GATA-binding factor 2
GATA3	GATA-binding factor 3
GLUT4	glucose transporter 4
GPX1	glutathione peroxidase transcript 1
HAART	highly active antiretroviral therapy
HAART+LD+	HAART-treated patients with lipodystrophy
HAART+LD-	HAART-treated patients without lipodystrophy
HbA _{1c}	glycosylated hemoglobin A _{1c}
HDL	high-density lipoprotein
HEXOK1	hexokinase 1
HIV-1	human immunodeficiency virus type 1
HOMA-IR	homeostasis model assessment of insulin resistance
HOXA10	homeobox A10
HOXC8	homeobox C8
HOXC9	homeobox C9
hs-CRP	high sensitivity C-reactive protein
HSL	hormone sensitive lipase
IAT	intra-abdominal adipose tissue
IL1B	interleukin 1β
IL6	interleukin 6
IL8	interleukin 8
IRS	insulin receptor substrate
ITGAM	integrin αM
KLF2	Kluppel-like factor 2
KLF7	Kluppel-like factor 7
LA	lipoatrophy
LD	lipodystrophy
LDL	low-density lipoprotein
LMF1	lipase maturation factor 1
LMNA	lamin A/C
LMNB	lamin B
LPL	lipoprotein lipase
MRI	magnetic resonance imaging
mRNA	messenger RNA
mtDNA	mitochondrial DNA
N/A	not applicable
NAFLD	non-alcoholic fatty liver disease
nDNA	nuclear DNA
NFKB	nuclear factor κB
NNRTI	non-nucleoside reverse transcriptase inhibitor

NRTI	nucleoside reverse transcriptase inhibitor
NS	not significant
OGTT	oral glucose tolerance test
P ₁	first systolic peak
P ₂	second systolic peak
p53	tumor protein p53
PAI1	plasminogen activator inhibitor 1
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PGC1A	peroxisome proliferator –activated gamma coactivator 1 α
PGC1B	peroxisome proliferator –activated gamma coactivator 1 β
PI	protease inhibitor
PI-3-kinase	phosphatidylinositol-3-kinase
PLIN1	perilipin 1
POLG1	DNA polymerase γ (catalytic subunit)
POLG2	DNA polymerase γ (accessory subunit)
PP	pulse pressure
PPARG	peroxisome proliferator –activated receptor γ
PPARG2	peroxisome proliferator –activated receptor γ subunit 2
PRDM16	PRD1-BF1-RIZ1 homologous domain containing 16
PREF1	preadipocyte factor 1
PWA	pulse wave analysis
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SAT	subcutaneous adipose tissue
SEM	standard error of mean
SHORT	short stature, hyperflexibility of joints and/or inguinal hernia, ocular depression, Reiger anomaly and teething delay
SHOX2	short stature homeobox 2
SMART	Strategies for Management of Antiretroviral Therapy
SOD1	superoxide dismutase 1 (cytosolic)
SREBP1C	sterol regulatory element-binding protein 1c
TFAM	mitochondrial transcription factor A
TNFA	tumor necrosis factor α
tNRTI	thymidine analogue nucleoside reverse transcriptase inhibitor
UCP1	uncoupling protein 1
VLDL	very-low-density lipoprotein
WAT	white adipose tissue
ZMPSTE24	zinc metalloprotease

INTRODUCTION

Since the introduction of combination antiretroviral therapy (cART) in the mid-1990s, morbidity and mortality rates of individuals infected with human immunodeficiency virus type 1 (HIV-1) have markedly decreased (265,307,315,321). However, with the spreading use of cART, various metabolic adverse effects have emerged (47,122). Cumulative exposure to cART has been demonstrated to be associated with increased incidence of myocardial infarction (108,109). Interestingly, the increase in relative risk of myocardial infarction could only partially be explained by conventional risk factors (108,109). The augmentation index, a non-invasively measured surrogate marker of systemic arterial stiffness, is known to predict cardiovascular events and mortality independent of dyslipidemia and other confounders in subjects not infected with HIV-1 (227,420,421). Its value in explaining the increased cardiovascular risk beyond that attributable to conventional risk factors has not been previously assessed in HIV-1-infected patients.

One of the most readily apparent side effects of antiretroviral treatment is that of cART-associated lipodystrophy. This condition is characterized by the loss (i.e., lipoatrophy) of subcutaneous adipose tissue (SAT) in the face, limbs and trunk, with or without concurrent accumulation (i.e., hypertrophy) of adipose tissue in the intra-abdominal and dorsocervical regions (47,122,164,193). These changes in body fat distribution are often accompanied by other metabolic derangements such as hepatic steatosis, insulin resistance and dyslipidemia (47,122,164,193). Upon its recognition in the late-1990s, it was estimated that after a year of exposure to cART one out of every two patients suffered from at least one physical abnormality indicative of lipodystrophy (47). At present, despite the increasing availability of less toxic antiretroviral

agents, cART-associated lipodystrophy remains the most common form of human lipodystrophy (39,114).

In recent years, much interest has been focused on unraveling the mystery of cART-associated lipodystrophy. To date, several potential pathogenetic mechanisms have been proposed (25,43,53,100,268,422). One of the most popular hypotheses has been the so-called “mitochondrial toxicity theory” (25). This theory states that nucleoside reverse transcriptase inhibitors (NRTI), a class of antiretroviral drugs, inhibit mitochondrial deoxyribonucleic acid (DNA) polymerase γ (POLG), an enzyme responsible for replication of mitochondrial DNA (mtDNA) in adipocytes. Inhibition of this enzyme decreases mtDNA copy number, resulting in depletion of transcripts of mtDNA-encoded genes such as those involved in electron transfer chain and adenosine triphosphate (ATP) production. This, in turn, leads to decreased lipogenesis, and to increased oxidative stress and apoptosis in affected adipocytes with the end result of adipose tissue inflammation and lipoatrophy (71,171,215). While this process may contribute to the development of lipoatrophy, the “mitochondrial toxicity theory” remains insufficient, as it does not explain why in cART-associated lipodystrophy adipose tissue atrophies in some anatomic locations, but remains preserved or even hypertrophies in others. Furthermore, there are no data comparing lipoatrophic and lipohypertrophic SAT from the same individual with cART-associated lipodystrophy.

Numerous therapeutic interventions, including thiazolidinediones (368) and leptin (206,275), have been attempted in an effort to alleviate cART-associated lipodystrophy. Results, however, have been disappointing. While modification of cART is currently recognized as the best choice to halt and revert lipoatrophy (367), there is no known effective treatment for cART-associated lipodystrophy during unchanged cART. Uridine has shown promise *in vitro* by reducing NRTI-induced toxicity in adipocytes despite the

continued presence of toxic antiretroviral agents (414).

The ensuing review of literature will summarize the current knowledge of clinical features and pathogenetic mechanisms underlying cART-associated lipodystrophy, and will contrast the

pathology inherent to this condition with the normal physiology of adipose tissue and insulin action in humans. The review will also recapitulate the already-explored and the yet-promising therapeutic options for the treatment of cART-associated lipodystrophy.

REVIEW OF THE LITERATURE

1. ADIPOSE TISSUE

1.1. Adipose tissue depots in humans

Approximately 10-50% of human body mass is composed of adipose tissue which can be divided into SAT and intra-abdominal adipose tissue (IAT). An average person with a body mass index (BMI) of 20-30 kg/m² has 10-30 kg of SAT and 0.5-4 kg of IAT (385). Women have significantly more SAT for a given body weight than men (385). There are two types of adipose tissue in humans, white and brown. White adipose tissue (WAT) constitutes the majority of adipose tissue within the body (65). Typical white adipocytes are unilocular and have few mitochondria, while brown adipocytes are multilocular and rich in mitochondria giving the tissue its typical appearance and coloration (65). Besides mature adipocytes, adipose tissue also contains preadipocytes, macrophages, endothelial cells, fibroblasts and leukocytes (432).

The main physiological function of WAT is to store free fatty acids (FFA) in the form of triglycerides through esterification to glycerol in times of energy surplus and release thereof via lipolysis during energy shortage. WAT also provides mechanical support and insulation to other organs. Furthermore, WAT is an active endocrine and paracrine organ that secretes factors involved in adipose tissue development and remodeling, regulation of food intake, energy expenditure and fat mass deposition, insulin sensitivity, lipid and cholesterol metabolism, angiogenesis and vascular function, as well as pro- and anti-inflammatory reactions and immune responses (112,316,432).

In animals, the main function of brown adipose tissue (BAT) is thermogenesis, i.e.,

utilization of fat stores for generation of heat by uncoupling of ATP production (203). It was long believed that in healthy humans functional BAT exists only in infancy and is mainly located in axillary, deep cervical and perirenal sites, whereas interscapular depot, typical in rodents, was considered quantitatively unimportant (203). Recently, however, fluorodeoxyglucose positron emission tomography (FDG PET) studies in healthy adult humans have shown functional BAT to exist in cervico-supraclavicular, para-aortic, paravertebral and suprarenal regions (73,283,399,410). In addition, islets of brown adipocytes have been found dispersed amid regular WAT in healthy adult humans (37,203). BAT has also been found in conditions such as hibernoma (24), a benign fatty tumor of BAT origin, and surrounding the capsule of pheochromocytoma (258), a neuroendocrine catecholamine-secreting adrenal tumor, although there is doubt whether the two processes are related or simply coincidental (255). The physiologic significance of BAT in adult humans remains unknown.

All WAT is not the same (8,406). Indeed, intra-abdominal adipocytes are smaller than subcutaneous ones (316,406). This difference could be a consequence of decreased FFA uptake following lower lipoprotein lipase (LPL) activity and triglyceride synthesis in intra-abdominal as compared to subcutaneous adipocytes (406). Furthermore, the rate of lipolysis is higher in IAT than in SAT (8). The intra-abdominal depot is also more sensitive to the lipolytic effect of catecholamines and less so to the anti-lipolytic action of insulin than the subcutaneous depot (8), notwithstanding the similar number of adrenergic and insulin receptors in both regions (406). Hormone sensitive lipase (HSL) does not offer an explanation for the increased rate of lipolysis in IAT as compared to SAT, as its gene expression and protein activity is, if anything, lower in IAT than in SAT (406). Gene expression of adipose triglyceride lipase is also similar in IAT and SAT (17). Interestingly, reduced signal transduction through the insulin

receptor substrate (IRS) 1 –associated phosphatidylinositol-3-kinase (PI-3-kinase) pathway has been reported in IAT as compared to SAT (452). As insulin is a key modulator of gene expression (299), its reduced action in IAT is thought to affect expression of a number of relevant downstream genes. Indeed, mRNA concentrations of insulin-regulated genes such as glucose transporter protein 4 (*GLUT4*), peroxisome proliferator-activated receptor γ (*PPARG*) and leptin are lower in IAT than in SAT (207). Impaired insulin action could also contribute to decreased triglyceride synthesis and, thus, to small adipocyte size seen in IAT (406). In addition, IAT has been found to have higher expression of the inflammatory cytokine interleukin 6 (*IL6*), plasminogen activator inhibitor 1 (*PAI1*) and glucocorticoid receptors than SAT does (406). Findings of differentially regulated adipogenesis, increased lipolysis and inflammation in IAT compared to SAT have been verified by microarrays (30,413). These depot-specific differences in molecular and physiological properties may explain as to why excessive IAT, which drains its secretions directly into the portal vein, appears to be more hazardous than SAT in terms of metabolic and cardiovascular complications (88,180). It should be noted, however, that arguments put forward in defense of IAT underscore the fact that only correlation, but not causality, has been demonstrated between accumulation of IAT and metabolic aberrations (105).

1.2. Adipogenesis

1.2.1. Adipogenesis of white adipose tissue

Molecular events of adipogenesis (Figure 1) are divided into two main phases, determination and terminal differentiation (194,304,326). During the determination phase, a multipotent mesenchymal stem cell becomes preadipocyte, a cell solely committed to adipocyte lineage, thereby losing its potential to differentiate into alternative cell types, e.g., myocytes,

osteoblasts or chondrocytes (194,304,326). In white adipogenesis, determination is initiated by bone morphogenetic protein 4 (BMP4) and, to some extent, bone morphogenetic protein 2 (BMP2) (304,390). BMP2 and BMP4 release preadipocytes from the suppressive action of anti-adipogenic transcription factors, e.g., transcription factor homologous to CCAAT-enhancer-binding protein (CHOP), CCAAT-enhancer-binding proteins γ (CEBPG), Klüppel-like factors 2 and 7 (KLF2 and KLF7), GATA-binding factors 2 and 3 (GATA2 and GATA3), preadipocyte factor 1 (PREF1) and forkhead proteins, thereby allowing the adipogenic program to proceed. In addition to BMPs, numerous other extracellular modulators, both activating (insulin, insulin-like growth factor 1, transforming growth factor β , fibroblast growth factors) and repressing (Wnt family, sonic hedgehog, tumor necrosis factor α ; TNFA), regulate adipogenesis (194,326). Notch signaling and the mitogen-activated protein kinase pathway may also be involved, although the evidence regarding their role is inconclusive (194,326). Together, all of these master regulators relate information about the suitability of extracellular conditions for adipocyte differentiation and, when appropriate, complement the BMPs in suppression of the anti-adipogenic factors (326).

After determination, committed preadipocytes are thought to undergo a number of cell divisions known as mitotic clonal expansion (194,326), although whether this proliferative phase truly occurs in human preadipocytes has been challenged (304). Finally, preadipocytes enter terminal differentiation, wherein sequential and partially reciprocal activation of pro-adipogenic transcription factors CCAAT-enhancer-binding proteins α , β and δ (CEBPA, CEBPB and CEBPD), sterol regulatory element-binding protein 1c (SREBP1C) and PPARG further suppresses the anti-adipogenic genes and converts preadipocytes to mature adipocytes. Once fully differentiated, mature adipocytes assume

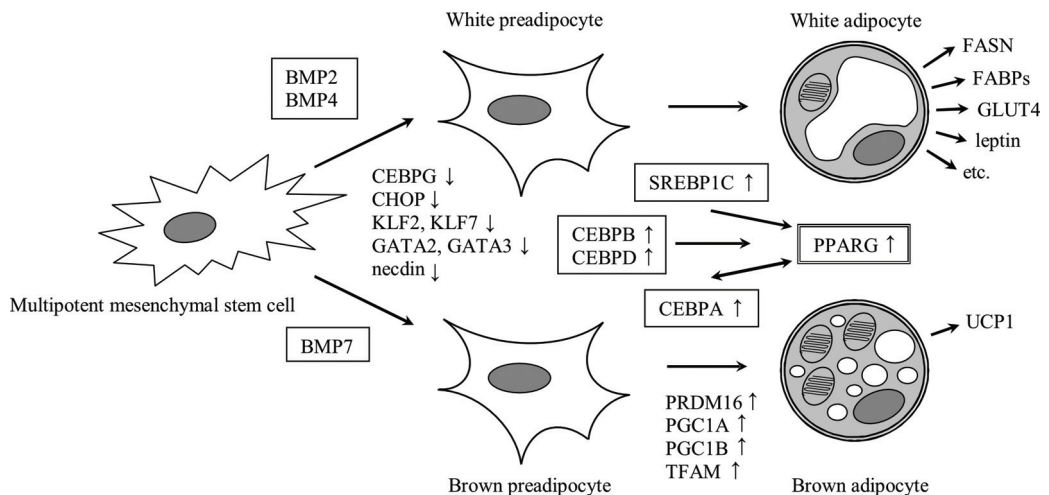
Figure 1.

Figure 1. Schematic representation of adipogenesis in white and brown adipocytes. Thick black arrows from mature adipocytes indicate proteins produced, but not necessarily secreted, by these cells. Bone morphogenetic proteins 2 and 4 (BMP2 and BMP4) and bone morphogenetic protein 7 (BMP7) drive the first step of adipogenesis, determination, in white and brown adipogenesis, respectively. They also suppress anti-adipogenic factors like CCAAT-enhancer-binding protein γ (CEBPG), transcription factor homologous to CCAAT-enhancer-binding protein (CHOP), Klüppel-like factors 2 and 7 (KLF2 and KLF7), GATA-binding factors 2 and 3 (GATA2 and GATA3), preadipocyte factor 1 (PREF1), forkhead proteins and neclin allowing committed preadipocytes to enter the last phase of adipogenesis, terminal differentiation. Sterol regulatory element-binding protein 1c (SREBP1c), CCAAT-enhancer-binding proteins α , β and δ (CEBPA, CEBPB and CEBPD) and peroxisome proliferator-activated receptor γ (PPARG) are the main pro-adipogenic transcription factors regulating terminal differentiation in both white and brown adipocytes. CEBPB and CEBPD are believed to regulate the early stages of terminal differentiation while SREBP1c, PPARG and CEBPA dominate the later stages of the process. In preadipocytes committed to brown lineage, BMP7 induces expression of PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16), peroxisome proliferator-activated gamma coactivator 1 α and 1 β (PGC1A and PGC1B) and mitochondrial transcription factor A (TFAM), i.e., genes promoting mitochondrial biogenesis. In the end of the adipogenic cascade, mature white adipocytes can finally produce proteins characteristic to them: fatty acid synthase (FASN), fatty acid-binding proteins (FABPs), glucose transport protein 4 (GLUT4), leptin, adiponectin and many others. Brown adipocytes specialize in producing uncoupling protein 1 (UCP1).

the physiological function of WAT, accumulating intracellular triglycerides and acquiring machinery for lipid synthesis and transport, insulin responsiveness and secretion of adipocyte-specific proteins (194,304,326). In addition to the main transcription factors orchestrating terminal differentiation, over 100 co-factors contributing to regulation of adipogenesis have been identified (326). These factors act in concert to, directly or indirectly, induce expression of adipogenic genes like fatty acid synthase (FASN), fatty acid-binding proteins (FABPs), insulin receptors, GLUT4, leptin and adiponectin (194,304,326). Interestingly, not only are

the key transcription factors of adipogenesis such as PPARG and CEBPA, necessary for adipogenesis itself, but these factors are vital also for maintenance of the functional differentiated state of adipocytes. Indeed, adipocyte cultures and murine models with genetically-induced lack of these transcription factors demonstrate loss of lipid accumulation and insulin sensitivity, and even de-differentiation (326).

1.2.2. Adipogenesis of brown adipose tissue

Adipogenesis of BAT features the same phases as that of WAT, but some of

the mediators involved differ (Figure 1) (390). While BMP2 and BMP4 are the main enhancers of determination in white adipogenesis (304,390), bone morphogenetic protein 7 (BMP7) acts exclusively to induce multipotent mesenchymal stem cells to become committed to brown adipocyte lineage (390). BMP7 suppresses expression of necdin, a negative modulator of brown preadipocyte differentiation, and increases that of “traditional” pro-adipogenic transcription factors (*PPARG*, *CEBPA*, *CEBPB*, *CEPBD*, *SREBP1C*). In addition, BMP7 specifically promotes mitochondrial biogenesis and increases mitochondrial density in the brown preadipocytes by stimulating expression of genes like PRD1-BF1-RIZ1 homologous domain containing 16 (*PRDM16*), peroxisome proliferator-activated gamma coactivator 1 α and 1 β (*PGC1A* and *PGC1B*) and mitochondrial transcription factor A (TFAM) (390).

Mature brown adipocytes are characterized by expression of uncoupling protein 1 (UCP1), a protein considered exclusive for mammalian BAT (284). UCP1 is located on the inner mitochondrial membrane and its function is to dissipate cellular energy as heat by uncoupling oxidative phosphorylation from ATP production in a process called non-shivering thermogenesis. UCP1 is activated, e.g., by noradrenaline and FFAs released in response to external stress stimuli such as cold exposure (284). There have been reports of UCP1 in WAT (280,301). However, the cells expressing UCP1 were also reported as having morphological appearance of brown adipocytes, suggesting that the findings represent brown adipocytes dispersed within WAT as opposed to WAT itself expressing UCP1.

Interestingly, rodent studies have suggested that white and brown adipocytes may be able to transdifferentiate into one another (65). The exact mechanisms, however, remain obscure, and the finding has not been verified in cultured human adipocytes or human adipose tissue *in vivo*.

2. INSULIN ACTION

2.1. Insulin action in adipose tissue

Insulin prevents triglyceride breakdown and stimulates its storage in adipose tissue *in vivo* (433). Adipose tissue lipolysis is the most sensitive of the biological processes controlled by insulin being inhibited by a much lower concentration of insulin (half-maximal effective dose, ED₅₀, for suppression of lipolysis measured using glycerol infusions is 13 mU/L) (297) than is required for inhibition of hepatic glucose production (ED₅₀ 26 mU/L) (297) or stimulation of glucose disposal by skeletal muscle (ED₅₀ ~100 mU/L) (443).

Insulin signals through binding and autophosphorylation of the insulin receptor, followed by sequential tyrosine phosphorylation of IRS proteins and inositol phosphorylation of PI-3-kinase, with consecutive activation of protein kinase B and other downstream protein kinases (299). Insulin, at least in part assisted by SREBP1C, also facilitates intravascular lipolysis by stimulating LPL, FFA transport and intracellular lipogenesis (299). Insulin accomplishes these actions by increasing expression of fatty acid transport protein (*FATP*) and acyl-coenzyme A synthase (*ACS*), and by repressing expression of genes involved in fatty acid oxidation (299). Insulin blocks lipolysis in mature adipocytes by suppressing protein kinase A-mediated phosphorylation and translocation of perilipin (PLIN) 1, a protein normally keeping HSL inactive by preventing its phosphorylation, thereby maintaining a barrier against triglyceride hydrolysis in the lipid droplets (433).

The adipose tissue of obese individuals, perhaps due to the presence of hypertrophic apoptotic adipocytes (66), harbors vast amounts of macrophages that are known to be the main source of inflammatory cytokines produced by the adipose tissue (447). These include monocyte chemoattractant protein 1 (protein encoded for by chemokine (C-C motif) ligand 2 gene; *CCL2*), macrophage inflammatory protein 1 α (protein encoded for by chemokine (C-C

motif) ligand 3; *CCL3*), TNFA and IL6 (447). The chronic inflammation in adipose tissue is worsened by low expression of anti-inflammatory adiponectin and high expression of proinflammatory leptin (112). Interestingly, a causal link between adipose tissue inflammation and insulin resistance has been suggested (448). Inflammatory cytokines such as TNFA hamper insulin signaling in adipocytes by inducing serine phosphorylation of the IRSs (331). This decreases the capacity of the IRSs to undergo phosphorylation on their tyrosine residue by the insulin receptor (331). This, in turn, leads to a decreased activation of their associated downstream kinases and opposes the production of proteins involved in triglyceride synthesis (LPL, FATP, ACS), while at the same time increasing lipolysis (331). As a net effect, more FFAs are mobilized from the adipose tissue and circulating FFA concentration increases (331). Increased FFAs, through toll-like receptor 4 –mediated activation of the nuclear factor κ B (NF κ B) pathway, then trigger adipose tissue-resident macrophages to produce auxiliary inflammatory mediators (351,448). These mediators can further inflame the adipose tissue, generating a vicious circle of worsening insulin resistance.

2.2. Insulin action in the liver

In the liver, the physiological action of insulin is to suppress endogenous glucose production (glycogenolysis and gluconeogenesis) in both basal and post-prandial states (433). Also, under normal conditions, insulin suppresses hepatic secretion of very-low-density lipoprotein (VLDL). This is accomplished via direct insulin-induced inhibition of assembly of the VLDL particles along with decreased availability of FFAs following insulin-induced inhibition of lipolysis in adipose tissue (433). In insulin resistance due to conditions such as hepatic steatosis, insulin fails to exert these physiological effects, leading to hyperglycemia and hypertriglyceridemia (441). The lack of insulin-stimulated LPL activity decreases

VLDL catabolism, resulting in further worsening of hypertriglyceridemia. The latter, in turn, encourages enrichment of high-density lipoproteins (HDL) and low-density lipoproteins (LDL) with triglycerides (441). This renders them better substrates for hepatic lipase and results in low circulating HDL cholesterol concentration and small, dense and atherogenic LDL particles (441). Due to prevailing hyperglycemia and in an attempt to compensate for insulin resistance, the pancreatic β -cells increase insulin secretion, resulting in hyperinsulinemia that, depending on the degree of insulin resistance, may or may not be sufficient to counteract it (441).

In obese subjects, adipose tissue –derived inflammatory cytokines, such as TNFA and IL6, directly blunt insulin signaling in the liver (by a mechanism analogous to that in adipose tissue), stimulate hepatic lipogenesis and aggravate hypertriglyceridemia (448). At the same time, these cytokines also stimulate adipose tissue lipolysis (331), mobilize excessive FFAs from inflamed and insulin resistant adipose tissue, and increase FFA delivery to the liver (441). In the liver, FFAs are metabolized into lipid intermediates such as ceramides, ceramide-derived gangliosides and diacylglycerols (DAG), all of which have been proposed to inhibit insulin action (223,339,366). Ceramides are thought to exert this action via caveolin-mediated activation of atypical protein kinase C and/or protein phosphatase 2A, both of which deactivate protein kinase B, rendering it unable to transmit normal insulin signaling to downstream components (223,366). It has been suggested that gangliosides, such as ganglioside monosialo 3, cause insulin receptor displacement from caveolin-rich domains of the cell membrane (223). Equally, gangliosides may also phosphorylate IRS proteins on their serine residue, thereby interfering with their ability to react with the insulin receptor and to recruit PI-3-kinase with the following signaling cascade (223). Finally, DAGs are thought to convey their insulin-desensitizing effects via

phosphorylation of IRS proteins on their serine residue, accomplished by activation of an isoform of protein kinase C, thereby preventing activation of the IRS-associated PI-3-kinase pathway (339). Circulating inflammatory cytokines promote the deleterious accumulation of ceramides and their derivatives by supplying the liver with adipose tissue –derived FFAs as well as by acting directly via toll-like receptor 4 –mediated activation of enzymes such as sphingomyelinase which are involved in the synthesis of ceramides (152,223). Collectively, these molecular events further decrease insulin sensitivity of hepatic gluconeogenesis and promote the development of hyperglycemia (195,254).

The degree of hepatic insulin resistance is closely associated with the amount of fat in the liver (188,332,346). The fatty acids in intrahepatocytic triglycerides are derived from i) dietary chylomicron remnants, ii) FFAs mobilized from adipose tissue, iii) post-prandial lipolysis of chylomicrons occurring in excess of what can be taken up the tissues (FFA spillover) and iv) *de novo* lipogenesis (441). The fatty liver has been found to contain increased amounts of saturated and decreased amounts of unsaturated fatty acids, with concentration of DAGs increasing with increasing intrahepatocytic triglyceride content (189). Intriguingly, *de novo* lipogenesis that yields only saturated fatty acids as a product, is increased in insulin resistant subjects (441). Ceramides, suggested as causative of insulin resistance, in turn, are built from saturated fatty acids only (223,366). Both increased intracellular concentrations of ceramides and of DAGs have (*vide supra*) been proposed to be “agents provocateurs” in the pathogenesis of insulin resistance (223,339,366).

2.3. Insulin action in skeletal muscle

In skeletal muscle, there are two main mechanisms by which insulin stimulates glucose uptake, direct and indirect (433). Insulin directly stimulates glucose transport and phosphorylation as well as glycogen synthesis in skeletal muscle

(433). Insulin promotes glucose uptake also indirectly by decreasing FFA availability via its anti-lipolytic effect and via stimulation of intravascular lipolysis (433). In insulin resistant states such as obesity, lipolysis in adipose tissue is enhanced and FFA transport into adipocytes decreased, leading to increased circulating FFA concentrations (441). FFAs are known to compete with and inhibit glucose uptake in skeletal muscle though inhibition of glucose transport activity, possibly as a consequence of reduced IRS-associated PI-3-kinase signaling and hampered GLUT4 activity (20).

Another factor possibly contributing to increased muscle lipid content is the activity of muscular LPL, which is increased rather than decreased in insulin resistant states (441). Accumulation of intramyocellular lipid may, through ceramides (223,366) and DAGs (339), interfere with insulin signaling at a post-receptor level (*vide supra*). As a result, translocation of GLUT4 to the cell membrane and glucose phosphorylation via hexokinase II are reduced, and glucose uptake impaired (195,254,441). Indeed, intramyocellular triglyceride content has been shown to closely correlate with insulin resistance in skeletal muscle (198,310).

3. ARTERIAL STIFFNESS

3.1. Definition of arterial stiffness

Arterial stiffness is a term used to describe the capacity, or lack thereof, of arteries to expand and contract during the cardiac cycle (288). In healthy, compliant vasculature, contraction of the left ventricle during systole increases pressure and dilates the arteries (288). The stiffer the vessels, the less their caliber changes with varying pressure conditions during a cardiac cycle, and the faster the pressure wave travels along the vessels as its energy is no longer dissipated in distending the elastic load-bearing elements of arterial walls (288). The lack of arterial compliance augments systolic blood pressure, resulting in increased left ventricular afterload

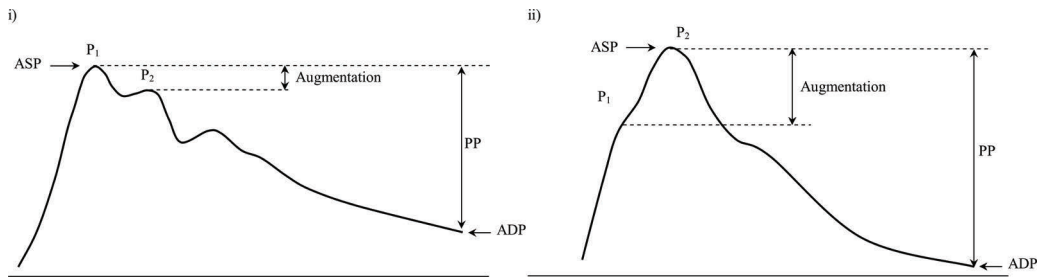
Figure 2.

Figure 2. Representative ascending aortic pulse waveforms during one cardiac cycle of a subject with i) compliant vasculature and ii) stiff vasculature. P_1 and P_2 denote the first and the second systolic pressure peaks, respectively. Augmentation is calculated as the difference between P_2 and P_1 . Augmentation index is defined as the ratio of augmentation and pulse pressure (PP), the latter being the difference between systolic and diastolic pressure. ADP, aortic diastolic pressure; ASP, aortic systolic pressure, equals the highest of the two systolic pressure peaks, P_1 or P_2 . Adapted from Sevastianova et al. *Antiviral Therapy* 2005; 10:925-35 (Study I), reproduced with permission of the copyright holder.

while simultaneously decreasing coronary perfusion, thereby aggravating coronary artery insufficiency (288).

3.2. Measurement of arterial stiffness

Many methods have been proposed as ways to assess arterial stiffness. These include sphygmomanometry, ambulatory arterial stiffness index, pulse wave velocity, ultrasonography and magnetic resonance imaging (MRI). Traditional measurement of pulse pressure by sphygmomanometry is widely available and simple, but prone to inaccuracy and human error, and is difficult to standardize. Ambulatory arterial stiffness index uses a 24-hour blood pressure monitoring to produce a stiffness estimate (217). While multiple readings taken by this approach increase its accuracy, this method is laborious and time-consuming. Applanation tonometer or even MRI techniques can be used to record pulse wave velocity (76,231,266). Ultrasonography and MRI, in particular, have been developed to enable measurements of not only arterial diameter and compliance properties, but also of thickness of different layers of the arterial wall, e.g. intima media (231). These methods, however, require expensive equipment and skilled personnel to perform. Results are also highly user-dependent and reproducibility may be of concern.

Pulse wave analysis (PWA) is a technique used to assess arterial stiffness by recording changes in arterial pressure occurring during one cardiac cycle (300). PWA utilizes a tonometer to record a pulse wave contour from peripheral artery, e.g., radial. In the arterial waveform (Figure 2), contraction of the left ventricle propelling blood along the arteries is seen as the first systolic pressure peak. This systolic pulse wave is then reflected back from the periphery causing the second systolic peak of the pressure wave. The stiffer the conducting vessels, the faster the pressure wave travels and the faster it is reflected back. Consequently, the return wave may superimpose with the forward-traveling wave, increasing the systolic pressure wave and decreasing the diastolic one. The degree of augmentation of the systolic pressure, a parameter evaluated by pulse wave analysis, is defined as the pressure difference between the second and the first systolic pressure peaks in the aorta. Another important parameter analyzed is the augmentation index which is defined as the ratio of the augmentation and pulse pressure, the latter being the difference between systolic and diastolic pressure (288,300).

3.3. Significance of arterial stiffness

In a recent meta-analysis of 18 longitudinal studies with a total of 15877 subjects and

a mean follow-up time ranging from 2.5 to 19.6 years, arterial stiffness, measured by aortic pulse wave velocity, was a strong independent predictor of future cardiovascular events and of cardiovascular and all-cause mortality (411). In studies using the aortic augmentation index as a marker of arterial stiffness, it has been found to predict the risk of future cardiovascular events in patients undergoing angiography for suspected coronary artery disease (420). Furthermore, in a study of 262 subjects undergoing percutaneous coronary intervention, aortic augmentation index added prognostic value above and beyond clinical risk factors, angiographic variables and medication use in a multivariable Cox-regression model for the risk of future cardiovascular events and mortality (421). The augmentation index has also been reported to predict cardiovascular death independent of age, gender, BMI, blood pressure, aortic pulse wave velocity, smoking status, lipid profile and other confounders in patients with end-stage renal disease (227). Most recently, in a community-based study involving 1272 participants and a 15-year follow up, the augmentation index predicted cardiovascular and all-cause mortality in men (417).

3.4. Causes of arterial stiffness

3.4.1. Age

Arteries stiffen as part of normal aging, making age the most important independent determinant of arterial stiffness (10,176). Arterial stiffening increases systolic blood pressure and decreases diastolic blood pressure, thereby increasing pulse pressure as seen with progressive age (176). There are several factors contributing to age-related changes in arterial stiffness. The first relates to the composition of the extracellular matrix, namely to the proportion of rigid collagen to extensible elastin within the tunica media of the arterial wall. It is known that with increasing age this ratio skews towards more collagen and less elastin, resulting in loss of stretch and in increased stiffness (288). It is not only the amount and

proportion of collagen and elastin, but also their disrupted cellular organization that makes the arterial wall more rigid (288). Other contributors to age-related arterial stiffening include progressive endothelial dysfunction (featuring decreased release of nitric oxide), dysregulation of matrix metalloproteases, intimal hyperplasia, deposition of calcium within the arterial wall, and decreases in vascular endothelial growth factors and telomere length, all associated with developing senescence (282,288).

3.4.2. Genetic factors

Genome-wide association scans have identified several loci linked to increased arterial stiffness, suggesting its heritability (263). Identified candidate genes include endothelial nitric oxide synthase, atrial natriuretic peptide, as well as several genes involved in cell adhesion, cell-cell interaction and cellular matrix formation (263). The exact pathogenetic mechanism, however, remains obscure (263).

3.4.3. Hypertension

While hypertension may be a consequence of normal ageing and concomitant arterial degeneration (*vide supra*), it can also accelerate arterial degeneration by causing overt mechanical strain on the load-bearing elastic lamellae of the arterial wall (288). Acting much like “accelerated aging”, hypertension leads to thinning, splitting, fraying, fragmentation and disruption of the orderly arrangement of the lamellae of the arterial tunica media (288). Indeed, hypertension has been documented to be associated with increased arterial stiffness at any given age indicating its independent detrimental impact on vascular health (288).

3.4.4. Dyslipidemia

Hypercholesterolemia due to increased LDL cholesterol is as a major risk factor for cardiovascular disease, recognized by large cohort initiatives such as the Framingham Heart Study (426), the

Prospective Cardiovascular Munster Study (9) and the Interheart Study (446). Thus, it can be expected that LDL cholesterol concentration correlates with measures of systemic arterial stiffness (35). Accordingly, in a study with 136 subjects with and without hypercholesterolemia, concentration of LDL cholesterol, but not of triglyceride or HDL cholesterol, was identified as an independent predictor of the aortic augmentation index despite covariates such as age, gender, peripheral mean arterial pressure and smoking status (425).

3.4.5. Hyperglycemia and hyperinsulinemia

One of the functions of insulin is to diminish the stiffness of large arteries and induce vasodilatation in the peripheral resistance vessels (441). This effect is blunted with obesity and other states characterized by insulin resistance (441). Indeed, perhaps the largest study evaluating measures of arterial stiffness in the general population (the Atherosclerosis Risk in Communities Study, n=4701 subjects) has reported both fasting glucose and insulin concentrations to be strong predictors of arterial stiffness in multivariate analyses controlled for age, BMI, smoking status, blood pressure and total cholesterol concentration (336). Moreover, among the non-diabetic subjects (~95% of the cohort), fasting glucose and insulin concentrations were found to correlate, independently and in synergy, with increased arterial stiffness in both males and females (336). In a study assessing arterial stiffness in 271 diabetic and 285 healthy subjects, multiple linear regression analysis carried out in all subjects identified the presence of type 2 diabetes as a predictor of arterial stiffness independent of age, gender, BMI, smoking, hypertension and dyslipidemia (378). Among the diabetic patients only, duration of diabetes was the most significant predictor of arterial stiffness along with age (378). Several other studies have confirmed these findings concluding that type 2 diabetes

is inextricably connected to arterial stiffening (208).

3.4.6. Inflammation

Inflammation has been suggested to be involved in the development of arterial stiffness and the associated cardiovascular complications. In particular, circulating concentration of high sensitivity C-reactive protein (hs-CRP), a marker of systemic inflammation and increased cardiovascular risk, has been shown to be independently related to measures of arterial stiffness both in healthy subjects (439) and in patients with untreated essential hypertension (234). Also other inflammatory mediators, such as IL6 and TNFA, were found to correlate positively with pulse wave velocity and aortic augmentation index in hypertensive patients (234). Moreover, causal relationship between acute inflammation and deterioration of arterial compliance has been postulated (412).

3.4.7. Smoking

Smoking has been documented to have both short-term (95,185,233) and long-term (219,233) harmful effects on arterial compliance. Even passive smoking, both in terms of acute (363) and cumulative (230) exposure, has been shown to stiffen the arteries. In a study assessing arterial stiffness in normotensive and hypertensive patients, current smoking was found to be associated with increased arterial stiffness irrespective of blood pressure levels (213). In a multivariate analysis of 138 otherwise healthy subjects with and without dyslipidemia, smoking was associated with increased arterial stiffening independent of age, gender, lipid profile and peripheral blood pressure (425).

3.4.8. Physical inactivity

Regularly performed physical exercise is associated with reduced risk of cardiovascular disease and even lower mortality rate (19). Individuals involved

in endurance sports have been reported to have lower aortic pulse wave velocity, augmentation index and blood pressure than their age-matched sedentary controls (376,377,391). Moreover, daily brisk walking has been documented to improve arterial compliance in previously sedentary men (377) and postmenopausal women (269) to levels observed in age-matched endurance exercise –trained and premenopausal peers, respectively, independent of changes in body mass, adiposity, arterial blood pressure, dietary intake and maximal oxygen consumption. This suggests that even moderate aerobic exercise may produce highly beneficial effects on cardiovascular health by minimizing or even reverting the age-related structural changes in the arterial wall (345). In contrast, the effects of resistance exercise (i.e., strength training or weight lifting) on arteries and cardiovascular health may be less favorable (18,264).

4. HIV-1 INFECTION

Since the beginning of the epidemic in 1981, more than 60 million people have been infected with HIV and nearly 30 million people have died of acquired immunodeficiency syndrome (AIDS) (428). In 2009, according to the World Health Organization, 33 million people were estimated to live with HIV, 2.6 million people were newly infected by the virus and, in 2009 alone, 1.8 million deaths occurred due to HIV/AIDS (428), making it the sixth leading cause of death worldwide (427). There are two subtypes of HIV, HIV-1 and HIV-2. The latter is less virulent than the former and is largely restricted to Western Africa. In the rest of this review, the focus is set on HIV-1 as it is the most prevalent and the most studied subtype of HIV.

4.1. Natural course of HIV-1 infection

HIV-1 is transmitted through unprotected sexual contact, contaminated needles or other injection equipment, transfusion of

contaminated blood products or through mother-to-child transmission that can occur *in utero*, *intrapartum* or postnatally via breast-feeding. As illustrated in Figure 3, HIV-1 enters target cells, often T-lymphocytes, that express CD4 receptor (subsequently referred to as CD4+ T-cells) using its envelope glycoprotein complex that attaches to CD4 receptors and to either chemokine (C-C motif) receptor 5 (CCR5) or chemokine (C-X-C motif) receptor 4 (CXCR4) as a co-receptor (141). The virus then releases its single-stranded ribonucleic acid (RNA) into the host cell's cytosol, where it is converted into double-stranded DNA using viral reverse transcriptase enzyme. The viral DNA is then transported into the host cell's nucleus, incorporated into the cell's own DNA and replicates by exploiting nuclear transcription mechanisms. Viral proteins are produced based on the instructions provided by the replicated DNA, cleaved into active form by viral protease enzyme and packed into new virions that are now ready to be released from the host cell to infect other cells (141).

During the acute viremia (2–4 weeks after encountering the virus) most HIV-1-infected individuals develop symptoms of primary infection featuring fever, lymphadenopathy, pharyngitis, rash, headache, myalgia, arthralgia and malaise (150). Initially, there is rapid rise in plasma viremia, often to levels in excess of 1 000 000 RNA copies/mL (150). This usually coincides with a rapid decrease in the number of CD4+ T-cells and commencement of antibody production against HIV-1, i.e., seroconversion (150). From this point onwards, circulating virions gradually become trapped by the follicular dendritic cell network in the lymphoid tissue germinal centers and plasma viremia subsides (141). HIV-1 infection then enters a chronic asymptomatic phase wherein the patients remain clinically stable and symptom-free for a variable duration of time, commonly years, while the virus keeps replicating within the lymphoid tissue gradually infecting and destroying more and more CD4+ T-cells (141). Over

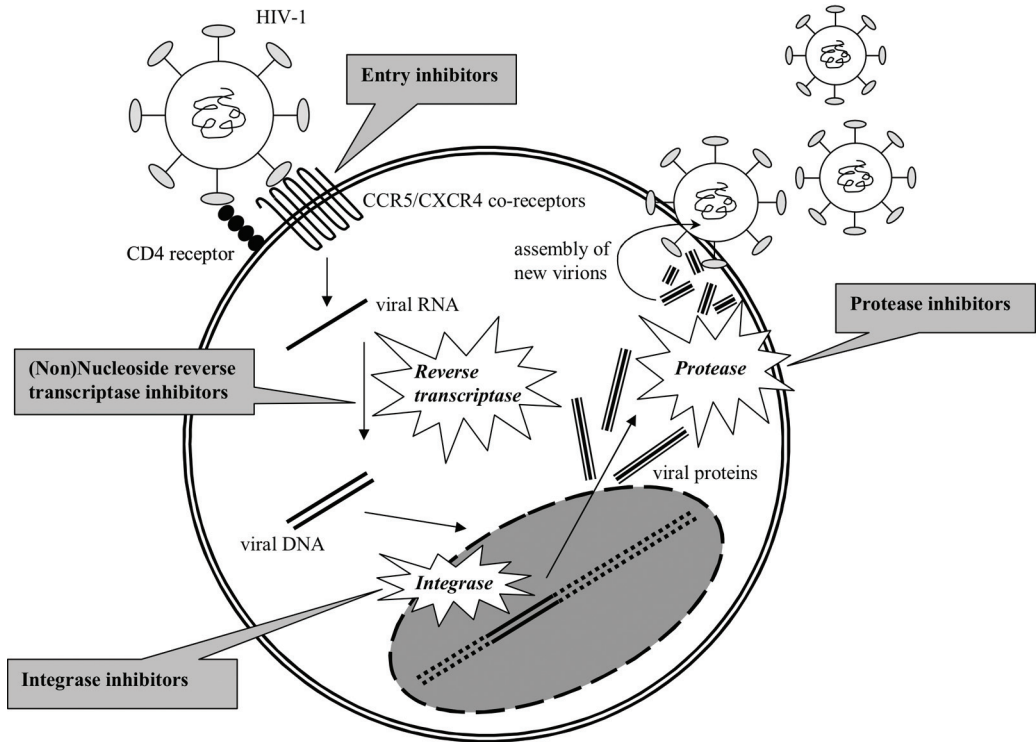
Figure 3.

Figure 3. Schematic diagram of life cycle of human immunodeficiency virus type 1 (HIV-1), key enzymes in viral replication and the targets of antiretroviral drugs within the host cell. CCR5, chemokine (C-C motif) receptor 5; CXCR4, chemokine (C-X-C motif) receptor 4; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

time, accelerating viral replication and advancing immunodeficiency ultimately compromise the body's ability to maintain effective immune responses, and patients develop non-specific symptoms such as fever, fatigue, night sweats, weight loss and dermatological or mucosal manifestations (141). The term AIDS is used to refer to the most advanced stage of HIV-1 infection. AIDS is defined by the European Centre for Disease Control and Prevention as occurrence of any of more than 20 opportunistic infections (e.g., *Pneumocystis jirovecii* pneumonia, esophageal candidiasis, cytomegalovirus retinitis) and neoplasms (e.g., Kaposi's sarcoma) (92).

Before the advent of contemporary antiviral medication, prognosis of people infected with HIV-1 was bleak - median time to development of AIDS was 8-11

years and median survival was 8-13 years from seroconversion (1).

4.2. Treatment of HIV-1 infection

Antiretroviral drugs have been used to battle HIV-1 infection since 1987 (98). Initially, they were used as mono-/dual/alternating agent therapy and, although reducing morbidity and mortality rates of HIV-1-infected patients at a statistical level, failed to have a significant impact on their life expectancy (149). The breakthrough took place in the mid-1990s when quantitative measurement of HIV-1 viremia became possible, enabling better evaluation and comparison of efficacy of individual drugs and combinations thereof. Only then the superiority, in terms of viral suppression and reversal of immunodeficiency, of triple-

Table 1. Antiretroviral agents available in Finland as of autumn 2011.

Nucleoside reverse transcriptase inhibitors	Non-nucleoside reverse transcriptase inhibitors	Protease inhibitors	Entry inhibitors		Integrase inhibitors
			Fusion inhibitors	CCR5 inhibitors	
abacavir didanosine emtricitabine lamivudine tenofovir stavudine zidovudine	efavirenz etravirine nevirapine	atazanavir darunavir fosamprenavir indinavir lopinavir ritonavir saquinavir tipranavir	enfuvirtide	maraviroc	raltegravir

CCR5, chemokine (C-C motif) receptor 5.

drug regimens over older therapeutic modalities became understood (34,129). Triple-drug combinations became known as highly active antiretroviral therapy (HAART), a term later changed to cART. Improved control of viral replication lead to gradual increases in CD4+ T-cell count, recovery of cell-mediated immunity and unprecedented improvements in the prognosis of HIV-1-infected patients (265,307,315,321). Over time cART has evolved to be more effective, better tolerated and has been simplified in terms of dosing. These therapeutic advances have transformed HIV-1 infection from being a fatal disease into a chronic condition.

At present, 6 classes of antiretroviral drugs are available: nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), fusion inhibitors, CCR5 inhibitors and integrase inhibitors (Table 1). NRTIs are structurally similar to natural nucleosides that DNA is built from. Unlike the natural nucleosides, however, NRTIs lack the ability to form a phosphodiester linkage with the following nucleoside halting the elongation of nascent viral DNA. Structurally, NRTIs can be divided into two subclasses, pyrimidine analogues (e.g., stavudine and zidovudine) and purine analogues (e.g., didanosine). Both subclasses act against HIV-1 in an identical manner in that they compete with the natural nucleosides for incorporation into the viral DNA and, once there, terminate further DNA elongation

(383). NNRTIs block the same process, but act through non-covalent binding to a substrate binding site of the reverse transcriptase enzyme (27). By altering the enzyme's three-dimensional structure, they block its active site and inhibit its action in a non-competitive fashion (27). PIs prevent cleavage of inactive viral protein precursors into their active form, thereby decreasing the infectivity of nascent virions (99). Entry inhibitors (fusion inhibitors and CCR5 inhibitors) block binding of viral envelope proteins with their receptors and co-receptors on the host cell membrane, hampering membrane fusion and entry of HIV-1 into the cell (70). The integrase enzyme facilitates the insertion of virally encoded DNA into the host cell genome. Integrase inhibitors target the catalytic domain and the ionic cofactors of HIV-1 integrase enzyme, interfering with merging of viral DNA with the host cell DNA (341). Schematic representation of the targets of action of each antiretroviral drug class is depicted in Figure 3.

Albeit highly efficient in preventing viral replication and decreasing infectivity, no combination of antiretroviral agents can eradicate the virus completely. Thus, the treatment for HIV-1 is life-long. While the life-expectancy of HIV-1-infected patients diagnosed in a timely fashion is approaching that of non-infected individuals (2,226,249,400), long-term and cumulative side effects of the antiretroviral agents are becoming increasingly important.

5. cART-ASSOCIATED LIPODYSTROPHY

Despite the undisputed positive impact that cART has had on the life of HIV-1-infected patients (265,307,315,321), multiple adverse effects have burgeoned as the use of cART has increased (47,122). Among the most worrisome of these effects is a constellation of metabolic disturbances (*vide infra*) collectively termed cART-associated lipodystrophy, features of which were first reported in cART-treated patients as early as 1997 (147,148). Originally, the prevalence of cART-associated lipodystrophy was reported to range from 2% (322) to 83% (54) depending on the characteristics of the cohort (age, gender, race), type and duration of antiretroviral therapy and diagnostic criteria for the condition. Large surveys indicated a prevalence of 50% of least one physical abnormality after 12-18 months of cART (47). Although with the spreading use of contemporary, less lipotoxic antiretroviral agents, the prevalence of cART-associated lipodystrophy seems to be declining (142,314), it still remains the most common form of human lipodystrophy. Other types of human lipodystrophies are extremely rare (97,114,340,357) and are summarized in Table 2.

5.1. Definition of cART-associated lipodystrophy

There are no uniform, universally accepted criteria for cART-associated lipodystrophy. A case definition (48) and a severity grading scale (50) have been proposed. These models include variables such as age, gender, time since HIV-1 diagnosis, stage of HIV-1 disease, serum HDL cholesterol concentration, anion gap, leg fat percentage, waist-to-hip ratio, and ratios of IAT-to-SAT and trunk-to-limb fat assessed by a single-slice computed tomography (CT) at the level of L4 vertebra and by dual-energy X-ray absorptiometry (DEXA), respectively (48,50). The case definition has 79% sensitivity and 80% specificity for diagnosis of cART-associated

lipodystrophy, but requires the use of CT and/or DEXA which are not always readily available in every day practice (48). Additionally, the case definition does not differentiate between the different aspects of lipodystrophy, i.e., lipoatrophy and lipohypertrophy (48). Therefore, the use of the proposed definition has been limited, and cART-associated lipodystrophy continues being diagnosed based on clinical assessment encompassing patients' self-reported signs and symptoms confirmed by physical examination. The concordance between the latter two has been reported to be as high as 98% (54). Of great clinical importance is to distinguish cART-associated lipodystrophy, in which adipose tissue is the affected organ and skeletal muscle is spared (373), from HIV/AIDS-related "wasting syndrome" which is characterized, predominantly, by loss of muscle mass (124).

5.2. Features of cART-associated lipodystrophy

5.2.1. Adipose tissue

cART-associated lipodystrophy is characterized by the loss of SAT (i.e., lipoatrophy) in the face, limbs, buttocks and, to a large extent, in the trunk (47,122,164,193). Excessive accumulation of adipose tissue (i.e., lipohypertrophy) may or may not associate with lipoatrophy (47,122,164,193). Regions that may hypertrophy include the subcutaneous dorsocervical fat pad (i.e., "buffalo hump"), breasts (both in males and females) and IAT (47,122,164,193). Collectively these alterations in body fat distribution are termed lipodystrophy.

During the first months following initiation of cART amounts of both SAT and IAT show an increase (83,239,276). This is most likely due to the general improvement in health and nutrition associated with suppression of HIV-1 viremia and reversal of the associated catabolic state (83,239,276). Subsequently, loss of peripheral SAT and accumulation of IAT take place (239,276). Initially, both

Table 2. Classification of non-HIV-1-associated human lipodystrophies (97,114,340,357).

Type of lipodystrophy	Model of inheritance	Gene involved	Etiopathogenesis
<i>Acquired</i>			
Generalized lipodystrophy	-	-	50% idiopathic, 25% antecedent panniculitis, 25% presence of concomitant autoimmune disease. In some cases, activation of classical complement pathway, featuring low complement factor C4, has been reported.
Partial lipodystrophy (Barraquer-Simons syndrome)	-	-	Activation of alternative complement pathway, low complement factor C3 and a circulating autoantibody C3 nephritic factor causing lysis of adipocytes (autoimmune, possibly, infection-triggered reaction).
Localized lipoatrophy	-	-	Injection site lipoatrophy (e.g., insulin, corticosteroids); pressure-induced lipoatrophy.
<i>Inherited</i>			
Congenital generalized lipodystrophy (Berardinelli-Seip syndrome)	Autosomal recessive	AGPAT2 BSCL2/seipin caveolin 1 Unknown	Mutation in AGPAT2 impair synthesis of triglycerides and membrane phospholipids; mutations in seipin and caveolin 1 impede formation of lipid droplets, but precise mechanisms leading to loss of adipose tissue are unknown.
Familiar partial lipodystrophy	Autosomal dominant	LMNA PPARG AKT2 Unknown	Disruption of lamin function hampers nucleocytoplasmic traffic and lamins' association with histones, chromatin and transcription factors such as SREBP1C; mutations in PPARG and AKT2 inhibit differentiation of adipocytes.
Dunningan variety			
PPARG-associated variety			
AKT2-associated variety			
Others			
Mandibuloacral dysplasia	Autosomal recessive	LMNA ZMPSTE24 Unknown	LMNA, <i>vide supra</i> ; dysfunctional ZMPSTE24 prevents posttranslational processing of lamins.
Type A (partial)			
Type B (generalized)			
Others			
Hutchinson-Gilford progeria syndrome (partial-to-generalized)	Autosomal dominant	LMNA	<i>Vide supra</i>
Atypical progeroid syndrome (partial-to-generalized)	Autosomal dominant	LMNA	<i>Vide supra</i>
Hypertriglyceridemia-associated lipodystrophy (partial)	Autosomal recessive	LMF1	Unknown
Neonatal progeroid syndrome (partial-to-generalized)	Autosomal recessive	Unknown	Unknown
SHORT syndrome (partial)	Autosomal dominant	Unknown	Unknown

AGPAT2, 1-acylglycerol-3-phosphate O-acyltransferase 2; AKT2, RAC- β serine/threonine protein kinase; BSCL2, Berardinelli-Seip congenital lipodystrophy 2; HIV-1, human immunodeficiency virus type 1; LMF1, lipase maturation factor 1; LMNA, lamin A/C; PPARG, peroxisome proliferator-activated receptor γ ; SHORT, short stature, hyperflexibility of joints and/or inguinal hernia, ocular depression, Reiger anomaly and teething delay; ZMPSTE24, zinc metalloprotease.

processes occur simultaneously, but over time accumulation of IAT plateaus while the amount of peripheral SAT continues to decrease (239,276) at a approximate rate of 0.10 kg of limb fat lost per month (54) or ~20% decrease in limb fat after 8-12 months of cART (83,276).

5.2.1.1. Adipose tissue composition

Upon comparison with non-lipoatrophic adipose tissue under light/electron microscope, lipoatrophic adipose tissue is characterized by smaller adipocytes (12,80,159,224), greater cell size variation (12,80,224), disruption of cell membranes (224), increased fibrosis (159) and vascularization (80,159), and signs of apoptosis as assessed by immunohistochemistry (80,159). Adipocyte size has been reported to be similar in aspiration biopsy samples of adipose tissue from both lipohypertrophic dorsocervical fat pad and lipoatrophic SAT in a small group (n=8) of patients with cART-associated lipodystrophy (165). Other histological parameters were not evaluated due to the nature of sample acquisition technique. Only one other study has addressed the issue of morphological differences between dorsocervical and abdominal SAT in cART-associated lipodystrophy using surgical biopsy specimens (224). The authors of this study reported changes similar to, but milder than, those seen in the lipoatrophic areas (*vide infra*) (224). The cited study, however, did not specify the location of origin of the hypertrophic adipose tissue samples and was limited in size (n=4).

5.2.1.2. Adipose tissue inflammation

On histological and immunohistochemical analyses, lipoatrophic adipose tissue of cART-treated patients is characterized by increased macrophage infiltration and increased number of lipogranulomata (i.e., lipid-laden macrophage clusters encircling adipocytes) when compared to that of NRTI-naïve (291,292) and HIV-1-negative subjects (80,159,224,291,292).

Prior to Studies III and IV, histological or immunohistochemical signs of inflammation in SAT of similarly treated patients with and without cART-associated lipodystrophy has not been contrasted.

Adipose tissue macrophages are involved in numerous immune functions including phagocytosis of cellular debris and foreign material as well as triggering immune responses via cytokine release and antigen presentation (447). Accordingly, profusely macrophage-infiltrated abdominal SAT, such as occurs in HIV-1-infected patients with cART-associated lipodystrophy, demonstrates increased gene and protein expression of proinflammatory cytokines (TNFA, IL6 and IL8) when compared to that of HIV-1-infected patients without cART-associated lipodystrophy (165,222) or HIV-1-negative subjects (12,165).

Comparative data on dorsocervical and abdominal SAT regarding adipose tissue inflammation were scarce. Prior to Study IV, only two studies on the matter existed and both reported that inflammation, as judged from mRNA expression of genes such as *TNFA* and *CD68* (126) and release of TNFA protein (165), is more severe in lipoatrophic abdominal than in dorsocervical SAT. These studies, however, were limited in size (n=8-10) and by nature of the samples. In one of the studies, the samples from the two adipose tissue depots were not taken from the same individuals (126). In the other study, adipose tissue was biopsied by a needle aspiration technique which does not allow assessment at a protein level by immunohistochemistry (165). Thus, further research of location-dependent differences between SAT depots was called for.

5.2.1.3. Mitochondrial DNA depletion

The mtDNA content has been shown to be significantly lower in the lipoatrophic SAT from lipodystrophic patients when compared to SAT from the corresponding location from cART-treated patients without lipodystrophy (352,415), HIV-1-infected cART-naïve (352) and HIV-1-negative subjects (352,415). Furthermore, hyperlactatemia, which is thought to

represent a marker of mitochondrial dysfunction, has been reported in patients with cART-associated lipodystrophy and associated with the severity of adipose tissue loss (21,50,51).

One study has characterized hypertrophic SAT from the dorsocervical depot of cART-treated patients in comparison to lipoatrophic abdominal SAT from other cART-treated patients or SAT from healthy controls (126). This study reported mtDNA content to be lower in both lipoatrophic and liphypertrophic SAT compared to that of controls (126). Furthermore, there was a tendency of the dorsocervical SAT to have even greater mtDNA depletion than the abdominal SAT (126). Prior to Study IV, however, no studies had compared the lipoatrophic (e.g., abdominal) and the remarkably unaffected (e.g., dorsocervical) SAT of the same otherwise lipoatrophic cART-treated patients.

5.2.2. Liver fat

In case of absence or malfunction of specialized energy stores (i.e., adipose tissue), triglycerides are deposited ectopically in non-adipose tissues such as the liver and skeletal muscle (441). This phenomenon has been clearly demonstrated in non-HIV-1-related lipodystrophies (114). In keeping with this, patients with cART-associated lipodystrophy have also been shown to have abnormal transaminase concentrations (51,369), higher liver fat content (369) and greater prevalence of non-alcoholic steatohepatitis (210) when compared to HIV-1-infected cART-treated patients without lipodystrophy.

5.2.3. Insulin resistance

Insulin resistance is a characteristic feature of non-HIV-1-related lipodystrophies (114). Patients with cART-associated lipodystrophy are no exception having higher fasting insulin concentrations than non-lipodystrophic cART-treated patients (54,369) and HIV-1-negative subjects (132). A close correlation between fasting

insulin concentration and liver fat content measured by proton magnetic resonance spectroscopy (¹H-MRS) has been reported in patients with cART-associated lipodystrophy (369). In a multivariate regression analysis that included 91 patients with cART-associated lipodystrophy, alanine aminotransferase (ALT) was a strong positive predictor of fasting glucose, 2-hour glucose, fasting insulin and insulin area under the curve following oral glucose tolerance test (OGTT) (64). These findings were maintained even after adjustment for confounding factors such as age, gender, BMI, use of PIs and co-infection with viral hepatitis (64). Furthermore, the ratio of IAT to abdominal SAT, both in terms of waist-to-hip ratio and absolute compartment volume measurements by CT, have been reported to correlate positively and strongly predict fasting hyperinsulinemia and insulin area under the curve during OGTT in HIV-1-infected men independent of BMI (257). Results from OGTTs performed in large cohort studies have indicated that 7% of patients with cART-associated lipodystrophy fulfill the criteria for type 2 diabetes and up to 35% can be diagnosed with some disorder of glucose metabolism (impaired fasting glucose, impaired glucose tolerance, diabetes) as compared to 0.5% and 7%, respectively, in HIV-1-negative subjects matched for age, gender and BMI (132). Among PI-treated patients, of whom 83% were lipodystrophic, 7% had type 2 diabetes and 16% were diagnosed with impaired glucose tolerance (54). Peripheral lipoatrophy in HIV-1-infected cART-treated patients has been reported to correlate closely with reduced peripheral insulin sensitivity as measured by hyperinsulinemic euglycemic clamp technique (279). A further study, complementing the hyperinsulinemic euglycemic clamp with the use of a glucose tracer, found patients with cART-associated lipodystrophy to have increased post-absorptive glucose production, reduced insulin sensitivity of peripheral glucose uptake and reduced suppression of lipolysis when compared to HIV-1-negative non-obese subjects (393). Finally, yet another hyperinsulinemic

euglycemic clamp study, combining the technique with indirect calorimetry, has confirmed the aforementioned findings and reported lower steady-state glucose infusion rate and decreased lipid oxidation during hyperinsulinemia in patients with as compared to those without cART-associated lipodystrophy (113).

5.2.4. Dyslipidemia

High triglyceride and low HDL concentrations are common features of cART-associated lipodystrophy (52,122,132,193,259). The severity of dyslipidemia, particularly that of hypertriglyceridemia, shows a strong positive correlation with the severity of lipodystrophy (54). The use of PI-containing therapy is linked with more atherogenic changes in lipid profile than the use of NRTI-NNRTI-based therapy (51,245). It must be remembered, however, that considerable differences exist between the individual antiretroviral agents, even within the same class. Some antiretrovirals may even exert a beneficial effect on lipid profile. For example, NNRTIs, particularly nevirapine, have been reported to increase HDL cholesterol concentrations (104,395,398).

5.2.5. Cardiovascular complications

Impaired flow-mediated vasodilation, indicative of endothelial dysfunction and arterial stiffening, has been reported in HIV-1-infected cART-treated patients to age-matched HIV-1-negative controls (402). A study comparing patients receiving cART regimens that include PIs against those which did not, found greater evidence of the aforementioned vascular anomalies among the PI users (364). The prevalence of atheromatous plaques, defined as thickening of intima media on ultrasound, has also been found to be increased in HIV-1-infected patients, particularly in those taking PIs, as compared to HIV-1-negative subjects (78,157,232,402). These studies, however, did not consider cART-associated lipodystrophy as a

group-defining characteristic, or even a factor that could potentially contribute to structural or functional atherosclerotic changes in the vasculature, despite the fact that the incidence of lipodystrophy has been strongly associated with the use of PIs (52). Of note, prior to Study I, PWA had not been used in HIV-1-infected patients to assess arterial stiffness.

Arterial stiffness and hypertension are reciprocally regulated processes (287,300). Indeed, much like the measures of arterial stiffness mentioned above, elevated blood pressure has also been associated with the use of PIs (63). Both systolic and diastolic blood pressure have been reported to be higher in patients with as compared to those without cART-associated lipodystrophy or to controls matched for age and gender (338). Lipodystrophy was found to be more prevalent among the hypertensive HIV-1-infected cART-treated patients than among the normotensive ones (115). In fact, after family history of hypertension and the presence of features of the metabolic syndrome, the presence of cART-associated lipodystrophy was one of the strongest predictors of hypertension in these patients (115). It should be pointed out, however, that controversial data does exist refuting the link between the use of cART, or any individual drug class, lipodystrophy and hypertension (16,170).

One more feature exacerbating the cardiovascular risk burden of patients with cART-associated lipodystrophy is that of coagulopathy as high concentrations of PAI1 and tissue-type plasminogen activator, both markers of fibrinolysis, have been reported in patients with cART-associated lipodystrophy (133,444).

5.3. Pathogenesis of cART-associated lipodystrophy

The etiology of cART-associated lipodystrophy, is unclear, but is likely to be multifactorial and to include combined effects of antiviral agents, HIV-1 infection and host-related factors (46,164,193,237,293). While several hypotheses regarding the pathophysiology

of cART-associated lipodystrophy have been put forward (25,43,53,100,268,422), none of them provide an exhaustive explanation, and some even contradict each other. Moreover, none of these hypotheses offer an explanation as to why adipose tissue atrophies in most anatomic locations while in the dorsocervical region it remains, at least seemingly, unaffected or even hypertrophies.

5.3.1. Antiretroviral agents

It is the current consensus that cART-associated lipodystrophy is mainly attributed to the use of NRTIs (220), particularly those with a thymidine-like structure, i.e., thymidine analogue NRTIs (tNRTI) (138,166,236). Of tNRTIs, stavudine has been identified as causing the highest prevalence of lipodystrophy (166,236) with higher rates of SAT loss when compared to regimens containing zidovudine (83,142), abacavir (314) or tenofovir (142). With regards to mitochondrial toxicity, studies of NRTIs in enzyme assays and cell cultures have demonstrated the following hierarchy of inhibition of the mtDNA-replicating POLG protein: zalcitabine > didanosine > stavudine > lamivudine > zidovudine > abacavir (171). Originally, NNRTIs were attributed little role in the development of lipodystrophy (46). Recently, however, this has been challenged as, irrespective of the NRTI backbone, efavirenz was reported as causing more limb fat loss than ritonavir-boosted lopinavir (142), although still less than nelfinavir (83). PIs are thought to be associated mainly with the development of features of the metabolic syndrome (hypertriglyceridemia, low HDL cholesterol and insulin resistance) and lipohypertrophy (220).

Over time it has become increasingly clear that the pathogenesis of cART-associated lipodystrophy needs to be investigated with regards to the impact of individual antiretroviral agents rather than entire drug classes. The effects of individual drugs, however, are difficult to assess in HIV-1-infected patients as they are always treated with combinations of antiretrovirals.

Thus, most of the data on the side effects of individual agents *in vivo* in HIV-1-infected humans have been produced by large multi-center cohort studies such as the Data Collection on Adverse Events of Anti-HIV Drugs (DAD) Study (108).

5.3.2. Lipodystrophy

In theory, loss of adipose tissue can arise through several mechanisms. These include decreased differentiation of new adipocytes, decreased ability of existing adipocytes to store triglycerides leading to decreased adipocyte size, increased adipocyte loss due to processes such as disrupted function of cellular organelles, inflammation and apoptosis, or any combination of the above.

5.3.2.1. Adipogenesis

Histological analyses of SAT of cART-treated patients have revealed smaller adipocytes, greater cell size variation, disruption of cell membranes and signs of apoptosis in lipodystrophic as compared to non-lipodystrophic SAT (80,159,224). Expression of transcription factors necessary for adipocyte differentiation, such as *CEBPA*, *CEBPB*, *SREBP1C* and *PPARG* (12,159,173), and that of genes involved in cellular lipid storage and metabolism such as *ACS* and *LPL* (159,173), was found to be lower in SAT of lipodystrophic as compared to that of non-lipodystrophic cART-treated patients (173) and HIV-1-negative subjects (12,159). Furthermore, expression of adipogenic genes was documented to correlate positively with the proportion of small adipocytes, suggesting that alterations in gene expression may be linked with abnormal differentiation of adipocytes in lipodystrophic SAT (12). Based on these findings, it can be speculated that in cART-associated lipodystrophy adipocyte size decreases and SAT is lost as a result of at least two processes, possibly occurring independent of each other. The first process is that of intracellular triglyceride depletion due to increased lipolysis, while the second is of decreased lipogenesis and

poor adipocyte differentiation aggravated by concomitant apoptosis. The causal relationship between lipoatrophy and reduced expression of genes involved in adipocyte differentiation and lipid uptake has been investigated in a prospective *in vivo* study that showed that the aforementioned changes in gene expression in SAT from cART-treated patients precede visible loss of adipose tissue by several months (197). A 6-month interruption of cART, on the contrary, has been reported to result in significant improvements in markers of adipose tissue biogenesis and mitochondrial function, as well as in decreased adipose tissue inflammation, although no visible improvements in body fat distribution were recorded (179).

As PIs were originally thought to be the causative agents of lipodystrophy, a large number of early *in vitro* studies explored the potential mechanisms by which PIs could instigate adipose tissue loss. These studies demonstrated that PIs suppress the differentiation of preadipocytes and reduce lipid accumulation in these cells (43,44,81,422,449). PIs were also found to inhibit *de novo* lipogenesis (320), increase lipolysis (15,212,320,329) and induce apoptosis in cultured mature adipocytes (81). A microarray analysis carried out in differentiating preadipocytes treated with several antiretroviral drugs indicated that PIs decrease expression of several master adipogenic transcription factors, adipokines and genes involved in modulation of cell cycle control (306). Exposure of HIV-1-negative subjects to indinavir for 4 weeks resulted in a statistically significant loss of total and truncal fat and a borderline significant loss of limb fat as measured by DEXA (294). Hypotheses contemplating both PPARG-dependent (12,43,44,53,81) and PPARG-independent (422) mechanisms of adipose tissue loss induced by PIs have been put forth. Ultimately, however, this body of evidence proved to have little clinical relevance as the role of PIs in causing cART-associated lipodystrophy was challenged and greater emphasis was set on the effects of NRTIs (220).

NRTIs have been implicated as exerting toxicity on adipose tissue both independently and in toxic synergy with PIs (46,186,236,394). In particular, tNRTIs (stavudine, zidovudine), but not other NRTIs (didanosine, abacavir, lamivudine, tenofovir), have been reported to exert a number of adverse effects on adipocytes. The effects include promotion of apoptosis (42), a decrease in adipocyte lipid content (42,200) and a decrease in insulin-induced lipogenesis *in vitro* (42). The latter appeared to be due to low expression of adipogenic genes such as *CEBPA*, *PPARG*, *FASN*, adipocyte lipid binding protein 2 and acyl-coenzyme A carboxylase (42). In line with these data, a 2-week exposure of HIV-1-negative subjects to NRTIs has been reported to result in decreased *PPARG* gene expression in SAT of these subjects (241).

5.3.2.2. Mitochondrial toxicity

Clinical trials have demonstrated that the absence or presence of tNRTIs in the backbone of antiretroviral regimens influences the individual's risk of developing lipoatrophy (166). Mechanistic studies *in vitro* have suggested that tNRTIs inhibit POLG resulting in impaired synthesis of mtDNA (71,171). This results in depletion of genes transcribed by it such as those involved in the mitochondrial respiratory chain and the generation of ATP, a major cellular fuel molecule (71,171). Impaired synthesis of proteins required for such processes as respiratory chain function and β -oxidation leads to failure of affected mitochondria to meet cells' energy requirements and, consequently jeopardizes the viability of these cells (293).

5.3.2.3. Adipose tissue inflammation

Macrophages, present in abundance in the lipoatrophic SAT of patients with cART-associated lipodystrophy (80,159,224,291,292), are known to be a source of proinflammatory cytokines and chemokines (447). The attraction of macrophages to the adipose tissue and release of inflammatory mediators by

them is stimulated by factors such as the presence of apoptotic cells within the tissue (447), also a recognized feature of cART-associated lipodystrophy (80). Endoplasmic reticulum stress caused by, e.g., FFA overflow (121) characteristic to cART-associated lipodystrophy (130,256), can activate the inflammatory pathways and cytokine response even further (305). Moreover, hypercytokinemia has been speculated to result from dysregulation of the TNFA system as a part of the immune reconstruction that follows initiation of cART (205). In a setting outside HIV-1, TNFA, the most extensively studied cytokine, has been found to inhibit expression of genes involved in adipogenesis (365), hamper LPL activity (106) and increase lipolysis by downregulation of PLIN1 (331,433), thereby leading to adipocyte apoptosis and consequent loss of adipose tissue mass (317). These mechanisms of cytokine-induced lipotoxicity seem to hold true also in patients with cART-associated lipodystrophy. Indeed, expression of *TNFA* mRNA has been reported to correlate inversely with gene expression of adipogenic transcription factors in SAT (12,159). Furthermore, the loss of limb fat has been associated with high serum concentrations of soluble TNFA receptors (165,279) and IL6 (165) *in vivo* in humans. Finally, the rates of release of TNFA and IL6 from in cultured human adipocytes have been reported to be associated with the loss of limb fat (165). It has been hypothesized that TNFA and cART may have independent and synergistic deleterious effects on adipose tissue metabolism, as exposure to TNFA of adipocytes cultured in the presence of PIs has been reported as enhancing the anti-adipogenic effects beyond what was seen with PIs alone (267).

5.3.3. Lipohypertrophy

While lipoatrophy has been studied extensively, lipohypertrophy remains poorly understood. Low circulating growth hormone concentrations have been reported in HIV-1-infected patients with

IAT accumulation (324), similar to what can be seen outside the HIV-1-setting in growth hormone deficiency, a condition characterized by abdominal obesity, lipid abnormalities and insulin resistance (67). Studies of the hypothalamic-pituitary-adrenal axis prompted by the similarities in body fat distribution between cART-associated lipodystrophy and Cushing's syndrome have ruled out the possibility that lipohypertrophy associated with exposure to cART is a form of systemic hypercortisolism (261,437). It has been suggested, however, that local increases in concentrations of glucocorticoids and/or their receptors could induce regional (dorsocervical, intra-abdominal) adiposity by stimulating LPL activity (193). In HIV-1-negative obese subjects, IAT is known to express significantly higher mRNA concentration of 11β -hydroxysteroid dehydrogenase, an enzyme that catalyzes the conversion of cortisol from its inactive metabolite cortisone. Furthermore, IAT from these subjects has been reported to have greater glucocorticoid receptor density than their abdominal SAT, perhaps explaining why IAT appears to be intrinsically predisposed to excessive growth (29). While adipose tissue samples from hypertrophic depots of patients with cART-associated lipodystrophy have never been studied in this regard, gene expression of 11β -hydroxysteroid dehydrogenase has been reported to be significantly higher in the lipoatrophic SAT of cART-treated patients than in SAT from the non-lipoatrophic ones (371).

Prior to Study IV, no studies had performed a comprehensive comparison of hypertrophic (e.g., dorsocervical depot) to lipoatrophic adipose tissue (e.g., abdominal subcutaneous depot) harvested from the same individuals. Furthermore, no studies had attempted to elucidate why the depots behave differently within the same individual even though they are under the influence of the same external factors. A study comparing lipohypertrophic and lipoatrophic samples from different patients with cART-associated lipodystrophy has reported hypertrophic SAT to harbor less

inflammation than lipotrophic SAT (126). The authors interpreted the relative lack of inflammation as a prerequisite for the preservation of the dorsocervical region unaffected by lipotrophy (126). A Dutch research group utilized a completely different approach and generated animal models in an attempt to elucidate the regional differences in susceptibility to the toxic effects of antiretroviral drugs. This group suggested that cART-associated lipodystrophy results from cART-induced selective damage to the autonomic innervation of adipose tissue depots, leading to variable alterations in hormonal signaling and adipose tissue homeostasis depending on the region of the body affected (100). Interestingly, switch studies (both away from PIs and from tNRTIs) have generated controversial results. These studies reported little to mainly no resultant decrease in IAT, provoking speculations whether the phenomenon of abdominal obesity in cART-treated patients may be due more to the currently ongoing obesity epidemic in the general population than be an actual side effect of cART (272). In keeping with this, a 4-week exposure of HIV-1-negative subjects to a PI indinavir did not increase the amount of IAT as measured by MRI (294).

5.3.4. Insulin resistance

Based on *in vitro* studies using 3T3-L1 and 3T3-F442A preadipocytes, it has been suggested that PIs reduce insulin-stimulated glucose uptake by selectively inhibiting transport activity of GLUT4 (15,278,329), interfere with protein kinase B phosphorylation (15,329), and decrease mitogen-activated protein kinase activation at a downstream step in postreceptor insulin signaling (44). These findings are supported by the *in vivo* data from HIV-1-negative subjects in whom short-term administration of PIs indinavir (294,296) and ritonavir-boosted lopinavir (295), but not of atazanavir (295), decreased insulin-stimulated glucose disposal rate as measured by hyperinsulinemic euglycemic clamp.

Obesity and lipodystrophy share several features that link both conditions to insulin resistance despite the differences in their phenotype. Liver fat accumulation is associated with insulin resistance in both conditions (191,369). Hepatic steatosis and non-alcoholic steatohepatitis in insulin resistant lipodystrophic cART-treated patients has been associated with high gene expression of *SREBP1C* and low gene expression of *PPARG* in the liver (210). This is in line with the stimulatory effect of insulin, typically high in insulin resistant subjects with fatty liver, on *SREBP1C* expression (353). Increased expression of *SREBP1C*, in turn, further contributes to increased hepatic lipogenesis, thus, linking hyperinsulinemia and increased *SREBP1C* expression with high triglyceride and low HDL cholesterol concentrations seen in cART-associated lipodystrophy (268). Reduction of insulin sensitivity of lipolysis that accompanies cART-associated lipodystrophy, results in high concentrations of circulating FFAs (130,256). Accordingly, circulating FFA concentrations in these patients have been shown to correlate with insulin concentrations both in the fasting state and in response to a glucose challenge (130,256). Moreover, pharmacological inhibition of lipolysis and FFA production with the anti-lipolytic agent acipimox has been demonstrated to improve insulin sensitivity in patients with cART-associated lipodystrophy (134). These data further support the causal relationship between increased circulating FFAs and insulin resistance in these patients, just as it has been described in individuals who are not infected with HIV-1 (195,254,441). Other features that cART-associated lipodystrophy and obesity have in common include low concentrations of adiponectin mRNA (126,372) and protein (372,387), as well as adipose tissue inflammation that has been verified histologically (80,159,224), immunohistochemically (291,292) and at the level of gene and protein expression (12,126,165,168,222). Both decreased adiponectin (12) and increased adipose tissue inflammation (279) have been positively correlated with measures of

insulin resistance in patients with cART-associated lipodystrophy (216,448). Furthermore, just as in obese subjects without HIV-1, these changes are likely to contribute to insulin resistance also in HIV-1-infected cART-treated patients.

In contrast to findings in obesity, leptin concentrations have been found to be low in SAT of patients with cART-associated lipodystrophy (12,52,159,281,369) and in other human lipodystrophies (114). Nonetheless, leptin does appear to play some role in insulin resistance in the lipodystrophic patients, as treatment of hypoleptinemic HIV-1-negative lipodystrophic patients with leptin has been seen to mitigate their insulin resistance (161,303,311).

5.3.5. Dyslipidemia

While a certain degree of dyslipidemia occurs in HIV-1-infected patients due to viral infection *per se* (125), a randomized, double-blind 2-week ritonavir-exposure study in HIV-1-negative subjects has reported an increase in concentrations of triglyceride and VLDL cholesterol, and a decrease in concentration HDL cholesterol in subjects on ritonavir as compared to those on placebo (318). These findings suggest that the detrimental effects of PIs on lipids can occur independently of HIV-1 infection (318). As mentioned in conjunction with lipohypertrophy, decreased circulating concentration of growth hormone documented in patients with cART-associated lipodystrophy is known to be associated with lipid abnormalities (67). *In vitro* studies have reported that PIs stimulate the production of triglyceride-rich particles in murine hepatocytes (211), increase lipolysis in mature adipocytes (15,212,320,329), perhaps by reducing LPL activity (211,320), and inhibit proteasome activity, inducing endoplasmic reticulum stress (308). Interestingly, all these effects are also known to represent a link between obesity, insulin resistance and dyslipidemia (121,305). Inhibition of proteasomal degradation by PIs has also been shown to ensue accumulation

of apolipoprotein B, leading to increased VLDL secretion that has been proposed as a potential mechanism by which PIs may induce dyslipidemia in the setting of cART-associated lipodystrophy (218). Moreover, patients with cART associated lipodystrophy have been reported to have fatty liver (369), a condition renowned for overproduction of VLDL (4) leading to high concentrations of circulating triglycerides and low HDL cholesterol (192). Dyslipidemia in cART-treated patients may also be a consequence of lipodystrophic changes in the adipose tissue rendering it unable to take up circulating triglycerides and excessively release the stored fatty acids as seen in lipodystrophy syndromes unrelated to cART (114).

SREBP1C has also been hypothesized to be involved in the pathogenesis of dyslipidemia seen in cART-associated lipodystrophy as i) treatment of mice with PIs increases the abundance of SREBP1C protein in the nucleus of their hepatocytes and induces hypertriglyceridemia, ii) transgenic mice overexpressing nuclear SREBP1C in adipose tissue were reported to show complete congenital lipodystrophy, severe dyslipidemia and insulin resistance (354), and iii) single nucleotide polymorphism in the *SREBP1C* gene was found to predispose cART-treated patients to hypertriglyceridemia (262). However, the exact pathogenetic mechanism involving SREBP1C remains obscure as controversial results of both high (12,286,323) and low nuclear SREBP1C protein concentrations (43,44) have been reported after exposure to PIs in adipocyte cultures, murine adipocytes and hepatocytes as well as in human adipose tissue *in vivo*. Attempts to explain this controversy have focused on functional differences between the SREBP1 isoforms and non-discrimination between them (154,155), as well as on varying cell culture protocols (286). Nonetheless, a speculative theory linking lipodystrophy, dyslipidemia and insulin resistance has been proposed based on the assumption of elevated concentrations of active SREBP1C protein in adipocytes and hepatocytes

being the driving force of this constellation of abnormalities (268).

5.3.6. Role of HIV-1 infection

HIV-1 infection itself does not seem to bring about lipodystrophic changes in body fat as the lipodystrophy syndrome seen in HIV-1-infected cART-treated patients has never been reported in untreated HIV-1-infected patients, even in those with a high viral load (46). Furthermore, in most cases cART-associated peripheral lipoatrophy is reversible after changes in cART, without changes in HIV-1 RNA load (140). Data challenging these arguments, however, exist suggesting that HIV-1 infection *per se* initiates reduction of gene expression of adipogenic transcription factors (*CEBPA*, *PPARG*), adipokines (leptin, adiponectin) and mitochondrial respiratory chain components, making adipose tissue vulnerable to the toxic effects of cART (117). Moreover, expression of inflammatory cytokines such as TNFA has been reported to be upregulated in patients with CD4+ T-cell count <50 cells/mm³ as compared to less immunosuppressed HIV-1-infected patients (407). These findings are in line with data implicating the severity of immunosuppression as a risk factor for the development of cART-associated lipodystrophy (51).

HIV-1 replication has been documented to have a direct, cART-independent impact on lipid parameters and glucose homeostasis in humans (86). In a study involving antiretroviral-naïve HIV-1-infected patients, higher triglyceride and VLDL cholesterol concentrations, lower HDL and LDL cholesterol concentrations, and decreased insulin sensitivity have been associated with lower CD4+ T-cell count and higher viremia (86). In another study comparing plasma lipids of patient with AIDS, latent HIV-1 infection and healthy controls, HDL cholesterol concentration and triglyceride clearance were lower, while triglyceride and FFA concentrations seemed unaffected in asymptomatic HIV-1-infected patients as compared to healthy controls (125). Progression to symptomatic

HIV-1 disease and, subsequently, to AIDS was found to lead to a further decline in HDL cholesterol concentration and to increases in triglyceride, VLDL cholesterol and FFA concentrations (125). Increased concentration of interferon α , a cytokine associated with the progression of HIV-1 infection, was proposed to contribute to high circulating triglyceride concentration by decreasing triglyceride clearance (125) and increasing hepatic lipogenesis (145). Moreover, antiretroviral treatment of HIV-1 infection, even with zidovudine monotherapy, has been reported as reducing both interferon α and triglyceride concentrations (124). Disturbances in lipid metabolism, thus, may represent a non-specific, probably cytokine-mediated, response to a chronic infection rather than to the HI virus specifically.

Unlike dyslipidemia, insulin resistance and type 2 diabetes were originally perceived to be rare in untreated HIV-1-infected patients (86,178). However, when matched for BMI and waist-to-hip ratio with HIV-1-negative subjects, HIV-1-infected antiretroviral-naïve patients were found to have up to a 2.2-fold increase in the relative risk of diabetes (26).

5.3.7. Host-related factors

Several cross-sectional and prospective studies have identified host factors that place individuals at a greater risk of developing lipodystrophic changes while on cART (46,122,238). These include increasing age and, more controversially, female gender, low body weight at the start of cART and greater CD4+ T-cell and HIV-1 RNA responses to commencement of cART (46,122,238). Furthermore, dysregulation of proinflammatory cytokine homeostasis, which can be seen during immune reconstruction associated with reversal of HIV-1 disease progression, may play a role in the development of cART-associated lipodystrophy (205,221). As some patients get severe side effects from cART and some do not, it has been hypothesized that genetic factors play a role in predisposition to these adverse effects. Indeed, several

single nucleotide polymorphisms in genes involved in lipid metabolism such as *SREBP1C* (262), apolipoprotein E and in the apolipoprotein A1/C3/A4/A5 cluster (379) have been identified in HIV-1-infected patients. Enrichment of these polymorphisms in certain populations might explain why patients of certain origin and, thus, genetic background, are more/less prone to develop dyslipidemia when exposed to cART.

5.4. Significance of cART-associated lipodystrophy

5.4.1. Psychological repercussions

Lipodystrophic disfigurement can be obvious to the most cursory observation and may lead to unwanted disclosure of HIV-1 infection and stigmatization (69). Lipodystrophy has a profound impact on patients' health-related quality of life causing discomfort, erosion of self-image and self-esteem, altered social, sexual and work-related habits and, at worse, can result in social exclusion, depression and seclusion (69,128). Together, these effects might jeopardize adherence to, and even lead to discontinuation of, otherwise efficacious antiretroviral therapy (128). These considerations, as well as the measurable metabolic disturbances, justify the ongoing research into pathogenesis and potential treatments of cART-associated lipodystrophy.

5.4.2. Liver complications

Ectopic accumulation of fat in the liver in the absence of excessive alcohol use or other underlying conditions and exceeding liver triglyceride content of 5.56% (375) is termed non-alcoholic fatty liver disease (NAFLD). NAFLD is, on one hand, associated with insulin resistance and type 2 diabetes (192) and, on the other hand, can progress to more severe conditions such as non-alcoholic steatohepatitis, cirrhosis and, even, hepatocellular carcinoma (253). Both increased liver fat (369) and greater prevalence of non-

alcoholic steatohepatitis (210) have been reported in lipodystrophic as compared to non-lipodystrophic cART-treated patients. In HIV-1-infected patients, an additional risk factor for the development severe liver diseases is the high prevalence of co-infection with hepatitis viruses B and C due to common modes of transmission.

5.4.3. Type 2 diabetes

As cART-associated lipodystrophy features accumulation of IAT and liver fat as well as development of insulin resistance (47,122,164,193), its presence increases the risk of developing type 2 diabetes. While hyperinsulinemia may initially compensate for insulin resistance and maintain normoglycemia, insulin production eventually becomes exhausted leading to frank hyperglycemia and type 2 diabetes. Indeed, the presence of cART-associated lipodystrophy has been reported as associated with a 9.1-fold risk of impaired glucose tolerance and a 6.5-fold risk of type 2 diabetes compared to that of HIV-1-negative subjects matched for age, gender and BMI, even after adjustment for waist-to-hip ratio (132). The prevalence and incidence of type 2 diabetes in HIV-1-infected cART-treated patients have been estimated as 2-5 times greater than in the general population despite the similar prevalence rates of overweight and obesity (337). Some of the studies included in the cited review, however, were carried out in patients on currently outdated antiretroviral regimens and, as less toxic drugs have become available, these prevalence figures may decrease in the future. In assessing the prevalence of type 2 diabetes in this patient population it is also important to keep in mind other possible confounders of such analyses, e.g., trends in obesity, nutrition, active as opposed to sedentary lifestyle and genetic predisposition to diabetes.

5.4.4. Cardiovascular disease

Atherogenic lipid profiles, diabetes, hypertension, abdominal obesity, impaired

fibrinolysis and a significant prevalence of smoking in patients infected with HIV-1 as compared to HIV-1-negative subjects translate into increased rates of cardiovascular morbidity (181,389) and mortality (68) in the former group. While the aforementioned risk factors, except for smoking, are all features of cART-associated lipodystrophy, the presence of lipodystrophy *per se* has not been associated with an increased cardiovascular risk independent of these factors (109). On a positive note, the prevalence of cardiovascular risk factors, such as smoking and dyslipidemia, and the incidence of myocardial infarction, seems to be decreasing in HIV-1-infected patients over time (119,333).

Antiretroviral drugs may directly increase accumulation of cholesteryl esters in macrophages and, thus, increase the risk of atheroma formation (82) independent of their dyslipidemic effects. Duration of antiretroviral therapy has been documented as increasing the risk of cardiovascular events (74,108,110,247), with a large DAD Study reporting up to a 26% increase in the risk of myocardial infarction per each year on cART (109). More recently, further analyses of the DAD Study have identified specific classes of antiretroviral drugs (mostly PIs) (108) and individual antiretroviral agents (PIs indinavir and ritonavir-boosted lopinavir and NRTIs didanosine and abacavir) (334,431) as associated with the greatest increase in the risk of myocardial infarction. As antiretrovirals have detrimental effects on cardiovascular health, the Strategies for Management of Antiretroviral Therapy (SMART) Study was undertaken to explore the effects of minimizing exposure to antiretroviral agents using intermittent therapy (85). Surprisingly, intermittent, as compared to continuous, cART resulted in a significant increase in the incidence of cardiovascular events and all-cause mortality, and the study had to be terminated early (85). The exploratory analyses that followed SMART revealed that intermittent, but not continuous, cART resulted in a decrease in total, large, medium and small HDL particles, and in an

increase in concentrations of hs-CRP, IL6 and D-dimer (84). These markers were all associated with higher cardiovascular risk status and correlated with an increase of HIV-1 RNA that followed cART interruption (84). Thus, increased cardiovascular morbidity was proposed as attributable to the development of an unfavorable ratio of total-to-HDL cholesterol concentrations, and activation of inflammation, thrombosis and fibrinolysis caused by increased viremia upon halting the antiretroviral treatment (84).

5.5. Treatment of cART-associated lipodystrophy

5.5.1. Management of lipoatrophy

5.5.1.1. Switch studies

As PIs were originally suspected to cause cART-associated lipodystrophy, several switch studies were performed to replace PIs, either with a NNRTI or abacavir. These studies documented alleviation of the metabolic disturbances following the switch, but little impact on IAT accumulation was seen, and even a tendency towards worsened peripheral lipoatrophy was reported (49,244,285,330). These results, however, are challenging to compare and interpret due to differences in study size and design, measurements available and duration of follow-up (140). With recognition of NRTI toxicity as a key factor in the pathogenesis of cART-associated lipodystrophy (25), attention has been drawn to switching away from NRTIs. Studies have shown that substituting tNRTIs (stavudine, zidovudine) with less toxic non-thymidine-based NRTIs (abacavir, tenofovir) or with a NRTI-sparing regimen altogether leads to a slow reversal of cART-associated lipoatrophy (23,56,163,243,251,273,274,381, 382). However, depending on the patients' history of antiretroviral use, issues such as HIV-1 drug resistance and tolerability may arise, and changes in antiretroviral regimens may not always be feasible. Furthermore, while switch studies away

from tNRTIs result in the gain of peripheral SAT, amount of IAT and disturbances in glucose and lipid homeostasis remain unchanged (56,163,243,251,273,274), if not deteriorate (23,382).

5.5.1.2. Thiazolidinediones

Thiazolidinediones are insulin-sensitizing agents that act as ligands for PPAR γ . Although efficient in improving glycemic parameters in diabetic patients (440), thiazolidinediones are known to have an untoward side effect in this predominantly overweight population, namely gain of, what has been proposed to be subcutaneous, fat mass (41,270,360). As inhibition of the PPAR γ pathway in adipocyte differentiation was hypothesized to be causative of lipoatrophy (12,53) and *in vitro* PPAR γ agonists had been shown to revert this inhibition (44), thiazolidinediones seemed an ideal choice for a pharmacological intervention to treat cART-associated lipodystrophy. This view was further fortified by data reporting that troglitazone ameliorated the metabolic disturbances and subcutaneous lipoatrophy in patients with non-HIV-1-related lipodystrophies (7).

Thiazolidinediones have been studied extensively for the treatment of cART-associated lipodystrophy (319,350,368). A number of randomized double-blind placebo-controlled studies of rosiglitazone or pioglitazone, using lipodystrophy/lipoatrophy as the main inclusion criteria and with an objective method for measurement of SAT (MRI, CT, DEXA or combination thereof) have been performed (55,57,135,359,370). Surprisingly, most (55,57,359,370), though not all (135), have failed to result in improvements of subcutaneous lipoatrophy in cART-treated patients. Moreover, while effective in reducing liver fat and alleviating insulin resistance, treatment with rosiglitazone, but not pioglitazone, tended to worsen proatherogenic dyslipidemia in these patients (370).

The available evidence summarized in recent reviews and meta-analyses

(319,350,368) does not support the use of thiazolidinediones for the treatment of cART-associated lipoatrophy. They may, however, be beneficial in a subgroup of lipoatrophic patients that have been switched away from the most lipotoxic antiretrovirals (55,359). Thiazolidinediones are recognized to be efficient in improving insulin sensitivity in patients with cART-associated lipodystrophy and can be used for that indication in this group of patients (319,350,368). Troglitazone and rosiglitazone have, however, been withdrawn from the market due to severe hepatotoxicity (101) and increased risk of cardiovascular complications (93), respectively. Pioglitazone remains a treatment option, although its safety has also been recently questioned (94).

5.5.1.3. Leptin

Concentrations of leptin mRNA (12,159) and circulating leptin protein (52,281,369) have been reported as lower in patients with cART-associated lipodystrophy, consistent with their reduced adipose tissue mass, as compared to non-lipodystrophic cART-treated patients (52,281) and HIV-1-negative subjects (12,159,369). Leptin treatment in hypoleptinemic HIV-1-negative lipodystrophic patients has resulted in the reversal of insulin resistance, dyslipidemia and hepatic steatosis (161,303,311). Unfortunately, in some (161,303), but not in all (311), studies leptin therapy also decreased weight and body fat percentage, although adipose tissue loss was not specified by body compartments. These data prompted investigations of leptin substitution therapy as a potential treatment also for cART-associated lipoatrophy (206,275). In these studies, leptin was well-tolerated and led to a decrease in intra-abdominal/truncal fat, but had no effect on peripheral or liver fat (206,275). Leptin also improved lipid profiles and whole-body insulin sensitivity of glucose and lipid metabolism (206,275). Thus, while ameliorating several metabolic disturbances, administration of

leptin provided no solution for lipoatrophy in cART-treated patients.

5.5.1.4. Uridine

Mitochondrial toxicity caused by NRTI-induced inhibition of mtDNA-replicating POLG has been proposed as the basis of the pathophysiology of cART-associated lipoatrophy (25). *De novo* synthesis of pyrimidine nucleotides, the building blocks of DNA, is dependent on an intact mitochondrial respiratory chain, a dysfunction of which can ultimately lead to a deficiency of intracellular pyrimidines, jeopardizing DNA synthesis (225). Theoretically, the consequences of mitochondrial toxicity can be overcome, even in the presence of the original toxic agents, by providing exogenous pyrimidines to replenish the endogenous depleted pyrimidine pool. Indeed, *in vitro* studies with uridine, a precursor of pyrimidine nucleotides, have demonstrated that uridine increases mtDNA concentrations and ameliorates signs of mitochondrial toxicity in adipocyte cultures (414). Although theoretically possible via a mechanism of competitive inhibition, uridine has not been shown in *in vitro* studies to compromise the antiretroviral efficacy of NRTIs. Prior to Study V, uridine had not been used for any indication in HIV-1-infected patients. In HIV-1-negative subjects, however, uridine had been used to prevent and revert 5-fluorouracil-induced cytotoxicity in the treatment of malignancies (177,396,397), and as supplementation for hereditary orotic aciduria, an inherited disorder of pyrimidine metabolism (13,118). Uridine was shown to be safe and reasonably well-tolerated with diarrhea being the dose-limiting side effect. Intravenous administration of uridine, however, had been associated with febrile reactions and phlebitis (177,396,397).

5.5.1.5. Restorative surgical interventions

Facial lipoatrophy is, understandably, the most stigmatizing element of cART-

associated lipodystrophy. While switch studies and other therapeutic strategies for the treatment of cART-associated lipodystrophy have demonstrated slow reversal of SAT (*vide supra*), restorative surgical interventions have emerged as a quicker and more satisfactory solution (40,127). These interventions have been shown to improve the quality of life of lipodystrophic patients and to decrease depression and anxiety as evaluated by validated surveys (127). Studies have also proven them to be cost-effective in terms of quality-adjusted life years (312).

There are several treatment options currently available: i) autologous fat transplantation, ii) injection of biodegradable (collagen, hyaluronic acid, poly-L-lactic acid, calcium hydroxyapatite) or non-biodegradable (silicone, polymethylmethacrylate, polyacrylamide, polyalkylimide) fillers and iii) placement of alloplastic implants made of polymeric silicone, polytetrafluoroethylene or polyethylene (40,127). While implants and non-biodegradable fillers are permanent, the filling effect of the biodegradable products is estimated to be 6-12 months, after which the effect wears off, and the procedure can be repeated with the same or a different agent (127). The data, however, are limited to the follow-up period of the studies ranging from 6 to 24 months (127).

The operative risks are greater with autologous fat transplantation and implant placement than with the use of injectable fillers, as the former techniques require invasive surgery necessitating anesthesia and longer post-procedure recovery time (40,127). Injection treatments have their risks also in form of acute and delayed adverse reactions attributable to either the procedure itself or the filling agent used. Acute events are mostly transient and include injection site reactions such as edema, tenderness, pain, erythema, pruritus, hematoma and bleeding (40,127). Some of the more serious acute adverse events are asymmetry, lumps caused by uneven filler distribution, infection or even tissue necrosis (40,127). Delayed events may occur from weeks to years after

product application and include filler/implant migration or, alternatively, rigid persistence in their placement while the facial contours change with age, scarring due to fibrosis and overt collagenization, skin discoloration, acute or chronic infections, and graft rejection reactions (40,127). One should remain cautious about the use of permanent fillers/implants as cART-associated lipodystrophy can resolve or progress over time rendering the fillings excessively prominent and noticeable. Moreover, from the safety point of view, the use of permanent fillers/implants is not recommended as the long-term complications of permanent fillers/implants can prove very difficult to treat (367).

5.5.2. Management of lipohypertrophy

5.5.2.1. Lifestyle modifications

Longitudinal studies assessing the effects of aerobic exercise and/or endurance training in cART-treated patients with and without lipodystrophy have reported preferential decreases in IAT (386) and waist circumference (79), increases lean body mass (438), but no changes in SAT (79,386) or body fat percentage (438) in the exercise group as compared to the observation follow-up group. Some studies, however, have documented decreases in body fat as a result of intensive exercise (167,328), but were not designed to specify whether the lost fat was IAT or SAT. Exercise was reported to improve lipid profiles, especially fasting triglyceride concentrations, but had no effect on insulin sensitivity (386). The combination of a hypocaloric diet low in saturated fat with an exercise regime has proved effective at reducing both IAT and SAT significantly but had no impact on glucose or lipids in cART-treated women with general obesity (90). Retrospective case-control studies have suggested that diets rich in protein and fiber are associated with a lower risk of accumulation of IAT in cART-treated patients (146). Overall, a supervised balanced diet and versatile

exercise programs can be concluded as worthwhile and beneficial in managing cART-associated lipohypertrophy and concomitant metabolic disturbances, but may reduce SAT in exceptionally lipoatrophic individuals.

5.5.2.2. Growth hormone -related treatments

Administration of components of the growth hormone axis (growth hormone, insulin-like growth factor, growth hormone releasing hormone and recombinant human growth hormone releasing factor also known as tesamorelin) has been investigated as a potential strategy to treat hypertrophic IAT in patients with cART-associated lipodystrophy (358). The basis of this research was data suggesting that HIV-1-infected patients with IAT accumulation have reduced circulating growth hormone concentrations (324) along with reports of the lipolytic effect of growth hormone analogue therapy being associated with reduced abdominal obesity, and improved insulin sensitivity and lipid profiles in HIV-1-negative obese subjects (162).

A recent meta-analysis of 10 randomized, double-blinded, placebo-controlled trials with growth hormone axis treatments for cART-associated lipodystrophy evaluated the efficacy and safety of these interventions (358). Although, when pooled together, the growth hormone axis treatments exerted a beneficial effect on body composition by decreasing IAT and improving glucose and triglyceride concentrations with no concurrent decrease in SAT, individual agents had varying effects (358). Of the different agents studied, tesamorelin has shown the greatest potential for clinical use (123). In two large phase III studies (96), tesamorelin significantly decreased the amount of IAT, triglyceride concentration and the ratio of total to HDL cholesterol concentrations whilst having no effect on fasting plasma glucose and fasting serum insulin concentrations, or OGTT results. A small, but statistically significant, increase

in glycosylated hemoglobin A_{1c} (HbA_{1c}) of 0.12% was seen (96). Of particular importance in the context of cART-associated lipodystrophy, the lipolytic effect of tesamorelin was limited to IAT with no decrease in SAT (96).

Although promising and having already received approval by the U.S. Food and Drug Administration (102), tesamorelin therapy has a number of concerns regarding its use. Firstly, tesamorelin is administered by daily subcutaneous injections. Secondly, treatment with tesamorelin is costly and reimbursement is limited. Thirdly, effects of tesamorelin are reversed after cessation of the therapy (96) while the long-term safety of the therapy remains to be established.

5.5.2.3. Restorative surgical interventions

Suction-, ultrasound- and laser-assisted lipectomy techniques have been used as treatment options for enlarged dorsocervical fat pads (302,349). The rate of recurrence after liposuction alone, however, is relatively high (349). Excisional lipectomy with or without concomitant liposuction may give more sustainable results (418).

5.5.3. Management of insulin resistance and type 2 diabetes

Metformin has been studied as a treatment for insulin resistance in cART-treated patients with or without lipodystrophy/-atrophy (131,183,246,277,335,356,403). In these studies, while ameliorating insulin resistance, this treatment worsened lipodystrophy. Nonetheless, metformin is currently the drug of choice for the treatment of type 2 diabetes in HIV-1-infected cART-treated patients unless contraindications exist (91). Pioglitazone can be an alternative, especially for lipodystrophic patients (91). Sulfonylureas and insulin can be used as mono or add-on therapy as in the general population (91). Newer anti-diabetic agents, such as incretin mimetics or gliptins, have yet to be studied in HIV-1-infected cART-treated

patients. If they are used, potential drug-drug interactions must be kept in mind.

5.5.4. Management of dyslipidemia

Switching the antiretroviral regimen to one causing less dyslipidemia is considered the best solution if patient's other conditions and drug resistance profile allow the switch (367). If lipid-lowering medication is needed, its potential interactions with the antiretrovirals have to be considered (e.g., those mediated by cytochrome P450 3A4 isoform). Pravastatin and rosuvastatin are safe and most widely used, while simvastatin is contraindicated with PIs (91). Atorvastatin can be used but requires close follow-up and careful dose titration (91). Fibrates, ezetimibe, plant stanols/sterols and fish oil supplementation can be used as in the general population (91).

5.5.5. Management of other risk factors

Angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers with and without diuretics are safe with regards to drug-drug interactions and are recommended for the treatment of hypertension in HIV-1-infected cART-treated patients (91). Some calcium channel blockers might interact with PIs and NNRTIs and are preferably avoided (91). B-blockers are mostly used in HIV-1-infected cART-treated patients with coronary artery disease (91).

The most important behavioral risk factors for cardiovascular disease are tobacco use, unhealthy diet and physical inactivity (429). Accordingly, HIV-1-infected patients should be motivated to stop smoking, if needed with the support of nicotine substitution and bupropion or varenicline therapy (91). Varenicline appears to be safer than bupropion with respect to drug-drug interactions (91). Diet recommendations for HIV-1-infected patients do not differ from those given for the general population (91). Finally, patients with cART-associated lipodystrophy should be motivated to engage in regular moderate-intensity exercise (91).

AIMS OF THE STUDY

The present studies were undertaken to answer the following questions:

- I.** Does the use of antiretroviral therapy or the presence of cART-associated lipodystrophy affect arterial stiffness, an independent predictor of cardiovascular mortality, as assessed by a technique of pulse wave analysis?
- II.** Is gene expression of macrophage markers and inflammatory cytokines upregulated in the abdominal SAT of lipoatrophic as compared to non-lipoatrophic cART-treated patients? Is adipose tissue inflammation correlated with liver fat content?
- III.** Is the amount of mtDNA, adipose tissue inflammation and gene expression involved in multiple cellular functions different in SAT of patients with and without cART-associated lipoatrophy? Is the effect similar between the lipoatrophic users of stavudine as compared to those treated with zidovudine?
- IV.** How does the abdominal SAT (lipoatrophic in cART-associated lipodystrophy) differ from the dorsocervical SAT (unaffected or hypertrophied in cART-associated lipodystrophy) in patients with and without cART-associated lipodystrophy?
- V.** Can oral uridine supplementation revert lipoatrophy and the associated metabolic disturbances without changing current cART?

SUBJECTS, STUDY DESIGNS AND METHODS

1. SUBJECTS

All study subjects (n=64) were recruited from the HIV outpatient clinic of the Helsinki University Central Hospital. Subjects had to be older than 18 years of age, infected with HIV-1, treated with cART for at least 18 months prior to enrollment and clinically stable with no signs or symptoms of current opportunistic infections. No

medication changes, either of cART or other medications, were allowed within the last three months preceding the studies. Exclusion criteria included pregnancy and signs, symptoms or biochemical evidence of active diseases other than HIV-1. Lactation, allergy to milk proteins (the uridine supplement used in Study V contained milk protein) and current use of didanosine (no evidence from *in vitro* studies indicated that uridine can abrogate lipotoxicity of didanosine which is a purine rather than a pyrimidine analogue (414,416)) were additional exclusion criteria in Study V. None of the study subjects were carriers of hepatitis virus B or C. Flow chart of patient recruitment to Studies I-V is given in Figure 4. Characteristics of the study subjects are provided in Tables 3a and 3b.

Figure 4.

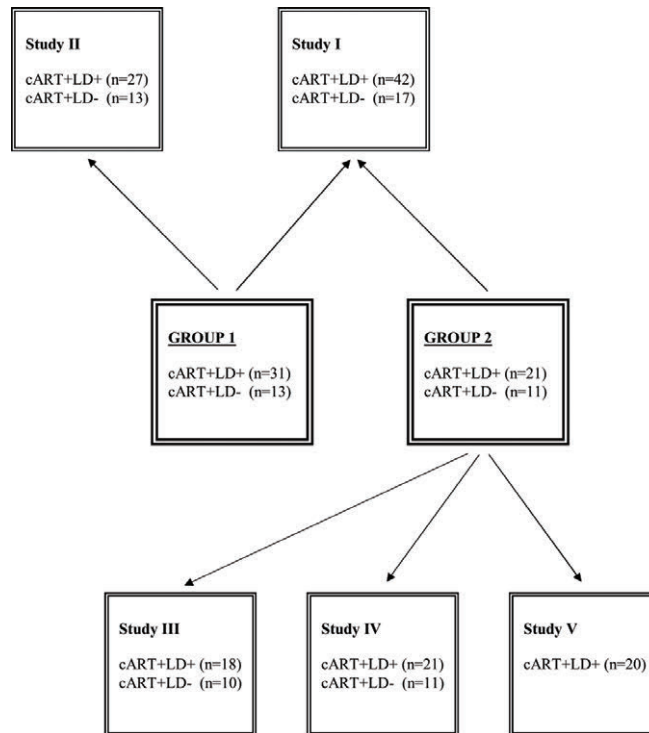


Figure 4. Flow chart demonstrating enrollment of HIV-1-infected patients with (cART+LD+) and without (cART+LD-) combination antiretroviral therapy –associated lipodystrophy to Studies I-V. Group 1 was recruited in 2001 and Group 2 in 2004-2005. The two groups had seven lipodystrophic and five non-lipodystrophic patients in common. In addition to patients from Group 1, eleven lipodystrophic and four non-lipodystrophic patients from Group 2 participated in Study I. For purposes of clarity, all study groups in the figure are termed as being treated with cART (an up-to-date term that has currently substituted the previously used term “highly active antiretroviral therapy, HAART” that, as mentioned in the text, was used in Studies I-III and V).

Table 3a. Characteristics of the study subjects (Studies I-III).

Variable	Study I		Study II		Study III	
	HAART+LD+	HAART+LD-	HAART+LD+	HAART+LD-	LA+	LA-
Number of subjects (male/female)	39/3	13/4	22/5	9/4	15/3	9/1
Age (y)	44 ± 1	41 ± 2	43 ± 2	39 ± 2	47 ± 2	43 ± 3
Body composition						
Body weight (kg)	72.0 ± 1.7	71.8 ± 3.0	73.8 ± 2.1	68.9 ± 3.5	73.6 ± 3.1	75.0 ± 3.4
BMI (kg/m ²)	23.3 ± 0.4	23.1 ± 0.9	23.6 ± 0.6	22.4 ± 1.1	23.5 ± 0.8	23.5 ± 0.9
Waist-to-hip ratio	0.99 ± 0.01***	0.90 ± 0.02	0.98 ± 0.1**	0.89 ± 0.3	1.00 ± 0.1***	0.91 ± 0.2
Total abdominal adipose tissue (cm ³)	N/A	N/A	3150 ± 280	2690 ± 460	3530 ± 390	2690 ± 450
Abdominal subcutaneous adipose tissue (cm ³)	N/A	N/A	1220 ± 170	1760 ± 280	1360 ± 290	1840 ± 300
Intra-abdominal adipose tissue (cm ³)	N/A	N/A	1930 ± 220*	930 ± 260	2170 ± 190**	850 ± 180
Liver fat (%)	N/A	N/A	5.0 (2.5 – 12.5)***	0.5 (0.5 – 1.75)	N/A	N/A
Features of insulin resistance						
fP-glucose (mmol/L)	5.8 ± 0.3	5.1 ± 0.1	5.6 ± 0.3	5.0 ± 0.1	N/A	N/A
fS-insulin (mU/L)	11.3 ± 1.2*	6.1 ± 0.8	11.0 ± 1.3*	6.5 ± 1.1	N/A	N/A
fS-triglycerides (mmol/L)	3.6 ± 0.4**	1.3 ± 0.2	2.8 (1.9 – 4.3)***	1.0 (0.75 – 1.6)	N/A	N/A
fS-HDL cholesterol (mmol/L)	1.1 ± 0.1***	1.6 ± 0.1	1.1 ± 0.1***	1.6 ± 0.1	N/A	N/A
HIV-1-related characteristics						
Time since diagnosis (years)	8.7 ± 0.6	8.0 ± 1.1	8.5 ± 0.7	8.7 ± 1.3	9.9 ± 1.1	6.9 ± 1.1
Total duration of ART (years)	6.2 ± 0.4	4.8 ± 0.8	N/A	N/A	N/A	N/A
Total duration of cART (years)	3.8 ± 0.3	2.8 ± 0.4	3.7 ± 0.2	3.1 ± 0.4	5.9 ± 0.4*	4.2 ± 0.6
Current NRTI (%)	100	100	100	100	100	100
Current NNRTI (%)	31	47	26	46	17**	80
Current PI (%)	78	59	74	62	100**	20
Most recent CD4+ T-cell count (cells/mm ³)	567 ± 40	533 ± 60	582 ± 59	516 ± 70	548 ± 64	543 ± 59
Undetectable HIV-1 viral load (%)	74	94	70	92	83	100

Data are shown as mean ± SEM or median (25% percentile – 75% percentile), as appropriate. p-values: * < 0.05, ** < 0.005, *** < 0.0005. ART, antiretroviral therapy (treatment with one or more antiretroviral drugs); BMI, body mass index; cART, combination antiretroviral therapy (treatment with three or more antiretroviral drugs used concurrently); HAART+LA+, HIV-1-infected patients with highly active antiretroviral therapy –associated lipodystrophy; HAART+LA-, HIV-1-infected patients without highly active antiretroviral therapy –associated lipodystrophy; HDL, high-density lipoprotein; HIV-1, human immunodeficiency virus type 1; LA+, lipotrophic HIV-1-infected patients; LA-, non-lipotrophic HIV-1-infected patients; N/A, not applicable; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor. Adapted from Sevastianova et al. *Antiviral Therapy* 2005; 10:925-35 (Study I), Sevastianova et al. *American Journal of Physiology: Endocrinology and Metabolism* 2008; 295:E85-91 (Study II) and Sievers et al. *Journal of Infectious Diseases* 2009; 200:252-62 (Study III), data are reproduced with permission of the copyright holders.

Table 3b. Characteristics of the study subjects (Studies IV-V).

Variable	Study IV		Study V	
	cART+LD+	cART+LD-	Uridine	Placebo
Number of subjects (male/female)	18/3	10/1	9/1	8/2
Age (y)	47 ± 2	44 ± 3	47 ± 2	49 ± 5
<i>Body composition</i>				
Body weight (kg)	73.8 ± 2.8	75.4 ± 3.1	76.7 ± 4.7	70.5 ± 3.2
BMI (kg/m ²)	23.6 ± 0.7	23.7 ± 0.8	24.4 ± 1.1	22.6 ± 0.9
Waist-to-hip ratio	1.00 ± 0.01*	0.92 ± 0.02	1.01 ± 0.02	0.99 ± 0.02
Total abdominal adipose tissue (cm ³)	3510 ± 250	2780 ± 410	3740 ± 560	3420 ± 520
Abdominal subcutaneous adipose tissue (cm ³)	1290 ± 250	1870 ± 270	1420 ± 420	1250 ± 390
Intra-abdominal adipose tissue (cm ³)	2220 ± 260**	910 ± 170	2230 ± 300	2180 ± 470
Liver fat (%)	3.0 (1.5 – 11.3)**	1.0 (0.75 – 1.5)	5.9 ± 2.8	12.4 ± 4.7
<i>Features of insulin resistance</i>				
fP-glucose (mmol/L)	5.1 (4.9 – 5.7)	5.3 (5.1 – 5.8)	6.2 ± 1.1	5.4 ± 0.5
fS-insulin (mU/L)	12.8 ± 2.4*	4.9 ± 0.5	12.3 ± 3.8	13.7 ± 5.7
fS-triglycerides (mmol/L)	3.4 ± 0.5*	1.6 ± 0.3	2.9 ± 0.4	3.0 ± 0.4
fS-HDL cholesterol (mmol/L)	1.2 ± 0.1**	1.8 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
<i>HIV-1-related characteristics</i>				
Time since diagnosis (years)	9.1 ± 1.0	7.8 ± 1.3	11.1 ± 1.4	8.7 ± 1.4
Total duration of cART (years)	5.9 ± 0.4*	3.8 ± 0.5	6.0 ± 0.6	6.3 ± 0.6
Current NRTI (%)	100	100	100	100
Current NNRTI (%)	20*	80	20	20
Current PI (%)	95**	20	100	90
Most recent CD4+ T-cell count (cells/mm ³)	545 ± 57	522 ± 44	594 ± 81	517 ± 88
Undetectable HIV-1 viral load (%)	86	100	90	80

Data are shown as mean ± SEM or median (25% percentile – 75% percentile), as appropriate. *p*-values: **p*<0.05, ***p*<0.005. BMI, body mass index; cART, combination antiretroviral therapy (treatment with three or more antiretroviral drugs used concurrently); cART+LA+, HIV-1-infected patients with combination antiretroviral therapy –associated lipodystrophy; cART+LA-, HIV-1-infected patients without combination antiretroviral therapy –associated lipodystrophy; HDL, high-density lipoprotein; HIV-1, human immunodeficiency virus type 1; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor. Adapted from Sevastianova et al. *Diabetes* 2011; 60:1894-900 (Study IV) and Sutinen et al. *Antiviral Therapy* 2007; 12:97-105 (Study V), data are reproduced with permission of the copyright holders.

Classification as a lipodystrophic patient (HAART+LD+ in Studies I-III and V; cART+LD+ in Study IV) required i) self-reported symptoms of loss of SAT with or without enlargement of abdominal girth, increase in breast size or accumulation of adipose tissue in the dorsocervical region (also known as buffalo hump) and ii) confirmation of these findings by a single investigator. Patients without lipodystrophy (HAART+LD- in Studies I-III and V; cART+LD- in Study IV) had received cART without developing the aforementioned changes in body fat distribution.

The purpose, nature and potential risks of the studies were explained to the patients before their written informed consent was obtained. The study protocols were approved by the Ethics Committee of the Department of Medicine at the Helsinki University Central Hospital. The clinical trial involving uridine (Study V) was investigator-initiated and undertaken independently of the manufacturer of the uridine supplement, Nucleomaxx® (Pharma Trade Healthcare, Spånga, Sweden). Study V was registered in the clinical trial registry of the Helsinki University Central Hospital and was compliant with

the revised CONSORT statement guidelines (www.consort-statement.org).

2. STUDY DESIGNS

2.1. Design of Study I

In this cross-sectional study, PWA was carried out in order to measure and compare arterial stiffness in HIV-1-infected cART-treated patients with (HAART+LD+, n=42) and without (HAART+LD-, n=17) cART-associated lipodystrophy. Multiple linear regression analysis was performed to identify determinants of arterial stiffness in the aforementioned patients.

2.2. Design of Study II

In this cross-sectional study, gene expression of macrophage markers (*CD68*; intergrin α M, *ITGAM*; epidermal growth factor –like module-containing, mucin-like, hormone receptor –like 1, *EMR1*; a disintegrin and metalloproteinase domain 8, *ADAM8*) and inflammatory cytokines (*CCL2*; *CCL3*; *TNFA*) in a needle aspiration biopsy of abdominal SAT was measured by real-time reverse transcriptase polymerase chain reaction (RT-PCR) and correlated with liver fat content, measured by ¹H-MRS, in HIV-1-infected cART-treated patients with (HAART+LD+, n=27) and without (HAART+LD-, n=13) cART-associated lipodystrophy.

2.3. Design of Study III

In this cross-sectional study, real-time RT-PCR –measured mtDNA content and gene expression of genes involved in mitochondrial energy metabolism (cytochrome c oxidase subunit III, *COX3*; cytochrome c oxidase subunit IV, *COX4*; 16S ribosomal RNA, *16SRNA*), mitochondrial biogenesis (DNA polymerase γ (catalytic subunit), *POLG1*; DNA polymerase γ (accessory subunit), *POLG2*; *TFAM*; *PGC1B*), adipogenesis (*CEBPA*; *CEBPB*; *LMNA*; lamin B, *LMNB*; *PPARG*, peroxisome proliferator

–activated receptor γ subunit 2, *PPARG2*; *SREBP1C*), lipid and glucose metabolism (*FASN*; *PLIN1*; *GLUT4*; hexokinase I, *HEXOK1*), and in apoptosis, inflammation and oxidative stress (factor of apoptotic stimulus, *FAS*; proliferating cell nuclear antigen, *PCNA*, tumor protein p53, *p53*; *TNFA*; *IL6*; interleukin 1 β , *IL1B*; glutathione peroxidase transcript 1, *GPX1*; superoxide dismutase 1, *SOD1*) were compared between HIV-1-infected cART-treated patients with (LA+, n=18) and without (LA-, n=10) cART-associated lipoatrophy. Also, immunohistochemical stainings indicative of inflammation (CD68) and tissue vitality (PLIN1) were compared between the same study groups. A subgroup analysis of the same parameters was conducted within the LA+ group comparing patients receiving stavudine (d4T+LA+, n=8) to those receiving zidovudine (AZT+LA+, n=10). Analyses were carried out in surgical adipose tissue biopsy specimens of the abdominal SAT.

2.4. Design of Study IV

In this cross-sectional study, surgical biopsy specimens of dorsocervical and abdominal SAT were compared in HIV-1-infected cART-treated patients with (cART+LD+, n=21) and without (cART+LD-, n=11) cART-associated lipodystrophy using histology, microarray and real-time RT-PCR. MRI technology was utilized to image both adipose tissue depots.

2.5. Design of Study V

In this investigator-initiated, double-blind, placebo-controlled trial, 20 HIV-1-infected patients with cART-associated lipoatrophy were randomized to receive either a dietary uridine supplement (36g tid for 10 consecutive days/month) or placebo for 3 months. Measurements of body composition were performed before and after the intervention. These included quantification of IAT and abdominal SAT by MRI, total body, trunk and limb fat by DEXA, and liver fat content by ¹H-MRS.

Secondary aims included evaluation of the effects of uridine on features of insulin resistance and safety parameters. For the purpose of these secondary analyses, blood samples were taken at baseline, after each 10-day course of treatment with uridine (days 11, 41 and 71) and at 3 months. Adherence to the supplement was verified by measuring blood concentrations of uridine at baseline, days 41 and 71 and at 3 months. Patients were seen by the study physician at baseline, day 41 and at 3 months.

3. METHODS

All subjects on all occasions were studied after an overnight fast. Unless otherwise stated, the methods below have been used in Studies I-V.

3.1. BODY COMPOSITION

3.1.1. Anthropometric indices

Body weight and height were measured barefoot in light clothing. Body weight was read via a digital scale with a precision of measurement of 0.1 kg and height via a non-stretchable tape with a 0.1 cm precision. Body circumferences were measured in triplicate using a non-stretchable tape and recorded to the nearest 0.5 cm. Circumferences were measured midway between the lower rib margin and the iliac crest for the waist, and over the greater trochanters for the hip (430). Skinfold thickness (mean values of triplicate measurements) was determined at five sites (triceps, biceps, iliac crest, thigh and cheek in Studies I-II and triceps, biceps, iliac crest, thigh and scapula in Study IV) (442).

3.1.2. Bioelectric impedance analysis

Body fat free mass and the percentage of body fat were determined (Studies I-II) using bioelectric impedance (BIA) analysis (BioElectrical Impedance Analyzer System model #BIA-101A;RJL Systems, Detroit, MI) (87,228).

3.1.3. Dual-energy X-ray absorptiometry

Limb, trunk and total body fat as well as lean body mass were measured (Studies III-V) using a DEXA scanner (Lunar Prodigy, Madison, WI).

3.1.4. Magnetic resonance imaging

Volumes of IAT and abdominal SAT (Studies II-V) were quantified by analyzing 16 T1-weighted two-dimensional fast low angle shot trans-axial MRI scans reaching 8 cm above and 8 cm below the 4th and 5th lumbar interspace (slice thickness 10 mm, field of view 375 x 500 mm², time of repetition 138.9 ms, echo time 4.1 ms). In addition, in Study IV the dorsocervical SAT volume was measured from scans covering the area from the interspace between the 2nd and 3rd cervical vertebra to the lower edge of the scapulae (slice thickness 5 mm, field of view 315 x 500 mm², time of repetition 159 ms, echo time 4.1 ms). Volumes of adipose tissue depots were calculated in a blinded fashion using an image analysis software (Alice 3.0, Parexel, Waltham, MA). A histogram of the pixel intensity of each scan was displayed and intensity corresponding to the nadir between lean and adipose tissue peaks was used as a cut-off threshold. Adipose tissue was defined as the area of pixels with intensity values above this threshold. In case of abdominal scans, this area equaled total abdominal adipose tissue. Thereafter, pixels corresponding to IAT were manually erased and the remaining area, covering abdominal SAT, was calculated. IAT volume was calculated by subtracting SAT volume from the total abdominal adipose tissue volume. In the case of dorsocervical SAT, additional limits defining the depot were set at the lateral-most point of the neck/shoulders or that of head of humerus, when visible, and the lateral-most point of the back below the level of armpits.

3.1.5. Proton magnetic resonance spectroscopy

Liver fat content was measured using ¹H-MRS (Studies II, IV-V). In this

procedure, localized single voxel ($2 \times 2 \times 2 \text{ cm}^3$) proton spectra were recorded using a 1.5 tesla whole body system (Magnetom Vision, Siemens, Erlangen, Germany) that consisted of a combination of whole body and loop surface coils for radiofrequency transmitting and signal receiving. T1-weighted high-resolution MRI images were used for localization of a voxel of interest that was carefully positioned in the right lobe of the liver, avoiding large vessels, bile ducts and the gall bladder. Subjects were required to lie on their stomach on the surface coil embedded in the mattress, in order to ensure a firm contact between the chest wall and the coil, and to minimize movement artifact due to breathing. The single voxel spectra were recorded using the stimulated echo acquisition mode sequence (echo time of 20 ms, repetition time of 3000 ms, mixing time 30 ms, 1024 data points over 1000 kHz spectral width with 32 averages). Water-suppressed spectra with 128 averages were recorded to detect weak lipid signals. Long repetition time and short echo time were chosen to minimize the effects of T1 and T2 relaxations, and ensure fully relaxed water signal which was used as the internal standard. Chemical shifts were measured relative to water at 4.80 parts per million. The methylene signal, representing triglycerides, was recorded at 1.4 parts per million (374). As there is no extrahepatic fat in the liver, the methylene signal can be directly extrapolated to represent the intrahepatic fat. Signal intensities were quantified using an analysis program VAPRO-MRUI (www.mrui.uab.es/mrui/). Spectroscopic intrahepatic triglyceride content was expressed as a ratio of the area under the methylene peak to that of the sum of the areas under the methylene and the water peaks ($\times 100 =$ percentage of liver fat). All spectra were analyzed by a single physicist blinded to other research data.

3.2. SUBCUTANEOUS ADIPOSE TISSUE BIOPSIES

3.2.1. Needle aspiration biopsy

A small area of skin in the abdominal

area was anesthetized with 1% lidocaine without epinephrine. A biopsy needle (14G), attached to a tight-fitting syringe, was inserted through the infiltrated skin and small samples of adipose tissue were aspirated by vacuum suction (Study II). The adipose tissue samples were thoroughly rinsed with sterile saline and immediately thereafter frozen in liquid nitrogen and stored in -80°C until analysis.

3.2.2. Surgical biopsies

Following local anesthesia with 1% lidocaine without epinephrine, surgical adipose tissue biopsies were taken from the midway between the iliac crest and the umbilicus as well as from the midline at the base of the neck (Studies III-V). The adipose tissue sample was thoroughly rinsed with sterile saline. Part of the biopsy was immediately snap-frozen in liquid nitrogen and stored in -80°C until analysis. Another part of the tissue sample was formalin-fixed and paraffin-embedded for subsequent histological (Study IV) and immunohistochemical (Study III) analyses.

3.3. MITOCHONDRIAL DNA CONTENT

In Studies III-IV, mtDNA content of the adipose tissue samples was quantified. Genomic DNA was extracted from adipose tissue with the QIAamp DNA isolation kit (Qiagen, Hilden, Germany). MtDNA and nuclear DNA (nDNA) copy numbers were determined by real-time RT-PCR using the ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). The mtDNA-encoded ATP synthase 6 gene was amplified between nucleotide positions 8981 and 9061. MtDNA was quantified with a FAM fluorophore -labeled probe (5'-6FAM-CCTAACCGCTAACATTACTGCAGGCCACC-TAMRA-3'). For the detection of nDNA, exon number 8 of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene between nucleotide positions 4280 and 4342 was selected and a VIC

fluorophore -labeled probe (5'-VIC-CCCTGCTCTACTGGCGCTGCC-TAMRA-3') used. Amplifications of mitochondrial and nuclear products were performed separately in optical 96-well plates (PE Applied Biosystems, Foster City, CA) using assays by the same manufacturer. All samples were run in triplicate. Absolute mtDNA and nDNA copy numbers were calculated using serial dilutions of plasmids with known copy numbers.

3.4. GENE EXPRESSION

3.4.1. RNA extraction and cDNA preparation

Frozen adipose tissue (50 - 150 mg) was homogenized using either the RNA STAT-60 (Tel-Test, Friendswood, TX; Study II) or the GeneClean kit (Bio101 systems, Obiogene Inc., Carlsbad, CA; Studies III-IV). Total RNA was extracted with the RNeasy Lipid Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. After the DNase treatment (RNase-free DNase set, Qiagen, Hilden, Germany), RNA was purified using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentrations were measured using the RiboGreen fluorescent nucleic acid stain (RNA quantification kit, Molecular Probes, Eugene, OR) and the quality of RNAs was assessed by agarose gel electrophoresis (Study II). Alternatively, in Studies III-IV, quantity and integrity of RNA were verified using RNA 6000 nanochips (Agilent 2100 Bioanalyzer, Palo Alto, CA). Average yields of total RNA were 3 ± 1 μ g per 100 mg of adipose tissue wet weight and did not differ between any of the study groups (data not shown). Isolated RNA was either transcribed into complementary DNA (cDNA) directly or stored at -80°C until further processing. Transcription of RNA into cDNA was done using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, U.K.; Study II), the Superscript II (Invitrogen, Carlsbad, CA; Studies III-IV)

and oligo(dT)12-18 primers (Invitrogen) or the SuperScript VILO cDNA synthesis kit (Invitrogen). cDNA was stored at -80°C until further analysis.

3.4.2. Real-time reverse transcriptase polymerase chain reaction

Quantification of messenger RNAs (mRNA) of all genes in Study II and of homeobox genes in Study IV was performed using TaqMan semiquantitative RT-PCR assays (TaqMan® Gene Expression Assays, PE Applied Biosystems, Foster City, CA) and commercially available primers (*vide infra*) according to the manufacturer's protocol using the ABI PRISM 7000 Sequence Detection System instrument and appropriate software (PE Applied Biosystems, Foster City, CA). Quantification of mRNAs of all genes in Study III and of *CD68* in Study IV was done using the LightCycler 480 (Roche, Penzberg, Germany) with SYBR® Green assays by Roche. Primer pairs were designed in an intron-spanning fashion to avoid non-specific amplification of contaminating genomic DNA using the universal probe library from Roche (www.universalprobelibrary.com). The conditions of real-time RT-PCR were optimized for linearity of amplification for all primers in a dilution series. Melting curve analysis was done to ensure that all investigated genes were represented by a single peak, indicating specificity. The real-time RT-PCR for *UCP1* (Study IV) was carried out as a pilot study in a subset of samples (n=6) from individuals with the largest "buffalo humps" on clinical evaluation on the ABI PRISM 7900HT instrument and software (PE Applied Biosystems, Foster City, CA), using the Power SYBR® Green PCR Master Mix by the same manufacturer.

All samples were run in duplicate. Expression of target genes was quantified (arbitrary units) by generating a six-point serially diluted standard curve (Studies II-IV) (419). The following housekeeping genes were used: β 2-microglobulin (*B2M*) (Study II), the mean of expression of actin β

Table 4. Primer sequences of the studied genes.

Gene	Forward primer 5' – 3'	Backward primer 5' – 3'
<i>16SRNA</i>	CGATTAAGTCCTACGTGATCTGA	AGGGAGGAATTTGAAGTAGATAGAAA
<i>36B4*</i>	CTGGAAAACAACCCAGCTCT	GAACACAAAGCCCACATTCC
<i>36B4**</i>	CATGCTCAACATCTCCCCCTTCTCC	GGGAAGGTGTAATCCGTCTCCACAG
<i>ACTB*</i>	ATTGGCAATGAGCGGTTC	GGATGCCACAGGACTCCAT
<i>ACTB†</i>	GAGCTACGAGCTGCCTGACG	GTAGTTTCGTGGATGCCACAG
<i>ATP6</i>	ACCAATAGCCCTGGCCGTAC	GGTGGCGCTTCCAATTAGGT
<i>B2M*</i>	TTCTGGCCTGGAGGCTATC	TCAGGAAATTTGACTTTCCATTCC
<i>B2M††</i>	GCCTGCCGTGTGAACCAT	TTACATGTCTCGATCCCACTTAACTAT
<i>CD68</i>	GAATTTTATAAATGTGACGAACTGC	GGAAAACCCCGTCAAAGATT
<i>CEBPA</i>	CAACACTTGTATCTGGCCTCTG	CCGAGCAAAAACAAAACAAA
<i>CEBPB</i>	AACCTGGAGACGCAGCAC	CAGCTGTCCACCTTCTTCT
<i>COX3</i>	TCCAAACATCACTTTGGCTTC	AACCACATCTACAAAATGCCAGT
<i>COX4</i>	CACCGCGCTCGTTATCAT	TGGCCACCCACTCTTTGT
<i>FAS</i>	TGAATCTCCAACCTTAAATCCTG	TGACTCCAGCAATAGTGGTGAT
<i>FASN</i>	CTGGTGATGAACGTCTACCG	GGCTCCTCAGGCTTGTC
<i>GADPH</i>	CGGGGCTCTCCAGAACATC	ATGACCTTGCCACAGCCT
<i>GLUT4</i>	CTGTGCCATCCTGATGACTG	CGTAGCTCATGGCTGGAAGT
<i>GPX1</i>	ATGTGTGCTGCTCGGCTA	CGAGAAGGCATACACCGACT
<i>HEXOK1</i>	GACCAAGTTTCTCTCTCAGATCG	CCTAGCTGCTGGAGGATAGC
<i>HOXA10</i>	GAGAGCAGCAAAGCCTCGC	CCAGTGTCTGGTGCTTCGTG
<i>HOXC9</i>	GCCTGCTGCCTCAGCACAG	GAACCTCCCAAATCGCAAG
<i>HOXC8</i>	TCTCCAACTCAGGCTACCAG	TGAGCCCCATAAAGGGACTGT
<i>IL1B</i>	CTGTCTGCGTGTGAAAGA	TTGGGTAATTTTGGGATCTACA
<i>IL6</i>	GATGAGTACAAAAGTCTGATCCA	CTGCAGCCACTGGTTCTGT
<i>LMNA</i>	AGCAAAGTGCGTGAGGAGTT	AGGTCACCTCCTTCTTGTT
<i>LMNB</i>	AGCTGGAGTGTTGTTGAGG	TTGGATGCTCTTGGGGTTC
<i>p53</i>	AGGCCTTGGAAGTCAAGGAT	CCCTTTTGGACTTTCAGGTTG
<i>PCNA</i>	TGTACACACAAGTAATGTCGATAAA	GAACTGGTTTCAATTCATCTCTATGG
<i>PGC1B</i>	GAAGAGGAAGAAGGGGAGGA	GGTAGGGGCAGTGGTTCAG
<i>PLIN1</i>	GGACACAGTGGTGCATTACG	GTCCCGGAATTCGCTCTC
<i>POLG1</i>	GAGAAGGCCAGCAGATGTA	ATCCGACAGCCGATACCA
<i>POLG2</i>	CCAGTGGCTTGATTCTGGT	AAGTTAGATGGACTCATGGCAAA
<i>PPARG</i>	GGCCCAGATACACTGACTACG	TGGCTTCATACTTGCTTTTCC
<i>PPARG2</i>	GACCTGAAACTTCAAGAGTACAAA	TGAGGCTTATTGTAGAGCTGAGTC
<i>SHOX2</i>	TGTTTCGTGTGGATGCACGTG	GAAACCAAACCTGCACTCGG
<i>SOD1</i>	TCATCAATTTTCGAGCAGAAGG	CAGGCCTTCAGTCACTCTTTT
<i>SREBP1C</i>	GACACACCAGCTCCTCGAC	GTAGCAGGAGGCCAGCAG
<i>TFAM</i>	GTTTCTCCGAAGCATGTGG	GCACAGCTCTGCTCCAGAC
<i>TNFA</i>	CAGCCTCTTCTCCTTCCTGA	GCCAGAGGGCTGATTAGAGA
<i>UCP1</i>	CTGGAATAGCGCGTGCTT	AATAAACTGGACGTCGGGC

*Used in Studies III and IV. **Used in Study IV as a housekeeping gene in homeobox gene analysis only. †Used in Study IV as a housekeeping gene in UCP1 analysis only. ††Used in Study II. For full names of the genes, please see text. Adapted from Sievers et al. *Journal of Infectious Diseases* 2009; 200:252-62 (Study III) and Sevastianova et al. *Diabetes* 2011; 60:1894-900 (Study IV), data are reproduced with permission of the copyright holders.

(*ACTB*), acidic ribosomal phosphoprotein P0 (*36B4*) and *B2M* (Study III and IV) and *36B4* or *ACTB* (Study IV). Gene expression was given relative to that of *B2M* (Study II) or calculated from the real-time RT-PCR efficiency (313) in relation to housekeeping genes (Studies III-IV). Non-regulation of the housekeeping genes was validated by geNorm version 3.4 (PrimerDesign, Southampton, U.K.; Study III) (404).

The assay ID of commercially available primers (PE Applied Biosystems, Foster City, CA) were: *CD68* (Hs00154355_m1), *ITGAM* (Hs00355885_m1), and *EMR1* (Hs00173562_m1), *ADAM8* (Hs00174246_m1), *CCL2* (Hs00234140_m1), *CCL3* (Hs00234142_m1), *TNFA* (Hs00174128_m1). The sequences of in-house primers are provided in Table 4.

3.4.3. Microarray

1 µg/sample of total RNA was indirectly labeled using the T7 amplification method (Amino Allyl MessageAmp™ II aRNA Amplification Kit, Ambion, Austin, TX) according to the manufacturer's instructions. The amplified RNA (aRNA, 5 µg/sample) was labeled using monoreactive Cy3 and Cy5 dyes (GE Healthcare, GE Life Sciences, Uppsala, Sweden) followed by purification according to the manufacturer's instructions. Labeled aRNAs were hybridized onto Whole Human Genome 4x44K microarrays (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. The slides were then washed according to the instructions with buffers from Agilent and scanned with the GenePix 4200 AL (Axon Instruments, Molecular Devices, Silicon Valley, CA) at the resolution of 5 µm/pixel and at 16 bit depth. The analysis was carried out as comparison of dorsocervical to abdominal SAT in the cART+LD+ and the cART+LD- groups separately. DAVID Bioinformatics Resources version 6.7 (<http://david.abcc.ncifcrf.gov>) was used to select over-represented pathways and gene categories (158).

3.5. HISTOLOGY

Histological appearance of adipose tissue samples was evaluated in Study IV. Formalin-fixed, paraffin-embedded adipose tissue samples were sectioned using a standard protocol. The slides were stained with hematoxylin and eosin and examined under the light microscope (Nikon Eclipse 80i, Nikon Corporation, Tokyo, Japan) for the amount of connective tissue, vascularization, lipogranulomata (i.e., lipid-laden macrophages encircling adipocytes), degree of adipocyte size variation, and for the presence of non-intact cells (due to membrane ruptures) and brown adipocytes. Images for publication were taken using computer software compatible with the microscope (NIS-Elements 3.0, Nikon Corporation, Tokyo, Japan).

3.6. IMMUNOHISTOCHEMISTRY

Adipose tissue biopsies were used for immunohistochemical analysis (Study III). CD68 served as a marker for macrophages and PLIN1 as a marker for viable adipocytes (66). Sectioning was performed using a standard protocol for formalin-fixed paraffin-embedded tissue blocks. Consecutive serial sections were treated with xylene, descending ethanol dilution series and distilled water to dewax the tissue samples. Thereafter, sections were microwave-treated in 10mM citrate buffer (pH 6.0) and washed alternately with distilled water, hydrogen peroxide and phosphate-buffered saline (PBS) with 0.25% Triton X-100 (pH 7.2) to inactivate endogenous staining. Non-specific staining was reduced by applying normal goat serum (1:5, Dako, Glostrup, Denmark) to the sections for 30 min. Samples were then incubated at room temperature with mouse monoclonal anti-CD68 (1:200; Novocastra Laboratories Ltd, Newcastle upon Tyne, U.K.) or guinea pig polyclonal anti-PLIN1 (1:1000; Acris Antibodies GmbH, Hiddenhausen, Germany). For negative controls of CD68 staining, mouse monoclonal isotypic

control (1:200; Abcam, Cambridge, U.K.) was used as a primary antibody. For PLIN1 negative control, the primary antibody was omitted. After rinsing in PBS-Triton X-100 buffer, sections were incubated with biotinylated anti-mouse (1:1500; Vector Laboratories Inc., Burlingame, CA) or anti-guinea pig (1:1500; Abcam, Cambridge, U.K.) secondary antibodies. Avidin-biotin peroxidase complexes (Vector Laboratories Inc., Burlingame, CA) were added followed by visualization with 3,3-diaminobenzidine tetrachloride (Vector Laboratories Inc., Burlingame, CA). Following washes with distilled water, ascending ethanol series and xylene, sections were counterstained with Harris hematoxylin (Histolab, Gothenburg, Sweden). For each sample, the number of macrophages, crown-like structures (CLS) and PLIN1-free adipocytes within the entire section were counted using light microscopy and normalized for the total section area. Macrophages were identified as CD68-positive cells and CLSs were defined as one PLIN1-free adipocyte surrounded by at least three macrophages (66). Measurement of total section area using arbitrary units was performed using Adobe Photoshop Elements version 1.0.1 (AdobeSystems Inc., San Jose, CA).

3.7. PULSE WAVE ANALYSIS

To determine central aortic pulse pressure and the augmentation index, pulse waves were recorded from the radial artery, with the wrist slightly extended and supported on a pillow, by applanation tonometry using a Millar tonometer (SPC-301, Millar Instruments, Houston, TX). Data were collected directly into a desktop computer and processed with the SphygmoCor Blood Pressure Analysis System (BPAS-1, PWV Medical, Sydney, Australia) which allows continuous online recording of the radial artery pressure waveform. The radial waveform was assessed visually to ensure that artifacts from movement and respiration are minimized. The mean of three recordings, each consisting of 15 to 20 sequential radial artery waveforms, was

used for subsequent analysis. The integral system software was used to generate an average radial artery waveform and the corresponding central ascending aortic pressure waveform applying a previously validated transfer factor (58,175,300). The aortic waveform was then subject to further analysis for calculation of aortic augmentation, the aortic augmentation index as well as central systolic, diastolic and pulse pressure (for definitions of the measurements, please see Figure 2). As the augmentation index is known to vary with heart rate (424), a correction formula was used to derive augmentation index values corrected for heart rate (AgI_{HR}) (424). Aortic, and not radial artery, data were used for further analyses as aortic characteristics are more predictive of cardiovascular morbidity. This is because the aorta, an elastic capacitance vessel, shows more pronounced changes in structure and function with ageing and co-morbidities than the muscular peripheral conduit vessels. As suggested by O'Rourke *et al.* (300), the radial blood pressure was calibrated against the sphygmomanometrically determined brachial blood pressure, ignoring the small degree of amplification between the brachial and radial sites. Pulse wave analysis and sphygmomanometric measurements were conducted after a minimum of 15 min of acclimatization and prior to blood sampling.

3.8. LABORATORY ANALYSES

Plasma glucose concentrations were measured using a hexokinase method (Studies I-II, IV-V), serum total (Studies I, IV-V) and HDL cholesterol (Studies I-II, IV-V) and triglyceride concentrations (Studies I-II, IV-V) with respective enzymatic kits from Roche Diagnostics using an autoanalyzer (Roche Diagnostics Hitachi 917, Hitachi Ltd., Tokyo, Japan). The concentration of LDL cholesterol (Studies I, IV) was calculated using the formula of Friedewald (107). Serum FFAs (Study II) were measured by fluorometric assay (260). Serum free

insulin concentrations were determined by radioimmunoassay (Phadeseeph® Insulin RIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) after precipitation with polyethylene glycol (Study II) and by time-resolved fluoroimmunoassay (Studies I, IV-V) using the Insulin Kit (AUTOdelfia, Wallac, Turku, Finland). C-peptide concentrations were measured using the C-peptide Kits on the AUTOdelfia apparatus (Studies I, IV). HbA_{1c} was measured on an autoanalyzer (DCA 2000 Analyzer, Bayer Ames Technicon, Tarrytown, NY; Study I) by high pressure liquid chromatography (Study V) using the fully automated Glycosylated Hemoglobin Analyzer System (BioRad, Richmond, CA). The homeostasis model assessment of insulin resistance (HOMA-IR; Studies II, IV-V) was calculated from the formula: fasting glucose (mmol/L) x fasting insulin (mU/L) / 22.5 (248). Serum ALT activity (Studies I, IV-V) was determined according to the recommendations of the European Committee for Clinical Laboratory Standards using the Roche Diagnostics Hitachi 917 analyzer. Plasma sodium (Studies I and V), potassium (Studies I and V), chloride (Study V) and bicarbonate (Study V) concentrations were determined with indirect ion-selective electrodes and plasma creatinine (Studies I and V) with an enzymatic kit from Roche Diagnostics using an autoanalyzer (Roche Diagnostics Hitachi Modulator, Hitachi Ltd., Tokyo, Japan). Anion gap (Study V) was calculated from the electrolyte concentrations using the formula ([sodium ion] + [potassium ion]) - ([chloride ion] + [bicarbonate ion]), with all units expressed in mmol/L. Venous blood gas analysis (Study V) was performed using specific electrodes with a blood gas analyzer (Ciba Corning 850, Medfield, MA). Plasma lactate concentration (Study V) was measured using an enzymatic kit from Roche Diagnostics which utilized an autoanalyzer (Cobas Integra 400/800, Roche Instrument Center, Rotkreutz, Switzerland). Serum CRP (Studies I-II, IV-V) was analyzed using a high sensitivity commercial kit (Ultrasensitive CRP Kit, Orion Diagnostica, Espoo, Finland).

CD4+ T-cell count was determined using a flowcytometric apparatus (FACSort/FACSCalibur, Beckton Dickinson, San José, CA). HIV RNA load was measured using either the Cobas HIV-1 Amplicor Monitor version 1.5, normal or ultra sensitive (Roche Diagnostics, Branchburg, NJ; Studies I-II) or the HPS Cobas TaqMan 48n HIV-1 Test (Roche Diagnostics, Branchburg, NJ; Studies I, III-V), with a detection limit of 1.7 log₁₀ copies/mL in both assays. Serum uridine concentrations (Study V) were measured using high performance liquid chromatography as previously described (453).

3.9. STATISTICAL METHODS

All data were tested for normality of distribution using a Kolmogorov-Smirnov test. In Studies I and II, logarithmic transformation was performed on data that were not normally distributed, and the unpaired t-test was used to assess the differences between the study groups. In Studies III-V, unpaired and paired t-tests, Mann-Whitney test and Wilcoxon matched pairs test for continuous variables were used, as appropriate. Categorical variables were compared using Fisher's exact test (Studies I-III, V) or Chi-square test (Study IV), as appropriate. In Study V, safety laboratory follow-up data were analyzed using ANOVA for repeated measures. Microarray (Study IV) results were analyzed using Benjamini and Hochberg *post hoc* correction for multiple comparisons.

Correlation analyses were performed with Pearson product-moment correlation coefficient after logarithmic transformation, when necessary (Studies II-III), or with Spearman's non-parametric correlation coefficient (Studies I,IV). Multiple linear regression analyses (Study I) were performed to identify the independent determinants of the augmentation index.

All calculations were carried out using Lotus 1-2-3 of Lotus SmartSuite Release 9.5 (Lotus Development Corporation, IBM Corporation, New York City, NY;

Studies I-V), SPSS version 11.0.1 (SPSS Inc., Chicago, IL; Study I), Sigma Stat for Windows software version 3.0 (Jandel Corporation, San Rafael, CA; Study III) or GraphPad Prism version 3.02 (Studies I, II, III, V) or version 4.03 (Study IV) (GraphPad Software Inc., San Diego, CA). Data are

expressed as mean \pm standard error of mean (SEM) or median (25% percentile – 75% percentile), as appropriate. Two-tailed p-values <0.05 were considered statistically significant in all analyses except for that of microarray in which the statistical significance was set to $p \leq 0.01$.

RESULTS

1. ARTERIAL STIFFNESS IN cART-ASSOCIATED LIPODYSTROPHY (STUDY I)

1.1. Study subjects

A total of 59 HIV-1-infected cART-treated individuals were studied. The physical and biochemical characteristics of the study subjects are shown in Table 3a. Both the HAART+LD+ and the HAART+LD- groups were similar with respect to age, gender and BMI. Smoking and alcohol consumption habits as well as the use of antihypertensive and lipid-lowering medication were comparable between the groups (Study I, Table 1). The HAART+LD+ group had several laboratory and anthropometric abnormalities indicative of lipodystrophy and insulin resistance. Study subjects in the HAART+LD+ group had greater central body adiposity as depicted by the waist-to-hip ratio (Table 3a), and thinner skinfolds indicating less SAT than their non-lipodystrophic counterparts have (Study I, Table 1). Serum insulin and triglyceride concentrations were significantly higher and HDL cholesterol concentrations significantly lower in the HAART+LD+ as compared to the HAART+LD- group (Table 3a). Serum ALT and hs-CRP concentration were significantly higher in the HAART+LD+ than in the HAART+LD- group (Study I, Table 1). Renal function was normal and comparable in both groups (Study I, Table 1) and, thus, could not be expected to influence the augmentation index.

Characteristics related to HIV-1-infection and antiretroviral treatment of the study groups are provided in Table 3a. The groups were comparable with respect to time since diagnosis of HIV-1 and the most recent viral load and CD4+ T-cell count (Table 3a). Total duration of treatment with one or more antiretroviral drugs (ART) and duration of HAART

(Table 3a) were slightly, although not significantly ($p=0.098$ and $p=0.057$, respectively), longer in the HAART+LD+ than in the HAART+LD- group. Cumulative exposure to NRTIs was greater in the HAART+LD+ group as compared to the HAART+LD- group (3880 ± 242 vs. 2730 ± 299 consumed daily doses, HAART+LD+ vs. HAART+LD-, $p<0.01$). The groups were comparable with regard to cumulative exposure to NNRTIs (240 ± 62 vs. 340 ± 98 consumed daily doses, HAART+LD+ vs. HAART+LD-, NS). Cumulative exposure to PIs tended to be longer in the HAART+LD+ than in the HAART+LD-, but did not quite reach statistical significance (1730 ± 202 vs. 1120 ± 254 consumed daily doses, HAART+LD+ vs. HAART+LD-, $p=0.096$).

1.2. Pulse wave analysis

There were no significant differences between the HAART+LD+ and the HAART+LD- groups with regards to peripheral systolic pressure (126 ± 2 vs. 124 ± 2 mmHg, HAART+LD+ vs. HAART+LD-, NS), diastolic pressure (79 ± 1 vs. 78 ± 2 mmHg, HAART+LD+ vs. HAART+LD-, NS), pulse pressure (47 ± 2 vs. 45 ± 2 mmHg, HAART+LD+ vs. HAART+LD-, NS) or mean arterial pressure (95 ± 1 vs. 94 ± 2 mmHg, HAART+LD+ vs. HAART+LD-, NS). The groups were also similar with respect to these parameters at the level of the aorta. Heart rate was comparable in both groups (66 ± 1 vs. 62 ± 2 beats/min, HAART+LD+ vs. HAART+LD-, NS). Augmentation (6.8 ± 0.8 vs. 5.4 ± 1.1 , HAART+LD+ vs. HAART+LD-, NS), augmentation index (18.2 ± 1.6 vs. 15.6 ± 3.0 , HAART+LD+ vs. HAART+LD-, NS) and the AgI_{HR} (19.2 ± 1.7 vs. 16.2 ± 3.2 , HAART+LD+ vs. HAART+LD-, NS) did not differ to a statistically significant degree between the groups.

Simple linear regression analysis combining both study groups was carried out to identify significant correlates of AgI_{HR} (Study I, Table 3). Variables which correlated with the AgI_{HR} in simple linear

Table 5. Selected models from multiple linear regression analysis explaining variation of the heart rate -corrected augmentation index.

Variable	Model 1 (R ² =0.20)	Model 2 (R ² =0.20)	Model 3 (R ² =0.25)	Model 4 (R ² =0.23)	Model 5 (R ² =0.26)	Model 6 (R ² =0.28)	Model 7 (R ² =0.25)
Age	0.061	0.095	0.191	0.086	0.124	0.098	0.041
Brachial systolic pressure	0.171	0.163	0.198	0.147	0.130	0.114	0.085
HbA _{1c}	0.762	0.935	0.875	0.649	0.704	0.789	0.287
Sodium	0.288	0.368	0.501	0.257	0.370	0.500	0.700
hs-CRP	0.737	0.713	0.608	0.566	0.761	0.566	0.909
Time since HIV-1 diagnosis	0.325	N/A	N/A	N/A	N/A	N/A	N/A
Nadir CD4+ T-cell count	N/A	0.269	N/A	N/A	N/A	N/A	N/A
Duration of ART	N/A	N/A	0.046	N/A	N/A	N/A	N/A
Duration of HAART	N/A	N/A	N/A	0.087	N/A	N/A	N/A
Cumulative exposure to NRTIs	N/A	N/A	N/A	N/A	0.032	N/A	N/A
Cumulative exposure to PIs	N/A	N/A	N/A	N/A	N/A	0.011	N/A
Duration of stavudine therapy	N/A	N/A	N/A	N/A	N/A	N/A	0.051

ART, antiretroviral therapy (treatment with at least one antiretroviral drug); HAART, highly active antiretroviral therapy (minimum of three antiretroviral drugs used concurrently); HbA_{1c}, glycosylated hemoglobin A_{1c}; HIV-1, human immunodeficiency virus type 1; hs-CRP, high sensitivity C-reactive protein; N/A, not applicable; NRTIs, nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors. Adapted from Sevastianova et al. *Antiviral Therapy* 2005; 10:925-35 (Study I), reproduced with permission of the copyright holder.

regression analysis to p-value of ≤ 0.20 were retained for the construction of multiple linear regression models. These included age, brachial systolic pressure, HbA_{1c}, plasma sodium, hs-CRP, time since HIV-1 diagnosis, nadir CD4+ T-cell count and duration of ART, HAART, lamivudine, stavudine, ritonavir and saquinavir therapy as well as cumulative exposure to NRTIs and PIs. In multiple linear regression analysis (Table 5), duration of ART and cumulative exposure to NRTIs and PIs were identified as independent predictors of the AgI_{HR}. Duration of HAART and duration of stavudine therapy showed borderline significance in the prediction of the AgI_{HR}, while the contribution of the other aforementioned HIV-1- and treatment-related factors remained insignificant.

2. SUBCUTANEOUS ADIPOSE TISSUE INFLAMMATION IN cART-ASSOCIATED LIPODYSTROPHY (STUDY II)

2.1. Study subjects

Characteristics of the HAART+LD+ and the HAART+LD- groups are provided in Table 3a. The groups were comparable with respect to age, gender and BMI. The HAART+LD+ group had 2-fold more IAT, 1.5-fold less SAT and 10-fold more liver fat than the HAART+LD- group. The sum of means of skinfold thicknesses taken at five body sites was significantly smaller in the HAART+LD+ than in the HAART+LD- group (38 ± 3 vs. 54 ± 5 mm, HAART+LD+ vs. HAART+LD-, $p < 0.01$). The HAART+LD+ group was also more insulin resistant than the HAART+LD- group as determined by

Figure 5.

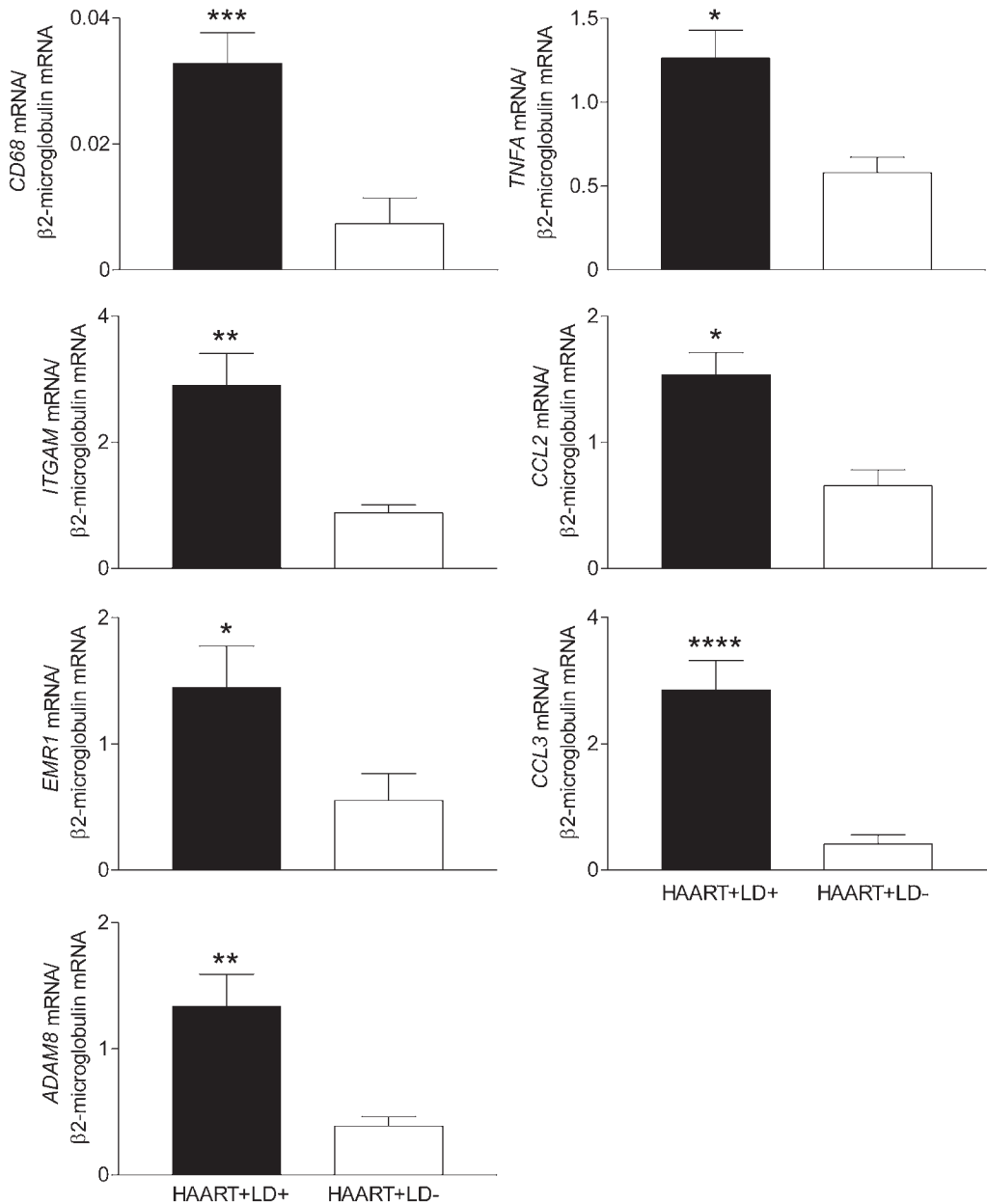


Figure 5. Expression of CD68, integrin α M (ITGAM), epidermal growth factor –like module-containing, mucin-like, hormone receptor –like 1 (EMR1), a disintegrin and metalloproteinase domain 8 (ADAM8), tumor necrosis factor α (TNFA), chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-C motif) ligand 3 (CCL3) mRNA in HIV-1-infected patients with highly active antiretroviral therapy –associated lipodystrophy (HAART+LD+; closed bars) vs. those without lipodystrophy (HAART+LD-, open bars). Data are given as mean \pm SEM. p-values: * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.00001$. Adapted from Sevastianova et al. *American Journal of Physiology: Endocrinology and Metabolism* 2008; 295:E85-91 (Study II), reproduced with permission of the copyright holder.

Figure 6.

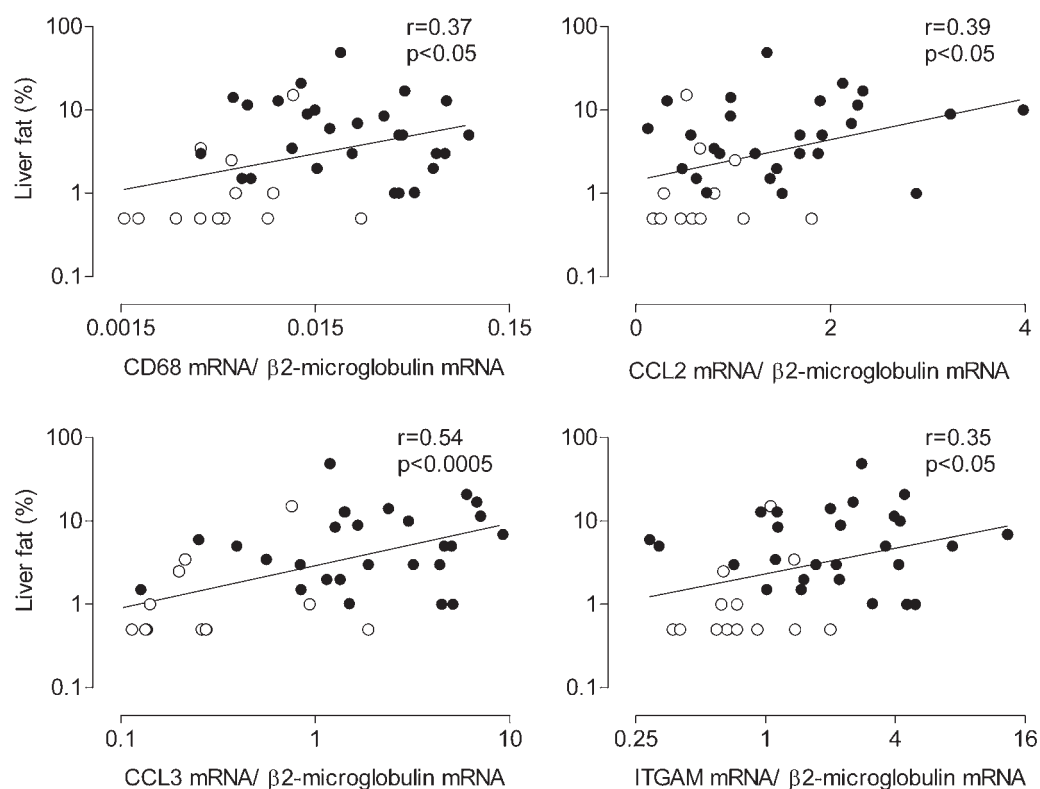


Figure 6. The correlation between adipose tissue mRNA expression of CD68, chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 3 (CCL3) and intergrin α M (ITGAM) with liver fat content. Closed circles, HIV-1-infected patients with highly active antiretroviral therapy –associated lipodystrophy; open circles, HIV-1-infected patients without highly active antiretroviral therapy –associated lipodystrophy. Adapted from Sevastianova et al. *American Journal of Physiology: Endocrinology and Metabolism* 2008; 295:E85-91 (Study II), reproduced with permission of the copyright holder.

serum insulin concentration (Table 3a) and the HOMA-IR index [2.0 (1.5 – 4.2) vs. 1.2 (1.0 – 1.4), HAART+LD+ vs. HAART+LD-, $p<0.005$]. HIV-1 RNA loads and CD4+ T-cell counts were comparable between the groups as were the classes of antiretroviral drugs used (Table 3a).

2.2. Expression of cytokines and macrophage-related genes

Gene expression analyses were carried out on samples from abdominal SAT. Expression of macrophage-related genes (CD68, ITGAM, EMR1 and ADAM8) and those encoding for inflammatory cytokines

(TNFA, CCL2 and CCL3) was significantly higher in lipoatrophic abdominal SAT of the HAART+LD+ group as compared to the HAART+LD- group (Figure 5). There was no difference between the study groups with respect to mRNA concentrations of B2M, the housekeeping gene (data not shown).

2.3. Correlation between adipose tissue gene expression and liver fat content

Expression of CD68, CCL2, CCL3 and ITGAM showed significant positive correlation with liver fat content (Figure 6). Correlations of expression of TNFA,

EMR1 and *ADAM8* with liver fat content remained non-significant. HOMA-IR showed a borderline significant positive correlation with *CD68* mRNA expression ($r=0.29$, $p=0.066$). Correlations between previously measured serum adiponectin concentrations (372) and currently reported inflammatory genes were as follows: *CD68* $r=-0.61$, $p<0.0001$, *CCL2* $r=-0.38$, $p<0.05$; *CCL3* $r=-0.65$, $p<0.0001$; *ITGAM* $r=-0.47$, $p<0.005$; *EMR1* $r=-0.49$, $p<0.001$; *ADAM8* $r=-0.58$, $p<0.0001$ and *TNFA* $r=-0.26$, NS.

3. MITOCHONDRIAL DNA, GENE EXPRESSION AND IMMUNOHISTOCHEMISTRY IN SUBCUTANEOUS ADIPOSE TISSUE IN cART-ASSOCIATED LIPOATROPHY (STUDY III)

3.1. Study subjects

The LA+ and LA- groups were comparable with respect to age, gender, weight, BMI, CD4+ T-cell count and HIV-1 RNA load (Table 3a), as well as with respect to truncal and total body fat (9550 ± 1170 vs. 9380 ± 1620 g, LA+ vs. LA-, NS and 13220 ± 1750 vs. 15680 ± 2550 g, LA+ vs. LA-, NS, respectively). Compared to the LA- group, the LA+ group had significantly less total limb fat (3240 ± 620 vs. 5750 ± 920 g, LA+ vs. LA-, $p<0.05$), a longer history of antiretroviral therapy and an almost statistically significantly longer duration of HIV-1 infection ($p=0.092$, Table 3a).

Of the 18 patients with lipoatrophy, eight received stavudine (d4T+LA+) and ten received zidovudine (AZT+LA+). Age, gender distribution and HIV-1-related characteristics were similar between the d4T+LA+ and the AZT+LA+ subgroups (Study III, Table 3). The subgroups were also similar with respect to use of antiretrovirals other than d4T and AZT (Study III, Table 3). When compared to the AZT+LA+ group, the d4T+LA+ group had a significantly lower weight (66.6 ± 3.5 vs. 79.2 ± 4.2 kg, d4T+LA+ vs. AZT+LA+, $p<0.05$) and lower BMI (21.5 ± 0.9 vs. 25.1 ± 1.0 kg/m², d4T+LA+ vs. AZT+LA+,

$p<0.05$). It also had less limb fat (1550 ± 520 vs. 4590 ± 810 g, d4T+LA+ vs. AZT+LA+, $p<0.01$), less truncal fat (6770 ± 880 vs. 11770 ± 1710 g, d4T+LA+ vs. AZT+LA+, $p<0.05$) and less total fat (8660 ± 1380 vs. 16880 ± 2440 g, d4T+LA+ vs. AZT+LA+, $p<0.05$) than the AZT+LA+ group. The amount of IAT was similar between the LA subgroups (Study III, Table 3).

3.2. Mitochondrial DNA content

The amount of mtDNA was significantly lower in the LA+ than in the LA- group (238 ± 129 vs. 585 ± 558 copies/cell, LA+ vs. LA-, $p<0.01$). Furthermore, the d4T+LA+ group had a significantly lower mtDNA copy number as compared to the AZT+LA+ group (139 ± 59 vs. 317 ± 115 copies/cell, d4T+LA+ vs. AZT+LA+, $p<0.001$).

In order to calculate the relative number of RNA transcripts per molecule of mtDNA template, expression of mtDNA-encoded genes was normalized for the amount of mtDNA molecules. Relative to the number of mtDNA copies per cell, transcription of mtDNA-encoded genes, *16SRNA* and *COX3*, was significantly higher in the LA+ compared to the LA- group (0.34 ± 0.04 vs. 0.15 ± 0.01 , LA+ vs. LA-, $p<0.005$ for *16SRNA* and 0.42 ± 0.05 vs. 0.25 ± 0.02 , LA+ vs. LA-, $p<0.005$ for *COX3*). This effect was more pronounced, albeit not quite to the level of statistical significance in the case of *16SRNA*, in the d4T+LA+ than in the AZT+LA+ group (0.52 ± 0.06 vs. 0.35 ± 0.03 , d4T+LA+ vs. AZT+LA+, $p<0.005$ for *COX3* and 0.42 ± 0.06 vs. 0.28 ± 0.02 , d4T+LA+ vs. AZT+LA+, $p=0.07$ for *16SRNA*).

3.3. Expression of genes involved in mitochondrial function and biogenesis

COX3, encoded by mtDNA, was slightly, but not significantly, lower and the nDNA-encoded *COX4* significantly higher in the LA+ compared to the LA- group (Table 6). These changes resulted in a significantly lower *COX3/COX4* ratio in the LA+ than in the LA- group (Table 6). The d4T+LA+

Table 6. Relative expression of selected genes in abdominal SAT from HIV-1-infected patients with and without cART-associated lipodystrophy.

Gene	LA+	LA-	d4T+LA+	AZT+LA+
<i>Mitochondrial function and biogenesis</i>				
COX3	0.87 ± 0.10	1.28 ± 0.24	0.64 ± 0.08†	1.06 ± 0.14
COX4	2.53 ± 0.23**	1.37 ± 0.14	2.77 ± 0.33	2.29 ± 0.32
COX3/COX4	0.33 ± 0.04***	0.89 ± 0.12	0.24 ± 0.03†	0.43 ± 0.06
16SRNA	0.70 ± 0.08	0.72 ± 0.11	0.50 ± 0.07†	0.86 ± 0.11
POLG1	1.21 ± 0.11	1.19 ± 0.04	0.93 ± 0.14†	1.41 ± 0.12
POLG2	1.24 ± 0.07	1.09 ± 0.04	1.20 ± 0.06	1.26 ± 0.11
TFAM	1.30 ± 0.12	1.22 ± 0.05	1.38 ± 0.24	1.24 ± 0.10
PGC1B	6.74 ± 1.68*	0.95 ± 0.11	6.52 ± 2.60	6.90 ± 2.32
<i>Adipogenesis</i>				
SREBP1C	0.40 ± 0.09***	1.98 ± 0.38	0.25 ± 0.19†	0.47 ± 0.10
CEBPA	1.96 ± 0.27	2.74 ± 0.41	1.29 ± 0.21†	2.50 ± 0.40
PPARG	1.82 ± 0.25	1.48 ± 0.15	1.58 ± 0.36	2.01 ± 0.35
LMNA	1.26 ± 0.14	1.05 ± 0.08	1.09 ± 0.21	1.44 ± 0.16
LMNB	1.39 ± 0.19*	0.61 ± 0.15	1.50 ± 0.32	1.33 ± 0.24
<i>Lipid and glucose metabolism</i>				
FASN	3.64 ± 0.92***	9.67 ± 0.94	3.00 ± 1.27	4.22 ± 1.36
GLUT4	1.42 ± 0.28*	2.87 ± 0.56	0.90 ± 0.20	1.78 ± 0.42
PLIN1	1.03 ± 0.28	1.37 ± 0.19	0.53 ± 0.27†	1.52 ± 0.43
HEXOK1	1.21 ± 0.13	1.42 ± 0.09	0.86 ± 0.18†	1.48 ± 0.13
<i>Inflammation, oxidative stress and apoptosis</i>				
IL1B	1.39 ± 0.04***	0.12 ± 0.03	0.38 ± 0.06	0.40 ± 0.05
SOD1	2.03 ± 0.20*	1.36 ± 0.06	1.71 ± 0.20	2.29 ± 0.30
PCNA	1.81 ± 0.09*	1.49 ± 0.13	1.94 ± 0.16	1.70 ± 0.09
GPX1	1.84 ± 0.26	1.31 ± 0.12	1.35 ± 0.15	2.18 ± 0.40
FAS	6.01 ± 0.73**	2.38 ± 0.39	6.65 ± 1.05	5.51 ± 1.02
P53	1.02 ± 0.08	1.09 ± 0.09	0.97 ± 0.16	1.05 ± 0.09

Gene expression values are calculated from the real-time reverse transcriptase polymerase chain reaction in relation to the mean expression of three housekeeping genes (actin β , ACTB; acidic ribosomal phosphoprotein P0, 36B4; β 2-microglobulin, B2M). Data are shown as mean \pm SEM. p-values: * <0.05 , ** <0.005 , *** <0.0005 for comparison of LA+ vs. LA-; † <0.05 for comparison of d4T+LA+ vs. AZT+LA+. AZT+LA+, lipodystrophic HIV-1-infected patients on zidovudine-containing antiretroviral regimen; cART, combination antiretroviral therapy; d4T+LA+, lipodystrophic HIV-1-infected patients on stavudine-containing antiretroviral regimen; HIV-1, human immunodeficiency virus type 1; LA+, lipodystrophic HIV-1-infected patients; LA-, non-lipodystrophic HIV-1-infected patients; SAT, subcutaneous adipose tissue. For full names of the genes, please see text. Adapted from Sievers et al. *Journal of Infectious Diseases* 2009; 200:252-62 (Study III), data are reproduced with permission of the copyright holder.

group had significantly lower expression of *COX3* and *16SRNA* as compared to the AZT+LA+ group (Table 6). Also, the *COX3/COX4* ratio was significantly lower in the d4T+LA+ compared to the AZT+LA+ group (Table 6). Expression of *POLG1* and *POLG2* did not differ between the LA+ and the LA- groups (Table 6). Expression of *POLG1*, but not of *POLG2*, was significantly lower in the d4T+LA+ compared to the AZT+LA+ group (Table 6). Expression of *TFAM* was comparable between all groups (Table 6). Expression of *PGC1B* was 7-fold higher in the LA+ compared to the LA- group (Table 6). There was no significant difference between the d4T+LA+ and the AZT+LA+ groups with regard to *PGC1B* (Table 6).

3.4. Expression of genes involved in adipogenesis

SREBP1C was significantly lower in the LA+ compared to the LA- group (Table 6). Among all study patients, there was an inverse correlation between expression of *SREBP1C* and *PGC1B* ($r=-0.58$, $p<0.005$) and a positive correlation between *SREBP1C* expression and mtDNA copy numbers ($r=0.55$, $p<0.05$). *CEBPA* and *SREBP1C* transcripts were lower in the d4T+LA+ than in the AZT+LA+ group (Table 6). There was no statistically significant difference between either LA+ vs. LA- or d4T+LA+ vs. AZT+LA+ with respect to expression of *PPARG* (Table 6). *LMNB* expression was significantly higher in the LA+ as compared to the LA- group (Table 6). Expression of *LMNA* and *LMNB* was comparable between the two LA subgroups (Table 6).

3.5. Expression of genes involved in lipid and glucose metabolism

The LA+ group had lower expression of *FASN* and *GLUT4* transcripts than the LA- group (Table 6). Expression of *GLUT4* was not significantly different between the LA subgroups, although it tended to be lower in the d4T+LA+ than in the AZT+LA+ group ($p=0.12$, Table 6). *PLIN1* and *HEXOK1* transcripts were significantly lower in the d4T+LA+ than in the AZT+LA+ group

(Table 6). Among all patients *GLUT4* expression correlated positively with both *FASN* ($r=0.73$, $p<0.001$).

3.6. Expression of genes involved in inflammation, oxidative stress and apoptosis

Expression of the inflammatory cytokine *IL1B* was higher in the LA+ than in the LA- group (Table 6). Expression of *IL1B* correlated inversely with mtDNA copy numbers ($r=-0.42$, $p<0.005$). Expression of *SOD1* and *PCNA* was higher in the LA+ than in the LA- group (Table 6). Expression of *FAS*, but not of *p53*, was increased in the LA+ compared to the LA- group (Table 6). There was no statistically significant difference in expression of markers of inflammation, oxidative stress and apoptosis between the d4T+LA+ and the AZT+LA+ groups (Table 6).

3.7. Immunohistochemistry

In abdominal SAT of the LA+ group, there were 7-fold more macrophages than in that of the LA- group (14.1 ± 13.1 vs. 2.3 ± 1.9 macrophages per 100 000 arbitrary area units, LA+ vs. LA-, $p<0.05$; Study III, Figure 1). The number of macrophages did not differ between the LA subgroups. The number of CLSs and PLIN1-free adipocytes did not differ between LA+ and LA- or between the LA subgroups (Study III, Figure 2).

4. DORSOCERVICAL SUBCUTANEOUS ADIPOSE TISSUE IN cART-ASSOCIATED LIPODYSTROPHY (STUDY IV)

4.1. Study subjects

Characteristics of the study patients are provided in Table 3b. The cART+LD+ and the cART+LD- groups were comparable with respect to age, gender and BMI. The cART+LD+ group had significantly higher ratios of dorsocervical to abdominal SAT and IAT to abdominal SAT than the cART+LD- group [0.8 ($0.5 - 1.3$) vs. 0.4 ($0.4 - 0.6$),

Figure 7.

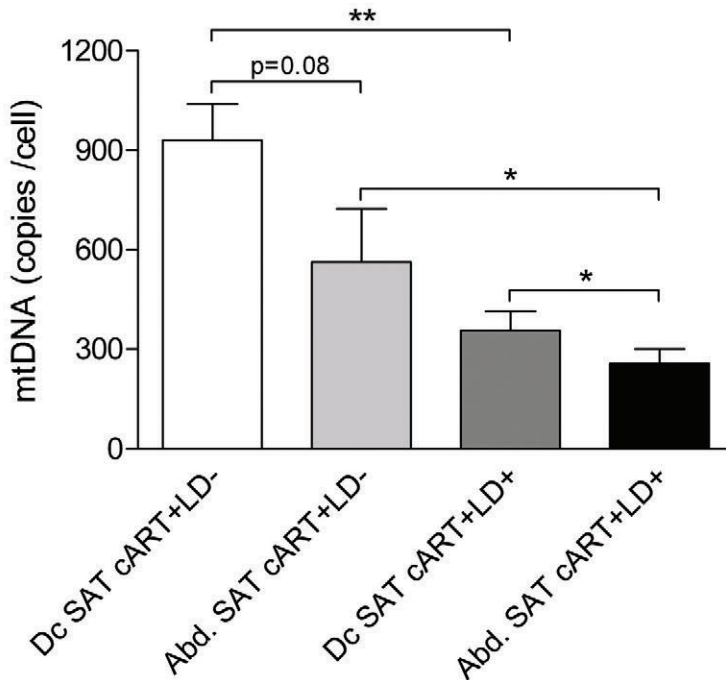


Figure 7. Number of mitochondrial DNA (mtDNA) copies per cell in dorsocervical (Dc SAT) and abdominal subcutaneous (Abd. SAT) adipose tissue of patients on combination antiretroviral therapy (cART) with (cART+LD+) and without (cART+LD-) treatment-associated lipodystrophy. Data are given as mean \pm SEM. *p*-values: **p*<0.05, ***p*<0.0001. Adapted from Sevastianova et al. *Diabetes* 2011; 60:1894-900 (Study IV), reproduced with permission of the copyright holder.

cART+LD+ vs. cART+LD-, *p*<0.05 and 2.3 (0.8 – 4.5) vs. 0.5 (0.5 – 0.7), cART+LD+ vs. cART+LD-, *p*<0.0005]. Fasting insulin and triglyceride concentrations were significantly higher and HDL cholesterol concentrations significantly lower in the cART+LD+ compared to the cART+LD- group (Table 3b).

4.2. Mitochondrial DNA content

The mtDNA copy number was significantly higher in dorsocervical compared to abdominal SAT in the cART+LD+ group (356 \pm 59 vs. 256 \pm 44 copies/cell, dorsocervical vs. abdominal SAT in cART+LD+, *p*<0.05). A similar trend was found in the cART+LD- group (930 \pm 109 vs. 563 \pm 161 copies/cell, dorsocervical vs. abdominal SAT in cART+LD-, *p*=0.08, Figure 7). The mtDNA copy number was 62% lower in the cART+LD+ than in

the cART+LD- group in dorsocervical (*p*<0.0001) and 54% lower in abdominal SAT (*p*<0.05, Figure 7).

4.3. Histology

Dorsocervical adipose tissue in both the cART+LD+ and the cART+LD- groups had significantly less adipocytes (55 \pm 6 vs. 77 \pm 5%, dorsocervical vs. abdominal SAT in cART+LD+, *p*<0.05 and 61 \pm 5 vs. 89 \pm 4%, dorsocervical vs. abdominal SAT in cART+LD-, *p*<0.00001) and more connective tissue (45 \pm 6 vs. 23 \pm 5%, dorsocervical vs. abdominal SAT in cART+LD+, *p*<0.05 and 39 \pm 5 vs. 11 \pm 4%, dorsocervical vs. abdominal SAT in cART+LD-, *p*<0.00001) than abdominal SAT (Study IV, Figure 2). The two depots were similar with respect to variation in adipocyte size, cell membrane rupture, presence of lipogranulomata and cells

morphologically typical of brown adipose tissue, irrespective of lipodystrophy status (data not shown).

Abdominal, but not dorsocervical, SAT from patients in the cART+LD+ group contained significantly more lipogranulomata ($p < 0.05$) than the corresponding tissue from the cART+LD- group (Study IV, Figure 2). Other histological parameters such as adipocyte size, cell membrane rupture and the presence of cells morphologically typical of brown adipose tissue did not differ significantly between the cART+LD+ and the cART+LD- groups in either depot (data not shown).

4.4. Microarray

Microarray analysis comparing dorsocervical to abdominal SAT within the cART+LD+ group identified 27819 probes for genes of which 99 were differentially expressed. These 99 probes corresponded to 75 unique Entrez gene IDs. Of these 75 genes, 61 were downregulated and 14 upregulated in dorsocervical as compared to abdominal SAT (Study IV, Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db11-0075/-/DC1>). The differentially expressed genes were involved in regulation of gene transcription, organ development and regionalization, immune response and inflammation, as well as in composition and organization of the extracellular matrix and cytoskeleton. Three of the top six genes downregulated in dorsocervical as compared to abdominal SAT were homeobox genes (*HOX*) *A10*, *HOXC9* and *HOXC8*. These genes are known to be transcription factors that provide cells with specific positional identities on the anterior-posterior axis. Among the genes upregulated in dorsocervical as compared to abdominal SAT was short stature homeobox 2 (*SHOX2*), a gene responsible for cranio-facial lateralization of the embryonic cells.

Microarray analysis comparing dorsocervical to abdominal SAT within the cART+LD- group identified 27900 probes for genes of which 18 were differentially expressed. Of these, 13 corresponded to

unique Entrez Gene IDs. Of these 13 genes, 9 were downregulated and 4 upregulated in dorsocervical as compared to abdominal SAT (Study IV, Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db11-0075/-/DC1>). The differentially expressed genes were involved in regulation of transcription, organ development and regionalization. The top three downregulated genes were also on the top of the list in the cART+LD+ group, namely *HOXA10*, *HOXC9* and *HOXC8*. In a similar fashion to the cART+LD+ group, *SHOX2* was among the genes that were upregulated to the greatest degree in dorsocervical as compared to abdominal SAT.

4.5. Gene expression by real-time reverse transcriptase polymerase chain reaction

Expression of *SHOX2* was 4-fold higher in dorsocervical as compared to abdominal SAT in the cART+LD+ and 6-fold higher for the respective comparison in the cART+LD- group. Expression of *HOXA10*, *HOXC9* and *HOXC8* was 18-, 8- and 11-fold lower in dorsocervical compared to abdominal SAT in the cART+LD+ group (Figure 8). In the cART+LD- group, expression of *HOXA10*, *HOXC9* and *HOXC8* were 21-, 9- and 10-fold lower in dorsocervical than in abdominal SAT. Fold change differences for any of the homeobox genes analyzed were not significantly different between the cART+LD+ and the cART+LD- groups (Figure 8).

UCP1 mRNA was detected in similarly minute quantities in some, but not all, of the dorsocervical and abdominal SAT samples from selected patients with prominent lipohypertrophy (“buffalo hump”) in the dorsocervical region (data not shown). The measured mRNA concentrations, however, were approximately 1000 times lower than those reported in human BAT previously (410).

Expression of *CD68* mRNA, used as a marker of macrophages and, thus, inflammation, was significantly lower in dorsocervical as compared to abdominal

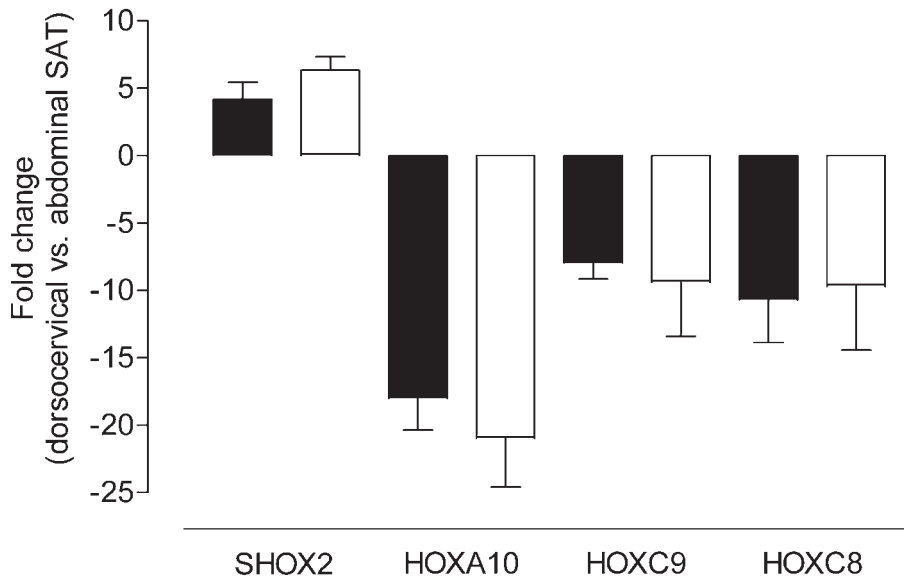
Figure 8.

Figure 8. Mean fold change in mRNA expression (measured by real-time reverse transcriptase polymerase chain reaction) in dorsocervical as compared to abdominal subcutaneous adipose tissue (SAT) in the combination antiretroviral therapy (cART) –treated lipodystrophic (cART+LD+ group, closed bars) and non-lipodystrophic (cART+LD- group, open bars) patients. Positive values on the Y-axis indicate upregulation and negative values downregulation of the gene in question in dorsocervical as compared to abdominal SAT. Error bars, SEM. Adapted from Sevastianova et al. *Diabetes* 2011; 60:1894-900 (Study IV), reproduced with permission of the copyright holder.

SAT in the cART+LD+ [0.39 (0.08 - 6.63) vs. 0.56 (0.19 - 6.48), dorsocervical vs. abdominal SAT in cART+LD+, $p < 0.05$]. Similar findings were noted in the cART+LD- group ($p < 0.05$). Expression of *CD68* mRNA did not differ between the study groups in either adipose tissue depot (data not shown).

5. TREATMENT OF cART-ASSOCIATED LIPOATROPHY WITH URIDINE (STUDY V)

5.1. Study subjects

A total of 39 patients were screened for the study. Of these, 15 did not meet the inclusion criteria and four refused to participate. Of the eligible 20 patients, ten were randomized to receive uridine supplementation and ten to placebo. One patient in the uridine group discontinued

the study after two days due to the taste of the product. One patient in the placebo group died of myocardial infarction. The remaining 18 patients completed the study. Antiretroviral and all other medications remained unchanged in all participants throughout the study. At baseline, the uridine and placebo groups were similar with respect to age, gender, body composition, metabolic parameters and HIV-1-related characteristics (Table 3b).

5.2. Effect of uridine treatment on body composition

Data on the effects of uridine and placebo on body composition in patients with cART-associated lipodystrophy are provided in Table 7. The mean increases in limb fat mass, total body fat mass and IAT volume were significantly greater in the uridine compared to the placebo

Table 7. Effects of uridine and placebo on body composition and metabolic parameters.

Variable	Uridine group		Placebo group	
	Baseline	3 months	Baseline	3 months
	(n=10)	(n=9)	(n=10)	(n=9)
Age (years)	47 ± 2		49 ± 5	
Male / female	9 / 1		8 / 2	
Weight (kg)	76.7 ± 4.7*	79.0 ± 5.4	70.5 ± 3.2	69.8 ± 3.4
Body mass index (kg/m ²)	24.4 ± 1.1*	25.1 ± 1.3	22.6 ± 0.9	22.4 ± 0.9
Total limb fat (g)	3080 ± 850***	4260 ± 940†	3060 ± 800	3340 ± 880
Total limb fat (%)	20.6 ± 5.9***	28.1 ± 6.0	24.0 ± 6.0	25.7 ± 6.4
Total truncal fat (g)	9980 ± 1730***	11570 ± 1910†	8660 ± 1350	8490 ± 1390
Total truncal fat (%)	24.1 ± 3.3**	27.2 ± 3.5†	23.5 ± 2.8	23.5 ± 2.9
Total fat (g)	13490 ± 2580***	16330 ± 2810††	12140 ± 2090	12280 ± 2250
Total fat (%)	17.3 ± 2.9***	20.6 ± 3.0	17.4 ± 2.7	17.8 ± 2.7
Intra-abdominal adipose tissue (cm ³)	2230 ± 300*	2520 ± 370†	2180 ± 470	1940 ± 460
Liver fat (%)	5.9 ± 2.8	7.7 ± 3.9	12.4 ± 4.7	8.7 ± 3.1
Serum insulin (mU/L)	12.3 ± 3.8	13.7 ± 5.7	10.8 ± 2.2	9.3 ± 2.4
HOMA-IR index	3.2 ± 0.9	3.2 ± 1.5	2.8 ± 0.7	2.0 ± 0.6
HbA _{1c} (%)	5.4 ± 0.4	5.1 ± 0.1	5.2 ± 0.3	4.9 ± 0.3
Serum triglycerides (mmol/L)	2.9 ± 0.4	3.0 ± 0.4	3.4 ± 0.8	3.0 ± 0.9
Serum cholesterol (mmol/L)	5.6 ± 0.4	5.7 ± 0.4	4.8 ± 0.3	5.3 ± 0.5
Serum HDL cholesterol (mmol/L)	1.23 ± 0.09	1.15 ± 0.08†	1.14 ± 0.05	1.22 ± 0.09

Data are mean ± SEM. *p*-values: **p*<0.05, ***p*<0.01, ****p*<0.001 for comparison between baseline vs. 3 months within the uridine or placebo group; †*p*<0.05, ††*p*<0.01 for comparison of change in the uridine vs. placebo group. HbA_{1c}, glycosylated hemoglobin A_{1c}; HDL, high-density lipoprotein, HOMA-IR, homeostasis model assessment of insulin resistance. Adapted from Sutinen et al. *Antiviral Therapy* 2007; 12:97-105 (Study V), reproduced with permission of the copyright holder.

group (Figure 9). After 3 months of treatment with uridine, total limb fat mass increased by 29%, truncal fat mass by 10%, and total body fat mass by 14% (Table 7). In addition, the percentage of limb, truncal and total body fat increased significantly in the uridine, but not in the placebo, group (Table 7). In the uridine group, limb fat accounted for 18 ± 3% and truncal fat for 78 ± 3% of total fat mass at baseline. After 3 months of treatment, the proportion of limb fat to total fat mass increased to 25 ± 2% (*p*<0.05), whereas

that of truncal fat to total fat mass decreased to 72 ± 2% (*p*<0.05). In the uridine group, the increase in total limb fat was similar in patients treated with both AZT (800 ± 240 g) and d4T (990 ± 110 g). Body weight and IAT increased significantly in the uridine group while it remained unchanged in the placebo group. There was no change in abdominal SAT (1350 ± 390 vs. 1480 ± 440 cm³, uridine group baseline vs. 3 months, NS; 1250 ± 390 vs. 1220 ± 440 cm³, placebo group baseline vs. 3 months, NS). In contrast

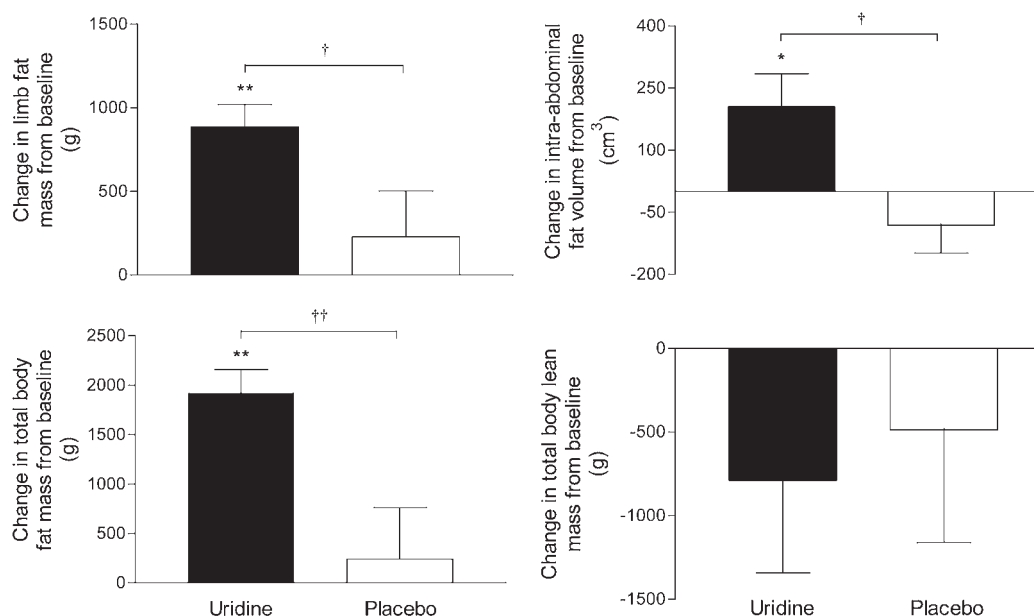
Figure 9.

Figure 9. Change from baseline in total limb fat mass, intra-abdominal fat volume, total body fat mass and lean body mass in the uridine (closed bars) as compared to placebo (open bars) group. Data are mean \pm SEM. *p*-values: †*p*<0.05 and ††*p*<0.01 for comparison of the change in the uridine vs. placebo group; **p*<0.05 and ***p*<0.001 for comparison between baseline and 3 months within the uridine group. Adapted from Sutinen et al. *Antiviral Therapy* 2007; 12:97-105 (Study V), reproduced with permission of the copyright holder.

to the significant increase in total body fat mass in the uridine group, there was no significant change in total lean body mass in either group (Figure 9). Liver fat content did not change significantly in either group (Table 7).

5.3. Effect of uridine treatment on metabolic parameters

Uridine or placebo treatment had no effect on fasting insulin, total cholesterol and triglyceride concentrations, HOMA-IR and HbA_{1c} (Table 7). Serum HDL cholesterol showed a non-significant decrease in the uridine group and a non-significant increase in the placebo group, with the mean difference between the groups being, however, statistically significant (Table 7). Although there were no statistically significant changes in blood gas analyses between the study groups, venous blood pH and base

excess increased significantly within the uridine group (Study V, Table 3). In the uridine group, a trend was observed in terms of an increase of the bicarbonate concentration (24.0 ± 0.3 vs. 24.9 ± 0.3 mmol/L, baseline vs. 3 months, *p*=0.052). Plasma ALT and lactate as well as serum hs-CRP concentrations or anion gap did not change significantly in either group (Study V, Table 3).

None of the patients, who had an undetectable HIV-1 viral load (<50 copies/mL) at baseline, lost virologic control during the study. HIV-1 viral load remained unchanged among patients with measurable viremia at baseline (one patient in the uridine group and two in the control group). CD4+ cell counts remained stable in both groups (594 ± 81 vs. 613 ± 92 cells/mm³, baseline vs. 3 months in the uridine group, NS; 517 ± 88 vs. 503 ± 93 cells/mm³, baseline vs. 3 months in the placebo group, NS).

5.4. Circulating uridine concentrations

At baseline, serum uridine concentrations did not differ between the groups (5.2 ± 0.5 vs. 4.4 ± 0.5 $\mu\text{mol/L}$, uridine vs. placebo group, NS) and were similar to those previously described by the same laboratory for HIV-1-negative subjects (5.6 ± 1.1 $\mu\text{mol/L}$) (405). On day 71, serum uridine concentrations increased to 16.9 ± 1.9 $\mu\text{mol/L}$ ($p < 0.001$) in the uridine group, but remained stable in the placebo group (5.0 ± 0.4 $\mu\text{mol/L}$, NS). The difference between the groups was highly significant ($p < 0.001$).

5.5. Safety and tolerability

Both uridine and placebo were well tolerated. One patient in the uridine group discontinued the study due to the taste of the product and one patient on placebo died of myocardial infarction. No other side effects were reported in either group during the 3 months of treatment. Total blood count, ALT, creatinine, sodium and potassium concentrations, which were measured additionally on days 11, 41 and 71 to assess the safety of the intervention, remained unchanged in both groups (data not shown).

DISCUSSION

1. SUBJECTS AND METHODS

1.1. Subjects

All of the patients in Studies I-V were infected with HIV-1 and treated with antiretroviral agents, but differed with respect to the presence or absence of cART-associated lipodystrophy. While this set-up allowed for an analysis of the effects of lipodystrophy on the parameters evaluated, the separate impact of the HIV-1 infection or cART on these parameters could not be determined. This is particularly important in the context of interpretation of expression of proinflammatory genes, as HIV-1 infection *per se* is known to induce expression of inflammatory cytokines (85,125,201). Furthermore, human preadipocytes have been shown *in vitro* to express receptors used by HIV-1 for cell entry (143). If this applies also to (pre)adipocytes *in vivo* in humans, HIV-1 may be able to influence directly the transcription of genes, including proinflammatory genes, in these cells. However, as the study groups in Studies I-V were highly similar with respect to time since the diagnosis of HIV-1 and the severity of immunodeficiency, as depicted by the nadir CD4+ T-cell count as well as the most recent CD4+ T-cell count and HIV-1 viral load, it can only be assumed that the potential effect of the HIV-1 infection, if present, is similar between the lipodystrophic/-atrophic study groups.

The majority of the study subjects were male. This does not represent the normal gender distribution among the HIV-1-infected patients in the HIV outpatient clinic of the Helsinki University Central Hospital or worldwide. Potentially, this aspect may have an impact on the generalizability of the results to the greater HIV-1-infected population. However, in the present studies, the proportion of

males to females was similar between the study groups compared. Furthermore, in Study I, the data analysis was repeated after omitting female study subjects, and no change in the outcome occurred.

Although case definitions have been proposed (48,50,54), at present there is no universal consensus on the diagnostic criteria for cART-associated lipodystrophy. Therefore, in the present studies, like in most others, allocation of study patients to either the lipodystrophic or the non-lipodystrophic group relied on the clinical definition of cART-associated lipodystrophy, i.e., self-reported and investigator-confirmed symptoms of loss of SAT with or without accumulation of adipose tissue intra-abdominally or in the dorsocervical upper-trunk.

1.2. Methods

1.2.1. Pulse wave analysis

In Study I, PWA was used for the first time to assess arterial compliance in HIV-1-infected cART-treated patients. PWA is a rapid, non-invasive, technically simple, relatively inexpensive and highly reproducible technique (355,423). It has the advantage of reflecting changes not only in an isolated artery, but also in the entire vascular tree (355,423). In PWA, a commercially available software uses a generalized transfer function to calculate aortic blood pressure from the applanation tonometry measurements from the radial artery. The transfer function has been validated and the results proven comparable with those of catheterization studies (58,175) and perioperative blood pressure recordings (309). No mathematical model is perfect, however, and it should be noted that the augmentation index is influenced not only by arterial stiffness/elasticity, but also by ventricular ejection and arterial reflecting sites (288).

1.2.2. Body composition analyses

In Studies I-V, body composition was measured by several independent

non-invasive methods including anthropometry, measurement of skinfold thickness, BIA, DEXA, MRI and $^1\text{H-MRS}$. Of these methods, the first three mentioned are readily available, portable, inexpensive and easy to operate. They are, however, prone to considerable inter-observer variation (392). In the present studies, a single investigator performed all measurements in an attempt to optimize the reproducibility of data. The BIA method has been validated in HIV-1-infected patients before the cART era (187), but although it is known to be more reliable in lean subjects with sparse SAT than in obese subjects (392), it has not been validated in lipodystrophic cART-treated patients. In fact, BIA might be particularly unsuitable in patients with uneven body fat distribution, as is the case in cART-associated lipodystrophy (344), for more than 90% of the measured whole-body impedance arises from the limbs that represent only <50% of fat free mass (103) and might particularly poorly represent the rest of the body.

DEXA was used to measure total body fat (in both absolute and percentage equivalents) as well as that of limbs and trunk separately. DEXA is a highly accurate method for the assessment of body composition, but its limited availability and cost makes it unpractical for wide-spread clinical use. The precision of body fat and lean body mass measurement by DEXA has been validated in animal models and by comparison with, e.g., neutron activation analysis (87). The reproducibility of repeated DEXA measurements is good if conducted on the same instrument, while inter-instrument difference may be substantial, presumably due to differences in device calibration (87).

As DEXA does not differentiate between SAT and IAT, MRI technology was needed to complement it in the present studies. With the 16 slices covering the area from 8 cm above to 8 cm below the 4th and 5th lumbar interspace, this approach can be estimated to capture approximately 60% of the total IAT volume. Accuracy of

measurements of adipose tissue mass by MRI has been validated against human cadaver dissection and has proved very precise (average difference of 0.076 kg) (3). The reproducibility of repeated measurements of IAT and SAT in our laboratory is 5% and 3%, respectively (369). The reproducibility data from our laboratory are also available for repeated measurements of liver fat using $^1\text{H-MRS}$, with reproducibility of 11% (369). $^1\text{H-MRS}$ data have also been validated against histologically determined lipid content (190) and against estimates of fatty infiltration in the liver by CT (332) and MRI (229), and have proven to be highly accurate.

1.2.3. Real-time reverse transcriptase polymerase chain reaction

Real-time RT-PCR is the most sensitive method for quantification of mRNA (31). It should, however, be emphasized that mRNA concentration does not necessarily reflect protein expression due to factors like post-transcriptional regulation and protein's secondary/tertiary structure assembly. It should also be remembered that gene expression detected in adipose tissue does not originate from adipocytes only, but also from preadipocytes, fibroblast, endothelial cells, macrophages and leukocytes, all of which reside in the adipose tissue and, thus, contribute to the observed mRNA and protein concentrations. Furthermore, a sufficient sample size is critical for the reliability of studies of gene expression in adipose tissue, as RNA yields from adipose tissue are up to 200-fold lower per weight of tissue than in, e.g., liver or muscle (240). This can be particularly challenging in the case of severely lipoatrophic individuals.

1.2.4. Microarray

Microarray analysis covering the whole human genome (20000 - 25000 genes) generates a vast amount of data. In order to avoid false positive findings, it is important to utilize stringent filtering

criteria on the acquired data (correction for multiple comparisons, using lower p-value as a “threshold” of statistical significance) and performing validation analyses (histology and real-time RT-PCR) on the findings.

1.2.5. Histology and immunohistochemistry

One considerable limitation of histological and immunohistochemical analyses is the requirement of a surgical biopsy specimen. This limited analyses in Study II, in which only needle aspiration biopsies of adipose tissue were available. In addition, histological and immunohistochemical analyses are prone to sampling error and are more robust in their sensitivity than, e.g., real-time RT-PCR. In Study IV, we encountered a discrepancy between the histological and the gene expression data. While an increased number of lipogranulomata (a histological indication of the presence of macrophages) was found in abdominal SAT from the cART+LD+ group in comparison to the corresponding tissue in the cART+LD- group, there was no such difference between the study groups in mRNA expression of CD68, a gene that is widely used as a marker for macrophages. This could be due to a limited sensitivity of the analytical methods, i.e., a type 2 error or, alternatively, the discrepancy between histological and real-time RT-PCR results may be a consequence of different macrophage phenotypes being detected by each method. While all macrophages are recognized by a CD68 antibody, only some macrophages are known to be proinflammatory and secrete cytokines such as TNFA and IL6 that were presently measured by real-time RT-PCR (22,120). Indeed, it is possible that macrophages resident in adipose tissue, and detected by the histological analysis of Study IV, are anti-inflammatory, functioning mainly in engulfment of cellular debris and tissue repair, and do not secrete inflammatory cytokines (22,120).

2. VASCULAR COMPLICATIONS IN cART-ASSOCIATED LIPODYSTROPHY

2.1. Arterial stiffness

Cumulative exposure to cART has been associated with an increased incidence of myocardial infarction (108,109). The increment in the relative risk of myocardial infarction seen in these studies could only partially be explained by conventional risk factors (108,109). The augmentation index, a surrogate of systemic arterial stiffness measured by PWA technique, is known to predict cardiovascular events and mortality independent of dyslipidemia and other confounders in HIV-1-negative subjects (227,420,421). Prior to Study I, its value in explaining increased cardiovascular risk beyond that attributable to conventional risk factors had not been assessed in HIV-1-infected cART-treated patients with and without cART-associated lipodystrophy. Despite the representativeness of the lipodystrophic and the non-lipodystrophic study groups in Study I, the presence of cART-associated lipodystrophy was not seen to have any effect on parameters measured by PWA. Following the publication of Study I, only one study has readdressed the issue of arterial stiffness in cART-associated lipodystrophy (401). This study, comparing 55 cART-treated non-lipodystrophic and 23 cART-treated lipodystrophic patients, confirmed the findings of Study I that there are, indeed, no differences between these groups with respect to arterial stiffness as depicted by pulse wave velocity (401).

In Study I, antiretroviral therapy *per se*, both in terms of duration of ART (treatment with at least one antiretroviral drug) and in terms of cumulative exposure to NRTIs and PIs, was found to be associated with the AgI_{HR} independent of age and brachial blood pressure. Duration of HAART (minimum of three antiretroviral drugs used concurrently, i.e., contemporary cART) and d4T therapy were notably, albeit only borderline

significantly, predictive of the AgI_{HR} . Age, as anticipated, was a strong, but not the sole, predictor of the AgI_{HR} , whereas laboratory and anthropometric measures related to the cART-associated lipodystrophy were not predictive of the AgI_{HR} . The association of cumulative exposure to cART and measures of arterial stiffness, e.g., pulse wave velocity and the aortic augmentation index, has been confirmed by some (209,342,401), though not all (151,202) subsequent studies by other groups. It should be noted, however, that the patients in the studies with discrepant results were notably different from those assessed in Study I: i) cART was initiated at a significantly higher CD4+ T-cell count, thus, underestimating the detrimental effects that active HIV-1 infection and immunodeficiency can have on the vasculature (*vide infra*) (151) and ii) exposure to cART was significantly shorter, which may not have permitted its full impact to become evident (202). Of importance, the results of Study I have been supported by large cohort studies which have established that HIV-1-infected cART-treated patients are at notable cardiovascular risk due to their medication (68,74,110,181,389) with up to 26% increase in the rate of myocardial infarction per year of exposure to cART, even independent of cART-associated lipodystrophy (109).

The mechanisms by which antiretroviral agents may cause cardiovascular complications independent of lipodystrophy are not, at present, completely understood. Atherogenic dyslipidemia, which may be seen in cART-treated patients also without adipose tissue redistribution, is hypothesized to occur due to PI-induced increase of the hepatic production of triglyceride-rich particles (211), increased lipolysis in mature adipocytes (15,212,320,329), and decreased activity of endothelial LPL (211,320), therefore, representing a plausible link between cardiovascular disease and cART. Mechanisms independent of dyslipidemia, however, are also believed to exist. For example, a

study exposing HIV-1-negative subjects to four weeks of monotherapy with the PI indinavir reported the development of significant endothelial dysfunction without any concurrent changes in blood lipids (348). In a similar fashion, PI-induced inhibition of proteasomal degradation of apolipoprotein B in the liver (218) has been proposed to contribute to the development of endothelial dysfunction, even in the absence of dyslipidemia (402). Furthermore, it has been suggested that PIs promote CD36-dependent accumulation of cholesteryl esters in monocyte-derived macrophages, thereby contributing to the formation of foam cells and, consequently, of atherosclerosis (82). Finally, PIs have been demonstrated to damage mtDNA and cause necrosis (450), impair vasomotor function, and decrease nitric oxide synthase gene and protein expression (111) in endothelial cells *in vitro*.

Although no association between HIV-1-related characteristics and markers of arterial stiffness were found in Study I, a number of other studies have reported increased arterial stiffness in untreated HIV-1-infected patients compared to HIV-1-negative controls (11,343). The literature on this topic indicates several possible pathways by which HIV-1 infection *per se* could cause vascular complications. Firstly, it has been suggested that HIV-1 infects endothelial cells *in vitro*, causing their enhanced adhesiveness, disrupted proliferation and apoptosis (60). This, in turn, leads to secretion of proinflammatory cytokines that have been suggested to act in synergy with viral proteins to induce further endothelial injury and dysfunction (60). Secondly, HIV-1 may contribute to endothelial damage and premature atherosclerosis by sustaining low-degree inflammation, in a manner similar to that of other infections such as *Chlamydia pneumoniae* and cytomegalovirus (384,451). Thirdly, immunodeficiency likewise immune reconstruction following initiation of cART are both associated with enhanced T-lymphocyte proliferation and activation (144), which could potentiate any pre-existing atherogenic

inflammation. Chronic inflammation, in turn, through the release of cytokines and growth factors, has been documented to stimulate migration and proliferation of smooth-muscle cells, and elaboration of the extracellular matrix of tunica media, resulting in thickened, poorly compliant arterial walls (327). Finally, infections in general can induce atherogenic changes in lipid profile, along with a pro-coagulant state of blood (77), and HIV-1 is no exception (86,125,133). The various detrimental effects of infection with HIV-1 are evident from clinical studies identifying low CD4+ T-cell count as a robust risk factor for arterial stiffness (151) and the development of atherosclerotic lesions (157,174). A further indicator of this detrimental relationship was reported in the SMART Study, in which the interruption, as opposed to continuous use, of cART was associated with an increased incidence of myocardial infarction (85). Recently, the effect of chronic inflammation on arterial structure was elegantly demonstrated in HIV-1-infected cART-untreated “elite controllers” (156). The term “elite controllers” refers to patients with undetectable viral load and preserved CD4+ T-cell count despite the long-term HIV-1 infection and no antiretroviral treatment. When compared to age-matched HIV-1-infected cART-treated patients and to HIV-1-negative subjects, the “elite controllers” were reported to have an increased intima media thickness, an important structural marker of atherosclerosis, most likely as a consequence of their permanent immune activation and inflammatory response (156).

2.2. Hypertension

As cART-associated lipodystrophy has a close resemblance with the metabolic syndrome, and hypertension is part of the international diagnostic criteria for the metabolic syndrome (5), it would be reasonable to expect a high prevalence of hypertension also in patients with

cART-associated lipodystrophy. In Study I, however, there were no differences between the lipodystrophic and the non-lipodystrophic groups in either brachial or aortic blood pressure, even in the presence of substantial differences in body composition, lipid profiles and measures of insulin resistance between the groups. Previous data regarding the link between cART-associated lipodystrophy and hypertension are controversial (16,63,115,170,338). It is interesting to note that the status of hypertension as a component of the metabolic syndrome has also become an area of controversy in recent years. Compelling epidemiological evidence suggests that obesity, or more specifically, accumulation of IAT may be the link between features of insulin resistance and hypertension (136,160). In fact, an insulin-independent, leptin-melanocortin pathway –mediated activation of sympathetic nervous system has been proposed as the mechanism by which obesity causes hypertension (137). In light of these findings, it seems comprehensible that lipodystrophic patients, typically lean and hypoleptinemic, may remain normotensive despite their marked insulin resistance.

Another plausible explanation for the absence of an association between high blood pressure and the presence of cART-associated lipodystrophy is the fact that changes in blood pressure may take longer to develop than the other features of cART-associated lipodystrophy. In HIV-1-negative subjects, alterations in arterial elasticity are known to precede the onset of clinically relevant hypertension by a substantial period of time (288,300). Therefore, a technique of PWA non-invasively evaluating the augmentation index, a measure of arterial elasticity (287,289,300,424) and a strong independent predictor of risk (298,420) and mortality (227) of cardiovascular disease, is a valuable tool in detecting arterial hardening before any sphygmomanometrically detectable changes in blood pressure occur.

3. BODY COMPOSITION IN cART-ASSOCIATED LIPODYSTROPHY

3.1. Adipose tissue compartments

Although the cART-treated lipodystrophic and non-lipodystrophic patients had similar BMI, the former group had significantly greater waist-to-hip ratio and thinner skinfolds, indicative of IAT accumulation in combination with the loss of SAT (Studies I-II, IV). Total body fat (assessed by BIA in Studies I-II and by DEXA in Studies III-IV) tended to be lower in the cART-treated lipodystrophic/-atrophic than in the non-lipodystrophic/-atrophic patients, although the difference did not reach statistical significance (Studies I-IV). In Studies II-IV, MRI was used to quantify the abdominal SAT and IAT. The cART-treated lipodystrophic/-atrophic patients had up to 2.5-fold more IAT and 1.4-fold less SAT than their non-lipodystrophic/-atrophic counterparts, even though they were comparable with respect to age, gender and BMI. This in line with previous data and the lipodystrophy-defining criteria (48,50).

3.2. Liver fat

Liver fat was measured using ¹H-MRS that showed the cART-treated lipodystrophic patients to have up to 10-fold more liver fat than the cART-treated non-lipodystrophic patients (Studies II and IV). Study V, including only lipoatrophic cART-treated patients, further supported the concept of pathologically high liver fat content in these patients. Liver fat content at study baseline averaged $9.1 \pm 2.7\%$ in all study participants, which is above the upper limit of normal (5.56%) (375). Yet, plasma ALT concentrations were 38.6 ± 6.0 U/L, i.e., within the normal range (10-70 U/L for men and 10-45 U/L for women). Uridine or placebo treatment had no effect on liver fat content or ALT concentrations in the study subjects. In Study I, in which liver fat content was not available, plasma ALT concentrations were higher in the

cART-treated lipodystrophic as opposed to the non-lipodystrophic group, likely also reflecting increased liver fat in the context of cART-associated lipodystrophy as described previously (122).

4. SUBCUTANEOUS ADIPOSE TISSUE IN cART-ASSOCIATED LIPODYSTROPHY

4.1. Histology

In cART-treated patients, lipoatrophic, as compared to non-lipoatrophic adipose tissue, is characterized by smaller adipocytes, greater cell size variation, disruption of cell membranes and signs of apoptosis as determined by immunohistochemical staining (80,159,224). On histological analysis of Study IV, irrespective of lipodystrophy status, less adipocytes and more connective tissue were seen in the dorsocervical than in the abdominal SAT. Thus, these characteristics seem to be intrinsic to the dorsocervical depot. Prior to Study IV, light microscopy data and the ultrastructural characteristics of adipose tissue from lipohypertrophic areas of HIV-1-infected patients on cART were reported only in one small study (n=4) (224). This study did not specify the anatomical location of the lipohypertrophic areas, and reported to have used as controls ten skin biopsies from patients with pigmented or bullous skin lesions, all of them containing SAT. The authors reported that in the lipohypertrophic samples, occasional adipocytes exhibited changes similar to those seen in the lipoatrophic SAT, although the changes were generally of milder degree than would be seen in a lipoatrophic depot (224). These changes could not be quantitated because of the small sample size. Although the ratio of adipocytes to connective tissue was not reported in the cited study, the finding of the lipohypertrophic adipose tissue to be "more normal" than the lipoatrophic one is in line with the present data on

lower CD68 expression and greater preservation of mtDNA copy number in the dorsocervical as compared to the abdominal SAT (*vide infra*).

4.2. Adipose tissue inflammation

In Study II, gene expression of macrophage markers (*CD68*, *ITGAM*, *EMRI*, *ADAM8*) and cytokines (*CCL2*, *CCL3*, *TNFA*) was found to be significantly higher in the lipoatrophic as compared to the non-lipoatrophic abdominal SAT from cART-treated patients. Accordingly, Study III showed upregulation of *IL1B* in the lipoatrophic compared to the non-lipoatrophic SAT, with *TNFA* and *IL6* showing a similar trend towards upregulation, but failing to reach statistical significance, possibly due to a large variation within the study groups. Studies III and IV further confirmed a greater degree of adipose tissue inflammation, in terms of lipogranulomata (lipid-laden macrophages encircling adipocytes) and number of immunohistochemically detected CD68-positive cells (macrophages), in the abdominal SAT from lipoatrophic cART-treated patients as compared that of those without cART-associated lipodystrophy/-atrophy. These findings are in line with previous gene and protein expression data as well as with histological and immunohistochemical detection of adipose tissue –resident macrophages indicative of increased adipose tissue inflammation in SAT from patients with as compared to those without cART-associated lipodystrophy/-atrophy (165,222) or HIV-1-negative subjects (12,80,159,165,224).

With regards to the comparison of two anatomically separate adipose tissue depots (preserved dorsocervical and atrophied abdominal SAT), expression of *CD68* was found to be significantly lower in the dorsocervical than in the abdominal SAT in both lipodystrophic and non-lipodystrophic patients (Study IV). Furthermore, microarray data suggested that genes involved in inflammatory processes are downregulated in the

dorsocervical as compared to the abdominal SAT in the lipodystrophic group. These findings are in keeping with previous reports indicating that inflammation, as judged by mRNA expression of, e.g., *TNFA* and *CD68* (126) and tissue release of *TNFA* (165), is more severe in the lipoatrophic abdominal than in the dorsocervical SAT. These studies, however, were small (n=8-10) and samples of both dorsocervical and abdominal SAT were not available from the same individuals.

The antiretroviral drugs most frequently associated with the development of lipodystrophy (tNRTIs such as d4T and AZT) are known to increase the release of proinflammatory *CCL2* and *IL6* from adipocytes *in vitro* (200), and incite increased macrophage infiltration of supra-iliac SAT *in vivo* in HIV-1-infected patients started on antiretroviral treatment (291,292). Study III was specifically designed to compare the effects of d4T and AZT on the abdominal SAT from lipoatrophic patients. No differences in the degree of inflammation were seen between the lipoatrophic abdominal SAT from users of d4T compared to those of AZT, implying that the differences observed in inflammatory status of lipoatrophic as compared to non-lipoatrophic group are not due to the individual tNRTIs, but rather are associated with lipodystrophy *per se*.

It cannot be determined from a cross-sectional study whether adipose tissue inflammation precedes cART-associated lipoatrophy or vice versa. Data from studies performed in settings outside HIV-1 imply that macrophage-secreted proinflammatory cytokines exert a number of effects on adipose tissue. The cytokines inhibit expression of genes involved in adipocyte differentiation (365), decrease lipogenesis due to disrupted activity of LPL (106) and poor synthesis of FATP and ACS (331), increase adipose tissue lipolysis via upregulation of HSL activity (305), and increase apoptosis (317). As a net effect, adipocyte size, number and total adipose tissue mass tend to decrease (317). Intriguingly, adipose tissue inflammation

has been implicated in the pathogenesis of insulin resistance (448), also a feature of cART-associated lipodystrophy (54,122,138).

4.3. Adipose tissue inflammation and liver fat

Study II reported a significant, positive correlation between expression of several inflammatory markers (*CD68*, *ITGAM*, *CCL2* and *CCL3*) in abdominal SAT and liver fat content. Such correlations, in isolation, do not prove causality, but there are data on potential molecular mechanisms linking adipose tissue inflammation to liver fat content and vice versa. Overexpression of *CCL2* in murine adipocytes has been shown to enhance macrophage infiltration into adipose tissue, increase concentrations of circulating FFAs, cause hepatic and intramyocellular triglyceride accumulation, and increase insulin resistance despite unchanged body weight (172). In contrast, induction of hepatic steatosis in mice by high fat diet or by transgenic manipulations was shown to lead to subacute hepatocellular inflammation, featuring increased number of CD68-positive cells within the liver, and cause both hepatic and peripheral insulin resistance (32). This pathway was postulated to involve NF κ B, a transcriptional factor mediating induction of inflammatory cytokine expression (32).

Similar associations seem to exist in humans, as gene expression of both *CD68* and *ITGAM* have been shown to be increased in SAT of obese as compared to non-obese subjects, and have been positively correlated with liver fat content independent of obesity (235). Yet another study has reported that expression of *CD68*, *CCL2* and *CCL3* in SAT of subjects with high liver fat content is higher than in SAT of carefully weight-matched subjects with normal liver fat content (184). In the latter study, gene expression of *CD68* was also positively and significantly correlated with liver fat content (184).

One possible link between adipose tissue inflammation and liver fat in

subjects with and without cART-associated lipodystrophy is serum FFA concentration. These were noted to be comparable between the HAART+LD+ and the HAART+LD- groups in Study II. However, fasting insulin concentrations have been consistently shown to be significantly higher in the cART-treated lipodystrophic patients relative to the cART-treated non-lipodystrophic ones (Studies I-II, IV). Since lipolysis mobilizing FFAs into the circulation is suppressed by even small increases in insulin concentration (297), the finding of similar FFA concentrations in both study groups suggests resistance to insulin-induced suppression of lipolysis in the lipodystrophic group. Indeed, increased FFAs, through activation of the NF κ B pathway, are known, on one hand, to trigger adipose tissue-resident macrophages to produce auxiliary inflammatory mediators further inflaming the adipose tissue (351,448) and, on the other hand, most likely via FFA-derived ceramides (223,366) and DAGs (169,445), to induce insulin resistance in the liver and peripheral tissues (195,254).

IAT has been proposed to be an important source of inflammatory cytokines and to have a higher rate of lipolysis than SAT (8,406). Moreover, inflammatory changes in IAT are more closely correlated with liver fat than those in SAT (36). Furthermore, in morbidly obese subjects macrophage accumulation in omental, but not in subcutaneous, adipose tissue has been reported to associate significantly with the severity of steatotic and fibroinflammatory lesions in the liver (36,388). However, gene and protein expression of inflammatory markers in IAT could not have been studied in Studies II-IV as it is ethically unacceptable to obtain samples from IAT for research purposes only. Moreover, in terms of fatty acid metabolism, there is evidence in favor of studying SAT and not IAT as the catheterization studies have shown the liver to receive most of its FFAs from the upper-body SAT rather than IAT even in abdominally obese subjects (290). However, since cART-associated

lipodystrophy encompasses simultaneous loss of SAT and gain of IAT, it is possible that the intra-abdominal depot of these patients contributes proportionally more to the hepatic FFA supply than it does in the non-lipodystrophic subjects.

Another potential mediator between inflamed adipose tissue and liver fat is adiponectin. Adiponectin is anti-inflammatory and has been shown to reduce liver fat and improve hepatic insulin sensitivity in mice (436). Recent data have suggested that adiponectin conveys its beneficial effects on insulin sensitivity by lowering the content of intrahepatocytic ceramides, molecules implicated causative of insulin resistance (223,366), by activating ceramidase, an enzyme catalyzing degradation of ceramides (153). In multiple human studies, changes in serum adiponectin induced by anti-diabetic agents from the thiazolidinedione class have been closely and inversely correlated with changes in liver fat (440). Patients with cART-associated lipodystrophy have been shown to have low adiponectin gene expression in SAT and low circulating adiponectin concentrations (126,372,387). Furthermore, low adiponectin has been shown to correlate inversely with liver fat in these patients (372). Study II is in line with these findings and extends them further reporting, on one hand, a positive correlation between liver fat and expression of proinflammatory genes in lipoatrophic abdominal SAT and, on the other hand, an inverse correlation between serum adiponectin concentration and expression of proinflammatory genes in lipoatrophic abdominal SAT. Thus, it can be speculated that low adiponectin may contribute to the inflammatory changes in lipoatrophic adipose tissue and to the accumulation of liver fat seen in this patient group.

4.4. Mitochondrial DNA content

In keeping with other studies (126,139,291,415), mtDNA copy numbers were significantly lower in abdominal adipose tissue of the lipoatrophic as compared to the non-lipoatrophic

cART-treated patients (Study III). Also confirming previous data (59,291), the mtDNA depletion was found to be more severe among the lipodystrophic users of d4T than among those using AZT. The dorsocervical adipose tissue had significantly more mtDNA than the abdominal SAT in the cART+LD+ group, with difference close to significance ($p=0.08$) also in the cART+LD- group. This is discordant with previous reports of a trend ($p=0.051$) towards lower mtDNA content in the lipohypertrophic dorsocervical than in the lipoatrophic abdominal SAT from cART-treated patients (126). One potential explanation for this controversy could be the fact that the aforementioned study compared SAT from different anatomic locations taken from different subjects, while in Study IV samples of dorsocervical and abdominal SAT from the same individual were available.

Mitochondrial toxicity of cART manifesting as mtDNA depletion has been suggested, in a similar fashion to what occurs in obesity, to contribute to impaired adipocyte differentiation, increased inflammation, and activation of apoptosis in adipose tissue (408). Supporting this theory, a positive correlation between mtDNA copy numbers and gene expression of an adipogenic transcription factor *SREBP1C* in SAT, and an inverse correlation between mtDNA copy number and expression of cytokine *IL1B* in SAT were found in Study III.

The most significant finding of Study IV, namely that mtDNA content is highly significantly lower (by 62%) in the dorsocervical SAT in the cART+LD+ as compared to the corresponding tissue in the cART+LD- group, although the two depots are phenotypically comparable, contradicts the prevailing theory that mtDNA depletion *per se* causes lipoatrophy. The improbability of simple linear association between mtDNA depletion and development of lipoatrophy is further supported by report of up to 17% of samples from the lipoatrophic adipose tissue from cART-treated patients having

normal mtDNA content while, on the contrary, some samples from the non-lipodystrophic cART-treated controls have subnormal mtDNA content (352). Moreover, if inhibition of mitochondrial POLG is proposed to be induced exclusively by NRTIs (25,171), and if lipoatrophy is caused directly by mtDNA depletion, then lipoatrophy would have only been found in NRTI-treated patients, which has not been the case (362,394). Although the mtDNA depletion theory has been challenged in a research letter as early as in 2001 (271), no study prior to Study IV has had a design that would have allowed it to compare the preserved SAT of otherwise lipoatrophic cART-treated patients to their lipoatrophic SAT and, thereby, perhaps to support or refute this challenge.

From inherited mitochondrial diseases it has been learned that the mitochondrial genome exhibits a considerable degree of redundancy, so that reductions in functional mtDNA to <15% of original are often required for diseases to manifest (61). Thus, it is also possible that in cART-associated lipodystrophy there is a certain threshold of cellular mtDNA content beyond which adipose tissue starts to atrophy. Based on the findings of Study IV, such a threshold could be suggested to lie at ~30% of the original mtDNA content. This could also explain the different phenotypes of dorsocervical and abdominal SAT: notwithstanding both depots are affected by the loss of mtDNA, the dorsocervical SAT simply has not yet reached the threshold required for the development of visible atrophy. It does not seem possible that the ultimate reason for this can be determined from mtDNA measurements alone. What can be concluded from the findings of Study IV, however, is that once the lipodystrophy syndrome develops, the derangements observed in adipose tissue seem to be systemic and not restricted to the lipoatrophic regions.

4.5. Mitochondrial function and biogenesis

In Study III, expression of selected genes

involved in mitochondrial function and biogenesis were quantified and compared between the lipoatrophic and the non-lipoatrophic cART-treated patients, and within the lipodystrophic group between the users of d4T and AZT. Gene expression of mtDNA-encoded mitochondrial respiratory chain subunit *COX3* as well as its ratio to that of nDNA-encoded *COX4* (*COX3/COX4*) was found to be significantly lower in cART-treated patients with as opposed to those without lipoatrophy. This is in keeping with data from other investigators reporting a lower ratio of expression of mitochondrial to nuclear respiratory chain subunit genes in cART-treated patients with as compared to those without cART-associated lipoatrophy (168,291). Furthermore, Study III not only confirmed, but also extended the previous findings of greater mtDNA depletion among the lipoatrophic patients using d4T as opposed to those on AZT (59,291), by showing decreased *COX3* expression and low *COX3/COX4* ratio to be more pronounced among the former than in the latter group of patients. Detection of decreased expression of *POLG1*, a key enzyme involved in replication of mtDNA, in the d4T+LA+ as compared to the AZT+LA+ group might offer an explanation for the more severe mtDNA depletion consistently seen in SAT of the d4T users. Increased expression of mitochondrial genes relative to the number of mtDNA copies per cell, along with increased gene expression of *PPARG* (326) and *PGC1B*, an activator of mitochondrial gene transcription (434), in the lipoatrophic as compared to the non-lipoatrophic cART-treated patients may be reflective of an attempt to compensate for the mtDNA depletion (Study III). Some previous publications agree with this theory (126,291), although contradictory data also exist (173). Compensatory upregulation of transcription of mitochondrial genes could serve as an alternative and/or an addition to the aforementioned “mtDNA threshold theory” in explaining why dorsocervical SAT is non-lipoatrophic despite is mtDNA depletion. Perhaps dorsocervical SAT has sufficient copy numbers of mtDNA in

order to support a compensatory effort via the upregulation of transcription, while the lipoatrophic abdominal SAT is so badly depleted of mtDNA that even the compensatory mechanisms are no longer enough.

Unlike other SAT depots in cART-associated lipodystrophy, dorsocervical SAT has been noted to remain unaffected by lipoatrophy, and can even accumulate in this region. Prior to Study IV, it was unknown how the preserved dorsocervical SAT differs from the abdominal SAT, both obtained from the same HIV-1-infected cART-treated lipodystrophic patient. Based on its anatomical location, the dorsocervical depot has been suggested to represent BAT (126,409). Hallmarks of BAT include ample mitochondria featuring high metabolic activity and high expression of *UCP1* (37,65,284). Previous studies reported significant amounts of *UCP1* mRNA in the “buffalo hump” of patients with cART-associated lipodystrophy, but not in healthy or lipoatrophic abdominal SAT (126,325). In the cited study, however, protein levels of *UCP1* were not quantified. In the dorsocervical SAT from the lipodystrophic patients in Study IV, increased copy numbers of mtDNA (suggestive of BAT) when compared to dorsocervical and abdominal SAT from the non-lipodystrophic individuals, could not be detected. Furthermore, no evidence of biologically significant *UCP1* mRNA expression was found using the same assay as previously used to verify the presence of *UCP1* in BAT in human supraclavicular adipose tissue studied by FDG PET (410). Finally, no typical BAT morphology was seen in the dorsocervical SAT samples, even in those from the clinically most prominent “buffalo humps”. This is in line with a recent study in subjects with cART-associated lipodystrophy reporting that their dorsocervical SAT lacked BAT morphology and was not activated by cold exposure like true BAT would (89). Thus, the present data support the view that dorsocervical SAT in cART-associated lipodystrophy is not BAT.

4.6. Adipogenesis

Study III confirms previous data on decreased *SREBP1C* mRNA expression in the lipoatrophic as compared to the non-lipoatrophic SAT in cART-treated patients (12,173). Also in line with previous data (168), this was found to be equally true for both d4T and AZT users. Low expression of *SREBP1C* gene detected is, however, discrepant with some (12,286,323), although not all (43,44), data on *SREBP1C* protein concentrations in cART-associated lipodystrophy/-atrophy. One possible explanation for the discrepancy between the gene and protein expression data could be sequestration of mature *SREBP1C* protein in the endoplasmic reticulum due to its retarded degradation by proteasome, whereby total cellular *SREBP1C* concentration is high and *SREBP1C* transcription autodownregulated (6,12). With regards to expression of other adipogenic genes, *PPARG* was unchanged as in some (117), but not all (12,173,241), previous studies. Expression of *CEBPB* was significantly lower in lipodystrophic as compared to non-lipodystrophic cART-treated patients. *CEBPA* followed the same trend, although the difference was not statistically significant. Downregulation of *CEBPA* and *CEBPB* was more prominent among users of d4T than AZT. These findings are in accordance with previous reports of decreased expression of *CEBPA* and *CEBPB* in SAT from patients with cART-associated lipoatrophy (12,197).

LMNA and *LMNB* expression was analyzed because it has previously been proposed that PI treatment may alter *LMNA* maturation, inhibiting translocation of *SREBP1C* from the cytoplasm to the nucleus, and contributing to deranged adipogenesis in cART-associated lipodystrophy/-atrophy (43). Study III could not confirm this hypothesis as no difference in *LMNA* expression and upregulation in *LMNB*, an alternatively spliced lamin gene, was seen in abdominal SAT of lipoatrophic as compared to that of non-lipoatrophic cART-treated patients. However, the relationship between altered

lamin maturation and stability, and disrupted SREBP1C translocation through nuclear pores has been challenged (199).

4.7. Glucose and lipid metabolism

In Study III, *GLUT4* gene expression was found to be decreased in cART-treated lipoatrophic patients as compared to those on cART but without lipoatrophy. This was in keeping with our own previous findings (173) and with those of others (12). Furthermore, the finding is in line with data from Studies I, II and IV as well as with the generally accepted notion (54,122,138) that lipodystrophic cART-treated patients have higher fasting concentrations of insulin, C-peptide and HOMA-IR than the non-lipodystrophic similarly treated patients. Although not necessarily associated with decreased gene expression, impaired assembly, translocation and transport activity of GLUT4 protein have been implicated in the pathogenesis of PI-induced insulin resistance (15,278,329).

Concordant with the presence of lipoatrophy, *FASN*, an enzyme involved in triglyceride synthesis, was found to be significantly lower in abdominal SAT of patients with as compared to those without cART-associated lipoatrophy. Furthermore, expression of *FASN* was significantly positively correlated with that of *GLUT4*, underpinning that both lipogenesis, represented by *FASN* expression, and glucose uptake, represented by *GLUT4* expression, share a common nominator such as sensitivity/resistance to insulin action.

Gene and protein expressions of PLIN1 were also assessed in Study III. PLIN1 is a molecule that normally prevents HSL phosphorylation and, thereby, excessive lipolysis in adipocytes (433). Indeed, increased lysosome-mediated proteolysis of PLIN1 has been documented to result in increased lipolysis in PI-treated cultured adipocytes (196). Thus, PLIN1 is considered as a marker of viable lipid-storing adipocytes (66). No differences were observed in *PLIN1* gene expression or in the number of PLIN1-free adipocytes

in the lipoatrophic as compared to the non-lipoatrophic SAT from cART-treated patients. Lower mRNA concentrations of *PLIN1*, were, however, seen in the d4T+LA+ as compared to the AZT+LA+ group. This is in line with the suggested differences in lipotoxicity between the drugs (83,142), although it should be noted that the immunohistological analysis did not confirm differences of similar degree. The contradiction between results of real-time RT-PCR and immunohistochemistry can, perhaps, be attributed to the limited study group size, unrepresentative sampling or the intrinsic differences in the sensitivity of the methods in question.

4.8. Oxidative stress and apoptosis

Upregulation of *SOD1* and *GPX1*, indicators of oxidative stress (28,242), evident in the lipoatrophic as compared to the non-lipoatrophic cART-treated group is in keeping with reports of increased oxidative stress in cell cultures treated with PIs (14,200) and NRTIs (45,200), drugs inextricably associated with the development of lipodystrophy (220). Expression of *SOD1* and *GPX1* tended to be higher in the AZT+LA+ group when compared to the d4T+LA+ group. This finding may reflect the particular propensity of AZT to induce production of reactive oxygen species (435). *FAS*, a key regulator of apoptosis in response to extrinsic stress stimuli, was found to be increased in the lipoatrophic as compared to the non-lipoatrophic cART-treated patients in keeping with previous reports of apoptosis in the context of cART-associated lipoatrophy both *in vitro* (42,81,414) and *in vivo* in humans (80,159,251). Expression of *p53* did not differ between the study groups, which is consistent with its predominantly post-transcriptional stress-induced activation (62) and confirmatory of stress-related pro-apoptotic mRNA alterations such as those seen in *FAS*.

4.9. Homeobox genes

The microarray data suggested that the dorsocervical and the abdominal SAT are

largely similar. Using stringent criteria to avoid false positive data, out of all genes in the genome less than 100 were differentially expressed between the two depots. The genes differentially expressed in both the cART+LD+ and the cART+LD- groups are most likely to be genes which are different due to the anatomic location of the tissues irrespective of lipodystrophy status. The number of genes differentially expressed in the dorsocervical as compared to the abdominal SAT was markedly greater in the cART+LD+ than the cART+LD- group. Keeping in mind that the dorsocervical SAT in the cART+LD+ group was preserved and, at least seemingly, unaffected by the lipoatrophy present elsewhere in SAT (e.g. abdominal region), and that both SAT depots in the cART+LD- group were equally normal, one could hypothesize that the genes found to be discrepant between the dorsocervical and abdominal SAT only within the cART+LD+ group, would, in fact, reflect the abnormality in the abdominal lipoatrophic SAT, rather than anything in the preserved dorsocervical depot.

The foremost difference between the dorsocervical and the abdominal SAT in Study IV was in expression of the homeobox genes. The difference was observed in both the lipodystrophic and the non-lipodystrophic SAT using microarray analysis, and was confirmed by real-time RT-PCR. Homeobox genes are highly conserved genes from the evolutionary point of view (347). They serve as transcription factors guiding cells of the embryonal neural tube to migrate to their destined locations, and determining the distinctive traits inherit to the tissues being formed (347). There are 4 homeobox gene clusters in mammals, HOXA, HOXB, HOXC and HOXD (347). Each cluster has several members numbered in ascending order starting from the anterior boundary of the central nervous system (347). Thus, the lower the "order number" of a homeobox gene, the greater its quantities in dorsocervical as compared to abdominal SAT (347). The findings of Study IV are in line with this, as "rostral" *SHOX2* was overexpressed

in the dorsocervical compared to the abdominal SAT. The reverse was true for the "caudal" homeobox genes *HOXA10*, *HOXC9* and *HOXC8*. This underscores the validity of the present microarray data. Depot-specific expression of homeobox genes has been previously reported in adipose tissue from HIV-1-negative subjects (38,75,116,413), and in human adipose tissue –derived preadipocytes (380) and stromal cells (214). These differences were hypothesized to reflect the differentiation stages and the different adipogenic potential of individual adipose tissue depots, as suggested also by work in murine models and cell cultures (72). Of interest, *HOXC4*, reported as exclusive for BAT (38), was not differentially expressed between the two adipose tissue depots in the microarray analysis of Study IV. This fortifies the premise that dorsocervical adipose tissue in cART-associated lipodystrophy is not BAT.

5. TREATMENT OF cART-ASSOCIATED LIPOATROPHY WITH URIDINE

Study V is the first randomized, double-blind, placebo-controlled trial to evaluate uridine supplementation as a potential means of preventing adipose tissue loss during unchanged concurrent cART. In this study, a significant increase in limb fat mass was observed after 3 months of uridine supplementation in HIV-1-infected cART-treated patients with lipoatrophy on unchanged cART. The average increase in total limb fat with uridine in Study V (890 g in 3 months) was greater than the increases (an average of 400-500 g after 6-12 months) described in switch studies (56,163,274,367,381,382). Despite these encouraging results, a randomized, double-blind, placebo-controlled study with 165 subjects that was carried out after the completion of Study V reported that uridine supplementation failed to have a significant and sustained effect with regards to increasing limb fat mass (252). Of note, the dropout rate in the cited study

was 41%, which was mainly attributed to the bad taste of uridine supplementation or diarrhea associated with its use. Yet another recent study has reported that uridine supplementation alongside concurrent tNRTI-based treatment was inferior to a switch from tNRTIs to tenofovir in a 48-week follow-up analysis with the primary aim of evaluating limb fat changes (250). Thus, switching away from tNRTIs remains to date the best documented treatment for cART-associated lipoatrophy (56,274,381,382).

Regardless of the significant beneficial effects uridine had on body composition, it did not change laboratory markers of insulin resistance, nor did it change liver fat content. This is discordant with data from murine models suggesting that uridine attenuates NRTI-induced steatohepatitis (204). It is widely recognized, however, that translation of murine data to humans is not always possible. As peripheral lipoatrophy in HIV-1-infected cART-treated patients has been associated with insulin resistance (279), one would expect the amelioration of lipoatrophy to translate to an improvement in insulin sensitivity. In Study V, however, despite the statistically significant increase in limb fat, no change in insulin sensitivity was seen. Potential reasons for this discrepancy could be insufficient sample size and duration of treatment, or too small gain in SAT. Of note, the power calculations for Study V were done for its primary aim, i.e., change in total limb fat, by extrapolating the effect size of uridine from switch study data on SAT gain after cART changes. Prior to Study V, uridine had never been used in HIV-1-infected cART-treated patients, and switch study data had not focused on insulin sensitivity, therefore it was not possible to estimate the effect size of uridine on insulin sensitivity and make accurate power calculations in this regard. Alternatively, the untoward 200 cm³ increase in IAT observed in the uridine group after 3 months of uridine supplementation therapy could have counteracted any beneficial changes in insulin sensitivity due to partial resolution

of lipotrophic features. This, along with the trend for a decrease of HDL cholesterol, could, ultimately, have a harmful effect on the cardiovascular risk of the patients.

In keeping with the presumed mechanism of action, i.e., improvement of mitochondrial function, there were a statistically significant increases from baseline in both venous pH and base excess in the uridine group. The changes in acid-base balance seen with uridine contrast with switch studies in which no such changes were observed (56,163). It should, however, be kept in mind that venous blood gas analysis and lactate concentration are only surrogate markers of mitochondrial function, and no direct measurements of mitochondrial function in tissue biopsies were performed in Study V.

Theoretically, supplementation with uridine could compromise the antiretroviral efficacy of pyrimidine NRTIs (i.e., tNRTIs) by increasing the concentration of intracytoplasmic natural pyrimidine triphosphates that compete with tNRTIs for the viral reverse transcriptase enzyme. However, phenotypic HIV-1-resistance assays, animal models and limited human data did not indicate that uridine or its metabolites interfere with tNRTIs at HIV-1 reverse transcriptase and, thus, with their antiretroviral efficacy (33,182,361). No adverse effect of uridine on the antiviral activity of tNRTIs was observed in Study V as none of the patients with undetectable viral load at baseline lost virological control.

Limitations of the Study V include its small sample size and fairly short duration, which were dictated by its nature as a pilot study in administering uridine to HIV-1-infected cART-treated patients. An important inclusion criterion of the present study was current use of pyrimidine-based NRTIs (d4T or AZT) based on the inability of uridine, a precursor of pyrimidine nucleotides, to mitigate cellular toxicity induced by purine analogue antiretrovirals (414,416). Therefore, it must be kept in mind that these results cannot be extended to patients who are not currently receiving tNRTIs.

SUMMARY

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

I. Duration of antiretroviral therapy and cumulative exposure to NRTIs and PIs, but not lipodystrophy itself or duration and severity of HIV-1 infection, are, independently of age and blood pressure, associated with increased arterial stiffness.

II. Expression of macrophage markers and inflammatory cytokines is increased in abdominal SAT of lipodystrophic as compared to non-lipodystrophic cART-treated patients. Expression of inflammatory genes is positively correlated with liver fat content.

III. mtDNA is depleted in abdominal SAT of patients with as compared to those without cART-associated lipoatrophy. Expression of mtDNA-encoded genes relative to template is significantly higher in patients with cART-associated lipoatrophy, perhaps in an attempt to compensate for mtDNA loss. Lipoatrophic SAT is inflamed as judged from increased numbers of adipose tissue –resident macrophages and expression of proinflammatory genes. Expression of adipogenic genes is lower, and that of apoptotic genes, higher in abdominal SAT of lipoatrophic as compared to non-lipoatrophic patients. The observed changes are more pronounced in patients treated with d4T than with AZT.

IV. Depletion of mtDNA is observed even in the non-lipoatrophic dorsocervical SAT of otherwise lipoatrophic individuals. This challenges the prevailing hypothesis that mtDNA depletion *per se* causes lipoatrophy. In lipodystrophic patients, non-lipoatrophic dorsocervical SAT is less inflamed than lipoatrophic abdominal SAT. Dorsocervical SAT bears no features of BAT. The most marked difference in gene expression between dorsocervical and abdominal SAT, irrespective of lipodystrophy status, lies in expression of homeobox genes involved in organogenesis and regionalization.

V. Uridine significantly increases SAT in patients with cART-associated lipoatrophy during unchanged tNRTI-based cART. Uridine does not improve features of insulin resistance or lead to a decrease in liver fat content, and may have the untoward effect of increasing IAT and decreasing HDL cholesterol. Uridine is well-tolerated and does not jeopardize the virological control of cART-treated patients.

CONCLUSIONS

Despite disparate phenotypes in terms of amounts of SAT, several features of patients with cART-associated lipodystrophy, namely accumulation of intra-abdominal and liver fat, adipose tissue inflammation, insulin resistance, dyslipidemia and arterial stiffness, resemble those of HIV-1-negative subjects with the metabolic syndrome. The risk of myocardial infarction in HIV-1-infected cART-treated patients increases with cumulative exposure to cART. Most, but not all, of the risk increase can be attributed to conventional cardiovascular risk factors. The augmentation index, a surrogate marker of systemic arterial stiffness, increases with the cumulative exposure to antiretroviral agents, representing an additional risk factor that contributes to the cardiovascular hazard burden of HIV-1-infected cART-treated patients.

Although multiple derangements of gene and protein expression have been described to characterize cART-associated lipodystrophy, the exact pathogenetic mechanisms underlying this condition remain elusive. Lipoatrophy has been hypothesized as being caused by NRTI-induced depletion of mtDNA. The present studies challenge this hypothesis, as mtDNA content is significantly decreased even in the non-lipoatrophic dorsocervical SAT of otherwise lipoatrophic patients as compared to the corresponding depot of non-lipodystrophic cART-treated patients. Instead, the observed disparate expression of homeobox genes in dorsocervical compared to abdominal SAT may contribute to different susceptibility of these adipose tissue depots to cART-induced toxicity.

Uridine significantly increases SAT in patients with cART-associated lipoatrophy, despite concurrent treatment with stavudine or zidovudine –containing cART. In the present day, with the introduction of newer, less toxic antiretroviral agents, use of stavudine/zidovudine can be avoided,

thereby alleviating or even preventing the development of lipoatrophy. Therefore, the need for uridine or any other lipoatrophy-targeting treatment has significantly decreased.

To conclude, cART-associated lipodystrophy is as a complex syndrome with multiple metabolic and cardiovascular aspects. While its detailed pathophysiology remains a mystery, it is of the utmost importance to recognize the contribution of individual antiretroviral agents to the side effects of treatment, avoid the use of those causing particular harm, and seek for alternative, less toxic agents whenever possible. Since cART is life-long, even the smallest increment in cardiovascular risk is of significance. Therefore, monitoring and treatment of conventional modifiable risk factors is warranted in HIV-1-infected cART-treated patients in order to improve their prognosis and quality of life.

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