# Molecular analysis of the HIV-therapy associated lipodystrophy-syndrome

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**ABBREVIATIONS**

| | |
|---|---|
| **ATP** | Adenosine triphosphate |
| **ADP** | Adenosine diphosphate |
| **AMP** | Adenosine monophosphate |
| **AMPK** | Adenosine monophosphate kinase |
| **A-2-P** | L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate |
| **aP2** | Fatty acid binding protein |
| **Akt (PKB)** | Protein kinase B |
| **AZT** | Zidovudine |
| **BSA** | Bovine serum albumin |
| **BCA** | Bicinchoninic acid |
| **C/EBP** | CCAAT/enhancer-binding protein |
| **CPT1** | Carnitine palmitoyltransferase 1 |
| **CHD** | Coronary heart disease |
| **CCCP** | Carbonyl cyanide m-chlorophenylhydrazone |
| **CD36** | Transporter of fatty acids |
| **d4T** | Stavudine |
| **DHEA** | Dihydroepiandrosterone |
| **dTTP** | Deoxythymidine triphosphate |
| **dCTP** | Deoxycytosine triphosphate |
| **dUTP** | Deoxyuridine triphosphate |
| **dsDNA** | Double-stranded DNA |
| **DMSO** | Dimethyl sulfoxide |
| **DMEM** | Dulbecco's modified eagle medium |

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|---|---|
| **dNTP** | Deoxyribonucleotide triphosphate |
| **DOC** | Dissolved organic carbon |
| **ddC** | Zalcitabine |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **Earle's BSS** | Earle's balanced salt solution |
| **EGTA** | Ethylene glycol bis(β-aminoethyl ether)-N,N,N',N-tetraacetate |
| **ERK** | MAP-kinase |
| **ECM** | Extracellular matrix |
| **FAS** | Fatty acid synthase |
| **FFA** | Free fatty acids |
| **FBS** | Fetal bovine serum |
| **FACS** | Fluorescence activated cell sorting |
| **FP** | Prostaglandin F receptor |
| **FCS** | Fetal calf serum |
| **GH** | Growth hormone |
| **HAART** | Highly active antiretroviral therapy |
| **HDL** | High-density lipoprotein |
| **HIV** | Human immunodeficiency virus |
| **HBV** | Hepatitis B virus |
| **HSL** | Hormone sensitive lipase |
| **HCV** | Hepatitis C virus |
| **IFN** | Interferon |
| **IDV** | Indinavir |
| **IL** | Interleukin |
| **IBMX** | 3-Isobutyl-1-methylxanthine |

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|---|---|
| **IPTG** | Isopropyl-β-D-thiogalactopyranosid |
| **IR** | Insulin resistance |
| **IGF** | Insulin-like growth factor |
| **JNK** | MAP kinase |
| **LPL** | Lipoprotein lipase |
| **LDH** | Lactate dehydrogenase |
| **LB** | Luria-Bertani |
| **LDL** | Low density lipoprotein |
| **LRP** | Low-density lipoprotein receptor–like protein |
| **mtDNA** | Mitochondrial DNA |
| **mRNA** | Messanger RNA |
| **MRC** | Mitochondrial respiratory chain |
| **MTR** | Mito Tracker Red |
| **NNRTI** | Nonnucleoside reverse transcriptase inhibitor |
| **NFV** | Nelfinavir |
| **NRTI** | Nucleoside reverse transcriptase inhibitor |
| **NADH** | *Reduced* nicotinamide adenine dinucleotide |
| **OD** | Optical density |
| **PI** | Protease inhibitor |
| **PPAR** | Peroxisome proliferator-activated receptor |
| **PAI1** | Plasminogen activator inhibitor-1 |
| **PBMC** | Peripheral blood mononuclear cell |
| **PCR** | Polymerase chain reaction |
| **PBS** | Phosphate buffered saline |
| **PEP** | K-phosphoenolpyruvate |

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|---|---|
| **pref** | Preadipocyte factor |
| **PS** | Penicillin/streptomycin |
| **PEPCK** | Phosphoenolpyruvate carboxykinase |
| **PDGF** | Platelet-derived growth factor |
| **PG** | Prostaglandin |
| **Rpm** | Revelations per minute |
| **PK** | Pyruvate kinase |
| **RTV** | Ritonavir |
| **ROS** | Reactive oxygen species |
| **RA** | Retinoic acid |
| **RT** | Reverse transcription |
| **SREBP** | Sterol regulatory element-binding protein |
| **SQV** | Saquinavir |
| **SDS** | Sodium dodecyl sulfate |
| **SCD** | Stearoyl-CoA desaturase |
| **SAT** | Subcutaneous adipose tissue |
| **TNF** | Tumor necrosis factor |
| **TNF-R** | Tumor necrosis factor-reseptor |
| **TZD** | Thiazolidinedione |
| **TG** | Triglycerides |
| **TK** | Thymidine kinase |
| **TGF** | Transforming growth factor |
| **WAT** | White adipose tissue |
| **X-Gal** | 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside |

**ABSTRACT**

The advent of highly active antiretroviral therapy (HAART) including protease inhibitors (PI) and nucleoside reverse transcriptase inhibitors (NRTI) in different combinations has significantly reduced the morbidity and mortality of HIV-infected patients. Unfortunately, HAART has been associated with severe adverse events such as fat redistribution, dyslipidaemia, insulin resistance and diabetes mellitus. The aetiology of this so called lipodystrophy syndrome appears to be multifactorial. It has been hypothesised to consist of drug effects on adipocyte proliferation and differentiation, alteration in adipokine and cytokine expression as well as impairment of mitochondrial functions. Specifically, mitochondrial DNA (mtDNA) depletion has been proposed as an important factor leading to peripheral lipoatrophy in HIV-patients receiving antiretroviral therapy.

We aimed in this study: 1) to explore the molecular mechanisms of the effects of different HAART drugs on different *in vitro* models of adipose tissue metabolism and physiology; 2) to investigate the effect of PI and NRTI on adipocyte proliferation, differentiation, viability and adipokine production; 3) to assess nucleoside-analogue reverse transcriptase inhibitor (NRTI)-mediated mtDNA depletion and to correlate mtDNA depletion with the activity of the respiratory chain complexes; 4) to investigate the effect of NRTI treatment on mtDNA content of various organs and tissues in a murine model as well as to establish a murine model of lipodystrophy.

3T3-L1 cell line, primary human subcutaneous fibroblasts and preadipocytes were exposed to different concentration of PI Indinavir (Crixivan®) and Ritonavir (Norvir®) and to NRTI including AZT (Retrovir®), d4T (Zerit®) and ddC (Hivid®) at different developmental stages for up to two months. Groups of C57BL/6 mice were treated with d4T, AZT or vehicle (5-20 mice per group) for up to 15 weeks with daily human doses adjusted for murine body surface area. MtDNA contend was determined by Real-Time PCR in liver, muscle, heart, brain, and fat tissue.

AZT and d4T impaired adiponectin production *in vitro* at therapeutic $C_{max}$ concentrations. Susceptibility of preadipocytes to mtDNA depletion was depending on cell proliferation and differentiation and mtDNA depletion occurred only after exposure to high drug concentration. Under these conditions, d4T led up to 80 % mtDNA depletion in both dividing and differentiating preadipocytes whereas AZT had some effect only in the latter. Despite mtDNA depletion by NRTI, activities of the respiratory chain complexes were found to be unimpaired. High doses of AZT impaired

differentiation process through inhibition of clonal expansion. Preadipocyte proliferation, clonal expansion and differentiation were unaffected by IDV. This drug contributed to adipose tissue atrophy of the *in vitro* model through promotion of adipocyte cell death. Over a time period of 15 weeks animals receiving d4T had less of both peripheral as well as central fat. Neither d4T nor AZT had and effect on insulin sensitivity. Analysis of mtDNA content revealed depletion of mtDNA in organs including the brain (-20%) and fat tissue (-27%) but the most profound and significant depletion was evident in muscle (-56%), liver (-64%), and heart (-45%).

Our results suggest that adipocyte proliferation and/or differentiation is required for significant mtDNA depletion. At the same time, these data do not support the hypothesis that mtDNA depletion leads to impairment of the respiratory chain function in adipocytes. Instead, we provide evidence that AZT contributes to atrophy by inhibiting clonal expansion rate and ultimately reducing the overall differentiation capacity. We conclude from our studies that IDV and perhaps other PI are able to affect cell viability of the 3T3-L1 cell line model of differentiation. This is the first study to show fat loss and hypoadiponectinema in mice after treatment with thymidine-analogues. d4T can cause fat loss without significant mtDNA depletion in fat tissue or insulin resistance in mice.

**ZUSAMMENFASSUNG**

Seit dem Beginn der hochaktiven antiretroviralen Therapie (HAART), die oft aus Kombinationen von Proteaseinhibitoren (PI) und nukleosidalen Reversetranskriptase-Inhibitoren (NRTI) besteht, ist es zu einem signifikanten Rückgang der Morbidität und Mortalität von HIV-Patienten gekommen. Leider kann diese Therapie mit unerwünschten Nebenwirkungen, wie z.B. Fettverteilungstörungen, Dyslipidämien, Insulinresistenz und Diabetes mellitus, verbunden sein. Die Ätiologie dieses sog. Lipodystrophie-Syndroms ist offenbar multifaktoriell. Verschiedene Hypothesen gehen davon aus, dass sich die Medikament negativ auf die Adipozytendifferenzierung und -proliferation, die Expression von Adipokinen und Zytokinen sowie die Mitochondrienfunktion auswirken. Besonders der Verlust von mitochondrialer DNA (mtDNA) ist als wichtiger Faktor für den peripheren Fettverlust antiretroviral behandelter HIV-Patienten postuliert worden.

In dieser Studie sollten die molekularen Mechanismen verschiedener HIV-Medikamente auf den Fettstoffwechsel in *in vitro* Modellen untersucht werden. Zweitens sollte der Effekt von PI und NRTI auf die Proliferation, Vitälität und Adipokinproduktion von Adipozyten analysiert werden. Drittens sollte die mtDNA-Depletion durch NRTI quantifiziert und mit der Aktivität der Atmungskettenkomplexe korreliert werden. Schließlich sollte in einem Mausmodell der Effekt einer NRTI-Behandlung auf den mtDNA-Gehalt in verschiedenen Organen bestimmt und ein Mausmodell zur medikameteninduzierten Lipoatrophie etabliert werden.

3T3-L1 Zellen, humane Fibroblasten aus dem Subkutangewebe und Präadipozyten wurden mit verschiedenen Medikamentenkonzentrationen zu unterschiedlichen Entwicklungsphasen mit den PI Indinavir (Crixivan®) und Ritonavir (Norvir®) bzw. mit den NRTI AZT (Retrovir®), d4T (Zerit®) und ddI (Hivid®) für bis zu zwei Monaten inkubiert. Darüber hinaus wurden C57BL/6 Mäuse (5-20 Tiere per Gruppe) mit d4T, AZT oder Plazebo für bis zu 15 Wochen mit Dosierungen behandelte, die den beim Menschen verwendeten Medikamentenmengen entsprechen. Anschließend wurde mittels Real-Time-PCR in den Organen der Tiere (Leber, Muskel, Herz, Gehirn und Fettgewebe) der Gehalt an mtDNA bestimmt.

Bei therapeutischen Cmax Konzentrationen inhibierten AZT und d4T die Produktion von Adiponektin *in vitro*. Die Depletion von mtDNA in Adipozyten war von ihrer Proliferation oder Differenzierung abhängig und trat nur bei höheren Medikamentenkonzentrationen auf. Unter diesen Bedingungen führte d4T zu einer 80%

mtDNA Depletion in proliferierenden oder differenzierenden Präadipozyten. AZT hatte nur einen nur schwachen Effekt auf den mtDNA-Gehalt differenzierender Adipozyten. Trotz dieser mtDNA Depletion ließen sich keine Funktionsbeeinträchtigungen der Atmungskettenkomplexe objektivieren. Hohe Konzentrationen von AZT reduzierten durch eine Inhibition der klonalen Expansion die Differenzierung von Präadipozyten. Die Proliferation, klonale Expansion und Differenzierung von Präadipozyten war durch Indinavir unbeeinträchtigt, jedoch kam es durch dieses Medikament zu einem vermehrten Zelltod der Adipozyten. Mäuse, die über 15 Wochen mit d4T behandelt wurden, wiesen weniger peripheres und zentrales Fett auf. Die mtDNA war in diesen Tieren im Gehirn (-20%) und im Fettgwebe (-27%), besonders aber im Skelettmuskel (-56%), in der Leber (-64%) und im Herzmuskel (-45%) vermindert.

Zusammenfassend deuten die Ergebnisse darauf hin, dass mtDNA Depletion in Adipozyten von ihrer Proliferation und/oder Differenzierung abhängig ist. Andererseits fanden sich keine Hinweise dafür, dass diese mtDNA Depletion notwendigerweise zu einer mitochondrialen Dysfunktion und mit eingeschränkter Atmungskettenaktivität führt. AZT trägt wahrscheinlich durch eine Inhibition der klonalen Expansion von Präadipozyten bei zur Lipoatrophie. Indinavir, wie vielleicht auch andere PI, verringern zusätzlich die Vitalität von Adipozyten. Schließlich gelang es in dieser Arbeit erstmals, ein Tiermodell für medikamenteninduzierte periphere Lipoatrophie und Hypoadiponektinämie ohne Zeichen der Insulinresistenz zu entwickeln.

# 1 INTRODUCTION

Clinical use of highly active antiretroviral therapy (HAART) including protease inhibitors (PI) and nucleoside reverse transcriptase inhibitors (NRTI) in different combinations has significantly reduced the mortality of AIDS patients. NRTI target the HIV enzyme reverse transcriptase that in the cytoplasm of the infected cell, reverse transcribes the viral RNA genome into double-stranded cDNA. The double-stranded cDNA migrates to the nucleus where it is integrated into the cell genome, becoming a provirus. NRTI differ from physiological nucleosides only by a minor modification in the ribose molecule and act as alternative substrate. Incorporation of NRTI induces abortion of DNA synthesis. Until now, all classical HAART regimens have contained two NRTI. PI inhibit the enzyme HIV protease which cuts the viral gag-pol polyprotein into its functional subunits. Inhibition of HIV protease leads to non-infectious virus particles. Combination of NRTI and PI for the first time led to efficient and sustained viral suppression and leads to significant immunologic reconstitution. Unfortunately, HAART has been associated with severe adverse events such as: fat redistribution, dyslipidaemia, insulin resistance and diabetes mellitus. The so called HAART associated lipodystrophy syndrome has been suspected to have a multifactorial aetiology. This aetiology probably includes: inhibition of adipocyte differentiation, alteration of mitochondrial functions, altered leptin and adiponectin production and altered cytokine expression. Adipose tissue appears to play a central role in HAART associated alterations of lipid metabolism and insulin sensitivity.

# 2 REVIEW OF THE LITERATURE

## 2.1 HAART associated lipodystrophy syndrome

HAART associated lipodystrophy syndrome can include the following clinical characteristics (1-22):

### 2.1.1 Morphological alterations

- Loss of subcutaneous fat (arms, legs and buttocks)
- Accumulation of visceral fat
- Dorsocervical fat pads (Buffalo hump)
- Breast enlargement (in females)

### 2.1.2 Metabolic alterations

- Insulin-resistance
- Diabetes mellitus
- Elevated serum triglycerides (TG)
- Elevated low-density lipoprotein (LDL)-cholesterol
- Elevated very low-density lipoprotein (VLDL)-cholesterol
- Elevated apolipoprotein B and E
- Reduced high-density lipoprotein (HDL)-cholesterol

### 2.1.3 Factors associated with the development of HAART-associated lipodystrophy

**Main factors**

- HIV infection is suspected to play a role in the lipid alterations (23)
- The use of PI is mainly associated with visceral fat accumulation and metabolic alterations (5;12;24-27)
- The use of NRTI is mainly associated with peripheral fat wasting (9;12;21;28)

**Additional factors**

- Duration of antiretroviral treatment (3;12;22;29)
- Nadir of CD4 T cells (29)
- Age (3;29)
- Nutrition (12)
- Elevated TG at baseline (12;22)
- Elevated C-peptide levels (22)
- Nutritional status (12)
- HCV co-infection (30;31)

### 2.1.4 Proposed pathophysiological mechanisms of lipodystrophy syndrome

#### 2.1.4.1 Retinoic acid binding protein-1 (CRABP-1) mediated inhibition of adipocyte differentiation

The catalytic domain of HIV-1 protease has been found to be highly homologous to the lipid-binding domain of low-density lipoprotein receptor-like protein (LRP) and to the retinoic acid-binding domain of cellular retinoic acid binding protein 1 (CRABP-1) (13). Retinoic acid isomerizes in cis-9-retinoic acid and activates the nuclear retinoid X receptor-α - PPARγ complex. This complex is involved in the regulation of adipocyte proliferation and differentiation (32). As CRABP-1 carries retinoic acid, its PI induced inhibition may result in ultimate alterations in the process of adipocyte proliferation and differentiation (33). Many *in vitro* studies however disproved this hypothesis. There is no structural similarity between the three-dimensional crystal structure of CRABP-1 and HIV-1 protease (34). PI bind directly to neither retinoid X receptor-α nor PPARγ (35;36).

#### 2.1.4.2 Lamin A/C disturbances

Phenotypic similarities between HAART lipodystrophy and familial partial lipodystrophies have been found in certain aspects. Defects in lamin A/C are considered to be the reason for autosomal dominant familial partial lipodystrophies (37). Mutations in 1-acylglycerol-3-phosphate O-acyltransferase-2 from other site is the genetic feature of autosomal recessive congenital generalized lipodystrophy. Therefore PI-induced alteration in the expression and maturation of these or some other homologous genes has been hypothesized to be involved in the pathogeneses of HAART lipodystrophy (38).

#### 2.1.4.3 Local changes in glucocorticoid concentrations

HAART lipodystrophy syndrome presents some similarities in body fat redistribution with Cushing's syndrome which led to the hypotheses of eventual participation of the hypothalamicpituitary-adrenal axis (39;40). Although some studies argue agains this hypotheses as a mechanism of HAART induced fat redistribution, cortisol could be locally produced in adipose tissue after 11β-hydroxysteroid dehydrogenase type 1 conversion of cortisone (41). The expression levels of this enzyme and of glucocorticoid

receptors are significantly higher in omental versus subcutaneous fat (42;43), which is consistent with the idea that although in patients on HAART serum and urine cortisol concentrations are normal, locally increased glucocorticoid levels may be responsible for regional adiposity, even without hypercortisolism. Whether or not this mechanism contributes to HAART lipodystrophy syndrome currently is not very clear.

#### 2.1.4.4 PI induced peripheral adipocyte apoptosis in HIV patients with lipodystrophy

Histopathologically subcutaneous adipose tissue has been characterized as nonencapsulated mature adipose tissue (44). The presence of fibrotic changes excludes neoplastic or dysplastic pathology. HAART lipodystrophy has been characterized with subcutaneous adipocyte apoptosis (45). Loss of peripheral adipocytes could lead to increased fat accumulation in visceral adipocytes with subsequent IR and may partially explain the occurrence of lipodystrophy syndrome (13).

#### 2.1.4.5 PI inhibited differentiation

Certain groups hypothesized that PI effects on sterol regulatory element-binding protein-1 (SREBP-1) may interfere with the process of adipocyte differentiation (46;47). In contrasts to these *in vitro* PI studies, a recent human study demonstrated that four weeks of IDV has no effect on adipogenic transcription factors expression in subcutaneous adipose tissue of healthy HIV-negative volunteers (48).

#### 2.1.4.6 NRTI induced mitochondrial toxicity

It has been shown that NRTI inhibit the DNA polymerase-γ thereby leading to inhibition of mtDNA synthesis resulting in mitochondrial DNA (mtDNA) depletion (49). NRTI induced mtDNA depletion may be a reason for a decrease in mitochondrial gene expression and ultimate impairment of function as mtDNA encodes 13 polypeptides, which are subunits of the respiratory chain (MRC) with active role in the process of oxidative phosphorylation. Subcutaneous fat samples from patients with lipodystrophy demonstrate mtDNA depletion (50-53).

##### 2.1.4.6.1 Depletion of mtDNA

Both treated and therapy naïve HIV positive patients have been demonstrated to have lower mtDNA in comparison to healthy controls (54-59). Pathologies related to

mitochondrial toxicity such as hepatic steatosis, myopathy and lactic acidosis occur in HIV-positive patients and have been associated with NRTI-induced mtDNA depletion (49;60;61). Numerous studies documented mtDNA depletion and mitochondrial ultra-structure abnormalities in NRTI treated patients (50-53). The fact that some patients with inborn mitochondrial diseases present with fat redistribution, directed the attention to the eventual role of mitochondrial toxicity in the process of fat redistribution (62;63), although marked differences of the phenotypic alteration are evident. Accumulating reports about the association of lipoatrophic changes with mtDNA depletion (50;51;64;65) led to the hypothesis that, NRTI induced white adipose tissue mtDNA depletion may play a role in lipodystrophy pathogenesis (49). Possible mechanisms for mtDNA depletion have been provided by *in vitro* analyses demonstrating direct NRTI inhibition of mitochondrial polymerase γ enzyme(51;66-71). Additionally to these *in vitro* experiments the model has been supported by some animal studies (72-76). According to DNA polymerase-γ hypothesis NRTI induce a shift in the balance of intra-mitochondrial pool of native nucleotides that affects the activity of DNA polymerase-γ and results in subsequent decrease in mtDNA, mtRNA and the expression of electron transport proteins encoded by mtDNA with ultimate effect on cell energetics. Assuming that adipose tissue would react in the way other tissues react to the above mentioned changes, OXPHOS deficiency in adipocytes may bring reduction of fatty acid synthesis, decrease in fatty acid oxidation and apoptosis (77). On the other hand the initiation of mtDNA replication depends on an RNA-primed DNA synthesis activity (78). It is tempting to hypothesise that at this point NRTI may inhibit DNA polymerase γ reverse transcription activity as well. In a principle situation a mtDNA decrease bellow a certain threshold for a definite period of time should results in an impairment of electron transport and ATP synthesis(79;80). Reduced ATP syntheses in the liver for example, requires a reduction of mtDNA below a threshold of about 20–30% of the normal mtDNA content (81). Whether this 'threshold hypotheses' applies also for adipocytes is currently not known. In comparison between lipoatrophic, non-lipodystrophic HIV-positive patients and HIV-negative control the first group turned out to have 36% and 43% less mtDNA in the subcutaneous fat than the second and the third group, respectively (51). However, at present it is still under discussion whether or not mtDNA depletion is involved in the pathogenesis of the HAART induced clinical symptoms and DNA polymerase-γ hypothesis alone seems insufficient to explain the lipodystrophy syndrome (82). Some studies describing HAART-related mitochondrial toxicity do not

find mtDNA depletion. Additionally, there is increasing discrepancy and controversy concerning the effect of NRTI-induced mtDNA depletion on mitochondrial function. Although some studies in HAART patients correlated positively mtDNA levels and mitochondrial activities (83-85) there are many that were unable to establish such correlation. The discrepancy has been deepened by recent study in lipodystrophic patients demonstrating preservation of multiple mitochondrial enzyme activities despite mitochondrial depletion (86). Some of the difficulties to establish correlation come from the fact that mitochondrial dysfunction and in particular OXPHOS deficiency can be compensated through certain mechanisms such as mitochondrial proliferation (79;87). Such proliferatory compensation has already been reported in adipose tissue of patients with lipoatrophy (50;88). No correlation has been found in a study paralleling mtDNA depletion, cytochrome c oxidase gene expression and enzyme activity (89). Contradictory results have been obtained from studies that were able to show an association of mtDNA depletion in blood cells of patients with hyperlactataemia and lipoatrophy (56;59;90), as others have failed to demonstrate such an effect (91-93).Taken together these studies suggest additional mechanisms, and several hypotheses have been proposed that oxidative damage of mtDNA could contribute to peripheral fat loss (94;95). Others suspected NRTI direct inhibition of mitochondrial respiration (96) as well as inhibition of ADP/ATP translocase (97), adenylate kinase (98), NADH oxidase (99), protein glycosylation (100) and a ‘bystander effect’ (101).

**2.1.4.6.2 Depletion of mRNA**

The cause-effect link between mtDNA depletion and mtRNA transcription has been questioned by some non-HAART-studies demonstrating normal transcription despite severe mtDNA depletion (102;103). This raised the concern that mtDNA depletion does not necessarily affect the quantity of mtRNA. On the other hand in peripheral blood mononuclear cells (PBMC) from HAART treated patients, decrease in mitochondrial gene expression was observed even without mtDNA depletion (89). Direct influence of NRTI on mitochondrial gene expression and/or inhibition of mtRNA syntheses has been suspected in these cases (104;105). NRTI may directly inhibit the activity of mtRNA polymerase or affect the regulation of mitochondrial transcription co-factors (as for some of them the regulation is through the process of phosphorylation) and thereby disturb the whole process of transcription (106). NRTI may influence nucleotide

syntheses and eventually ribonucleotide synthesis in a similar way. The above mentioned effects of NRTI would potentially result in altered mtRNA levels.

**2.1.4.6.3 Disturbances of the nucleotide pool**

NRTI cellular and mitochondrial internalization, transport, homeostasis and phosphorylation are critical steps in their effect on cell metabolism. Their intra-mitochondrial concentration is absolutely critical for their effect on mtDNA replication and transcription, but measurement of intracellular and intra-mitochondrial concentrations of NRTI and their metabolites is hampered by technical issues. Although it is clear, that NRTI pharmacological effects are exerted by their triphosphates it is more and more believed that their toxicological effects are exerted by some of their other forms as well. There are three steps in the phosphorilation of most of NRTI, particularly the thymidine analogues. Thymidine kinase (TK1, and TK2) phosphorylates NRTI to NRTI-MP and then to NRTI-DP. NRTI-DP is phosphorylated to NRTI-TP by nucleoside diphosphate kinase (107-110). The balance phosphorylation /dephosphorylation is maintained by phosphatases and if disrupted could impact mtDNA replication. A critical relation exists between endogenous deoxyribonucleotide triphosphate (dNTP) and NRTI triphosphate pools. NRTI act as competitive inhibitors and/or chain terminators (111-113) in order to inhibit both HIV-reverse transcriptase and mammalian DNA polymerase-γ (113-115). Competitive inhibition results from the competition of NRTI and endogenous nucleotides at the nucleotide-binding site of DNA polymerase-γ leading to inhibition of mtDNA replication. MtDNA abnormalities have been documented in patients with inherited thymidine phosphorylase deficiencies (116;117) suggesting that eventual imbalances in the mitochondrial nucleotide pool may result in mtDNA disturbances. A wide variety of nucleic acid pathways depend on the endogenous nucleoside/nucleotide pools and could be affected if NRTI disrupt the balance. AZT in its non-phosphorylated form has been shown to inhibit thymidine phosphorylation in certain organs which eventually may disrupt the substrate supply of deoxythymidine triphosphate (dTTP) for mtDNA replication (118-120). The proper function of MRC is very important for the synthesis of pyrimidine nucleotides (121) and if disrupted may result in decrease in ATP production with subsequent impairment of pyrimidine synthesis and phosphorylation (122). Uridines ability to rescues *in vitro* mtDNA depletion induced by the pyrimidine analogues such as d4T, AZT, and ddC supports this hypotheses (57;123)

#### 2.1.4.6.4 Mutations

Apart from the quantity, mtDNA quality is also a very important feature of this genetic information. In the process of cell aging mitochondrial DNA mutations accumulate gradually (62;79;80). This accumulation is explained with the fact that mitochondrial DNA is much more vulnerable than nuclear DNA. As mitochondria are powerful source of reactive oxygen species (ROS), mtDNA is exposed to very strong oxidative stress. Oxidative phosphorylation is based on the negative membrane potential. This negative membrane potential at the matrix side leads to high concentration of lipophilic cations inside mitochondria and makes drugs that are lipophilic cations prone to damage this region. In connection to HAART some groups reported mtDNA mutations in patients blood (124), others found mtDNA deletions (125;126) and still others suggested NRTI impairment of mitochondrial energy-production, cellular redox misbalance and affected mtDNA integrity(127).

#### 2.1.4.6.5 Physical interference

Interestingly, AZT has been found to induce *in vitro* reduction in mitochondrial membrane potential even without mtDNA depletion (128) and to have a short term effect on the MRC (76) and ATP production (127). These observations led to the speculation that NRTI interfere physically with the membrane structure. ddC increased the production of reactive oxygen species (ROS) and decreased ATP in a murine model long before any detectable mtDNA depletion (129).

### 2.1.4.7 Cytokines and adipocytokines

Misbalanced cytokine expression has been suggested to play a role in the pathogeneses of peripheral lipoatrophy. Although in the normal course of HIV progression the number of Tumor necrosis factor-α (TNF-α) producing T cells progressively decreases through apoptosis (130), HAART alleviates this natural process and leads to their accumulation (131). HAART has been associated with dysregulation of TNF-α producing T cells, and this association has been very persuasive in the settings of lipodystrophy syndrome. As the physiological regulation of TNF-α synthesis involves apoptosis, the increased potential of TNF-α production of some CD4 and CD8 T cells helps them to escape activation-induced apoptosis, which explains their HAART associated accumulation. The amount of TNF-α CD8 T cell precursors has been

associated with TG, cholesterol and apoB/Apo-A1 ratio in the setting of HAART lipodystrophy (131). *In vitro* TNF-α affects mitochondria, impairs the function of MRC, and increases ROS production (132;133). The mechanism has been hypothesised to be either TNF-α binding to its cell surface receptor or to a 60 kD protein of the inner mitochondrial membrane (134). Although TNF-α overexpression in mice was associated with some heart mtDNA damage, suggesting impaired capacity of mtDNA repair or increase in ROS formation, to date there is no confirmation that direct interaction of mitochondrial components and TNF-α is responsible for any kind of mitochondrial dysfunction (135).

*In vitro* models of preadipocyte differentiation demonstrated antiadipogenic effect of inflammatory cytokines. Changes in adipose tissue as in the state of obesity or lipoatrophy are associated with altered cytokine and adipocytokine secretion. Similar alterations are observed in HAART lipodystrophy. Expression profiles of fat biopsies from lipodistrophic HAART treated patients revealed increase in TNF-α and IL-6 expression. The increased expression of these cytokines was positively correlated with cell apoptosis (136). Certain TNF-α gene polymorphisms have been also associated with lipodystrophy establishing once more the link between this cytokine and the pathology (137). TNF-α is able to directly inhibit the autophosphorylation of insulin receptors (138). It has been suggested that TNF-α inhibitory effect on adipogenesis is mediated by TNF-α-R1 which activates ERK pathway (139). *In vitro* TNF-α is able to inhibit the expression of PPARγ and C/EBPα(140), as well as GLUT4 and lipoprotein lipase (LPL), which very well correspond to the induced inhibition of differentiation (141). TNF-α-induces lipolysis through TNF-α-R1, JNK and p44/42 mediated activation of MAPK family (142). In HIV as in other viral infections plasma interferon-α (IFN-α) levels have been found to increase (143;144). Plasma concentrations of IFN-α in HAART patients are much higher than in controls and have been associated with lipodystrophy (145;146). The immune function of interferons is to provide resistance to viral infections and to regulate the immune response (147). On the other hand IFN-α possesses a very complex metabolic function in the regulation of lipid metabolism: it reduces TG clearance, suppresses LPL activity, and stimulates lipolyses and liver lipogenesis. It also influences glucagon, cortisol and noradrenaline metabolism, which makes the involvement of IFN-α in lipodystrophy syndrome highly probable (39;40;42;43;148;149). Last but not least the expression of critical adipokines such as leptin and adiponectin has been found to be reduced under HAART (46;136;150-152).

#### 2.1.4.8 HAART induced selective autonomic neuropathy

Adipose tissue is innervated both by sympathetic as well as parasympathetic nerves. Activation of these nerves decreases or increases fat accumulation. It has been hypothesized that selective autonomic neuropathy may be the reason for the fat redistribution in HAART-lypodistrophy (153). Indeed, in the hypothalamic area, sympathetic and parasympathetic nuclei are organized in a way that, they separate different sets of neurons towards intra-abdominal and subcutaneous fat (154). HAART neurotoxicity in some particular sets of neurons may result in selective fat loss or/and fat accumulation in certain parts of the body (155).

## 2.2 Adipose tissue

Studying the mechanism of adipocyte differentiation helps understand the cellular and molecular mechanisms of adipose tissue development and behaviour in physiological and pathophysiological states and may be an important tool in the development of therapeutic strategies for the treatment and prevention of adipose pathology.

### 2.2.1 Functions

#### 2.2.1.1 Secretory function

Mature adipocytes store energy under hormonal control. They secrete a wide variety of factors that play a role in processes such as appetite regulation, vascular diseases and immunological reactions. Adipocytes have been characterized as endocrine as well as paracrine/autocrine cells. White adipose tissue (WAT) not only plays a role in numerous physiological processes, but autoregulates its own growth and development. Adipocyte secreted hormone leptin participates in the regulation of body fat mass. Eventual decrease/increase in fat stores is accompanied by corresponding decrease/increase in leptin levels followed by a subsequent increased/decrease in food intake (156;157). Leptin is able to modulate insulin effects at the level of liver (158). It modulates adrenocortical steroidogenesis (159) and the ovary steroid production (160). Leptin is involved in haematopoietic and immune system development as well as in the processes of reproduction (161-164). Both human and murine obesity have been characterized with high levels of leptin. Another adipocyte secreted hormone is adiponectin (165). Adiponectin plasma blood levels have been positively correlated with adipose content

(166). There are two receptors AdipoR1 and R2 (167). In murine models adiponectin enhances free fatty acids (FFA) oxidation at the level of muscle (168). Patients with type 2 diabetics demonstrate low adiponectin levels (169). Adiponectin is considered to predominantly promote the effect of insulin. Adipocytes secret TNF-α, which contribute to the development of IR, inhibits adipocyte differentiation and has a negative impact on the specific adipocyte genes expression (138;170-177). Adipocytes secret immune system-related proteins such as acylation stimulation protein (ASP), which results from the interaction of complement factors (D, B and C3) (178). This factor stimulates glucose transport and TG synthesis (179-181). Some adipocyte secreted proteins have a vascular function. Angiotensinogen regulates the blood supply of adipose tissue and the efflux of FFA (182;183). Angiotensin II a derivative of angiotensinogen stimulates mature adipocytes to produce prostacyclin. This is considered to be a paracrine/autocrine mechanism to promote adipocyte differentiation (184). Angiotensin II influences positively the process of lipogenesis and modulates adipocyte lipid synthesis and subsequent storage (185). Another factor with vascular function is plasminogen activator inhibitor type 1 (PAI-1 ) (183). Subcutaneous fat has lower levels of PAI-1 than intra abdominal. PAI-1 levels have been found to be increased in obesity (186;187).

#### 2.2.1.2 Metabolic function

In addition to their secretory and endocrine functions adipocytes take part in energy metabolism as well. WAT, depending on the situation, synthesises and accumulates and/or degrades TG (188). Lipid accumulation is either through direct uptake of circulating lipoprotein-TG particles after LPL hydrolyses or through *de novo* synthesis from glucose. LPL hydrolyses lipoprotein-TG particles into FFA and with the help of fatty acid binding proteins they are transported into the adipocyte. GLUT4 transport the glucose for the *de novo* synthesis of TG. First pyruvate and glycerol-3-phosphate are synthesised. Pyruvate takes part in the formation of acetyl-CoA. Acetyl-CoA carboxylase (ACC) controls the conversion of acetyl-CoA into malonyl-CoA. Fatty acid synthase (FAS) takes part in the formation of long chains of fatty acids. Insulin controls these anabolic pathways. Hormone sensitive lipase (HSL) hydrolyzes TG into FFA and monoacylglycerol. Monoacylglycerol is also finally converted into glycerol. Lipolysis is controlled hormonally by noradrenaline and adrenaline when the insulin levels are low (189).

### 2.2.2 Adipose tissue heterogeneity and plasticity

WAT varies in volume and cell number. Anabolic or catabolic conditions may influence the size of adipocytes and induce either hypertrophy or hypotrophy (190;191). The number of adipocytes depends on preadipocyte proliferation and differentiation as well as on cell loss. Differentiation has been found to take place even in adult mammals (192). In adults differentiation is age and fat depot volume dependant. The volume of the fat depot depends on lipolytic factors (catecholamines, GH and TNF-α) and on lipogenic factors (insulin and glucocorticoids). The Adipocyte number depends on preadipocyte proliferation, differentiation, dedifferentiation and apoptosis(74;188;193). *In vitro* adipocyte apoptosis has been demonstrated to be induced by TNF-α (194) and growth factor deprivation. On the other hand insulin prevents this process (195). Different fat depots react on a different way to factors such as GH, insulin, catecholamines, IL-6 and TNF-α (196).

### 2.2.3 Origin of adipocytes

Adipocyte lineage derives from embryonic stem cell precursors. These precursors have the potential to differentiate into several mesodermal cell types (myocytes, osteoblasts, chondrocytes and adipocytes). The precise molecular mechanism of the commitment to adipocyte lineage is not very clear. WAT development starts at birth and depends on the species and the fat depot (197-200). Different fat pads demonstrate a wide variety of molecular and biochemical differences (43;201-205). Fat cell size and fat cell number increase after birth and adipogenesis persists throughout the whole life (206-209). *In vitro* experiments demonstrate that fat cell precursors isolated from adult WAT can still be differentiated (210-216).

### 2.2.4 Adipocyte differentiation

#### 2.2.4.1 Models of differentiation

In order to dissect the molecular and cellular events characterizing differentiation scientists need good models. Preadipose cell lines and isolated primary preadipocytes are already committed to the adipocyte lineage. 3T3-F442A and 3T3-L1 are the most widely used cell lines (217-219). Ob17 cells generated from (ob/ob) mice are another cell model (220). Mature adipocytes can be generated through *in vitro* differentiation of

embryonic stem cells (ES) (221). It is worth mentioning that *in vitro*-differentiated adipocytes possess many of the characteristics of the *in vivo* adipocytes (222;223). Of course primary cultures have to be used to validate results from preadipose cell lines, they are diploid and reflect better the *in vivo* situation. Although adipocyte primary cultures have the disadvantage that they are comprised of heterogeneous stromal vascular preadipose cells, 100% differentiation is possible under proper stimulation (216;224;225). At proliferation stage both preadipocyte lines and primary preadipocytes are fibroblasts like cells. In order to acquire the biochemical and morphological characteristics of mature adipocyte they require proper stimulation. Although very often different adipocyte cell models require different stimulation, there are some cell lines that are stimulated in the same manner as the primary precursors. For example 3T3-L1 cell line and primary human preadipocytes require the same stimulation: insulin, glucocorticoids and 3-isobutyl-1-methylxanthine (IBMX) (203;213;214;216;224;226-229).

#### 2.2.4.2 Process of differentiation

The beginning of preadipocyte conversion requires them to exit the cell cycle. Acquisition of an adipocyte phenotype is characterized by early, intermediate, and late markers of differentiation as well as progressive TG accumulation. These changes are predominantly achieved at the transcriptional level, and in rare particular cases at the level of posttranscriptional regulation (230;231). Differentiation process consists of: activation of genes necessary for differentiation and repression of genes inhibiting adipogenesis. Most of the information about the changes in gene expression during different stages of adipocyte differentiation has been derived from preadipose cell lines. To date there is only limited information about the molecular events accompanying primary preadipocyte differentiation.

##### 2.2.4.2.1 Growth arrest

It is believed that in both primary preadipocytes and preadipose cell lines, differentiation requires growth arrest(232). Transcription factors such as C/EBPα and PPARγ are involved in the process of growth arrest. C/EBPα contributes through its antimitotic activity (233), it increase mRNA and protein levels of p21/SDI-1 and thus inhibits the cell growth (234). PPARγ is able to induce growth arrest in fibroblasts most probably in cooperation with C/EBPα (235). It has been hypothesised that although

C/EBPα and PPARγ expression levels increase in the process of differentiation, they are already high enough in preadipocytes to induce growth arrest.

#### 2.2.4.2.2 Clonal expansion

The continuation of differentiation process after preadipocyte growth arrest depends on the availability of appropriate mitogenic and adipogenic signals. Preadipose cell lines undergo one or two rounds of DNA replication and cell division after growth arrest(232). It has been shown that inhibition of DNA synthesis at this stage inhibits the differentiation into fat cells(236-238). The drug rapamycin is able to inhibit the process of clonal expansion. This inhibition interferes with the subsequent adipocyte differentiation confirming the critical role of clonal expansion in 3T3-L1 differentiation model. Isolated primary human preadipocytes differentiate without cell division, giving rise to the speculation that probably they have already undergone this critical clonal expansion *in vivo* (239). Clonal expansion is characterized with temporal increase in the levels of the phosphatase inhibitor HA2. If constitutive expressed during clonal expansion this inhibitor blocks differentiation. Constitutive expressions during later stages of differentiation doesn't have this effect (240).

#### 2.2.4.2.3 Early changes in gene expression

Growth arrest and clonal expansion are accompanied with changes in the pattern of gene expression. The expression of LPL increases spontaneously at confluence (192;215;236;238;241-243). This factor is secreted by mature adipocytes and controls lipid accumulation (244;245). The early phases are characterised with a temporal increase in C/EBPβ and C/EBPδ expression (246-248). An increase in the not adipocyte specific PPARδ expression precedes that of PPARγ (249). Although PPARγ expression reaches its maximum in mature adipocytes, it increases already somewhere about day second after the hormonal induction of differentiation (246;250). The early induced C/EBPβ and C/EBPδ expression decreases at the moment when C/EBPα expression increases followed by a rise in the expression of a wide variety of adipocyte-specific genes (246;248;251). SREBP-1c is also induced in early differentiation process. SREBP-1c takes part in cholesterol metabolism and influences adipocyte gene expression (252-254). Preadipocyte factor-1 (pref-1) which maintains the preadipocyte phenotype, decreases during differentiation (255-257). Adipocyte differentiation is

characterised with drastic changes in shape, morphology and adipocyte-specific gene expression. Expression of genes such as actin and tubulin decreases (258). The occurring changes in cytoskeletal and Extracellular matrix (ECM) components eventually affect the expression and action of PPARs and/or C/EBPs.

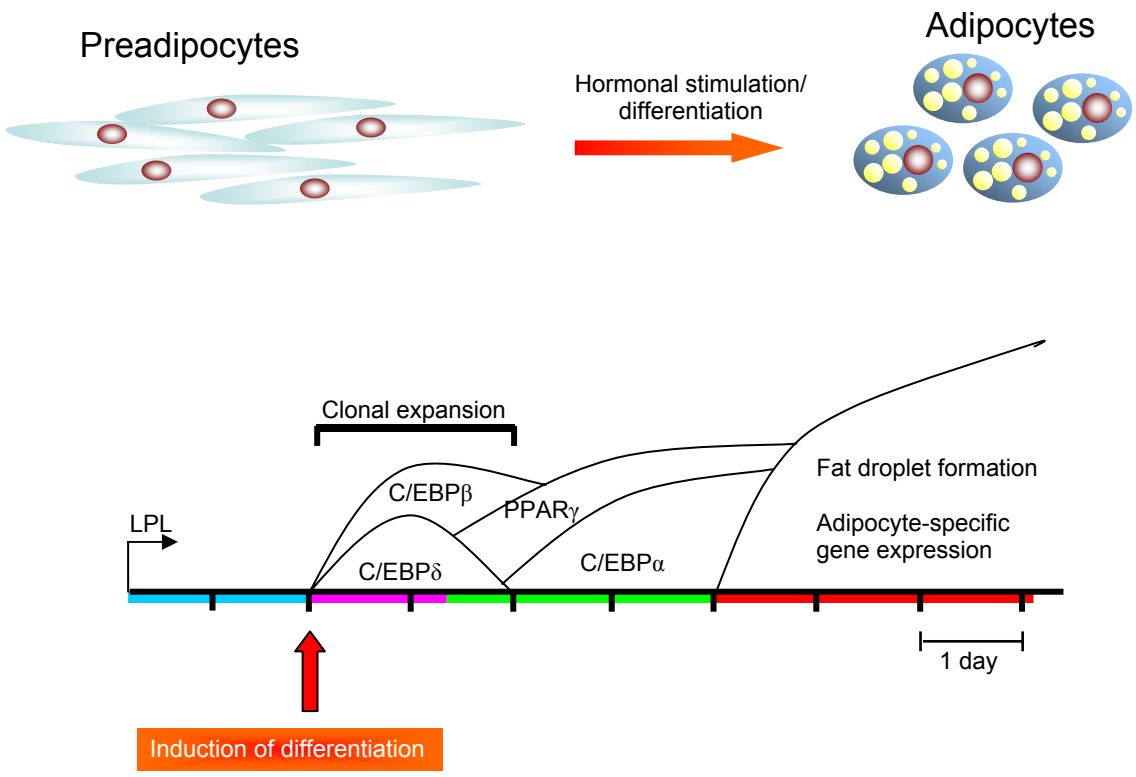



**Figure A** Progression of 3T3-L1 preadipocyte differentiation. The major identified events of preadipocyte differentiation are presented chronologically. Areas labelled by gene names represent periods of gene expression during the differentiation program. The distinct stages of differentiation (very early, early, intermediate and late) are also provided (259).

### 2.2.4.2.4 Terminal Differentiation

Acquisition of insulin sensitivity and increase in *de novo* lipogenesis are characteristic features of terminal differentiation. This stage presents marked increase in mRNA, protein level and activity of many enzymes involved in TG metabolism: ATP citrate lyase, glycerol-3-phosphate acyltransferase, malic enzyme, fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CoA desaturase, glycerol-3-phosphate dehydrogenase (260-262). The syntheses of certain factors such as perilipin (protein associated with lipid droplets), fatty acid binding protein (aP2) and transporter of fatty acids (CD36) increases (262-266). The occurring increase in insulin sensitivity is accompanied with concomitant increase in glucose transporters and the amount of insulin and adrenergic

receptors (267-271). Adipocytes secrete a lot of factors such as adiponectin, leptin, adipsin, monotubulin, PAI -1 and angiotensinogen II (185;187;242;272-275). The expression of many genes such as leptin, GLUT4, stearoyl-CoA desaturase (SCD1), aP2 and phosphoenolpyruvate carboxykinase (PEPCK) is under the control of PPARγ and C/EBPα (276-280). A process called dedifferentiation has also been suggested. During this process adipocytes reacquire preadipocyte characteristics (173;175;281). However to date it is not clear, whether or not reversion to a preadipose phenotype exists *in vivo.*

**2.2.4.2.5 Factors influencing differentiation**

GH promotes differentiation and turns preadipocytes sensitive to the mitogenic effects of insulin-like growth factor-I (IGF)-I which are necessary for clonal expansion (282-284). GH is also hypothesised to be involved in adipose conversion through the promotion of antimitogenic state (282;285). GH possesses lipolitic activity and eventually antiadipogenic. IGF-I from other site plays a role in the differentiation of 3T3-L1 preadipocyte as well as primary cultures (216;286-289). Insulin and growth factors activated serine/threonine kinase Akt (PKB) also takes part in differentiation process. Akt is a downstream factor of phosphatidylinositol 3-kinase pathway. Constitutive expression of Akt in 3T3-L1 preadipocytes has been shown to induce spontaneous differentiation even in the absence of differentiation stimulus (Dexamethasone(DEX)/IBMX/insulin) (290). Growth factors and cytokines are mostly inhibitors of differentiation. Transforming growth factor (TGF)α for example has been shown to inhibit differentiation (291;292). EGF I has also been characterised as an inhibitor of differentiation (292-294). Contradictory reports exist concerning the effect of platelet-derived growth factor (PDGF) ranging from inhibition to stimulation of differentiation (288;295-297). TGFβ has been found to indisputably inhibit differentiation process (210;294;298-300). Other inhibitors of differentiation are interleukin-1β and interferon-γ (301-303). TNF-α is a negative regulator of LPL and inhibitor of adipocyte differentiation (173;174) Its antiadipogenic activity may be due to its downregulation of C/EBPα and PPARγ expression, and upregulation of *myc* expression (171;304-307). The effect of the superfamily of nuclear hormone on adipocyte differentiation is not very clear. In principle they act through binding to intracellular hormone receptors. DEX (a synthetic glucocorticoid) is a component of the routine differentiation cocktail (203;210;211;213;214;308-311). Glucocorticoids induce

expression of C/EBPδ followed by the formation of C/EBPδ-C/EBPβ heterodimer and ultimately expression of PPARγ (247). Interestingly, at supraphysiological concentrations they inhibit adipocyte differentiation (213;312). Retinoic acids (RA) inhibit C/EBPβ expression with subsequently inhibition of C/EBPα and PPARγ expression (250;313;314). On the other hand 3,3',5-triiodothyronine effect on preadipocyte differentiation is restricted to Ob17 cell line and it has no effect on other models (213;214;225;227;228;288;315-317).

Adipocytes as well as preadipocytes are able to produce prostaglandins (PGs) (318;319) with various functions. Prostaglandin $E_2$ has been shown to have an antilipolytic effect. $PGF_{2\alpha}$ and $PGI_2$ have been characterized to act as modulators of differentiation process. Prostaglandin $D_2$ functions as an adipogenic signals with a stimulatory effect on differentiation (246;320). Prostacyclin ($PGI_2$) function as an adipogenic effector as well (294;321;322) with clear activatory effect on PPARs (246;323;324). On the other hand prostaglandin $F_{2\alpha}$ inhibits differentiation (294;319;325-328) and prostanoid receptor (FP) is also involved in the inhibition of differentiation (328;329). $PGE_2$ and $PGD_2$ have no clearly defined role in the process of preadipocyte differentiation (176;329).

Concerning the effect of cAMP on adiposity differentiation we have to mention that IBMX is used *in vitro* as a differentiation stimulus. IBMX increases the expression of C/EBPβ (330). And although it is suspected that IBMX acts through stimulation of cAMP accumulation (331;332) the mechanism of its action is still unknown.

Some studies demonstrated that pref-1, which is an EGF repeat-containing transmembrane protein, is able to inhibit adipocyte differentiation (333). It has been hypothesised that pref-1 links the cell interior to the differentiation signals from the extracellular environment (256;257;334). In mature adipocytes pref-1 is almost undetectable. Its expression is downregulated by FCS and it is suspected that pref-1inhibits adipocyte differentiation through unique mechanism (333).

There is a link between exogenous hormones and transcriptional regulators. In contrast, knowledge of the molecular mechanisms regarding the effect of the ECM on adipocyte differentiation is lacking. The molecular mechanisms of ECM effect on adipocyte differentiation are very important for our understanding of how is differentiation really going in tissues. ECM components as well as cell adhesion molecules modify the interaction between cells and environment and affects differentiation and migration. ECM alters cell situation and exposes the cell surface to different factors of the environment (335). Components of ECM interact with growth factors (336). Some

authors hypothesised cytoskeleton connection between nuclear matrix and ECM (337). To date there is not much information about how ECM influences adipocyte differentiation. During adipocyte differentiation, there are changes in cytoskeletal components, cell morphology, and the type of secreted ECM components (338).

## 2.3 Adipocyte and HAART

*In vivo* studies in HIV patients have three main disadvantages:

- Complexity
- Simultaneous use of several anti-retroviral drug classes
- Difficulty in obtaining tissue material

*In vitro* studies:

- Can be performed with well characterized adipocyte cell lines (3T3-L1 or 3T3F442A) to investigate drug effects on individual differentiation steps and events.
- Clarify the influence of individual molecules on adipocyte functions
- Are easy to perform, however, do not always parallel the *in vivo* situation

### 2.3.1 Effects of PI

*In vitro* PI have been found to affect adipogenesis, adipocyte functions, insulin sensitivity and adipocyte apoptosis. In terms of insulin sensitivity it has been shown that short nelfinavir (NFV) incubation, inhibits insulin signalling (339;340), and that IDV has a rapid and reversible inhibitory effect on GLUT4, independent of insulin signalling (339;341;342). As several PI have been found to inhibit *in vitro* adipocyte differentiation (35;36;343-345), some groups proposed that PI interfere with SREBP-1 maturation (47;346) with subsequent effect on expression of genes such as PPARγ, C/EBPα and FAS (47;346;347). For example, IDV inhibits *in vitro* the expression LPL and FAS, both downstream factors of SREBP-1 (344). On the other hand some PI were suggested to impair *in vitro* lamin A/C maturation leading to disruption of nuclear architecture. The disrupted nuclear architecture has been hypothesised to induce SREBP-1 nuclear dislocation and ultimately adipocyte dysfunction (38).

### 2.3.2 Effects of NRTI

#### 2.3.2.1 Effects of NRTI on adipogenesis

Subcutaneous abdominal adipose tissue from lipoatrophic HAART treated patients contains higher percent of small adipocytes in comparison to control fat and demonstrates decreased expression of the adipogenic transcription factors C/EBPβ, C/EBPα, PPARγ and SREBP-1c (348). The *in vitro* experiments targeting elucidation of the above mentioned changes in adipose tissue brought however some contradictory results. One group demonstrated no effect of d4T on 3T3-L1 lipid accumulation even in combination with some PI (346), when other showed that d4T and didanosine (ddI) affect the lipid accumulation and increase the rate of adipocyte cell death in 3T3-F442A adipocytes without effect on differentiation process and insulin sensitivity (349). A third group however found NRTI to interfere with 3T3-F442A adipocytes differentiation (345). In both cell lines d4T and AZT intracellular phosphorylation has been confirmed (350).

Some increase in the level of adipocyte apoptosis in subcutaneous adipose tissue from HAART patients has been reported (351) which could be a result from HAART-induced cytokine overexpression and/or mitochondrial toxicity.

#### 2.3.2.2 Effects of NRTI on mtDNA

Numerous pathological alterations related to mitochondrial toxicity such as hepatic steatosis, myopathy and lactic acidosis have been diagnosed in HAART treated patients and have been predominantly associated with NRTI-induced mtDNA depletion (49;60;61). *In vitro* studies demonstrated that d4T concentrations near $C_{max}$ for 4 days reduce the mtDNA content in CEM cells by 50% (352) and 25 days d4T (36 μM) incubation reduce the mtDNA content of HepG2 hepatoma cells by half (353). Some inborn mitochondrial diseases have been associated with fat redistribution although not similar to the HAART associated one (62;63). As mtDNA depletion and mitochondrial ultra-structure abnormalities have been found in NRTI treated patients (50-53), NRTI induced WAT mtDNA depletion has been suggested to play a role in lipodystrophy pathogenesis (49). Some studies correlated (51), but others did not, the lipoatrophy with subcutaneous fat mtDNA depletion, suggesting that mtDNA depletion may not be the main mechanism of NRTI induced lipoatrophy (50;53). Several studies found normal mtDNA levels in HAART treated patients with lipodystrophy (126;354-356). So far the

link between NRTI-induced mtDNA depletion and impairment of oxidative phosphorylation (OXPHOS) hasn't been established. Eventual OXPHOS deficiency in adipocytes could be accompanied by reduction in fatty acid synthesis, decrease in the oxidation of fatty acids and apoptosis (77). Some studies suggest that mitochondrial dysfunction like impaired OXPHOS may be compensated through mechanisms such as mitochondrial proliferation (79;87). Such compensation has been reported in adipose tissue of lipoatrophic HAART treated patient (51;88).

#### 2.3.2.3 Effects of NRTI on mitochondria (besides mtDNA depletion)

It is considered that NRTI may cause mtDNA oxidative damage (95;357). Furthermore some heteroplasmic mtDNA point mutations as well as multiple deletions in mtDNA have been reported in connection to NRTI treatment (124;355;356). On the other hand NRTI have been also related to direct inhibition of mitochondrial respiration (96). Another mechanism contributed to NRTI adverse effects on mitochondria is inhibition of ADP/ATP translocase (97). Some *in vivo* alteration in fatty acid and lipid metabolism unrelated to mtDNA content have been also documented (74;81).

### 2.3.3 Other factors contributing to mitochondrial dysfunction in WAT

TNF-α has been found to be able to affect mitochondrial function and to cause mtDNA damage. On the other hand the protease inhibitor RTV has been reported to induce mtDNA damage in human endothelial cells (358). Diabetes has also been involved in mtDNA damage as different tissues of diabetic patient presented mtDNA damage such as mtDNA depletion and multiple mtDNA deletions (359-361).

## 2.4 Metabolic complications

Although HAART has enormously improved the prognosis and survival of HIV infected patients, it has been associated with metabolic complications like IR, dyslipidemia, and fat redistribution and the term HAART-lipodystrophy syndrome has been introduced to in order to describe these pathological complications.

### 2.4.1 Insulin resistance

IR and impaired glucose tolerance are among the major metabolic abnormalities in HAART-lipodystrophy syndrome (362;363). These alterations have been associated with atherogenic lipid profiles which contribute to an independent risk of cardiovascular

disease (364). Even diabetes has been reported in association with the therapy (149). Disturbances in glucose homeostasis are very often accompanied by fat redistribution in HAART treated patients (149;365). *In vitro* studies demonstrated direct effect of some PI on insulin sensitivity(366). However, recent new results surprisingly demonstrated increased glucose uptake from subcutaneous adipose tissue (SAT) from lipoathrophic patients on a PI based regimen (367). This observation is not in line with hypothesis in which lipoatrophy is induced through PI generated blockade of insulin-stimulated glucose uptake in adipose tissue. This increased glucose uptake in subcutaneous fat of lipoatrophic patients may even compensate for the fat loss. Nevertheless the whole picture is that in the settings of HAART-induced lipodystrophy, IR correlates with decrease in subcutaneous and increase in intra abdominal fat (368;369), and there are plenty of studies confirming such correlation (149;368;370-372). The apparent increase in lipolysis and the inability of insulin to decrease FFA under such conditions argue in favour of eventual IR at the level of adipose tissue as well (373;374). Even genetic forms of lipoatrophy (Beradinelli-Seip syndrome) present IR (348).

### 2.4.2 Dyslipidemia

Lipid disturbances are commonly seen in HIV-infected patients with alterations like hypertriglyceridemia reported even before the introduction of HAART (144). Decreased TG clearance in combination with increased syntheses of VLDL in the liver were also commonly reported (375). In the era of HAART hypertriglyceridemia has been associated with PI use and has been induced by RTV in HIV-negative individuals (26). The HAART-associated lipodystrophy has been also associated with hypertriglyceridemia, and decrease of high-density lipoprotein (HDL) and increase in LDL cholesterol (362-364). Fat redistribution itself is frequently accompanied with hypertriglyceridemia and low HDL (149;365). The combination of high LDL together with low HDL is a constellation well known to be associated with an increased risk of coronary heart disease (CHD).

## 3 AIMS OF THE STUDY

The aims of our study were to address the following issues:

Do NRTI and PI affect adipocyte morphology, differentiation and TG accumulation?

Is the gene expression during adipogenesis altered as a result of antiretroviral drugs effect on *in vitro* models of differentiation?

Do NRTI deplete mtDNA content in adipocyte cell line models, adipocyte primary cultures and *in vivo* murine models?

What influence does adipocyte developmental stage have on NRTI-induced mtDNA depletion?

Does mtDNA depletion correlate with impairment of respiratory chain enzyme activity, mitochondrial membrane potential and cell viability?

Do antiretroviral drugs affect circulating concentration of adiponectin and its expression in adipocyte and murine models?

# 4 MATERIALS AND METODS

## 4.1 MATERIALS

### 4.1.1 PCR

| | |
|---|---|
| DNeasy Tissue Kit (50) | Qiagen, Hilden |
| RNeasy Lipid Tissue Mini Kit (50) | Qiagen, Hilden |
| RNeasy Protect Mini Kit (50) | Qiagen, Hilden |
| Omniscript RT Kit (200) | Qiagen, Hilden |
| QuantiTect SYBR Green PCR Kit (1000) | Qiagen, Hilden |
| QIAshredder Kit (50) | Qiagen, Hilden |
| RNeasy-Free DNase set (50) | Qiagen, Hilden |
| RNase A | Qiagen, Hilden |
| Taq PCR Core Kit (250 U) | Qiagen, Hilden |
| BCA™ Protein Assay Kit | PIERCE |
| TA Cloning® Kit with pCR®2.1 | Invitrogen, USA |
| Taq PCR Core Kit (250 U) | Qiagen, Hilden |

**4.1.2Primers** All the primers are given in the 5’ to 3’ direction

Real time PCR

Actb;D:11461

*FORWARD PRIMER* TGT TAC CAA CTG GGA CGA CA

*REVERS PRIMER* GGG GTG TTG AAG GTC TCA AA

LPL;ID:16956

*FORWARD PRIMER* TGT CAG CTG GCT ACA CCA AG

*REVERS PRIMER* GGT CAG ACT TCC TGC TAC GC

C/EBPα;ID:12606

*FORWARD PRIMER* TGG ACA AGA ACA GCA ACG AG

*REVERS PRIMER* TCA CTG GTC AAC TCC AGC AC

C/EBPβ;ID:12608

*FORWARD PRIMER* ACT TCA GCC CCT ACC TGG AG

*REVERS PRIMER* GGC TCA CGT AAC CGT AGT CG

PPARγ;ID:19016

*FORWARD PRIMER* CGA GTC TGT GGG GAT AAA GC

*REVERS PRIMER* GGA TCC GGC AGT TAA GAT CA

Adiponectin; ID:11450

*FORWARD PRIMER* GGA ACT TGT GCA GGT TGG AT

*REVERS PRIMER* CCA AGA AGA CCT GCA TCT CC

Tfrc; ID:22042

*FORWARD PRIMER* TCC GCT CGT GGA GAC TAC TT

*REVERS PRIMER* ACA TAG GGC GAC AGG AAG TG

**Primers for nested PCR**

GH; ID: 2688

*FORWARD PRIMER* GGT CTC CAG CGT AGA CCT TG

*REVERS PRIMER* CAA TCC TCC AGG CCT TTC TC

*FORWARD PRIMER* GCA TCC ACT CAC GGA TTT CT

*REVERS PRIMER* AAG AGA AGG AGA GGC CAA GC

mtDNA; NC_001807

*FORWARD PRIMER* GAG GAG ACA AGT CGT AAC ATG G

*FORWARD PRIMER* GTG CAC TTG GAC GAA CCA G

*REVERS PRIMER* GGG TTT GGG GCT AGG TTT AG

mtDNA; AY172335

*FORWARD PRIMER* CCG TCA CCC TCC TCA AAT TA

| | |
|---|---|
| *REVERS PRIMER* | GGG CTA GGA TTA GTT CAG AGT G |
| GH; ID: 14599 | |
| *FORWARD PRIMER* | CTG GCT GCT GAC ACC TAC AA |
| *REVERS PRIMER* | TCC CCT ACC CCA TAG TTT CC |

**Primers for cloning**

| | |
|---|---|
| GH; ID: 2688 | |
| *FORWARD PRIMER* | TAC AGG TAA GCG CCC CTA AA |
| *FORWARD PRIMER* | GTC CTG TGG ACA GCT CAC CT |
| *REVERS PRIMER* | AAG AGA AGG AGA GGC CAA GC |
| mtDNA; NC_001807 | |
| *FORWARD PRIMER* | AAG GTG TAG CCC ATG AGG TG |
| *FORWARD PRIMER* | ACC ACC TCT TGC TCA GCC TA |
| *REVERS PRIMER* | GGC AGG TCA ATT TCA CTG GT |

### 4.1.3 ELISA

| | |
|---|---|
| Mouse Adiponectin/Acrp30 Quantikine ELISA Kit | R&D Systems, Wiesbaden |
| Human Adiponectin/Acrp30 Quantikine ELISA Kit | R&D Systems, Wiesbaden |
| Mouse Leptin Quantikine ELISA Kit | R&D Systems, Wiesbaden |
| Human LeptinQuantikine ELISA Kit | R&D Systems, Wiesbaden |

### 4.1.4 Media

| | |
|---|---|
| F-12 Nutrient Mixture | GIBCO, Eggestein |
| Hepes | GIBCO, Eggestein |
| Newborn calf serum | GIBCO, Eggestein |

| | |
|---|---|
| Foetal calf serum | PAA Laboratories GmbH |
| DMEM | GIBCO, Eggestein |
| Trypsin EDTA | GIBCO, Eggestein |
| HBSS | GIBCO, Eggestein |
| NEAA | GIBCO, Eggestein |
| $H_2O$ DNAse, RNAse Free | GIBCO, Eggestein |
| MEM | GIBCO, Eggestein |

### 4.1.5 Buffers and solutions

Primary preadipocytes

| | |
|---|---|
| Buffer I | HANKS |
| Buffer II | 500ml HANKS<br>25ml 10%BSA<br>Filter sterilized |
| Primary preadipocyte cell culture medium (500ml) | DMEM/F12 428ml.<br>FBS 50 ml (final conc. 10%)<br>P/S 100U/100mg/ml<br>A-2-P 500µl (500mg+10mlPBS) |
| Differentiation medium for primary human preadipocytes | Primary cell culture medium 500ml<br>DEX 9,5ml (1mg in 1ml ethanol and 1:50 with DMEM)<br>IBMX 5,5ml (100mg in 10ml pure ethanol)<br>Indomethacin 895µl (20mg in 1ml pure ethanol)<br>Insulin 50µl (10mg in 1ml acetic acid) |
| 3T3-L1 propagation medium | 90% DMEM containing<br>4 mM L-glutamine<br>1.5 g/L sodium bicarbonate<br>4.5 g/L glucose<br>P/S 100U/100mg/ml<br>and 10% FCS |

| | |
|---|---|
| 3T3-L1 differentiation medium | 3T3-L1 propagation medium<br>520 µM IBMX<br>1 µM DEX<br>167 nM insulin<br>8 µg/ml biotin |
| HUH 7 propagation medium | 90% MEM (Eagle) containing<br>2 mM L-glutamine<br>Earle's BSS<br>1.5 g/L sodium bicarbonate<br>0.1 mM NEAA<br>1.0 mM sodium pyruvate<br>P/S 100U/100mg/ml<br>and 10% FCS |
| Hep G2 propagation medium | 90% MEM (Eagle) containing<br>2 mM L-glutamine<br>Earle's BSS<br>1.5 g/L sodium bicarbonate<br>0.1 mM NEAA<br>1.0 mM sodium pyruvate<br>P/S 100U/100mg/ml<br>and 10% FCS |
| C2C12 propagation medium | 90% DMEM containing<br>4 mM L-glutamine<br>1.5 g/L sodium bicarbonate<br>4.5 g/L glucose<br>P/S 100U/100mg/ml<br>and 10%; FCS |

**Enzyme activities**

| | |
|---|---|
| Hepes buffer | 110 mM NaCl<br>2.6 mM KCl<br>1.2 mM $KH_2PO_4$<br>1 mM $CaCl_2$<br>1.2 mM $MgSO_4$ $7H_2O$<br>25mM Hepes<br>pH 7.4 |

**Complex I**

| | |
|---|---|
| Modified buffer | Baker water<br>50mM KCl<br>60mM Saccharose<br>50mM Triethanolamin-HCl<br>2mM $MgCl_2$<br>1mM EDTA<br>pH 8 |

| | |
|---|---|
| 100mM NADH | 70mg/1ml modified buffer |
| 100µM Cytochtrom C | 20mg/330µl water |
| Rotenone | 0,1mg/1ml ethanol |
| **Complex II+III** | |
| 50mM $KH_2PO_4$ | 680,5mg/100ml Baker water |
| 250mM $NaN_3$ | 0,0163g/1ml Baker water |
| 100µM Cytochtrom C | 20mg/330µl water |
| Rotenone | 0.1mg/1ml ethanol |
| 1M Succinate (acid) | 1.18g/10ml Baker water |
| Antimycin | 0,001g/1ml ethanol |
| **Complex IV** | |
| 10mM $KH_2PO_4$ | 0,1365g/100ml Baker water<br>pH 7 adjusted with KOH |
| D-Cytochrome C | 0,01g Cytochtrom C<br>1trace ascorbic acid<br>3ml $KH_2PO_4$-buffer<br>Dissolve and dialyse for 3X6h |
| 100mM K-ferricyanide | 32,92g/1l Baker water |
| **Complex V** | |
| Modified buffer | 500ml Baker water<br>50mM KCl<br>60mM Saccharose<br>50mM Triethanolamin-HCl<br>2mM $MgCl_2$<br>1mM EDTA |
| MgATP (200mM) | 0,6g ATP in 3ml 500mM $MgCl_2$-Baker water<br>(Adjust pH 8 with KOH ) |
| 50mM PEP | 0.103g/5ml Baker water<br>(Adjust pH 7 with KOH ) |
| 100mM NADH | 0,07g/1ml modified buffer |

| | |
|---|---|
| LDH | 1100U/mg |
| PK | |
| 50mM EGTA | 0,95g/50ml $H_2O$<br>(Adjust pH 8 with KOH) |
| KCN | 0,02/Modiffied buffer |
| Oligomycin | 10mg/1ml ethanol |

**Citrate synthase**

| | |
|---|---|
| Tris buffer (0,1M Tris-HCL) | 1,6g/100ml Baker water<br>(pH 8 adjust with KOH) |
| Oxaloacetate | 1,32mg/1ml Baker water |
| Acetyl-CoA | 5mg/350µl Baker water |

**Cloning**

| | |
|---|---|
| LB (Luria Bertani) medium | 1.0% Tryptone<br>0.5% Yeast Extract<br>1.0% NaCl<br>pH 7.0 |
| LB agar plates | LB medium<br>15 g/L agar before autoclaving |
| pCR®2.1, linearized | 25 ng/µl in 10 mM Tris-HCl, 1 mM EDTA, pH 8 |
| 10X PCR Buffer | 100 mM Tris-HCl, pH 8.3 (at 42°C)<br>500 mM KCl<br>25 mM $MgCl_2$<br>0.01% gelatin |
| 10X Ligation Buffer | 60 mM Tris-HCl, pH 7.5<br>60 mM $MgCl_2$<br>50 mM NaCl<br>1 mg/ml bovine serum albumin<br>70 mM β-mercaptoethanol<br>1 mM ATP<br>20 mM dithiothreitol<br>10 mM spermidine |
| 50 mM dNTPs | 12.5 mM dATP<br>12.5 mM dCTP |

| | |
|---|---|
| | 12.5 mM dGTP<br>12.5 mM dTTP<br>(adjusted to pH 8.0) |
| T4 DNA Ligase | 4.0 Weiss units/µl |
| Sterile Water | Deionised, autoclaved water |
| Control DNA Template | 0.1 µg/µl in 10 mM Tris-HCl, 1 mM EDTA, pH 8 |
| Control PCR Primers | 0.1 µg/µl each in 10 mM Tris-HCl, 1 mM EDTA, pH 8 |
| S.O.C. Medium | 2% Tryptone<br>0.5% Yeast Extract<br>10 mM NaCl<br>2.5 mM KCl<br>10 mM $MgCl_2$<br>10 mM $MgSO_4$<br>20 mM glucose (dextrose) |
| pUC19 Control DNA | 10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8 |
| Glycerol stocks | Medium<br>15% glycerol |
| Oil Red O stock solution | 300 mg of Oil Red O powder<br>100 ml of 99% isopropanol |
| Oil Red O working solution | 3 parts Oil Red O stock solution<br>2 parts deionised water |
| NRTI and PI solutions | All drugs were dissolved in DMSO.<br>The highest concentration of the solvent used in the incubation experiments (DMSO 0.1%) did not affect cellular viability and preadipocyte differentiation. |

### 4.1.6 Chemicals

| | |
|---|---|
| DNA ladder | Invitrogen, USA |
| $H_2O$ DNAse, RNAse Free | Invitrogen, USA |
| AZT (3′-Azido-3′-deoxythymidine) | Sigma-Aldrich, Munich |
| AZT pills | GlaxoSmithKline-GmbH |

| | |
|---|---|
| D4T (2′,3′-Didehydro-3′-deoxythymidine ) | Sigma-Aldrich, Munich |
| D4T pills | Bristol-Myers Squibb GmbH |
| IDV | Merck Sharp&Dohme |
| TNF-α | Provided by Prof. D. Neumann, Pharmacology, MHH |
| IBMX (3-Isobutyl-1-methylxanthine) | Sigma-Aldrich, Munich |
| Insulin | Sigma-Aldrich, Munich |
| d-Biotin | Sigma-Aldrich, Munich |
| D-Lactose | Sigma-Aldrich, Munich |
| Uridine | Sigma-Aldrich, Munich |
| Dexamethasone | Sigma-Aldrich, Munich |
| DdC (2′,3′-Dideoxycytidine) | Sigma-Aldrich, Munich |
| Oil Red O | Sigma-Aldrich, Munich |
| MitoProbe JC-1 | Molecular Probes, Eugene, OR, USA |
| MitoTracker Red 580 | Molecular Probes, Eugene, OR, USA |
| Annexin V | Molecular Probes, Eugene, OR, USA |
| Trypan blue | ICN Biomedicals, Aurora, USA |
| Propidium iodide | Sigma-Aldrich, Munich |
| Hoechst 33342 | Sigma-Aldrich, Munich |
| PEP (K-phosphoenolpyruvate) | Sigma-Aldrich, Munich |
| Cytochrome C | Sigma-Aldrich, Munich |
| Antimycin A | Sigma-Aldrich, Munich |
| Oligomycin B | Sigma-Aldrich, Munich |
| Rotenone | Sigma-Aldrich, Munich |
| Acetyl CoA | Sigma-Aldrich, Munich |
| Camptothecin | Sigma-Aldrich, Munich |

| | |
|---|---|
| D-Pantothenic acid | Sigma-Aldrich, Munich |
| Amphotericin B | Sigma-Aldrich, Munich |
| Collagenase | Sigma-Aldrich, Munich |
| 2-Deoxy-D-glucose | Sigma-Aldrich, Munich |
| Glucose 40 Braun | Braun, Melsungen, Germany |
| Cytochalasin B | Sigma-Aldrich, Munich |
| Rapamycin | Sigma-Aldrich, Munich |
| NADH (Nicotinamide adenine dinucleotide, reduced) | Roche |
| PK (Pyruvate Kinase) | Roche |
| L-Lactatdehydrogenase | Roche |
| FGF (Fibroblast Growth Factor) | Promocell |
| 2,6-Diisopropylphenol | Sigma-Aldrich, Munich |
| Amobarbital Sodium salt | Sigma-Aldrich, Munich |
| Butilated hydroxyanisole | Sigma-Aldrich, Munich |
| Carbonyl cyanide | Sigma-Aldrich, Munich |
| 6 hydroxy-2,5,7,8-tetramethylchroman 2 carboxylic acid | Merck, Darmstadt |
| Carboxy $H_2$-DCFDA | Molecular Probes |
| L-Ascorbic acid | Sigma-Aldrich, Munich |
| Indomethacin | Sigma-Aldrich, Munich |
| MgATP | Sigma-Aldrich, Munich |
| BSA (Albumin from bovine serum) | Sigma-Aldrich, Munich |
| PBS (Phosphate buffered saline) | Biochrom, Berlin AG |
| Trypsin-EDTA | GIBCO, Eggestein |
| Biocoll separating solution | Biochrom, Berlin AG |
| PS (penicillin streptomycin) | Biochrom, Berlin AG |

| | |
|---|---|
| $KH_2PO_4$ (Potassium phosphate monobasic) | Sigma-Aldrich, Munich |
| $CaCl_2$ (Calcium chloride) | Sigma-Aldrich, Munich |
| $MgSO_4$ (Magnesium sulphate) | Sigma-Aldrich, Munich |
| Sucrose (β-D-Fructofuranosyl-α-D-glucopyranoside) | Sigma-Aldrich, Munich |
| Ethidium Bromide | Sigma-Aldrich, Munich |
| Agarose | Invitrogen, USA |
| $MgCl_2$ (Magnesium chloride) | Sigma-Aldrich, Munich |
| Formalin (formaldehyde) | Sigma-Aldrich, Munich |
| CCCP (Carbonyl cyanide 3-chlorophenylhydrazone ) | Sigma-Aldrich, Munich |
| KOH (Potassium hydroxide) | Sigma-Aldrich, Munich |
| EDTA (Ethylenediaminetetraacetic acid) | Sigma-Aldrich, Munich |
| $NaN_3$ (Sodium azide) | Sigma-Aldrich, Munich |
| Succinate | ICN Biomedicals, Aurora, USA |
| Acetic acid | Sigma-Aldrich, Munich |
| Ethanol | J.T. Baker |
| KCN (Potassium cyanide) | Sigma-Aldrich, Munich |
| EGTA Ethylene glycol-bis (2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid | Sigma-Aldrich, Munich |
| Tris-HCl (TRIS hydrochloride) | Sigma-Aldrich, Munich |
| Oxaloacetate | Merck, Darmstadt |
| DOC (dissolved organic carbon) | Sigma-Aldrich, Munich |
| Baker water | J.T. Baker |
| TCA (Trichloroacetic acid) | Sigma-Aldrich, Munich |
| IPTG (Isopropyl β-D-1-thiogalactopyranoside ) | Sigma-Aldrich, Munich |

| | |
|---|---|
| Ampicillin | Sigma-Aldrich, Munich |
| Kanamycin | Sigma-Aldrich, Munich |
| X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) | Sigma-Aldrich, Munich |
| Glycerol | Sigma-Aldrich, Munich |
| Tryptone | Sigma-Aldrich, Munich |
| Yeast Extract | Sigma-Aldrich, Munich |
| NaOH (Sodium hydroxide) | Sigma-Aldrich, Munich |
| Agar | Sigma-Aldrich, Munich |
| Gelatin | Sigma-Aldrich, Munich |
| β-mercaptoethanol | Sigma-Aldrich, Munich |
| DTT (dithiothreitol) | Sigma-Aldrich, Munich |
| Spermidine | Sigma-Aldrich, Munich |
| Isopropanol | J.T. Baker |

### 4.1.7 Consumables

| | |
|---|---|
| Conical Filter Paper | Whatman GmbH, Germany |
| Cell culture dishes | Becton Dickinson, Heidelberg |
| Tubes | Greiner, Frickenhausen |
| Cell culture flasks | Greiner, Frickenhausen |
| 96 well-round bottom plates | Greiner, Frickenhausen |
| 96 well-sharp bottom plates | ICN Biomedicals, Aurora, USA |
| 24 well cell culture plates | Corning Costar, Sigma-Aldrich, Munich |
| 12 well cell culture plates | Corning Costar, Sigma-Aldrich, Munich |
| 6 well cell culture plates | Corning Costar, Sigma-Aldrich, Munich |
| Petri-dishes | Sarstedt, Nümbrecht |

| | |
|---|---|
| Syringes | Braun, Melsungen, Germany |
| Needles | Braun, Melsungen, Germany |
| One touch test strips | Bayer, Germany |
| Sticking plastic film for the plates | Nunc, Wiesbaden |
| PS (Polyester)-tubes (0,6ml) | Greiner, Frickenhausen |
| Sterile filter (Easy flow TM) | Becton Dickinson, Franklin, USA |
| Safe Lock tube 1,5ml | Eppendorf, Hamburg |
| PCR tubes | Eppendorf, Hamburg |
| Optical 96-well reaction plates | Applied Biosystems, USA |
| Optical Adhesive Cover Starter Kit | Applied Biosystems, USA |
| Pipette tips 1000µL | Eppendorf, Hamburg |
| Pipette tips 200µL | Eppendorf, Hamburg |
| Pipette tips 100µL | Eppendorf, Hamburg |
| Pipette tips 10µL | Eppendorf, Hamburg |
| Optical tape | BioRad, Hercules, USA |
| Safe Skin Purple Nitrile | Kimberly-Clark, Roswell, USA |
| Safe Skin Satin Plus | Kimberly-Clark, Roswell, USA |
| FACS Flow 20l | Becton Dickinson, Heidelberg |
| Cryo-Box | NeoLab, Heidelberg |
| Cryo Tube | Roth, Karlsruhe |

### 4.1.8 Equipment

| | |
|---|---|
| iCycler | BioRad, Hercules, USA |
| Thermocycler | Biometra, Goettingen |
| Counter (MicroBeta) | PerkinElmer |

| | |
|---|---|
| Cell harvester | Inotech, Rockvill, MD |
| Gel Doc 2000 | BioRad, Hercules, USA |
| Lamina Air Flow (Gelaire) | Flow Laboratories Gmbh, Germany |
| Centrifuge 5415C | Eppendorf, Hamburg |
| Multifuge 3s-r | Heraeus, Osterode |
| Verifuge3,2RS | Heraeus, Osterode |
| Centrifuge (AvantiTM30) | Beckman-Coulter, Krefeld |
| Centrifuge J2-21 | Beckman-Coulter, Krefeld |
| Vortex (Stuart) | Bibby Sterilin LTD, Staffordshire, UK |
| Shaker | GFL (Gesellschaft fur Labortechnik mbH) |
| Photometer UV-1202 | Shimadzu Europa GmbH, Duisburg |
| Spectrophotometer 8453 | Hewlett-Packard |
| Photometer | Labsystems Multiskan PLUS, Helsinki |
| Scales | Sartorius Gmbh, Göttingen |
| Ascensia Elite Sensor Glucometer | Bayer, Germany |
| FACSCalibur | Becton Dickinson, Heidelberg |
| Pipette research 1000 | Eppendorf, Hamburg |
| Pipette research 200 | Eppendorf, Hamburg |
| Pipette research 100 | Eppendorf, Hamburg |
| Pipette research 10 | Eppendorf, Hamburg |
| Pipette research 2,5 | Eppendorf, Hamburg |
| Microscope | Carl Zeiss, West Germany |
| Microscope | Carl Zeiss, West Germany |
| Incubator | PLP,Heraeus, Australia |
| Water bath | Jurgens, Burgwedel |

| | |
|---|---|
| Hand counter | Omnilab, Bremen |
| Dual Timer | Omnilab, Bremen |

### 4.1.9 Cell lines and animals

| | |
|---|---|
| C2C12 | LGC Promochem GmbH, Germany |
| HUH 7 | Gift from Herpetology and Gastroenterology |
| 3T3-L1 | LGC Promochem GmbH, Germany |
| Hep G2 | LGC Promochem GmbH, Germany |
| Primary fibroblasts | Isolated from human |
| Primary preadipocytes | Isolated from human |
| One Shot® TOP10F´ Chemically Competent E. coli | Invitrogen, USA |
| One Shot® TOP10 Chemically Competent E. coli | Invitrogen, USA |
| C57BL/6 mice | Charles River Laboratories, USA |

## 4.2 METODS

### 4.2.1 DNA Isolation

DNA isolation was done using DNeasy Tissue Kit (50) Cat. No 69504 (QIAGEN GmbH, Deutschland) following the manufactures procedure. Briefly, total cellular DNA purification was achieved without organic extraction or ethanol precipitation, but using silica-gel-membrane technology. Selective binding of DNA to the DNeasy membrane took place after cell lyses. Proteinase K was used in the initial sample lyses. Contaminants and enzyme inhibitors (proteins and divalent cations) were removed by centrifugation. DNA binds selectively the DNeasy membrane and contaminations go through. Two wash steps removed the left over contaminations and enzyme inhibitors. Finally DNA was eluted. DNA A260/A280 ratio was between 1.7 - 1.9. The isolated fragments are between 100bp and 50 kb in size.

### 4.2.2 Total RNA Isolation

#### 4.2.2.1 Total RNA Isolation from lipid tissues

Total RNA was isolated using RNeasy Lipid Tissue Mini Kit (50) Cat. No74804 (QIAGEN GmbH, Deutschland) following the manufactures procedure. Briefly, in this kit phenol/guanidine-based lysis is combined with silica-gel membrane purification of total RNA. QIAzol Lysis Reagent consists of single-phase solution of guanidine thiocyanate and phenol. This solution is designed to improve fatty tissues lysis and inhibit RNases. After samples homogenization with QIAzol Lysis Reagent (QIAGEN GmbH) chloroform was added and the homogenate separated by centrifugation into organic and aqueous phases. Proteins are in the lower (organic phase) and to the interphase, DNA is in the interphase and RNA is in the upper (aqueous phase). Ethanol was added to the upper, aqueous phase to ensure appropriate binding conditions. Total RNA binds the membrane of the RNeasy Mini Spin Column making possible, phenol and the other contaminations to be washed away. RNase-free water was used for the elution of total RNA. With this method all RNA molecules that are longer than 200 nucleotides were isolated. RNAs such as 5.8S rRNA, 5S rRNA, and tRNAs which are <200 nucleotides were not isolated.

#### 4.2.2.2 Total RNA isolation from no lipid tissues and cells

Total RNA from no-lipid tissues and cells was isolated using RNeasy Protect Mini Kit (50) Cat. No 74124 (QIAGEN GmbH, Deutschland) following the manufactures procedure. Briefly, this is a combination of microspin technology and selective binding properties of a silica-based membrane. Guanidine-thiocyanate-containing buffer (which inactivates RNases) was used for denaturation, lyses and homogenization. Total RNA binds to the membrane of RNeasy Mini spin column and ethanol provides appropriate binding conditions leaving the contaminations to be washed away. RNA was then eluted in water. The membrane binds RNA molecules longer than 200 nucleotides. RNAs such as 5.8S rRNA, 5S rRNA, and tRNAs which are <200 nucleotides were not isolated.

Reliable gene expression analyses require RNA stabilization as an absolute prerequisite. Specific and nonspecific RNA degradation as well as transcriptional induction occur after harvesting the samples, which leads to significant changes in the gene expression pattern. RNA later RNA Stabilization Reagent (QIAGEN GmbH, Deutschland) is new technology for preservation of the gene expression pattern. After submerging in RNA later RNA Stabilization Reagent, it permeates the tissues and

stabilizes and protects RNA. With Stabilization Reagent the cellular RNA remains intact and undegraded even at elevated temperatures. RNA later RNA Stabilization Reagent was used only for tissue samples, as it does not stabilize RNA in animal cells, whole blood, plasma and serum.

RNA concentration was determined by the spectrophotometric absorbance at 260 nm (A260). The relationship between absorbance and concentration (A260 reading of 1 = 40 µg/ml RNA) determined for an extinction coefficient calculated for RNA in water was used. The principle of photometry requires readings between 0.15 and 1.0 in order to have significant results. Photometric absorbance is the same for DNA and RNA. DNA contamination results in higher A260 values. RNA purity was estimated by the ratio of absorbance values at 260/280 nm. RNA had an A260/A280 ratio of 1.9– 2.1. Protein contaminations result in lower ratio. For RNA purity the absorbance was measured in 10 mM Tris·Cl, pH 7.5.

### 4.2.3 Reverse Transcription

Reveres transcription of mRNA was done using Omniscript RT Kit (200) Cat. No 205113 (QIAGEN GmbH, Deutschland) following the manufactures procedure. Briefly, Real-Time PCR requires reverse transcription of mRNA when quantifying RNA. Using oligo-dT primers that specifically hybridize to the poly-A tail of mRNAs, RNA was transcribed into single-stranded cDNA. The multifunctional enzyme reverse transcriptase has 3 distinct enzymatic activities, which *in vivo* in retroviral infections allow transcription of the single-stranded RNA genome into double-stranded DNA:

- RNA-dependent DNA polymerase (reverse transcriptase). Transcribes cDNA from an RNA template (reverse transcription). This activity is used to synthesize cDNA.
- Hybrid-dependent exoribonuclease (RNase H). Specifically degrades only the RNA in RNA:DNA hybrids. It affects RNA that is hybridized to cDNA with no effect on pure RNA. RNase H activity during reverse transcription improves the sensitivity of subsequent PCR.
- DNA-dependent DNA polymerase

*In vitro* activity 1 and 2 are utilized to produce single-stranded cDNA

A negative control was used to test for DNA contamination. Genomic DNA cannot be completely eliminated for certain genes. There are pseudogenes and genes lacking introns. To detect DNA contamination a control reaction with no reverse transcription

was performed. The control RT reaction contained template RNA and all other components, except reverse transcriptase enzyme. An aliquot of this reaction was used in the PCR.

### 4.2.4 Real-Time PCR

Expression analyses as well as gene quantification were performed using QuantiTect SYBR Green PCR Kit (1000) Cat. No 204145 (QIAGEN GmbH, Deutschland) following the manufactures procedure and instructions. Briefly, the primers were designed using free available computer software Primer3, Steve Rosen & Helen Skaletsky, 2000 ( www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi ) and following the general guidelines for PCR Primer: GC content about 40–60%; primer length of 18–30 nucleotides; to use an annealing temperature of an average 5°C below Tm; primer pairs to be with similar Tm values; the length of the PCR product to be < 300 bp; to avoid sequences of 3 or more Cs or Gs at the 3' end; to avoid a 3'-end T which gives greater tolerance of mismatch; to avoid mismatches between the target-template sequence and primer 3' end; to reduce primer-dimer formation so that not to allow for complementarities of more than two bases at 3' ends of primer pairs; to avoid complementarity's sequences between the primers pair within the primer sequence. For gene expression analyses one half of the primer was designed to hybridize to the 3' end of one exon and the other half to the 5' end of the adjacent exon, so that the primers anneal to cDNA, but not to genomic DNA. In all cases when it was impossible, Real-Time PCR primers were designed to flank a region that contains introns and in this case the products were not amplified from genomic DNA under our reaction conditions, as they were much longer. All the expression analyses were done under the following reaction conditions:

| | Time | Temp |
|---|---|---|
| Initial activation step | 15 min | 95C |
| 3-step cycling | | |
| Denaturation | 15 sec | 94C |
| Annealing | 30 sec | 55C |
| Extension | 30 sec | 72C |
| Number of Cycles | 42 | |
| Final Extension | | |

Primer stock solutions were made by dissolving lyophilized primers in TE. The working solutions contained 10 pmol/µl. All the primer solutions were stored at –20°C. Quality check for the primers was performed on a denaturing polyacrylamide gel and estimated by the appearance of a single band.

In the Real-Time PCR reaction SYBR Green I dye was used. SYBR Green I dye binds to the minor groove of double-stranded DNA (dsDNA). SYBR Green I excitation and emission maximums are correspondingly at 497 and 520 nm. Once bound to dsDNA, the fluorescence of SYBR Green I greatly increases and that's how product accumulation during Real-Time PCR is detected. At denaturation step, DNA becomes single-stranded and SYBR Green is free in solution, therefore produces little fluorescence. At annealing the primers hybridize to the target sequence leading to the syntheses of a dsDNA to which SYBR Green I binds. At elongation phase as PCR primers are extended more and more DNA becomes double-stranded and then a maximum amount of SYBR Green I binds. Signal could also be generated from nonspecific dsDNA (spurious PCR products, primer-dimers). The specificity of the Real-Time PCR reaction is determined entirely by the specificity of the primers pairs as SYBR Green fluorescence depends mainly on the presence of dsDNA, which means that any double-stranded products generated during the reaction are detected by the instrument. Primer-dimer formation, extension of primers bound to non-target DNA sequences and other spurious PCR products are not initially distinguishable from the target amplicon as the increase in fluorescence detected in these cases is not due to an increase in the concentration of the target PCR product. Proper primer design minimizes the effects of any unwanted reactions. During the optimization of the assay, PCR products were analyzed on a gel to check for the product of interest and for correlation between the gel and fluorescence data.

Absolute amount of the target nucleic acid of interest was determined using external standards. These standards contained sequences that are the same as, or differ only slightly from the target sequence, and their primer binding sites are always identical to the target sequence. This ensured equivalent amplification efficiencies of standard and target molecules, which is essential for absolute quantification. A standard curve (plot of CT values/crossing points of different standard dilutions against log of amount of standard was generated using a dilution series of the standards. Amplification of the standard dilution series and of the target sequence was carried out in separate

tubes. Comparing the CT of unknown amounts of target with the standard curve allowed calculation of the initial amount of target used in Real-Time PCR. It was important to select an appropriate standard for the type of nucleic acid to be quantified.

The relative quantification performed, determines the ratio between the amount of target and a reference molecule in the samples. The reference molecule (housekeeping gene) was chosen in such a way so that its expression level to be almost constant. When using an RNA standard for gene expression analyses, the variable efficiency of reverse transcription had been taken into account. The target and the reference were amplified with comparable efficiencies. Amplification efficiencies in addition to the setup of the PCR reaction itself depend on factors as: pipetting, instrumentation, presence of inhibitors, effect of RT reaction volume. Target and reference amounts were quantified by using standard curves for both genes. All wells defined as standards were used for the generation of a standard curve. The CT values were plotted against the log of the template 'amount' to generate a straight line. CT values for the samples and the standard curve were then used to calculate the absolute or relative amount of starting template in experimental samples. One standard curve for the target gene and one standard curves for the reference molecule (housekeeping gene) were prepared by diluting the reference sample in 5-fold dilutions. The amount of unknown target fell within the range of the standard curve points. Reactions were carried out in triplicate. No template control was used for detection of contaminations. No template control contained all the components of the reaction, but no template. To determine amplification efficiency, Real-Time PCR with a dilution series of the sequence of interest, was performed. Standard curve was constructed using the obtained CT values. The amplification efficiency (E) was calculated using the following equation:

$E = 10^{(-1/S)} - 1$ (S is the slope of the standard curve)

Well factors were used in the iCycler to compensate for any system or pipetting differences.

Data analyses were performed according to the recommendations provided by the manufacturer of the iCycler detection system. Data were produced in the form of amplification plots where the number of cycles was plotted against fluorescence. The threshold cycle (CT) was used to calculate the starting template amount in each sample. The noise level in early cycles is the baseline. Normally it is between cycles 3-15 because there is still no detectable increase in fluorescence. The baseline was subtracted from the PCR products fluorescence. The threshold is a value in log-linear range of the

PCR above the baseline. The threshold is set using a logarithmic amplification plot, because in this case the log-linear range of the curve is easy to identify. The cycle at which the amplification plot crosses the threshold is the threshold cycle (CT).

Melting curve analyses was performed. This option is built into the software of iCycler. The temperature was increased very slowly from a 65ºC to 95ºC. All PCR products are double stranded at low temperatures; SYBR Green I dye binds to them and gives high fluorescence. At high temperatures denaturation takes place and there is a rapid decrease in fluorescence. When the temperature was increasing the fluorescence was measured and plotted against the temperature. At higher temperatures there was a rapid decrease in fluorescence as nonspecific and specific PCR products reached their melting temperatures.

### 4.2.5 ELISA

**Mouse adiponectin**

Mouse adiponectin quantification was performed using Mouse Adiponectin/Acrp30 Quantikine ELISA Kit Cat# MRP300 (R&D Systems) and following the manufactures procedure. Briefly, this assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse adiponectin has been pre-coated onto a microplate. Standards, control, and samples were pipetted into the wells and any mouse adiponectin present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse adiponectin was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the stop solution was added. The intensity of the colour measured is in proportion to the amount of mouse adiponectin bound in the initial step. The sample values were then read off the standard curve.

**Human adiponectin**

Human adiponectin quantification was performed using Human Adiponectin/Acrp30 Quantikine ELISA Kit Cat# DRP300 (R&D Systems) and following the manufactures procedure. Briefly, this assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for adiponectin had been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any adiponectin

present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for adiponectin was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of adiponectin bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

**Mouse leptin**

Mouse leptin quantification was performed using Mouse Leptin Quantikine ELISA Kit Cat# MOB00 (R&D Systems) and following the manufactures procedure. Briefly, this assay employs the quantitative sandwich enzyme immunoassay technique. An affinity purified polyclonal antibody specific for mouse leptin had been pre-coated onto a microplate. Standards, controls, and samples were pipetted into the wells and any mouse leptin present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse leptin was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the stop solution was added. The intensity of the colour measured was in proportion to the amount of mouse leptin bound in the initial step. The sample values were then read off the standard curve.

**Human leptin**

Human leptin quantification was performed using Human Leptin Quantikine ELISA Kit Cat# DLP00 (R&D Systems) and following the manufactures procedure. Briefly, this assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for leptin had been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any leptin present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for leptin was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of leptin bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

### 4.2.6 Cell lines

**Hep G2**

Hep G2 was from ATCC® Number HB-8065. This is a human hepatoma derived cell line with epithelial morphology. For subculturing, culture medium was removed and discarded, the cell layer briefly rinsed with 0.25% (w/v) trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. Then 2.0 to 3.0 ml of trypsin-EDTA solution added to flask and the cells observed under an inverted microscope until cell layer was dispersed (usually within 5 to 15 minutes). In order to avoid clumping it's advisable not to agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. As a next step 6.0 to 8.0 ml of complete growth medium were added and cells aspirated by gently pipetting. Appropriate aliquots of the cell suspension were added to the new culture vessels and the cultures incubated at 37°C. The normally used subcultivation ratio was 1:6 with a medium renewal twice per week. For preservation complete growth medium supplemented with 5% (v/v) DMSO was used as a freezing medium. The frozen cells were stored in the liquid nitrogen vapour phase. Propagation was done at 37°C in 90% minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and 10% fetal bovine serum.

**HUH 7**

HUH 7 was gift from the Department of Gastroenterology, Hepatology and Endocrinology at MHH. This is a human hepatoma derived cell line with epithelial morphology. For subculturing, culture medium was removed and discarded, the cell layer briefly rinsed with 0.25% (w/v) trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. Then 2.0 to 3.0 ml of trypsin-EDTA solution added to flask and the cells observed under an inverted microscope until cell layer was dispersed (usually within 5 to 15 minutes). In order to avoid clumping it's advisable not to agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. As a next step 6.0 to 8.0 ml of complete growth medium were added and cells aspirated by gently pipetting. Appropriate aliquots of the cell suspension were added to the new culture vessels and the cultures incubated at 37°C. The normally used

subcultivation ratio was 1:7 to 1:10 with a medium renewal twice per week. For preservation complete growth medium supplemented with 5% (v/v) DMSO was used as a freezing medium. The frozen cells were stored in the liquid nitrogen vapour phase. Propagation was done at 37°C in 90% minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and 10% fetal bovine serum.

**C2C12**

C2C12 was from ATCC® Number CRL-1772. This is a mus musculus derived cell line with fibroblast morphology. C2C12 is a subclone (produced by H. Blau, et al.) of the mouse myoblast cell line established by D. Yaffe and O. Saxel. (376). The C2C12 cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins (377)**.** When subculturing, the cells were never allowed to become confluent as this may deplete the myoblastic population in the culture. Myotube formation is enhanced when the medium is supplemented with 10% horse serum instead of fetal bovine serum. For subculturing, culture medium was removed and discarded, the cell layer briefly rinsed with 0.25% (w/v) trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. Then 2.0 to 3.0 ml of trypsin-EDTA solution added to the flask and the cells observed under an inverted microscope until cell layer was dispersed (usually within 5 to 15 minutes). In order to avoid clumping it's advisable not to agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. As a next step 6.0 to 8.0 ml of complete growth medium were added and cells aspirated by gently pipetting. Appropriate aliquots of the cell suspension were added to the new culture vessels normally inoculation of 1.5 X $10^5$ to 1.0 X $10^6$ viable cells/75 $cm^2$ and the cultures incubated at 37°C with a medium renewal every two to three days. For preservation complete growth medium supplemented with 5% (v/v) DMSO was used as a freezing medium. The frozen cells were stored in the liquid nitrogen vapour phase. Propagation was done at 37°C in 90% dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 90% and 10% fetal bovine serum.

**3T3-L1**

3T3-L1 was from ATCC® Number CL-173. This is a mus musculus derived cell line with fibroblast morphology. 3T3-L1 is a continuous substrain of 3T3 (Swiss albino) developed through clonal isolation. The cells undergo a pre-adipose to adipose like conversion as they progress from a rapidly dividing to a confluent and contact inhibited state. Culture was never allowed to become completely confluent and was subcultured before 80% confluence or when cells reached 5 to 6 $X10^4$ viable cells/sq cm. For subculturing, culture medium was removed and discarded, the cell layer briefly rinsed with 0.25% (w/v) trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. Then 2.0 to 3.0 ml of trypsin-EDTA solution added to flask and the cells observed under an inverted microscope until cell layer was dispersed (usually within 15 to 25 minutes). In order to avoid clumping it's advisable not to agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. As a next step 6.0 to 8.0 ml of complete growth medium were added and cells aspirated by gently pipetting. Appropriate aliquots of the cell suspension were added to the new culture vessels, normally an inoculum of 2 to 3 X $10^3$ cells/sq cm. The normally used subcultivation interval was every three days with medium renewal 2 to 3 times per week. For preservation complete growth medium supplemented with 5% (v/v) DMSO was used as a freezing medium. The frozen cells were stored in the liquid nitrogen vapour phase. Propagation was done in 90% dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose and 10%; bovine calf serum. Cells were maintained at 37°C in 95% atmosphere air and 5%; carbon dioxide ($CO_2$). As a requirement for growth conditions the cells were grown in plastic flasks as they do not grow well on some types of glass surfaces. During differentiation protocol committed preadipocytes have to withdraw from cell cycle before undergoing adipose conversion. For preadipose cell lines as well as for primary preadipocytes, growth arrest is required for adipocyte differentiation and is normally achieved through contact inhibition when confluence of cells is reached (333;378). For differentiation, cells were incubated two days after confluence when a saturation density of approximately 50000 cells per sq cm was reached (designated day 0) in DMEM-FCS supplemented with a mixture of hormones composed of 520 µM IBMX, 1 µM DEX and 167 nM insulin for 72 h. Cells were then incubated in DMEM-FCS and 167 nM insulin for another 72 h, after which they were maintained in DMEM-FCS with a medium

change every 72 h. Cell culture media were supplemented with 100U/100mg/ml penicillin/streptomycin and 8 µg/ml biotin.

**Preadipocyte primary culture**

For isolation of primary human preadipocytes Buffer I and Buffer II were used. Both buffers were filter sterilized. To prepare the collagenase 20mg were dissolved in 10 ml Buffer I and filter sterilized. The fat tissue was dissected and minced on a Petri dish (about 5ml tissue). Then it was pipetted into 10ml collagenase solution in a 50ml vials and vortexed (to reach total volume of about 15ml). The 50ml vial was put into shaking water bath at 90 cycles/minute and incubated for 1h. As a next step the solution was homogenized by vortexing and separated into two 50ml vials, which were then filled up with buffer I and centrifuged 5min/4°C/800rpm. Then the upper fat part was transferred into another 50ml vial, the middle part thrown away and the pellet added to the upper part in the 50ml vial. Next the 50ml vial was filled up with buffer II and centrifuged 10min/4°C/1500rpm. After centrifugation the supernatant and the floating fat were thrown away and the vial with the pellet filled up with buffer II and centrifuged 10min/4°C/1500rpm. The supernatant was thrown away and the pellet resuspended in primary cell culture medium. The medium was changed every 24-48h. For subculturing, culture medium was removed and discarded, the cell layer briefly rinsed with 0.25% (w/v) trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. Then 2.0 to 3.0 ml of trypsin-EDTA solution added to flask and the cells observed under an inverted microscope until cell layer was dispersed (usually within 20 to 30 minutes). As a next step, 10ml of primary cell culture medium was added followed by centrifugation at 10min/4°C/1500rpm. Appropriate aliquots of the cell suspension were added to the new culture vessels. The normally used subcultivation ratio was 1:2. Propagation was done in primary cell culture medium. For differentiation, differentiation medium for primary human preadipocyte was used. For preservation complete primary cell culture medium supplemented with 5% (v/v) DMSO was used as a freezing medium. The frozen cells were stored in the liquid nitrogen vapour phase.

**Human primary skin fibroblasts**

Human primary skin fibroblasts were provided by Dr. Thomas Lücke from Department for Pediatric Kidney and Liver Diseases and Metabolic Disorders, Hannover Medical School, Hannover; Germany. Propagation, preservation and cell culture medium were

as explained for 3T3-L1 preadipocytes. Subculturing was performed by splitting culture before confluence.

### 4.2.7 Oil Red O staining

Oil Red O staining was performed following the protocol from Cambrex Bio Science.
I order to fix adipogenic culture, media was aspirated and the plate was rinsed with 2 ml of sterile PBS. Then PBS was aspirated and 2 ml of 10% formalin added, followed by 1/2h incubation. To prepare Oil Red O stock solution 300 mg of Oil Red O powder was dissolved in 100 ml of 99% isopropanol. For the working solution 3 parts Oil Red O stock solution were mixed with 2 parts deionised water and left to stay 10 minutes at room temperature (stable for 2 hours). Finally Oil Red O working solution was filter sterilized. To stain the adipogenic culture formalin was removed and 2 ml of sterile water added in order to rinse the cells. The water was removed and 2 ml of 60% isopropanol added and incubated for 5 minutes. Then the isopropanol was removed and 2 ml of Oil Red O working solution pipetted and incubated for 1h. As a next step cells were rinsed with water left wet and analysed on a phase contrast microscope where the lipids appeared red.

### 4.2.8 Mitochondrial functions

#### 4.2.8.1 Membrane potential

To evaluate the integrity of mitochondrial functions we used the cationic dye JC-1 Assay Kit (Cat N# M34152; Molecular Probes, Eugene, OR, USA) following the manufactures procedure. JC-1 cationic dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) signals the loss of the mitochondrial membrane potential. The normal potential is associated with bright red staining. The lipophilic dye, bearing a delocalized positive charge enters the mitochondrial matrix using the negative charge of the intact mitochondrial membrane potential and accumulates there. The red fluorescence appears when JC-1 reaches critical concentration and forms J-aggregates. When mitochondrial membrane potential collapses, JC-1 cannot accumulate within the mitochondria, remains in the cytoplasm in monomeric form and florescence is green. The absorption/emission maximum of the aggregate red form is 585/590 nm and of the green monomeric form is 510/527 nm. To stain for flow cytometry for each sample, cells were suspended in 1ml warm medium at

1 x $10^6$ cells/mL. For the control tube, 1µl of CCCP were added and the cells incubated at 37°C/5min. Then 10µl of 200µM JC-1 were added and the cells incubated at 37°C/5%$CO_2$/ for 15-30min. As a next step cells were pelleted by centrifugation and ressuspend in 500µl PBS. Then cells were analysed on a flow cytometer with 488nm excitation using emission filters appropriate for Alexa Fluor 488 dye and R-phycoerythrin. The cells were gated, excluding debris. Standard compensation was done using the CCCP –treated sample.

#### 4.2.8.2 Mitochondrial mass

To evaluate the mitochondrial mass we used MitoTracker Red 580 (MTR, Cat N#M-22425; Molecular Probes, Eugene, OR, USA) following the manufactures procedure. The reduced probes do not fluoresce until they enter an actively respiring cell, where they are oxidized to the corresponding fluorescent mitochondrion-selective probe and then sequestered in the mitochondria. These probes are well retained after fixation and permeabilization. To stain the cells for flow cytometry, the medium was removed and prewarmed medium containing MitoTracker Red 580 added. After 30-45 min incubation the loading medium was replaced with fresh medium and the cells pelleted by centrifugation. The pellet was ressuspend in 500µl PBS and analysed on a flow cytometer. The excitation/emission maximum of the MitoTracker® Red 580 is 581/644.

### 4.2.9 Propidium iodide staining

To evaluate cell death we used propidium iodide solution (Propidium iodide Cat N# P3566; Molecular Probes, Eugene, OR, USA) following the manufactures procedure. Propidium iodide is membrane impermeant and generally excluded from viable cells. It is commonly used for identifying dead cells. Propidium iodide binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. Propidium iodide also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold. To stain cells for flow cytometry single cell suspension was prepared in buffer (e.g. PBS + 0.1% BSA), the cells were washed twice and pelletted. 1.5 mM propidium iodide staining solution was prepared by diluting the 1 mg/mL (1.5 mM) stock solution 1:500 in PBS and 300 µL of this staining solution was directly pipetted onto the specimen. The specimen was incubated in the dark for 30 minutes at room temperature

and washed twice with PBS. Then propidium iodide fluorescence (FL2 on the FACS) was determined and signals compensated accordingly. Fluorescence excitation/ emission maximums for nucleic acids bound propidium iodide are 535/617 nm. Propidium iodide fluorescence is detected in the FL2 channel of flow cytometers. Double staining propidium iodide and hoechst or annexin V as well as trypan blue dye exclusion were performed according to the manufactures protocols.

### 4.2.10 Enzyme activities

The examination of enzyme activity has been done in control and NRTI treated cells. The cell lines and primary cultures used in these experiments were from early passages. Cells were plated to confluence on Petri dishes (diameter 30mm). For the performance of spectrophotometric measurement cells were washed twice with a Hepes buffer and then incubated in 1ml of buffer containing 10mM glucose for 15min at room temperature. Respiratory chain enzyme activities were assayed as described in (379-383). Cells were subjected to sonication 2X10sec in a 'bandelin-probe' sonicator. The sonicater was set up at power 20W with 0.3sec duration of the single pulses and inter-pulse duration of 0.7 sec. We determined complex I (NADH dehydrogenase), complex II+III (succinate-ubiquinone reductase and ubiquinol-cytochrome *c* reductase), complex IV (cytochrome *c* oxidase), complex V (ATP synthase) and citrate synthase activity (used as marker of mitochondrial content). Complexes I+III activity were measured spectrophotometrically at 37 °C using wavelength of 340nm. Rotenone was used to inhibit complex I (380). Complexes II+III activity was measured at wavelength 550 nm and antimycin A was used as inhibitor (384). Complex IV activity was measured at a wavelength of 550nm (381). Complex V was measured at 340nm (379;382) and oligomycine-was used as inhibitor. Enzyme activity was expressed in nmol/min/mg of cell protein for individual enzyme complexes of the MRC. The measurement was performed at 37°C in 1ml cuvette in appropriate medium. Enzyme activity of all complexes was calculated by subtracting the remaining residual activity after the addition of the complex specific inhibitor (rotenone for complex I, antimycin A for complex II+III activity; KCN for complex IV activity; and oligomycin for complex V). The photometric measurement and the calculation have been done using UV Visible Chemstation Software-Alien Technologies (HP 845xUV visible system).

**Complex I+III**

The cuvettes were filled with 840µl modified buffer and 10µl NADH (stable for 3-4h). The photometer was set up at 340nm, y-scaling 0-2, run time 600s, cycle time 5, type Delta AU. 850µl modified buffer was used as a blank. Then the kinetic measurement was started at 340nm. Cytochrome C (5µl) was added and mixed well, and after 30 sec 150µl cell suspension was added. After another 180 sec 10µl rotenone were added and measurement continued till the end of the 600 seconds. To analyse the data the photometer offline was used. For the calculation of respiration, analyses started from the first point of the straight line. Interval of 100 units was given and walk with this interval along the straight line with a step of 10 units performed in order to find the negative value that is most different from zero (A). For the calculation of inhibition, analyses started from the first point of the straight line after the addition of rotenone. Interval of 100 units was given and walk with this interval along the straight line with a step of 10 units performed in order to find the negative value closest to zero (B). The calculation was done using the formula: Enzyme activity (nmol/min/mg protein) = (-1000*(A-B)*0.6)/(6.22*protein mg/ml*cell volume ml).

**Complex II+III**

The cuvettes were filled with 750µl $KH_2PO_4$ and 10µl $NaN_3$. The photometer was set up at 550nm, y-scaling 0-2, run time 2000sec, cycle time 5, type Delta AU. 750µl $KH_2PO_4$ and 10µl $NaN_3$ was used as a blank. Then the kinetic measurement was started at 550nm. 20µl Cytochrome C and 5µl rotenone were added and mixed well. After 30 sec 200µl cell suspension was added and measurement performed for 15min. Then 20µl succinate was added and measured for another 6min. Finally 10µl antimycin was added and measured for another 12 min. To analyse the data the photometer offline was used. For the calculation of respiration, analyses started from the first point of the straight line after the addition of succinate. Interval of 100 units was given and walk with this interval along the straight line with a step of 10 units performed in order to find the positive value that is most different from zero (A). For the calculation of inhibition, analyses started from the first point of the straight line after the addition of antimycin. Interval of 100 units was given and walk with this interval along the straight line with a step of 10 units performed in order to find the positive value closest to zero (B). The calculation was done using the formula: Enzyme activity (nmol/min/mg protein) = (1000*(A-B)*0.6)/(18.5*protein mg/ml*cell volume ml)

**Complex IV**

The cuvettes were filled with 650µl $H_2O$ and 100µl $KH_2PO_4$. The photometer was set up at 550nm, y-scaling 0-2, run time 2000sec, cycle time 5, type Delta AU. 650µl $H_2O$ and 100µl $KH_2PO_4$ were used as a blank Then the kinetic measurement was started at 550nm. 50µl D-Cytochrome C was added and mixed well. After 100 sec 200µl cell suspension was added and measurement performed for 10min. Then 10µl K-ferricyanide was added and measured for another 1min. To analyse the data the photometer offline was used. For the calculation of respiration the first point of the straight line was taken. For the calculation of inhibition the lowest point of the straight line after the addition of K-ferricyanide was taken. The values for respiration and inhibition were summed, divided by 2 and the corresponding middle absorption was found. Next, calculation was performed to find how many seconds took the reaction to come to the middle absorption. The seconds were transferred in minutes (Z). Then the formula: 0,69/Z = K; Enzyme activity (nmol/min/mg protein) = (K x 38,36)/( protein mg/ml *cell volume ml) was used.

**Complex V**

The cuvettes were filled with 850µl from the following mixture: 5ml modified buffer, 150µl ATP, 150µl PEP, 15µl NADH, 3µl PK, 15µl LDH, 200µl EGTA and 20µl KCN. The photometer was set up at 340nm, y-scaling 0-2, run time 600sec, cycle time 5, type Delta AU. 850µl modified buffer were used as a blank. Then the kinetic measurement was started at 340nm. After 30 sec 150µl cell suspension was added and measurement performed for 5min. Then 9µl oligomycin was added and measured for another 10 min. To analyse the data the photometer offline was used. For calculation of respiration, analyses started from the first point of the straight line. Interval of 100 units was given and walk with this interval along the straight line with a step of 10 units performed in order to find the negative value that is most different from zero (A). For the calculation of inhibition, analyses started from the first point of the straight line after the addition of oligomycin. Interval of 100 units was given and walk with this interval along the straight line with a step of 10 units performed in order to find the negative value closest to zero (B). The calculation was done using the formula: Enzyme activity (nmol/min/mg protein) = (-1000*(A-B)*0.6)/(6.22*protein mg/ml *cell volume ml).

**Citrate Synthase**

The quartz cuvettes were filled with 925µl Tris Buffer. The photometer was set up at 232nm, y-scaling 0-2, run time 1200sec, cycle time 5, type Delta AU. Quartz cuvettes filled with 925µl Tris Buffer were used as a blank. Then the kinetic measurement was started at 232nm. After 30 sec 20µl oxaloacetate and 5µl acetyl-Co A were added. After 10 min measurement 100µl cell suspension was added and measure for another 5min. To analyse the data the photometer offline was used. For calculation of respiration, analyses started from the first point of the straight line. Interval of 100 units was given and walk with this interval along the straight line with a step of 10 units performed in order to find the negative value that is most different from zero (A). The calculation was done using the formula: Enzyme activity (nmol/min/mg protein) = (-1000*(A)*0.6)/(5.4*protein mg/ml *cell volume ml).

**Protein measurement**

Protein measurement was done using BCA™ Protein Assay Kit Cat# 23225 (PIERCE) following the manufactures procedure. The BCA™ Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of $Cu^{++}$ to $Cu^{+}$ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation ($Cu^{+}$) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg/ml). The BCA™ method is not a true end-point method; that is, the final colour continues to develop. However, following incubation, the rate of continued colour development is sufficiently slow to allow large numbers of samples to be assayed together. To analyse the total cellular protein of the probes 250µl cell suspension, 750µl Baker water and 50µl 1% dissolved organic carbon (DOC) were mixed. For the DOC, 1000µl Baker water and 150µl DOC 1% were mixed. For the control, 250µl control solution, 50µl Baker water and 150µl DOC 1% were mixed. The probes, DOC and control were subjected to 5min incubation at room temperature, followed by addition of 200µl 50% trichloroacetic acid (TCA) and 15 min incubation on ice. Then the probes, DOC and control were subjected to 3 min centrifugation at 13500rpm, the supernatant was discarded and the pellet resuspended in 1000µl 0,2% DOC for Probes and DOC samples

and in 250µl DOC for control samples. From this point the protocol explained at: http://www.piercenet.com/files/ 1296dh4.pdf was followed.

### 4.2.11 Cloning

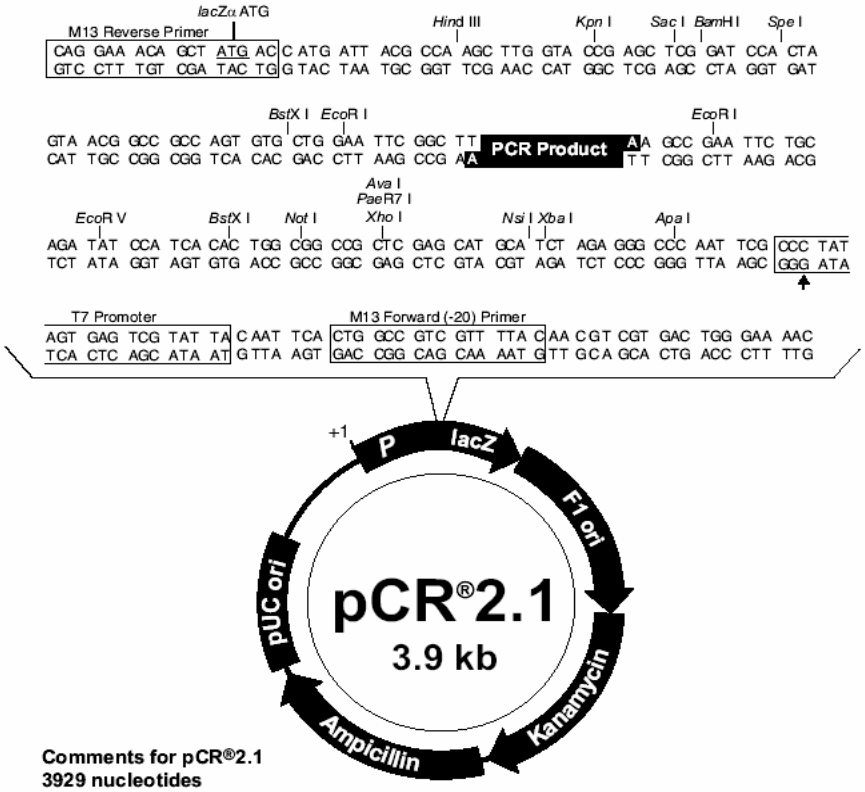
Cloning has been performed following the general recommendation from the cloning kit and from QIAGEN® PCR Cloning Handbook. TA Cloning® Kit with pCR®2.1 was used. This kit provides a quick, one-step cloning strategy for the direct insertion of a PCR product into a plasmid vector. Taq polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized vector supplied in this kit has single 3′ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. To clone a gene of interest, the PCR product was ligated into pCR®2.1 and transformed into competent cells. Since the PCR product can ligate into the vector in either orientation, individual recombinant plasmids were analyzed to confirm proper orientation. The correct recombinant plasmid was purified for further subcloning and characterization. Briefly, the PCR product was amplified using Taq polymerase and specially designed primers and parameters. Then the PCR product was ligated into pCR®2.1 and this ligation transformed into competent E. coli. Colonies were selected, plasmid DNA isolated and the plasmid DNA analysed for the presence and orientation of the PCR product by PCR using the plasmid and insert primers. DNA amount between 10 and100 ng was used as a template for the PCR and 30 cycles of amplification in order to achieve optimal ligation efficiencies. Total DNA was isolated from Hep G2 cell line (ATCC® Number: HB-8065) and the appropriate primers were used in the PCR for the creation of mtDNA and GH fragments to be cloned. The PCR reaction was performed using Taq PCR Core Kit (250 U) Cat. No 201223 (QIAGEN) and the following amplification conditions:

| | Time | Temp |
|---|---|---|
| Initial activation step | 15 min | 95C |
| 3-step cycling | | |
| Denaturation | 1 min | 94C |
| Annealing | 30 sec | 55C |
| Extension | 2 min | 72C |
| Number of Cycles | 30 | |
| Final Extension | 10 min | 72C |

The PCR was optimized to eliminate multiple bands and smearing and a single, discrete band was obtained. Special care was taken to avoid sources of nuclease contamination and long exposure to UV light. For optimal ligation efficiencies, fresh (less than 1 day old) PCR products were used as the single 3′ A-overhangs on the PCR products may be degraded over time, reducing ligation efficiency. The amount of PCR product needed to ligate with 50 ng (20 fmoles) of pCR®2.1 vectors was calculated using the formula:
X ng PCR product = ((Y bp PCR product)(50ng pCR® 2.1 vector))/(size in bp of the pCR® 2.1 vector~3900).
Where X ng is the amount of PCR product of Y base pairs to be ligated for a 1:1 (vector:insert) molar ratio. In general, 0.5 to 1.0 µl of a typical PCR sample with an average insert length (400-700 bp) gives the proper ratio of 1:1 (vector: insert). The ratio of 1:1 (vector: insert) gives the best efficiency of ligation. No more than 2-3 µl of the PCR sample was used in the ligation reaction as salts in the PCR sample may inhibit the T4 DNA Ligase. Ligation was performed at 14°C as higher or lower temperatures may reduce the ligation efficiency. One vial of pCR®2.1 was centrifuged to collect all the liquid in the bottom of the vial. The volume of PCR sample needed for the required amount of PCR product was determined (as explained above). The 10 µl ligation reaction was set up as follows (Fresh PCR product X µl; 10X Ligation Buffer 1 µl; pCR®2.1 vector (25 ng/µl) 2 µl; Sterile water to a total volume of 9 µl; T4 DNA Ligase (4.0 Weiss units) 1 µl; Final volume 10 µl. Then the ligation reaction was incubated at 14°C for a minimum of 4 hours. After the ligation of the insert into pCR®2.1, the construct was transformed into competent *E. coli*. TOP10F′ does express the lac repressor ( lacIq), which represses transcription from the lac promoter. To perform blue-white screening for inserts, isopropyl-β-D-thiogalactopyranosid (IPTG) was added to the plates to express LacZα. Kanamycin was used to select for transformants containing pCR®2.1 construct. To prepare for transformation, the water bath was equilibrated to 42°C and S.O.C. medium brought to room temperature. For TOP10F′ cells, LB plates containing antibiotic were taken and equilibrated at 37°C for 30 minutes. 40 µl of 100 mM IPTG and 40 mg/ml X-Gal were spread onto the plates and let to soak. Then the vials containing the ligation reactions were centrifuged briefly and placed on ice. One 50 µl vial of frozen One Shot® Competent Cells was thawed for each transformation. 2 µl of each ligation reaction were pipetted directly into the vial of competent cells and mixed by stirring gently with the pipette tip. The vials were incubated on ice for 30 minutes. The cells were heat shocked for 30 seconds at 42°C without shaking and

immediately transferred on ice. Then 250 µl of room temperature S.O.C. medium was added to each vial and shacked horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator. As a next step 10 µl to 50 µl from each transformation vial was spread on LB agar plates containing X-Gal and 50 µg/ml of kanamycin or 100 µg/ml ampicillin. IPTG was included for the TOP10F´ cells. The plates were incubated overnight at 37ºC and then shifted to +4°C for 2-3 hours to allow for proper colour development. On X-Gal/IPTG plates, an average of 50-200 colonies per plate was obtained with approximately 80% white colonies. To analyse the positive clones at least 10 white colonies were picked for plasmid isolation and analysis. The colonies were grown overnight in 2-5 ml LB broth containing either 100 µg/ml of ampicillin or 50 µg/ml kanamycin. Plasmid was isolated and analyzed by PCR for orientation of the insert.

**Map of pCR®2.1** The map of the linearized vector, pCR®2.1, is shown below. The arrow indicates the start of transcription for the T7 RNA polymerase. **The complete sequence of pCR®2.1 is available from our Web site (www.invitrogen.com) or by contacting Technical Service (page 18).**




**Comments for pCR®2.1**
**3929 nucleotides**

*LacZα* gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

The next procedure was used for plasmid preparation from bacterial culture. For the starter culture, a single colony was inoculated from a fresh selective plate into 10 ml LB medium with appropriate antibiotic. The culture was shacked vigorously~300 rpm 8 h at 37°C. As a next step the culture was diluted 1/500 - 1/1000 into LB medium and grown for 12–16 h at 37°C with vigorous shaking ~300 rpm. The cells were harvested by 15 min centrifugation at 6000 x *g* at 4°C and the supernatant was removed. *E. coli* was resuspended in Tris·Cl–EDTA buffer containing RNase A and lysis performed with NaOH/Sodium dodecyl sulphate (SDS) for 5 minutes. The lysate was neutralized with acidic potassium acetate and cleared from the debris by centrifugation. (Traditionally performed by cesium chloride (CsCl) ultracentrifugation). Concentration, desalting, and recovering of the nucleic acids was achieved using alcohol precipitation. Isopropanol in ratio 7/10 was added to the DNA solution at room-temperature and centrifugation performed for 15–30 min at 15,000 x *g* at 4°C. The supernatant was decanted and the pellet washed with 70% ethanol, followed by centrifugation for 5–15 min at 15,000 x *g* at 4°C. The supernatant was decanted and the pellet was air-dried for 5–20 min and redissolved in a suitable buffer (pH 8.0).

DNA was quantified spectrophotometrically. Plasmid DNA concentration was determined through absorbance measurement at 260 nm (A260) using spectrophotometer and quartz cuvette. A260 readings was adjusted to be between 0.1 and 1.0.A relationship A260 = 1 x 50 µg/ml, valid for measurements in neutral pH, (Tris·Cl, pH 7.0) was used.

### 4.2.12 Animals

Young male C57BL/6 mice weighing on average 23g at the six weeks of age were purchased from Charles River Laboratories. All the animals were fed *ad libitum* on a normal diet and were housed 5 or 10 mice per cage in an air conditioned facility with 12 h light/dark cycle. At the end of one week of adaption, the animals were randomly divided into three groups (n=5 in the pilot and n=20 in the second experiment). For some experiments, mice were deprived of food for the last 18 h of the treatments. Food and water intake was monitored by weighing the pelleted diet every week and the water bottles every 72h in each cage and calculating the mean intake/mouse/day. All experiments were performed after ethics approval by the local animal ethics committee.

### 4.2.13 Drug administration

Groups of mice were treated with d4T, AZT or vehicle (5-20 mice per group) for up to 20 weeks with daily human doses adjusted for murine body surface area, 13.5 mg/kg of d4T and 100 mg/kg of AZT. The body area is 0.026 and 0.30 $m^2$/kg of body weight in humans and mice, respectively (81). The plasma levels of d4T and AZT under such administration conditions have been reported by another group (74;81). d4T (Bristol-Myers Squibb GmbH) and AZT (GlaxoSmithKline-GmbH) were extracted from capsules put in PBS and homogenized directly before being given, as they are poorly soluble in PBS. d4T and AZT were administered by gavage rather than in drinking water. The reasons for such administration were two: First the content of drug pills used to extract the drugs was practically insoluble in water and second the constant intake of the drug from the drinking water would not allow us to parallel the pharmacokinetics in human's blood. Control mice received an equal volume of PBS.

### 4.2.14 Assessment of body fat mass distribution

The determination of subcutaneous mouse fat distribution (e.g. inguinal and axillary fat pads) was performed by macroscopic examination. In addition, pictures of the fat depots of the mice were evaluated by an independent person blinded for the respective treatment groups. This assessment was performed in male C57BL/6 mice on the last day of the 15 week treatment to compare the phenotypic body parameters at the end of treatments.

### 4.2.15 Collection of tissues

In anesthetised mice, blood was collected from the tail vain by bleeding in a 2ml tube. Serum was isolated by centrifugation and stored for analysis at -80°C. After sacrificing the mice subcutaneous adipose tissue, liver, heart, muscle and brain were excised, washed in PBS and aliquots immediately frozen at -80 for DNA analyses.

### 4.2.16 Glucose tolerance test (GTT)

Mice were fasted 18 hours before the GTT with free access to drinking water. Then the baseline glucose level for each mouse was recorded (before the GTT). At the beginning of the GTT the mouse was challenged with a glucose load of 1.5 g glucose/kg body weight. The glucose solution was injected intra-peritoneally using a syringe and a 25

gauge needle. The next glucose measurements were performed 30, 60 and 120 min after the injection using a glucometer.

### 4.2.17 $^{3}$H thymidine Assay

Cells were incubated for 24 h in DMEM containing 10% FCS (DMEM-FCS) and 100U/100mg/ml penicillin/streptomycin and the indicated drug. The labelled nucleotide was incubated for additional 24 hours with the cells, where only proliferating cells incorporate $^{3}$H thymidine during cell division. Cells were harvested and washed through a filter. The filters were placed into the β counter where the radioactivity and thereby the incorporated amount were measured. Assays were performed in eightplicates to build the arithmetic mean of the results.

### 4.2.18 Statistical methods

*Statistics*. All analyses represent at least triplicate experiments with triplicate plates in each experiment. The statistical analyses were done by unpaired and paired Student's t-test. The level of significance was set at $P < 0.05$. All data ± SEM. All calculations were performed using GraphPad Prism version 2.01

# 5 RESULTS

## 5.1 Relationship of mitochondrial DNA depletion and respiratory chain activity in preadipocytes treated with nucleoside-analogue reverse transcriptase inhibitors

**5.1.1 Influence of NRTI on adipocyte morphology, differentiation, and triglycerol accumulation**. First we examined the longitudinal effects of NRTI on differentiating 3T3-L1 adipocytes in regard to their phenotype. Preadipocytes were induced to differentiate in the presence of AZT (6μM($C_{max}$) or 180μM), d4T (3μM($C_{max}$) or 90μM) and ddC (0.1μM($C_{max}$) or 3μM) under standard conditions. Adipocytes were treated with vehicle (DMSO) or the indicated drug concentrations from day -7 until day +15 (from differentiation). Adipocytes exposed to high AZT concentrations retained less Oil Red O stain when compared with vehicle-treated cells. No changes were observed at $C_{max}$ drug concentrations (data not shown). Surprisingly, cells exposed to d4T or ddC did not differ from control neither at $C_{max}$ (not shown) nor at high concentration in terms of acquisition of a spheric shape and lipid droplet accumulation (Figure 1). Microscopic examination confirmed decreased triacylglycerol droplets, increased numbers of non-differentiated cells and some patches devoid of cells due to loss of detached cells during the media changes in AZT-treated adipocytes (Figure 1). Trypan blue staining revealed that up to 50% of detached cells were dead. In contrast, adipocytes exposed to vehicle, d4T and ddC remained attached, contained normal lipid droplets, and demonstrated normal cell viability (Figure 1).

**5.1.2 Molecular analysis of adipocyte differentiation.** To extend the above observations we examined important factors of differentiation including C/EBPα and PPARγ in differentiating 3T3-L1 adipocytes (338;385;386). Preadipocytes were induced to differentiate in the presence of vehicle or the indicated drugs. Measurement of C/EBPα and PPARγ expression on day nine demonstrated that AZT significantly inhibited the expression but only at high drug concentration (Figure 2C and D) corresponding to the inhibition of differentiation as described above. Similarly, d4T and ddC had no significant effect on C/EBPα and PPARγ expression at both concentrations (Figure 2). Taken together, none of the tested NRTI had a negative impact on adipocyte differentiation *in vitro* at therapeutic $C_{max}$ concentrations. Only AZT reduced adipocyte

maturation at high concentrations as indicated by increased preadipocyte numbers, premature cell loss, and reduced levels of crucial factors regulating adipogenesis.

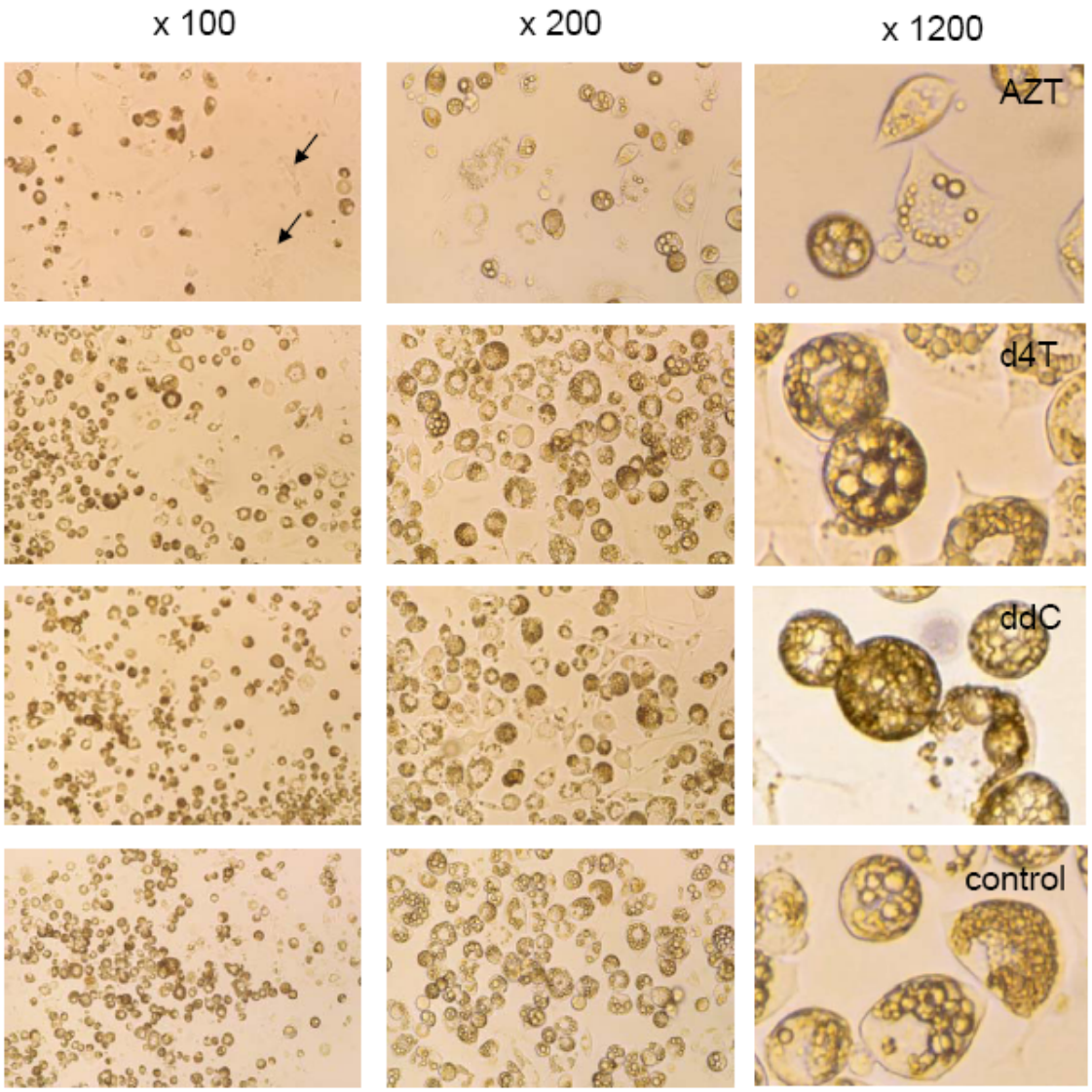



**Figure 1. Impaired adipocyte differentiation after exposure to AZT**
3T3-L1 adipocyte phenotype (unstained cells) at day 15 after initiation of differentiation in the presence of AZT (180µM), d4T (90µm), ddC (3µM), or control. 3T3-L1 cells in the d4T and ddC treated culture have acquired a mature phenotype with round shape and multiple lipid droplets. AZT exposed cultures present decreased numbers of triacylglycerol droplets, increased numbers of non-differentiated cells (arrows) compared to control and some patches devoid of cells.

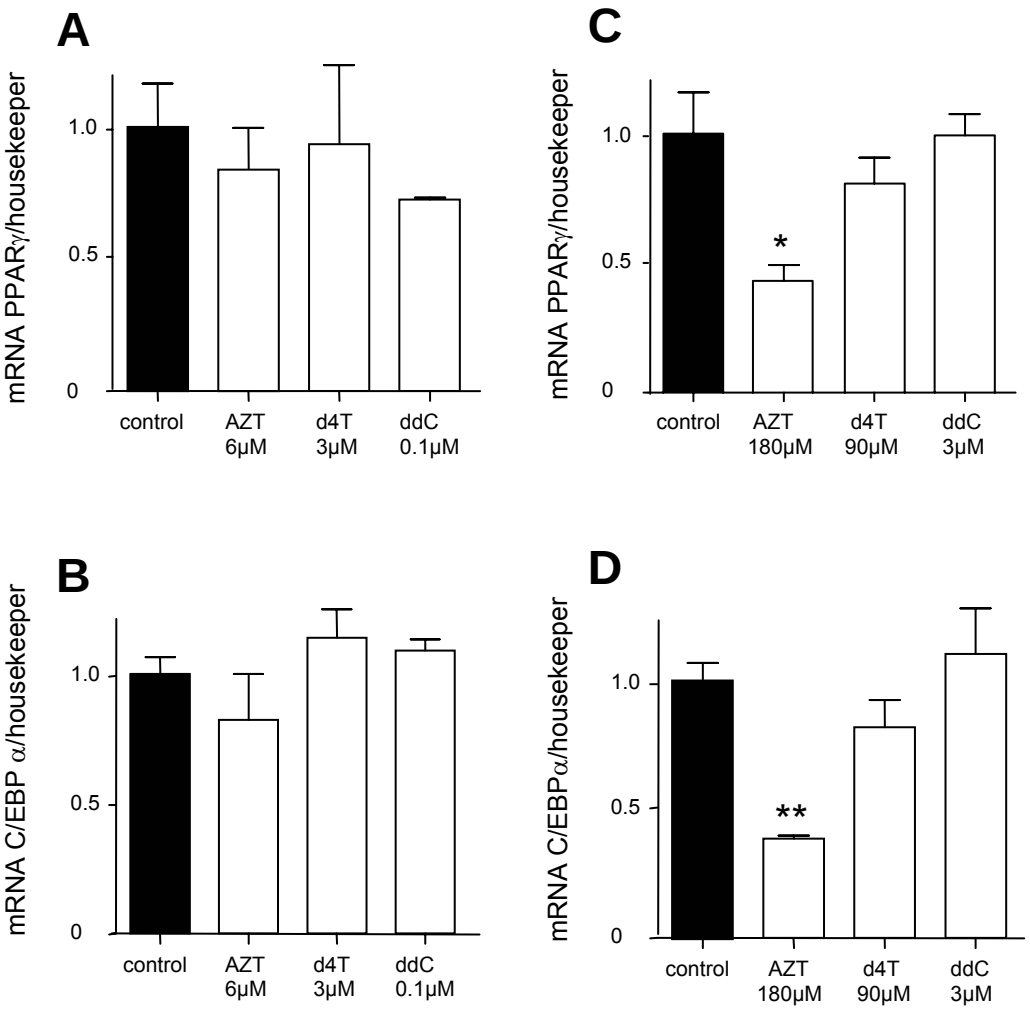



**Figure 2. Molecular analysis of adipocyte differentiation**
The panels reflect the effects of therapeutic drug concentration (A and B) or high concentration of AZT (180µM), d4T (90µm), ddC (3µM) (C and D) on the expression of the important factors of differentiation PPARγ (A and C) and C/EBPα (B and D) in differentiating 3T3-L1. Preadipocytes were cultured in the presence of vehicle (black column) or the indicated drugs from day -7, induced to differentiate on day 0 and differentiated until day + 9 when total RNA was isolated, reverse transcribed and expression levels measured with Real-Time PCR. Like in the morphological analysis (Figure 1), d4T and ddC had no significant effect on C/EBPα and PPARγ expression at both concentrations whereas AZT led to significant impairment only at high drug concentrations. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

**5.1.3 Effect of NRTI on adiponectin production**. We next examined an additional adipocyte function that depends on full adipocyte maturation and contribute to glucose homeostasis *in vivo*. The analysis of adiponectin in the supernatant after culture with different NRTI revealed that d4T reduced the adiponectin production by more than 50% with AZT having an even more pronounced effect (Figure 3A). Even after adjusting for the cell numbers of differentiated adipocytes as the only producers of

adiponectin, both drugs inhibited adiponectin production (Figure 3B). In contrast, adiponectin levels were unaffected by ddC. The substantial effect of d4T and AZT on adiponectin release was detectable using NRTI drug concentrations within the range of the plasma drug $C_{max}$ levels in HIV-patients (Figure 3C).

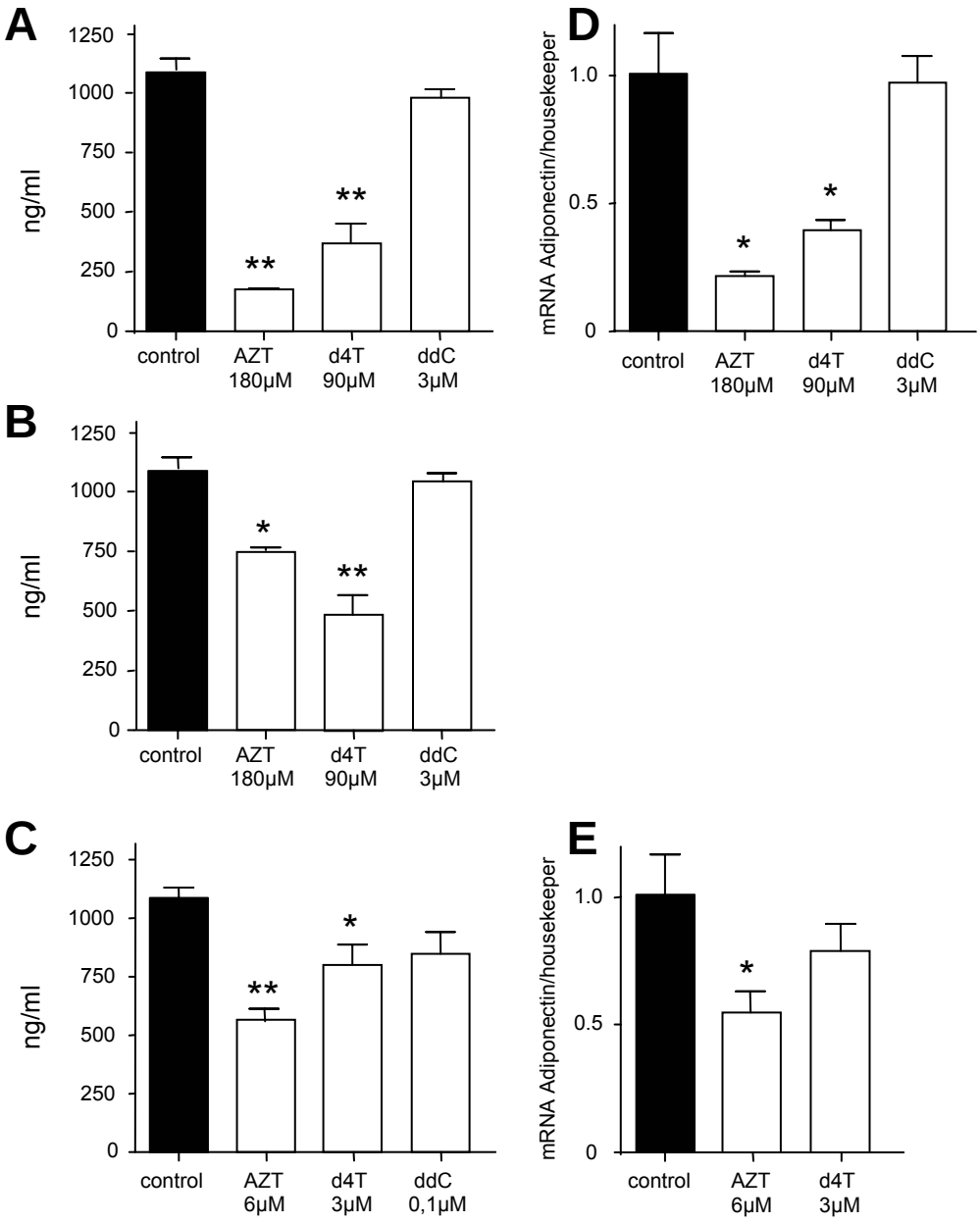



**Figure 3. Effect of NRTI on adiponectin production**
Preadipocytes were differentiated in the presence of AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column) and at day +9 supernatant (A) or adipocytes (D) assessed for adiponectin by ELISA or mRNA. (B) After adjustment for matured adipocyte numbers (see Figure 1) of the AZT exposed cells, AZT still leads to significant reduced adiponectin release into the supernatant. Significant reduction in adiponectin production after AZT and d4T exposure was evident also at therapeutic drug concentrations as confirmed by ELISA (C) and mRNA (E). Values are the mean ± SEM of three to five experiments. * $p<0.05$, ** $p<0.01$.versus control.

All these observations were confirmed by analysis of adiponectin mRNA expression (Figure 3D+E). These data are in line with the idea that NRTI may have impact on adipocyte function in addition to mitochondrial toxicity.

**5.1.4 Effect of NRTI on mtDNA content in non-proliferating cells**. We looked for NRTI-induced changes in the mtDNA content of adipocytes depending on their developmental stage and proliferation activity. First 3T3-L1 growth arrested (confluent) preadipocytes were incubated with AZT, d4T and ddC for 21 days and mtDNA content measured at the end of culture. Surprisingly, quantification by Real-Time PCR revealed no statistically significant differences in the mtDNA content even at the highest concentration tested compared to control (Figure 4A). In addition, analysis of cell viability excluded a toxic effect on resting preadipocytes (not shown). We next assessed the effect on mtDNA content in fully differentiated adipocytes. Preadipocytes were differentiated under the standard protocol and from day +8 incubated with AZT, d4T and ddC for 21 days. Similarly to the growth arrested preadipocytes, the Real-Time PCR measurements revealed no depletion of mtDNA at any drug concentration. Incubation with d4T even led to an increase in mtDNA copies in mature adipocytes (Figure 4B). Importantly, no profound effect on cell viability, attachment, triglyceride content, size or number of cell droplets per cell could be observed. Taken together, these results provide evidence that non-dividing preadipocytes or fully mature adipocytes clearly have a reduced susceptibility to NRTI-induced mtDNA depletion.

**5.1.5 Effect of NRTI on mtDNA content in differentiating or proliferating preadipocytes**. We next wanted to evaluate the effect of NRTI on mtDNA in dividing and differentiating cells. By splitting the preadipocyte culture before reaching confluence every third day in the presence of drugs the cells were kept proliferating without differentiating into fully mature adipocytes. After 21 days of drug treatment with high levels of d4T a significant depletion (reduction by 80%) of mtDNA similar to the effects in the ddC culture could be observed (Figure 4C). In contrast, AZT treatment had no effect on mtDNA content in proliferating preadipocytes. Together with the results of the resting preadipocytes, these experiments provide evidence that only d4T and ddC but not AZT impair mtDNA synthesis in preadipocytes. No significant mtDNA depletion in preadipocytes was observed at therapeutic drug levels (data not shown).

Finally, we measured the mtDNA content of adipocytes differentiated in the presence of NRTI (Figure 4D). Culture with d4T led to a significant reduction of mtDNA under these conditions whereas ddC had no substantial effect. AZT reduced the mtDNA content but differences were not statistically significant. Considering the morphological changes in the AZT-treated adipocyte culture (Figure 1) and the lack of any AZT-induced mtDNA depletion in resting or dividing preadipocytes, this mtDNA depletion is possibly a result of loss of maturing adipocytes and a proportional increase in immature preadipocytes. In contrast, the substantial mtDNA depletion by d4T in differentiating adipocytes was not accompanied by any detectable morphological alteration or increased apoptosis as compared to untreated cells. Experiments performed under similar conditions but at therapeutic drug concentrations revealed no mtDNA depletion (data not shown). In summary, d4T led to significant mtDNA depletion in both preadipocytes and adipocytes during differentiation whereas AZT had some effect only in the latter. In addition, NRTI-associated mitochondrial toxicity as measured by mtDNA depletion is evident only in adipocytes undergoing proliferation or differentiation.

**5.1.6 Effect of NRTI on mtDNA content in HUH 7 cell line**. We next wanted to evaluate the effect of NRTI on mtDNA in human hepatoma (HUH 7) cell line in order to examine whether the effect of NRTI is cell line and cell type specific. By splitting the HUH 7 before reaching confluence in the presence of drugs the cells were kept proliferating. After 10 days of drug treatment, incubation with high levels of d4T led to a significant depletion (reduction by 40%) of mtDNA (Figure 5). In the ddC cultures for 10 days the depletion observed was almost approaching 50% of the original content. (Figure 5). In contrast, AZT treatment led to increase in mtDNA content for 10 days. Similar results were obtained using Hep G2 cell line. These experiments provide evidence that d4T and ddC are able to impair significantly mtDNA synthesis in hepatoma cell lines.

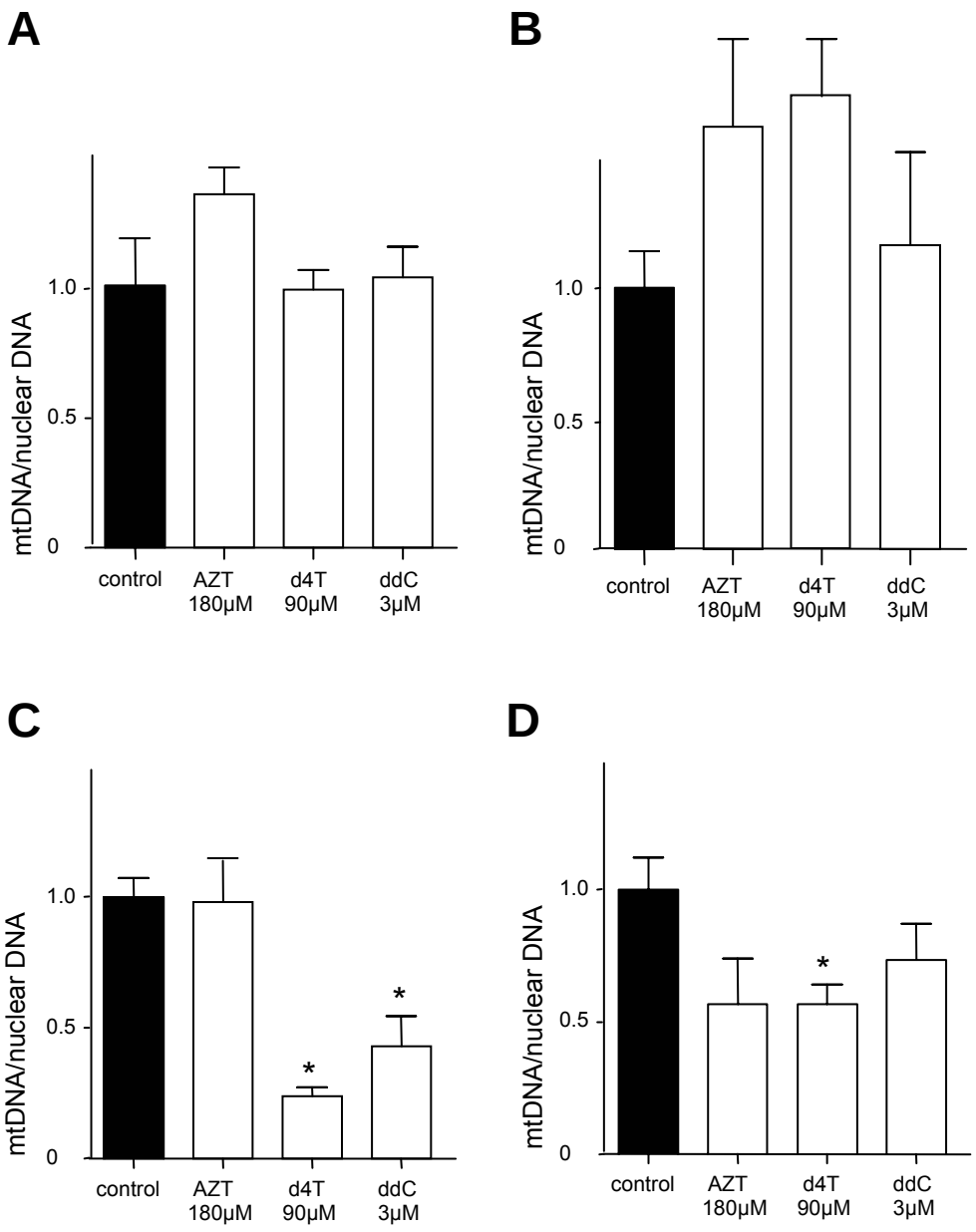



**Figure 4. Effect of NRTI on mtDNA content of non-proliferating cells and differentiating or proliferating adipocytes**

MtDNA content of growth arrested confluent preadipocytes (A) and fully differentiated adipocytes (B) was assessed 21 day after exposure to AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). The increase in mtDNA of fully matured adipocytes was statistically significant ($p<0.05$). MtDNA content measured by Real-Time PCR in (C) continuously proliferating preadipocytes and (D) preadipocytes differentiated in the presence of AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). Only d4T lead to a significant reduction of mtDNA in preadipocytes and preadipocytes undergoing maturation. Values are the mean ± SEM of three to five experiments. * $p<0.05$, ** $p<0.01$.versus control.

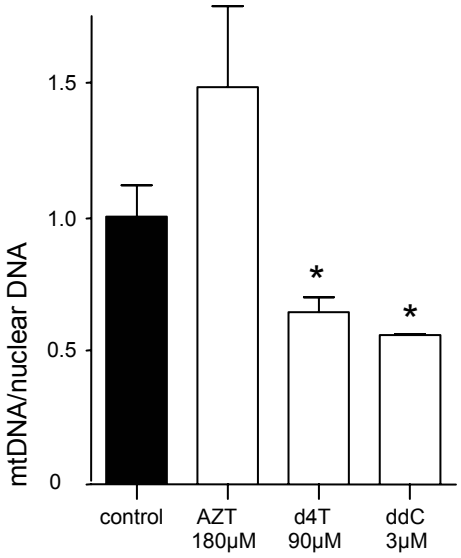



**Figure 5. Effect of NRTI on mtDNA content of HUH 7**
MtDNA content of HUH 7 after 10 days exposure to AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). MtDNA content was measure by Real-Time PCR. After 10 days of incubation only d4T and ddC lead to a significant reduction of mtDNA content. On the contrary an increase in mtDNA was observed under AZT incubation. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control

**5.1.7. Association of mtDNA depletion with respiratory chain function**. Given the profound mtDNA depletion that we observed after d4T and ddC treatment we wanted to assess whether this translates into functional impairment of respiratory chain complexes. As depicted in Figure 6, in conditions leading to an approx. 80% reduction in mtDNA (d4T), we did not detect an impairment of respiratory chain complexes. Similarly, AZT treatment, which was not associated with mtDNA depletion under these conditions, did not reduce enzymatic complex activity but rather increased the activity of complex I and IV (Figure. 6). Even long term culture of preadipocytes with NRTI for 2 months at therapeutic $C_{max}$ drug concentrations were not associated with any significant reduction in activities of the respiratory chain. Finally, citrate synthase activity as mitochondrial marker enzyme was not affected in NRTI treated cultures compared to control (not shown) indicating that the mitochondrial content in cells remained unchanged. The enzyme activity assays were tested using serial dilutions of the enzymes and demonstrated very good sensitivity to detect minor differences in the enzyme amount (data not shown).

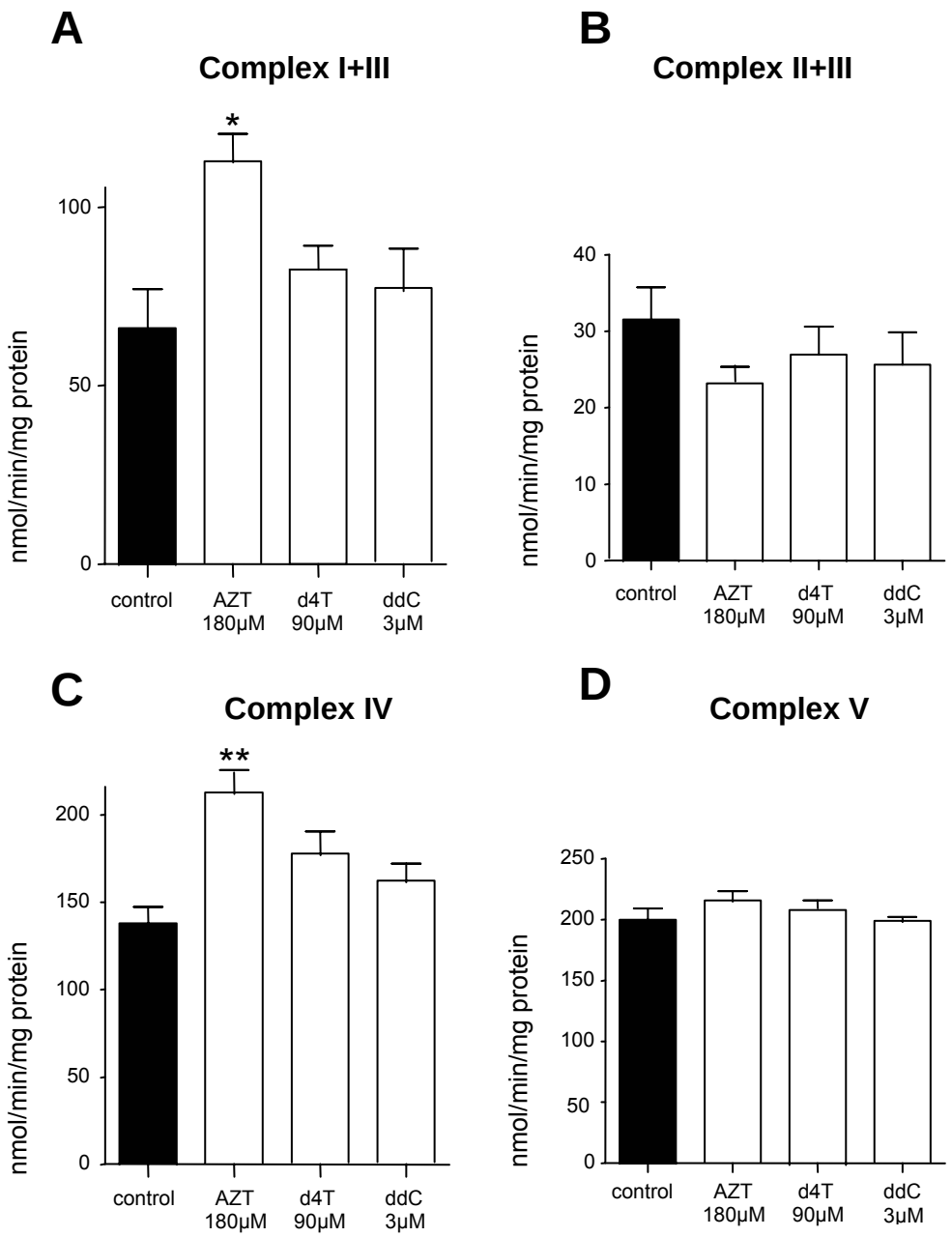



**Figure 6. Effect of NRTI on the respiratory chain complex activity in proliferating preadipocytes**
Preadipocytes were cultured for 21 days in the presence of AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). Cells were sonicated and individual substrates added to the cell lysates followed by photometric measurement of substrate metabolisation by the single respiratory chain complexes I, II+III, IV, and V. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

**5.1.8 Effect of NRTI on mtDNA content in human subcutaneous fibroblasts**.
We then evaluated the effect of NRTI on mtDNA in human subcutaneous fibroblasts in order to examine the effect in a system as close as possible to the *in vivo* situation. By splitting the fibroblasts culture before reaching confluence in the presence of drugs the cells were kept proliferating. After 21 and 42 days of drug treatment, incubation with

high levels of d4T led to a significant depletion (reduction by 60-70%) of mtDNA (Figure 7). Cultures with ddC for 42 days led to a depletion down to almost 10% of the original content (Figure 7). In contrast, AZT treatment had only slight effect on mtDNA content for 21 days. These experiments provide evidence that mainly d4T and ddC, and to a lesser extent AZT are able to impair significantly mtDNA synthesis in human primary fibroblast cultures.

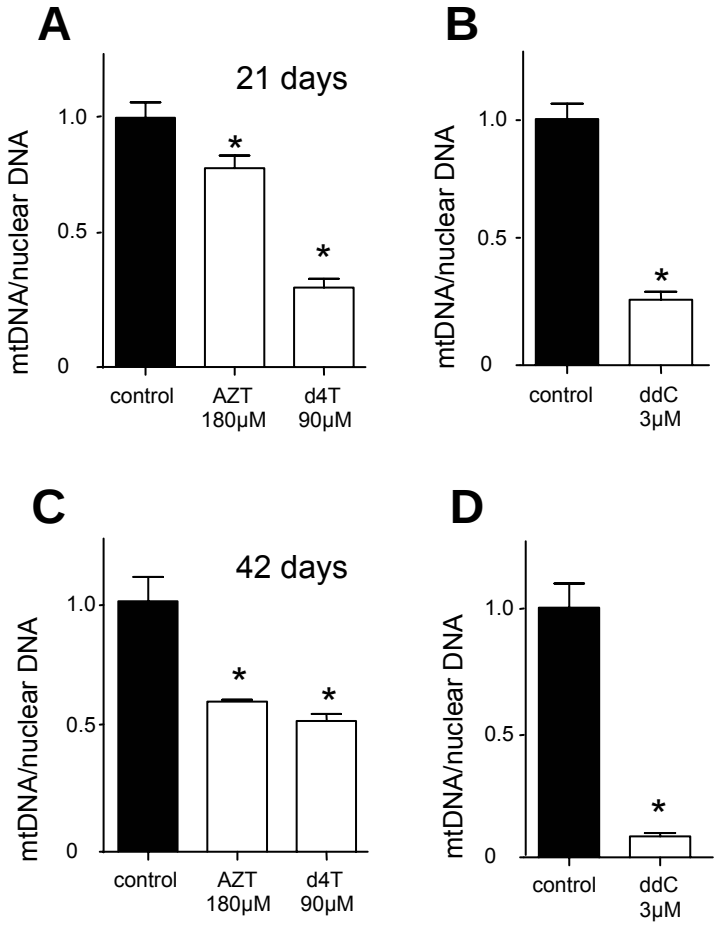



**Figure 7. Effect of NRTI on mtDNA content of human primary fibroblasts**
MtDNA content of human primary fibroblasts after 21 days (A and B) and after 42 days (C and D) exposure to AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). MtDNA content was measure by Real-Time PCR. After 21 days of incubation only d4T and ddC led to profound reduction of mtDNA content. In order to induce substantial mtDNA depletion under AZT incubation we needed 42 days exposure to high drug concentrations. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

**5.1.9 Association of mtDNA depletion with respiratory chain function in human primary fibroblasts**. Given the profound mtDNA depletion that we observed after d4T and ddC treatment after 21 and 42 days of incubation we wanted to assess whether this translates into functional impairment of complex activity in the respiratory

chain. To this end, proliferating human primary fibroblasts were exposed for 42 days to NRTI at high concentrations. Cells were then sonicated, substrate of the individual respiratory chain complexes was added to the cell lysates and complex activity quantitated by photometric measurement. As depicted in Figure 8, in conditions leading to an approx 90% reduction in mtDNA (ddC), we did not detect a measurable impairment of any respiratory chain complexes. AZT treatment, which was associated with the weaker mtDNA depletion under these conditions, had a slight effect on enzymatic activity of complexes II+III and V (Figure 8). Finally, citrate synthase activity as a measurement for the overall content of mitochondrial function was not affected in NRTI treated cultures compared to control (Figure 8).

**5.1.10 NRTI effect on mitochondrial mass and function in primary human fibroblasts**. As we were unable to associate the profound mtDNA depletion after 42 days of d4T and ddC treatment with impairment of complex activity in the respiratory chain in primary human fibroblasts, we decided to examine whether this depletion translates into impairment of mitochondrial potential and mass. To this end, proliferating human primary fibroblasts were exposed for 42 days to NRTI at high concentrations, washed and in order to evaluate mitochondrial membrane potential and to measure mitochondrial mass incubated with the cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolo carbocyanine iodide) and MitoTracker Red 580 (MTR) respectively (387;388)**.** None of the NRTI affected MitoTracker labelling (Figure 9B), indicating no effect of NRTI on mitochondrial mass. The mitochondrial membrane potential seemed to be preserved as shown by FACS fluorimetric quantification (Figure 9A) of JC-1 aggregates and monomers. The JC-1 aggregate/monomer ratio, which is an index of mitochondrial potential (388) was not significantly affected by NRTI incubation and the subsequent mtDNA depletion. These results indicate that d4T, AZT and ddC affect neither the number of mitochondria nor the mitochondrial dysfunction under these experimental conditions.

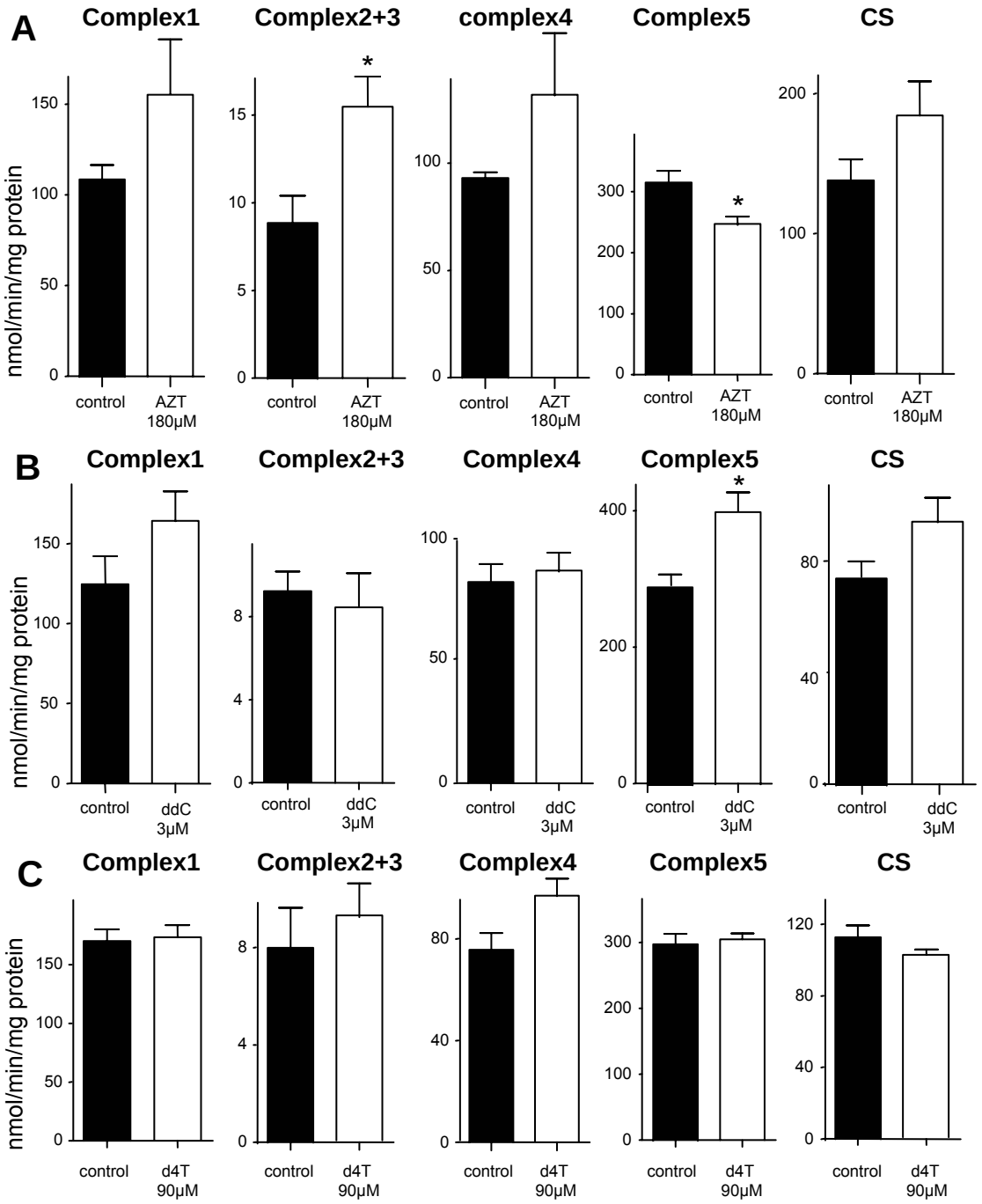



**Figure 8. Effect of NRTI on the respiratory chain complex activity in human primary fibroblasts**
Human primary fibroblasts were cultured for 42 days in the presence of AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). Cells were then sonicated and individual substrates added to the cell lysates followed by photometric measurement of substrate conversion by the single respiratory chain complexes I, II+III, IV, V and citrate synthase. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

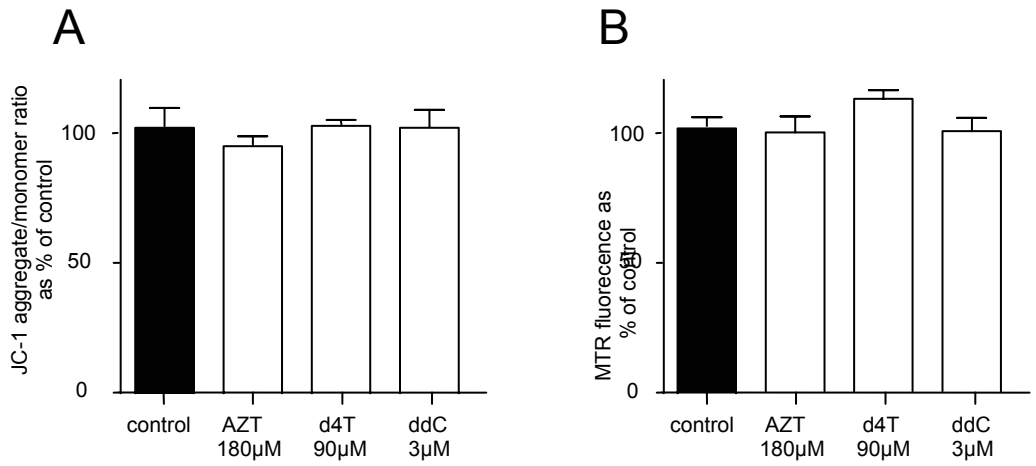



**Figure 9. NRTI effect on mitochondrial mass and function in primary human fibroblasts**
Human primary fibroblasts were cultured for 42 days in the presence of AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). Cells were stained for mitochondrial membrane potential and mitochondrial mass. The mitochondrial membrane potential was not significantly affected by NRTI incubation and the subsequent mtDNA depletion (A). MitoTracker labelling demonstrated no difference in the amount of mitochondrial mass in comparison to vehicle treated cells (B). Values are the mean ± SEM of three experiments.

### 5.1.11 Effect of NRTI on mtDNA content in human primary preadipocytes.

In order to bring our model as close as possible to the *in vivo* species and tissue situation we evaluated the effect of NRTI on mtDNA in human primary preadipocytes. The cells were kept in a proliferating state. After 40 days of drug treatment, incubation with high levels of d4T and ddC led to a significant depletion of mtDNA (Figure 10). In contrast, AZT treatment had no significant effect on mtDNA content for 40 days. These experiments provide evidence that d4T and ddC are able to impair significantly mtDNA content in human primary preadipocytes.

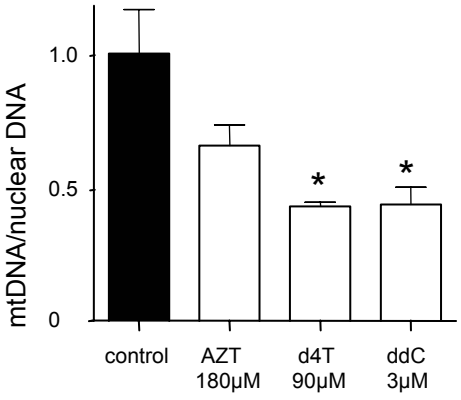



**Figure 10. Effect of NRTI on mtDNA content of human primary preadipocytes**
MtDNA content of human primary preadipocytes after 40 days exposure to AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). MtDNA content was measure by Real-Time PCR. After 40 days of incubation only d4T and ddC lead to a significant reduction of mtDNA content. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

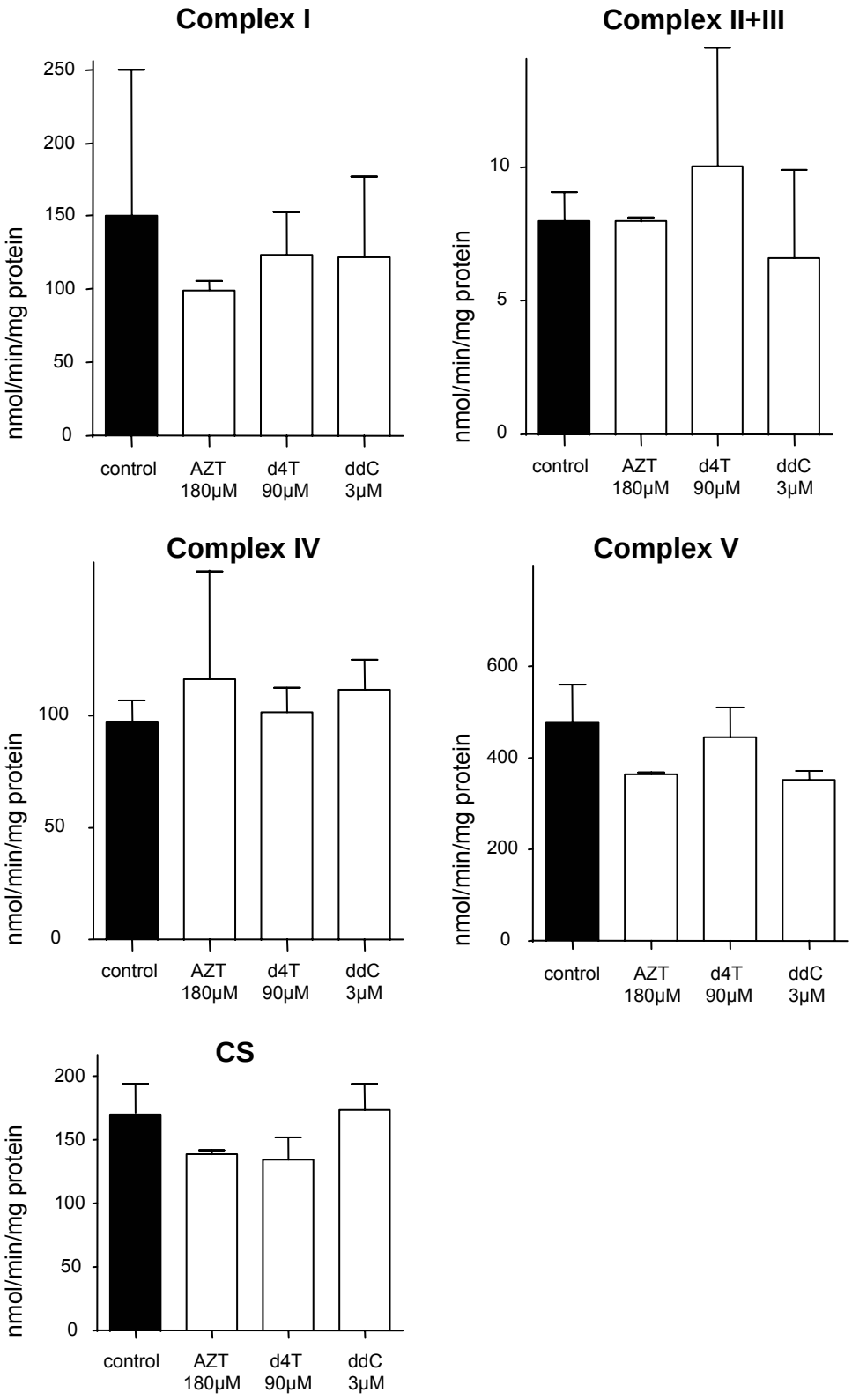



**Figure 11. Effect of NRTI on the respiratory chain complex activity in human primary preadipocytes**

Human primary preadipocytes were cultured for 40 days in the presence of AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). Cells were then sonicated and individual substrates added to the cell lysates followed by photometric measurement of substrate conversion by the single respiratory chain complexes I, II+III, IV, V and citrate synthase. Values are the mean ± SEM of three experiments.

**5.1.12 Association of mtDNA depletion with respiratory chain function in human primary preadipocytes**. Given the profound mtDNA depletion that we observed after d4T and ddC treatment after 40 days of incubation we performed experiments to assess whether this translates into functional impairment of complex activity in the respiratory chain. To this end, proliferating human primary preadipocytes were exposed for 40 days to NRTI at high concentrations. Cells were then sonicated, substrate of the individual respiratory chain complexes was added to the cell lysates and complex activity quantitated by photometric measurement. As depicted in Figure 11, in conditions leading to an approx 60% reduction in mtDNA (d4T), we did not detect a measurable impairment of any respiratory chain complexes. Finally, citrate synthase activity as a measurement for the overall content of mitochondrial function was not affected in NRTI treated cultures compared to control (Figure 11).

## 5.2 Zidovudine inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells

**5.2.1 AZT perturbs adipocyte mass through an effect on differentiation-dependent TG accumulation.** Adipocyte size and number as well as the rates of preadipocyte differentiation and adipocyte loss determine the mass of adipose tissue. Alteration in any of these processes may result in changes in adipose tissue mass. The 3T3-L1 cell line is a well characterized model for studying adipocyte differentiation and function. After stimulation 3T3-L1 preadipocyte change the pattern of gene expression and acquire adipocyte characteristics like spherical shape and accumulation of TG-rich lipid droplets as signs of differentiation. Therefore we aimed to determine whether AZT affects this process. Preadipocytes were induced to differentiate in the presence of AZT (6μM or 180μM) under standard conditions. Adipocytes were treated with vehicle, drug or TNF-α (250pM) as a positive control for differentiation impairment (140;141;389). Adipocytes exposed to high AZT concentrations failed to accumulate cytoplasmic TG compared to vehicle-treated cells, which was an effect of impaired differentiation (Figure 1). At low AZT doses the effect was less pronounced and they did not appear to differ noticeably from vehicle-treated cells. Microscopically AZT treated adipocytes revealed affected number of TG droplets and abundant patches devoid of cells (Figure 1). The acquisition of adipocyte phenotype and cell density was affected already at day 4, immediately after the completion of clonal expansion (Figure 12). Thus, under these

conditions AZT exerted a clear inhibitory effect on adipocyte differentiation. In contrast, 3T3-L1 adipocytes exposed to vehicle proceeded unaffected through the differentiation process (Figure 1 and 12).

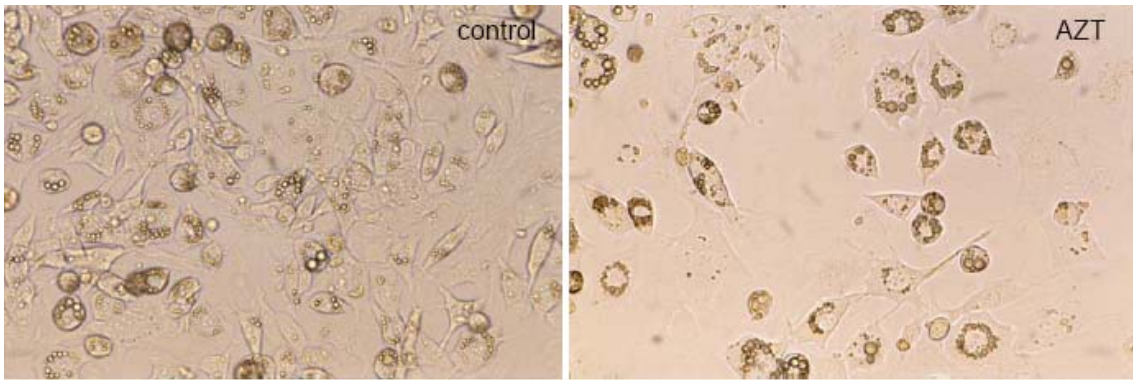



**Figure 12. Impaired adipocyte differentiation after exposure to AZT**
3T3-L1 adipocyte phenotype (unstained cells) at day 4 after initiation of differentiation in the presence of AZT (180μM) and control. 3T3-L1 cells in the control culture have expanded properly started acquiring a mature phenotype with round shape and multiple lipid droplets. AZT exposed cultures present decreased numbers of cells, decreased amount of triacylglycerol droplets and retarded acquisition of mature phenotype compared to control.

**5.2.2 Mitotic clonal expansion is affected by AZT.** The cascade of gene induction and the critical mitotic clonal expansion (390) are the first events characterizing adipogenesis. Mitotic clonal expansion takes place within the first three days of the *in vitro* adipogenic program. After roughly two divisions preadipocytes exit the cell cycle and continue with the expression of adipogenic cascade of transcription factors leading to the acquisition of adipocyte characteristics (Figure A). Given the known antiproliferative effects attributed to AZT (391-401) we were interested to determine whether AZT treatment affects mitotic clonal expansion. Preadipocytes were induced to differentiate in the absence or presence of different concentrations of AZT (6μM and 180 μM) and Rapamycin (10nM) as a positive control for impaired clonal expansion (402). Cell number was also determined from dishes of confluent preadipocytes without induction of differentiation, incubated with AZT and vehicle for an identical period of time. The number of vehicle-treated differentiating preadipocytes increased almost 3-fold, corresponding to roughly two rounds of mitosis (Figure 13A). The increase in cell number in preadipocytes induced to differentiate in the presence of AZT, and Rapamycin was significantly lower (Figure 13A). In case of confluent preadipocytes not induced to differentiate the number of cells remained almost the same,

with and without the presence of AZT. The very sensitive $^{3}$H-thymidine incorporation assay confirmed the inhibitory effect of AZT on the cell division during the phase of clonal expansion even at $C_{max}$ concentration (Figure 13B). Therefore, it appears that AZT significantly affects 3T3-L1 differentiation at the stage of mitotic clonal expansion, even without major difference in cell viability in comparison to control.

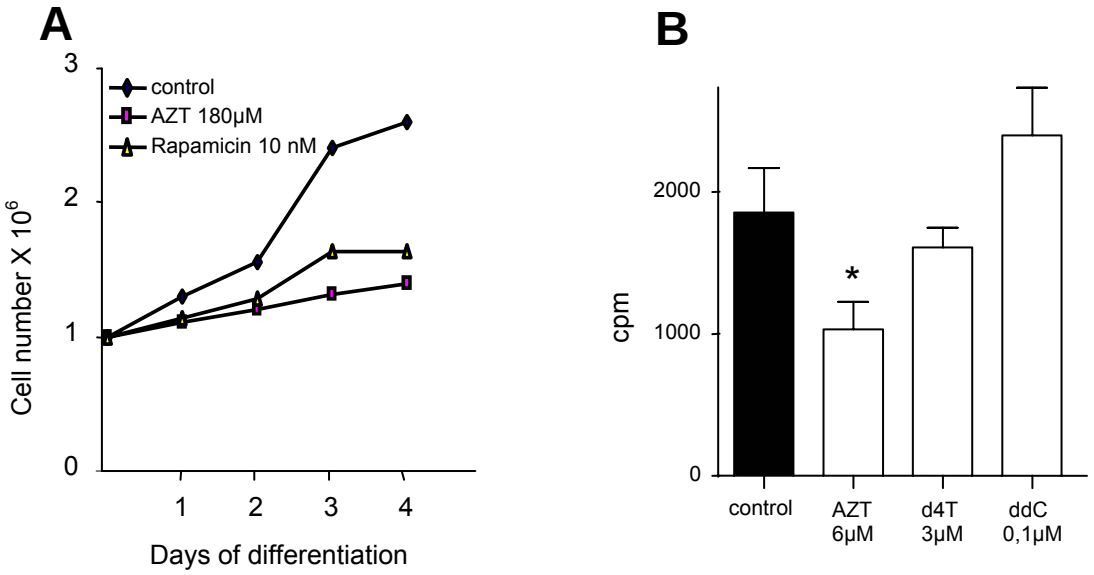



**Figure 13. Effect of AZT on mitotic clonal expansion**
Mitotic clonal expansion, takes place within the first three days of the differentiation program. Preadipocytes were induced to differentiate in the absence or presence of AZT (180 µM) and Rapamycin (10nM). Cell number increased almost 3-fold in vehicle-treated differentiating preadipocytes. Significantly diminished increase in cell number was observed in preadipocytes induced to differentiate in the presence of AZT, and Rapamycin. $^{3}$H-thymidine incorporation assay confirms the inhibitory effect of AZT on the cell division during the phase of clonal expansion even at $C_{max}$ concentration (Figure 13B). No inhibition has been observed in case of d4T (3µm), ddC (0, 1µM), or vehicle (black column) treated cells. Values are the mean ± SEM of five to ten experiments. * $p<0.05$, ** $p<0.01$.versus control.

**5.2.3 Precursors proliferation is affected by AZT.** $^{3}$H-thymidine incorporation assay demonstrated AZT anti-proliferative effect on primary human and 3T3-Ll fibroblasts as well as on human preadipocytes suggesting that this effect is not restricted to the cell line and could be expected in an *in vivo* system. The effect was readily observed at physiological concentrations even after 3h incubation (Figure 14B) and was strikingly persuasive at higher concentrations (Figure 14). The AZT anti-proliferative effect on 3T3-Ll was also confirmed using a total cellular protein assay (Figure 15).

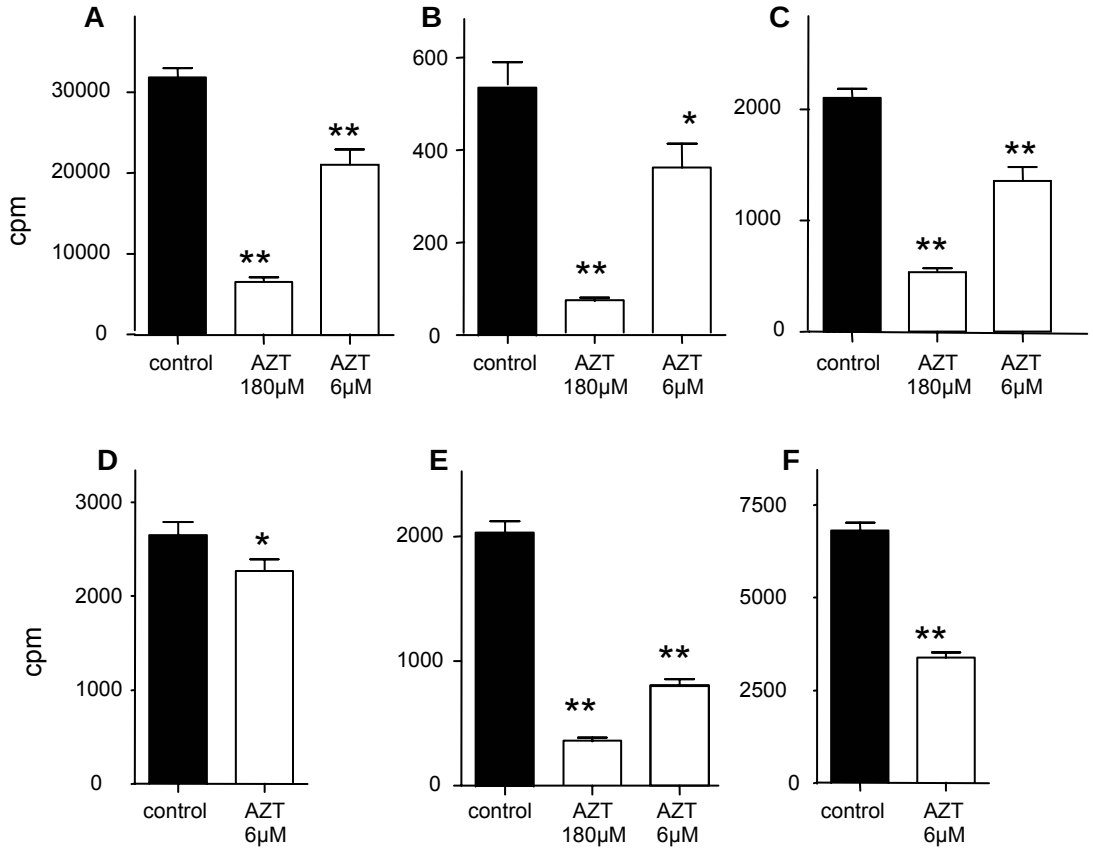



**Figure 14. Effect of AZT on precursor proliferation**
Primary human (C) and 3T3-L1 (A and B) fibroblasts , human preadipocytes (D) as well as HUH 7 (E) and C2C12 (F) cell lines were cultured in absence or presence of different concentrations of AZT (6µM and 180µM) or vehicle (black column). $^{3}$H-thymidine incorporation assay demonstrated antiproliferative effect of AZT on all the cell lines as well as on the primary human fibroblasts and preadipocytes suggesting that this effect is not restricted to species, tissue or cell line specificity and could be expected in an *in vivo* system. The effect was readily observed at physiological concentrations (6µM) and was strikingly persuasive at higher concentrations (180µM). Values are the mean ± SEM of eight experiments. * $p<0.05$, ** $p<0.01$.versus control.

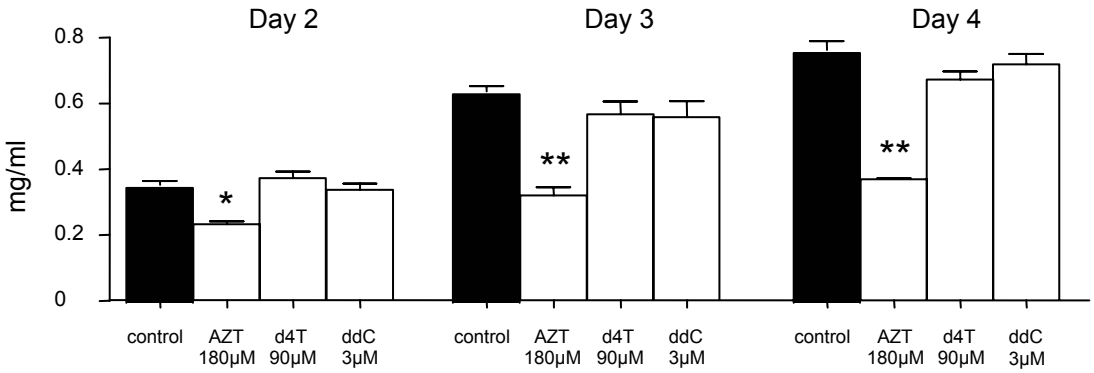



**Figure 15. Effect of AZT on cell proliferation measured through total cellular protein**
Equal number of 3T3-L1 preadipocytes were cultured in the presence of AZT (180µM), d4T (90µm), ddC (3µM), or vehicle (black column). Cells were then sonicated and total cellular protein measured at days 2, 3, and 4. AZT affected the amount of total cellular protein in a progressive way speaking for an inhibition of cell proliferation. Values are the mean ± SEM of five experiments. * $p<0.05$, ** $p<0.01$.versus control.

**5.2.4 Effects of AZT on adipogenic marker expression.** A comprehensive examination of the effects of AZT on adipogenic transcription factors expression in 3T3-L1 adipocytes was undertaken. We addressed the hypotheses that AZT inhibits differentiation-associated expression of these markers. Preadipocytes were induced to differentiate in the absence or presence of different concentrations of AZT (6μM and 180μM), rapamycin and TNF-α. mRNA was isolated every second day starting from day +1 of the differentiation protocol. The mRNA was reverse transcribed and Real-Time PCR analysis performed to assess expression levels of several transcription factors involved in a cascade during preadipocyte differentiation (Figure A). Increase in C/EBPβ expression starts from day one and peaks by day 2 (Figure A). To determine the very early effect of AZT on C/EBPβ induction before its complete effect on clonal expansion we measured C/EBPβ mRNA expression levels at day +1. Neither the cultures with AZT nor our positive control with TNF-α, showed statistically significant alteration in the expression of this factor at this time point under our experimental conditions (Figure 16). Expression levels of the adipogenic transcription factors PPARγ and C/EBPα after clonal expansion at days +3 and +5 respectively were markedly lower in AZT-treated cultures when compared to those in vehicle-treated ones (Figure 16). When considered together our Real-Time PCR analyses indicate that AZT treatment induces disruption of the expression patterns of the two main adipogenic factors C/EBPα and PPARγ, which expression normally picks after the successful completion of clonal expansion (Figure A). Our results are in line with a recent studies revealing trends towards reduced expression of adipogenic markers in 3T3-L1 and 3T3-F442A cell lines (349;403). The differentiation process, as measured by TG accumulation and expression pattern of adipogenic transcription factors, was disrupted by AZT. Similar results were obtained with rapamycin and TNF-α (Figure 16).

**5.2.5 Impaired differentiation is confirmed by decrease in adipokines expression and secretion.** As we observed a decrease in the expression of C/EPBα during AZT incubation we decided to assess whether it affects the expression of downstream factors such as adiponectin, as C/EPBα is a key transcription factor for the complete activation of the expression of adiponectin gene in mature adipocytes through interaction with response elements in the intronic enhancer (404). At day +9 of the differentiation program there was a significant reduction in the expression and

production of adiponectin which is normally expressed by mature adipocytes and is a marker of successful differentiation and complete maturation (Figure 3).

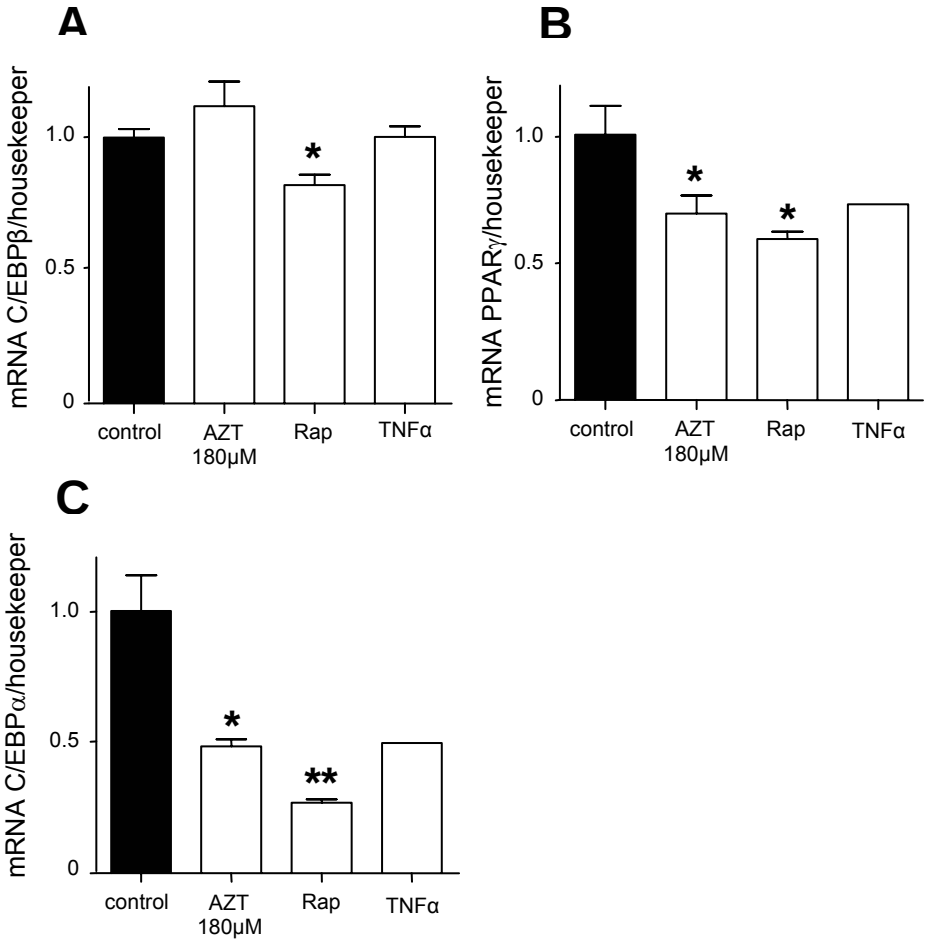



**Figure 16. Molecular analysis of adipocyte differentiation**
The panels reflect the effects of high concentration of AZT (180μM), Rapamycin (10nM) and TNF-α (250pM) on the expression of the important factors of differentiation: C/EBPβ (A), PPARγ (B) and C/EBPα (C) in differentiating 3T3-L1. Preadipocytes were cultured in the presence of vehicle (black column) or the indicated drugs from day 0, induced to differentiate on day 0 and differentiated until day + 9. Total RNA was isolated at days +1 (for C/EBPβ), +3 (for PPARγ) and +5 (for C/EBPα), reverse transcribed and expression levels measured with Real-Time PCR. Like in the morphological analysis (Figure 1), AZT led to significant impairment of C/EBPα and PPARγ expression at high drug concentrations. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

**5.2.6 AZT effect on adipocyte viability.** At first we examined AZT effects on the viability of 3T3-L1, which were maintained in a nondifferentiated state. Preadipocytes cultured for 21 days in the presence of AZT (6μM) demonstrate neither morphological difference nor different trypan blue staining in comparison to vehicle treated ones. These results indicated that long-term AZT (6μM) treatment did not significantly affect

the viability of 3T3-L1 preadipocytes. Similar results were obtained even when we increased the AZT concentration up to 180μM for a period of 21 days. To confirm whether this lack of cell toxicity is something restricted to the cell line model we used, we incubated primary human fibroblasts for 42 days with 180μM and found no major cell death (Figure 17B). It appears that the observed changes in proliferation and differentiation are not due to the cellular toxicity of the drug as 3T3-L1 retained normal fibroblast morphology and were viable even after 21 days under supraphysiological concentrations. As apoptosis of subcutaneous adipocyte from lipodystrophic HAART treated patients has been reported (351), we tried to determine whether AZT induces signs of apoptosis in 3T3-L1 adipocytes. At first experiments with trypan blue dye exclusion were performed in order to determine whether differentiating adipocytes remain viable after AZT treatment. Exposure of differentiating adipocytes to high AZT concentrations during and after initiation of differentiation indicated no significant effect on cell viability and although 50% of the detached cells appeared to be dead as a percent of the total cell population we found only a slight increase in cell death in comparison to control (Figure 17A), which is unlikely to account for any of the other effects observed. Therefore we concluded that although high doses of AZT affect 3T3-L1 proliferation and differentiation they have no effect on cell viability over the differentiation changes. Annexin V/ Propidium iodide staining demonstrated apoptosis levels which were in accordance with trypan blue staining.

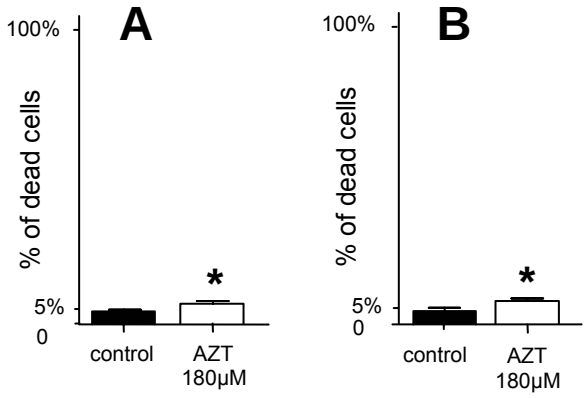



**Figure 17. Effect of AZT on cell viability**
Primary human and 3T3-L1fibroblasts were cultured in the presence of AZT (180μM) or vehicle (black column) for 42 and 21 days respectively. Both cultures demonstrated only slight increase in cell death. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

## 5.3 Adipocyte viability and function but not preadipocyte differentiation is compromised by IDV

**5.3.1 IDV affects adipocyte mass but does not affect differentiation-dependent accumulation of TG.** The rates of preadipocyte differentiation and adipocyte loss as well as adipocyte size determine adipose tissue mass. Alteration in any of these processes may in theory bring changes in adipose tissue mass. 3T3-L1 preadipocyte differentiation is induced by hormonal mixture (see the Introduction and Figure A) and appropriate culture conditions and they undergo specific pattern of gene expression and assume adipocyte characteristics like accumulation of cytoplasmic TG. We conducted experiments to determine whether the protease inhibitor IDV affects this process. Preadipocyte differentiation was induced as described above (see Methods). Drug incubation was initiated at days:-10,-5, 0, 8, adipocytes were exposed to different concentrations of IDV (5-100μM). Adipocytes were treated with vehicle or drug. Every second day of the experimental protocol, cells were stained with Oil Red O. Adipocytes exposed to IDV retained less Oil Red O then vehicle-treated cells. That was more an effect of decreased cell number leading to decrease in total cytoplasmic TG per culture flask than decrease in total cytoplasmic TG per cell (Figure 18). This effect was most pronounced in adipocytes treated with higher doses of IDV. Cells incubated with low doses IDV did not appear to differ from vehicle-treated ones. The number of TG droplets per cell did not differ between adipocytes exposed to IDV and vehicle-treated ones, but in the treated cultures there were many patches devoid of cells (Figure 18). Thus the inhibitory effect of IDV on adipocyte differentiation was not observed in any of the experimental conditions. In the incubations with high IDV concentrations an increase in the number of floating cells was observed. A big proportions of these floating cells were dead. In contrast, adipocytes exposed to vehicle were present in a continuous monolayer and were well attached to the culture dish (Figure 18).

**5.3.2 IDV does not affect mitotic clonal expansion.** The initial cascade of gene induction and the critical mitotic clonal expansion are the main steps in determining adipogenesis. The clonal expansion takes place within the first three days of the differentiation program.

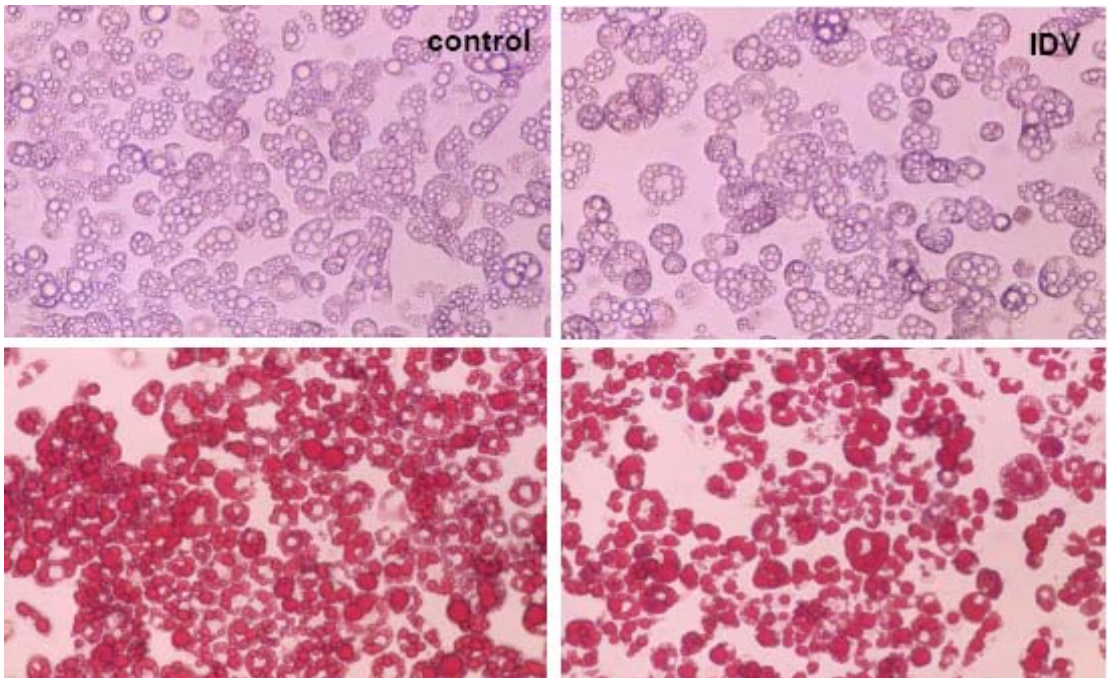



**Figure 18. Adipocyte differentiation after exposure to IDV**
3T3-L1 adipocyte phenotype (unstained and Oil Red O stained cells) at day 9 after initiation of differentiation in the presence of IDV (100μM) or control. 3T3-L1 cells in the both IDV treated and control cultures have acquired a mature phenotype with round shape and multiple lipid droplets. IDV exposed cultures present neither decreased numbers of TG droplets nor increased numbers of non-differentiated cells. The only difference compared to control is some patches devoid of cells.

After expanding preadipocytes exit the cell cycle and the continuing adipogenic gene expression leads to the acquisition of adipocyte characteristics. Therefore, it was of interest to determine whether IDV treatment affects mitotic clonal expansion. Preadipocytes were induced to differentiate in the absence or presence of different concentrations of IDV (30 and 100 μM) and IDV incubation started at different time points: days -10, -5, 0 and starting at day 0 cell number was determined. Cell number was also determined from cultures of confluent preadipocytes which were not induced to differentiate, but were maintained in the absence or presence of IDV for an identical period of time. The cell numbers of preadipocytes not induced to differentiate, in the presence IDV or vehicle were similar. Cell number increased almost 3-fold in vehicle-treated differentiating preadipocytes, corresponding to roughly approximately two rounds of mitosis (Figure 19). A similar expansion of cell numbers was observed also in preadipocytes induced to differentiate in the presence of IDV (Figure 19). At high IDV concentration the cell numbers were lower in comparison to control, but this was more a result of initial cell loss on day 1, followed by unaffected clonal expansion. The number of cells present under differentiating conditions with and without IDV has increased in

comparison to confluent preadipocytes that were not induced to differentiate. Therefore, it appears that IDV does not significantly affect the mitotic clonal expansion phase of preadipocyte differentiation despite its effects on cell viability.

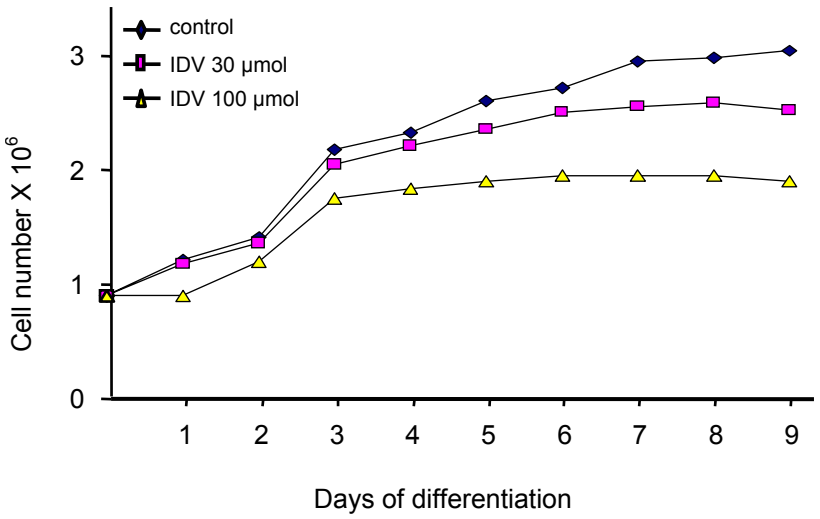



**Figure 19. Effect of IDV on clonal expansion**
Preadipocytes were differentiated in the presence of IDV (30 and 100µM) or vehicle (black column) and cell numbers were counted every day till day +9. IDV leads to significant reduction in cell numbers on day +1, which seems to be preserved during the clonal expansion. Significant reduction in cell numbers after IDV exposure was evident also at therapeutic drug concentrations. Values are the mean ± SEM of five experiments. * $p<0.05$, ** $p<0.01$.versus control.

**5.3.3 Effects of IDV on adipogenic marker expression.** Effects of IDV on adipogenic transcription factor expression in 3T3-L1 adipocytes were examined in details. The probable inhibitory effect of IDV on differentiation-associated expression was addressed. Preadipocytes were induced to differentiate in the absence or presence of different concentrations of IDV (30 and 100µM) starting incubation at different time points: days -10,-5, 0 and mRNA was isolated every second day starting from day –1 of the differentiation protocol. To assess the expression levels of several markers involved in preadipocyte differentiation mRNA was reverse transcribed and Real-Time PCR analysis performed (Figure 20). The drug did not affect LPL expression in one day post confluent cells (Figure 20 A). C/EBPβ mRNA expression levels at days +1 were similar in vehicle and IDV-treated cells demonstrating that IDV did not affect early induction of C/EBPβ (Figure 20 B). When compared to vehicle-treated cells IDV treated cells did not demonstrate different expression levels of the adipogenic transcription factors C/EBPα

and PPARγ (Figure 20 C and D). When considered together, our Real-Time PCR analyses indicate that IDV treatment does not disrupt the transcription factor cascade (C/EBPβ, C/EBPα, PPARγ) associated with preadipocyte differentiation (Figure 20). In other words, the differentiation process, as measured by the expression of adipogenic factors and by TG accumulation, was not disrupted by IDV. TNF-α a well known inhibitor of 3T3-L1 differentiation was used as a positive control for inhibition of adipogenic markers expression (Figure 21).

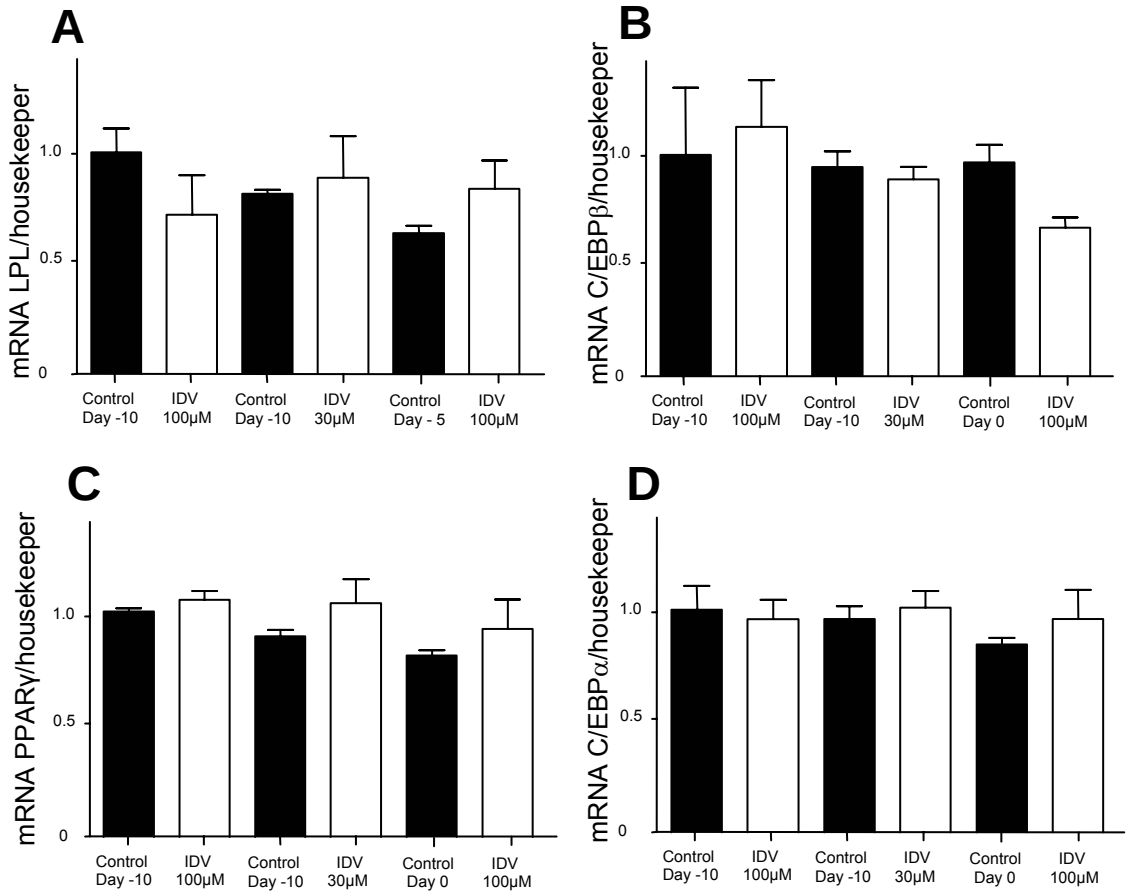



**Figure 20 Molecular analysis of adipocyte differentiation**
The panels reflect the effects of therapeutic (30µM) and high (100µM) IDV concentration on the expression of the important early and late factors of differentiation Lipoprotein lipase (LPL) (A), C/EBP β (B), PPARγ (C) and C/EBP α ( D) in differentiating 3T3-L1. Preadipocytes were cultured in the presence of vehicle (black column) or the indicated drugs from day -10, -5 and 0, induced to differentiate on day 0 and differentiated until day + 9. Total RNA was isolated on days -1, +1, +3, +5, reverse transcribed and expression levels measured with Real time PCR. Like in the morphological analysis (Figure 18), IDV had no significant effect on LPL, C/EBPβ, C/EBPα and PPARγ expression at both concentrations and in all time courses of incubation. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

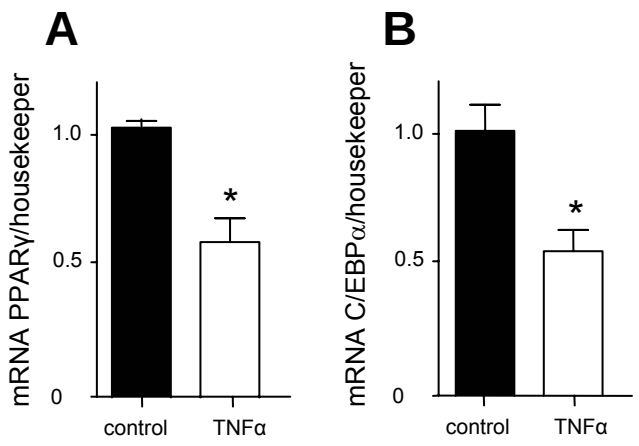



**Figure 21. TNF-α as a positive control for inhibition of differentiation**
The panels reflect the effects of (250pM) TNF-α on the expression of the important late factors of differentiation PPARγ (A) and C/EBP α (B) in differentiating 3T3-L1. Preadipocytes were cultured in the presence of vehicle (black column) or TNF-α from day 0, induced to differentiate on day 0 and differentiated until day + 9. Total RNA was isolated on days +3, +5, reverse transcribed and expression levels measured with Real-Time PCR. TNF-α had significant effect on C/EBPα and PPARγ expression. Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

**5.3.4 IDV perturbs adipocyte function**. Although differentiation was not significantly affected at day +9 there was a significant reduction in the expression of adiponectin as evidence for impaired adipocyte function (Figure 22). To separate this effect of IDV from the differentiation processes we incubated fully differentiated adipocytes for 9 days and found that adiponectin expression was also decreased although not to the same extend like in cell at day +9 of differentiation protocol (Figure 22).

**5.3.5 IDV promotes loss of adipocyte viability.** In the first set of experiments, we examined IDV effect on the proliferation and viability of 3T3-L1, maintained in a nondifferentiated state. Short-term (24h) IDV (30 and 100μM) treatment affected neither the proliferation nor the viability of 3T3-L1. Even after 10 days of culture in the presence of 100μM IDV preadipocytes looked morphologically similar to vehicle treated ones and did not show differences in terms of trypan blue staining. The cell number was similar at confluence in both untreated and treated cells. These results suggest that neither short nor long-term IDV (up to 100μM) treatment affect growth and viability of 3T3-L1 preadipocytes. As 3T3-L1 preadipocytes proliferation was normal in preconfluence and during clonal expansion (Figure 19), and they maintained normal fibroblast morphology in the presence of IDV we can conclude that IDV is not generally toxic to them. These results correspond to earlier report by another group (347).

Samples from HIV-infected patients with lipodystrophy demonstrate subcutaneous adipocyte apoptosis (351). We therefore performed experiments to determine whether IDV induces signs of apoptosis in 3T3-L1 adipocytes by trypan blue dye exclusion experiments. When exposed to high doses of IDV during and after initiation of differentiation adipocytes exhibited trypan blue staining in comparison with vehicle treated cultures which exhibited little or no trypan blue staining, indicating that a part of the cells were either dead or dying. Therefore, high doses of IDV induce extensive loss of cell viability over a differentiation changes and in differentiated adipocytes eventually indicating that IDV induces cell death only after preadipocytes have been initiated to differentiate into adipocytes. Hoechst/ Propidium iodide staining demonstrate results in accordance with trypan blue staining.

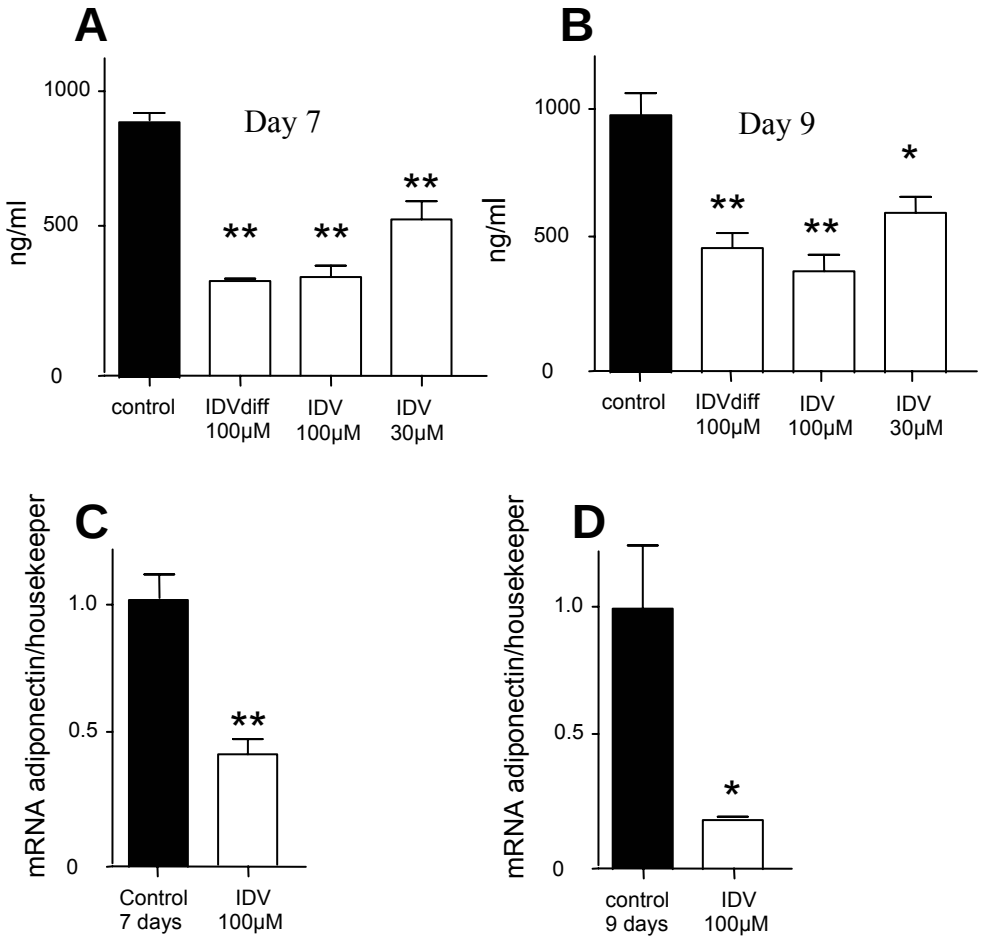



**Figure 22. Effect of IDV on adiponectin production**
Preadipocytes were differentiated in the presence of IDV (100µM) and (30µm) or vehicle (black column) and at day +7 and +9 culture medium supernatant (A and B) or adipocytes (C and D) assessed for adiponectin by ELISA or mRNA. Another bunch of preadipocytes designated (diff) were differentiated prior to 9 days of IDV incubation and subjected to the same analysis. IDV leads to significantly reduced adiponectin release into the culture medium (after adjustment for cell numbers) no matter whether cells were differentiated prior or during the drug incubation. Significant reduction in adiponectin production after IDV exposure was evident also at therapeutic drug concentrations as confirmed by ELISA (A and B). Values are the mean ± SEM of three experiments. * $p<0.05$, ** $p<0.01$.versus control.

**5.3.6 *In vivo* analyse of HAART on adiponectin production.** After analyzing the AZT and d4T effects on adiponectin release in culture we wished to confirm these observations *in vivo*. We analyzed the frozen serum samples of 12 randomly selected HIV-patients from our outpatient clinic before and mean 9 months after initiation of HIV-therapy consisting of AZT, 3TC and IDV for levels of adiponectin. Interestingly, we found a reduction in adiponectin levels in all but one patient leading to a significant overall reduction of mean adiponectin values (7.3 µg/ml versus 5.4 µg/ml) after start of therapy and before clinical evidence for lipodystrophy (Figure 23).

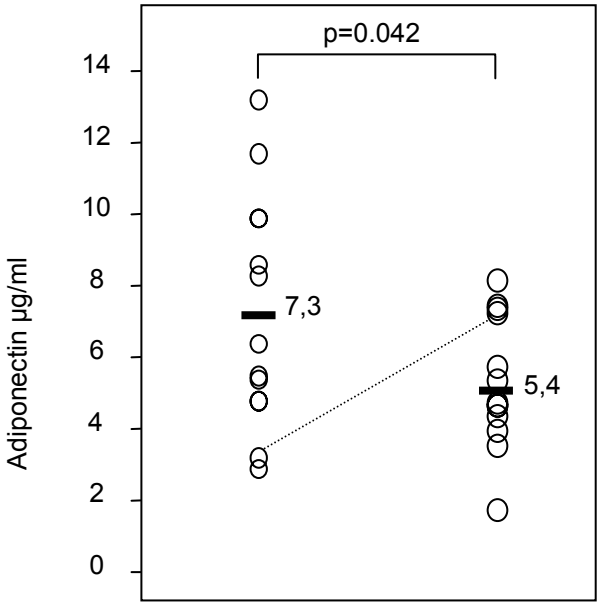



**Figure 23. Adiponectin levels in patients before and after start of AZT, 3TC and IDV therapy**
Frozen serum samples from randomly selected patients receiving the same antiretroviral regimen were assessed for adiponectin levels before and mean 9 months after start of therapy. In all but one patient (dotted line) we observed a decrease in serum adiponectin levels leading to an overall significant reduction.

## 5.4 Lipoatrophy and ubiquitous mtDNA depletion in mice following long-term NRTI traement

**6.4.1 Effects of AZT and d4T on fat amount and distribution in treated mice.** The *ex vivo* determination of body fat mass and distribution was performed by dissection and macroscopic examination of different fat depots. Mice treated for 15 weeks with therapeutic d4T or AZT doses similar to those used in humans presented fat

atrophy in axillary and inguinal subcutaneous fad pads as well as marked reduction in intra-abdominal fat.

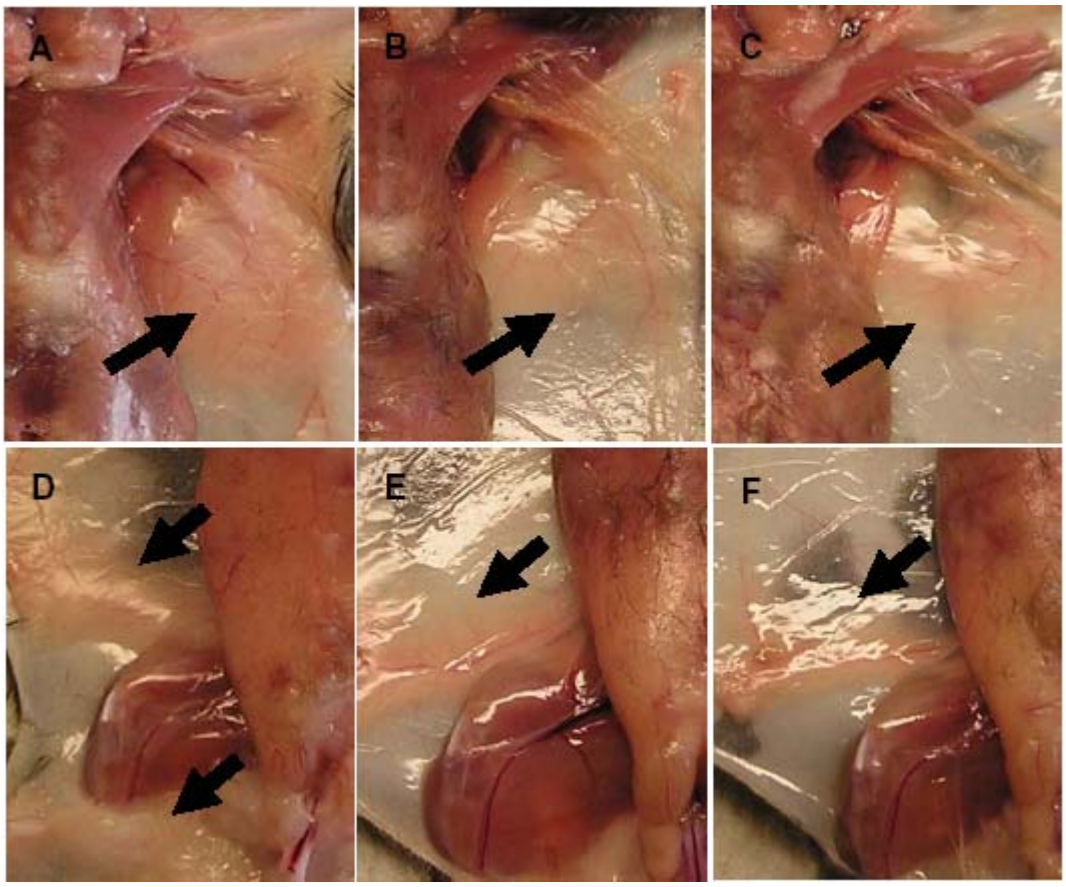



**Figure 24. NRTI induced atrophy in murine model**
Effect of 15 weeks d4T (B and E) and AZT (C and F) or vehicle (A and D) treatment on axillary (A,B and C) and inguinal (D,E and F) fad pads. Arrows show the regions with fat depletion. Each picture is representative from 14 pictures per group.

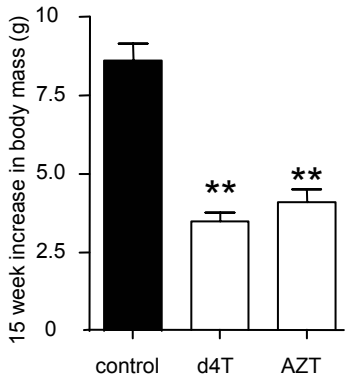



**Figure 25. Effect of d4T and AZT on the body weight acquisition in C57BL/6 mice**
Over a time period of 15 weeks mice receiving d4T or AZT gained less weight (d4T 3.5 ± 0.3g; AZT 4.1 ± 0.4g) as compared to control animals (8.6 ± 0.5g). Values are the mean ± SEM of 17 to 20 mice per group. * $p<0.05$, ** $p<0.01$.versus control.

**5.4.2 Effect of d4T and AZT on of body weight acquisition, in young male C57BL/6 mice.** We assessed the acquisition of body mass in young male C57BL/6 mice during 15 week treatment with pharmacological drug levels of d4T and AZT (Figure 25). A substantial increase in body weight was observed in mice treated with vehicle, but this increase was significantly attenuated in d4T and AZT treated mice (Figure 25).

**5.4.3 Effect of d4T and AZT on water and food consumption.** Given the evident effect on body weight acquisition (Figure 25) and on both peripheral and central fat (Figure 24) that we observed after d4T and AZT treatment we wanted to assess whether this was due to differences in water and food consumption. We compared the weekly consumption of food and the 72h consumption of water during the treatment period and calculated the average daily consumption per mouse (Figure 26).

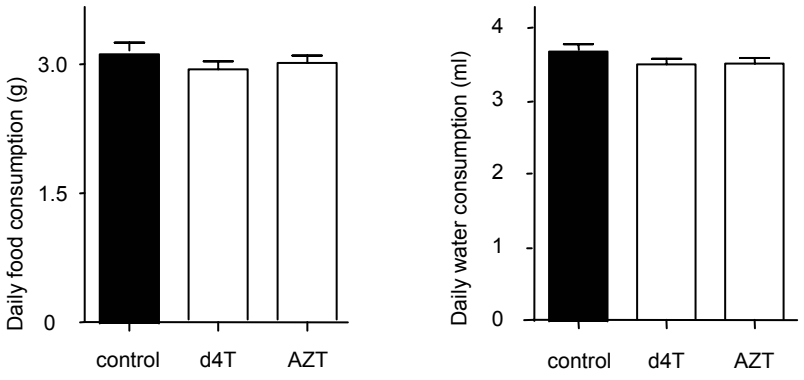



**Figure 26. Effect of d4T and AZT on the average daily water and food consumption**
d4T and AZT treatment resulted in no difference in daily water and food consumption. These results demonstrate that these drugs impair body weight acquisition without an effect on water and food intake.

**5.4.4 Effects of therapeutic drug levels d4T on mtDNA content in different tissues.** MtDNA depletion has been proposed to be an important factor leading to peripheral lipoatrophy in HIV-patients receiving antiretroviral therapy and this factor could have accounted for the profound lipoatrophy observed in our murine model (Figure 24). We therefore wanted to evaluate the effect of therapeutic d4T levels on mtDNA content in subcutaneous fat. Tissue samples were taken from subcutaneous fat, liver, heart, muscle and brain, DNA isolated and mtDNA quantified using Real-Time PCR. Analysis of mtDNA content revealed that 5 months of treatment with therapeutic d4T levels caused depletion of mtDNA in organs including the brain (-20%) and fat

tissue (-27%; Figure 27 E and A), but the most profound and significant depletion was evident in muscle (-56%), liver (-64%), and heart (-45%, Figure 27 D, B, and C). Together with the results of the macroscopic examination, these experiments provide evidence that d4T exerts a wasting effect on subcutaneous fat with only limited effect on mtDNA content. These data expand earlier observations reported by Fromenty *et al* (405) and indicate that mtDNA depletion is an unspecific event during treatment with thymidine-analogues.

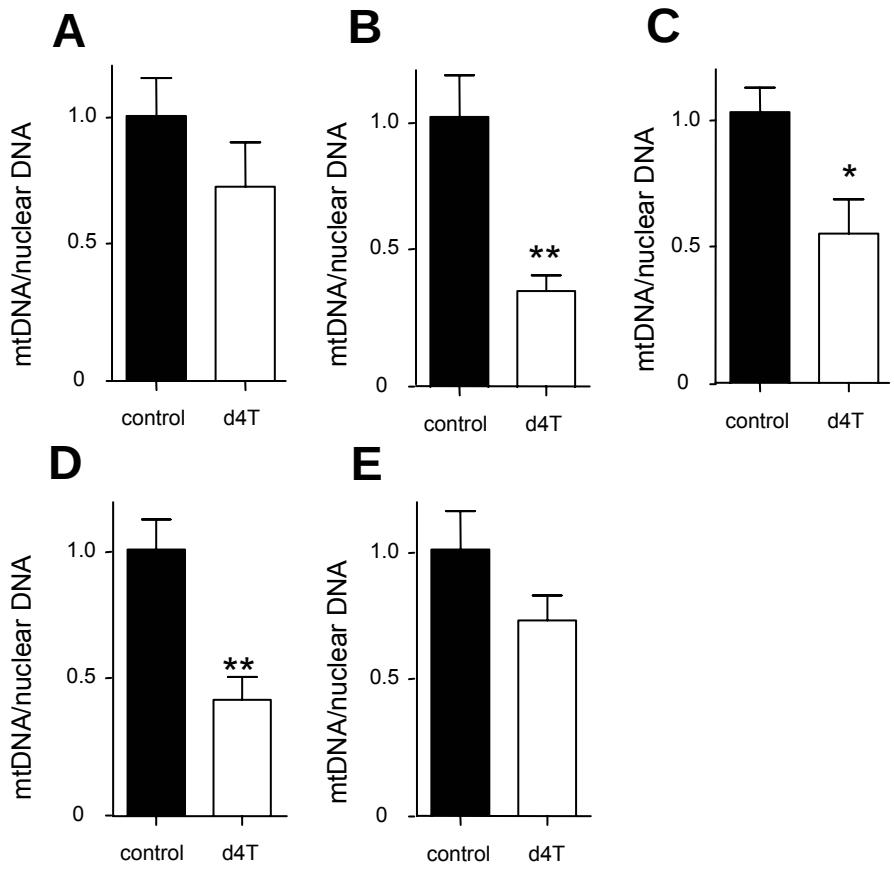



**Figure 27. Effect of d4T on mtDNA content of various mouse organs and tissues**
MtDNA content of subcutaneous fat, liver, heart, muscle and brain of 5 months treated mice with therapeutic drug levels d4T. Groups of mice were treated with d4T or vehicle (4 mice per group) for up to 5 months with daily human doses adjusted for murine body surface area. The figure demonstrates tendency for depletion of mtDNA in organs such as brain (E) and fat tissue (A) and significant depletion in muscle (D), liver (B), and heart (C). Values are the mean ± SEM of four mice per group. * $p<0.05$, ** $p<0.01$.versus control.

**5.4.5 Effect of d4T on adipokine production.** As adipose mass has been associated with adipokine production (406), and some studies reported decreased serum adiponectin in a murine model of lipoatrophy (407), we next examined blood adiponectin and leptin concentrations. The analysis of adipokine levels in mouse serum

revealed that d4T significantly reduced adiponectin production with an effect on leptin production as well (Figure A and B).

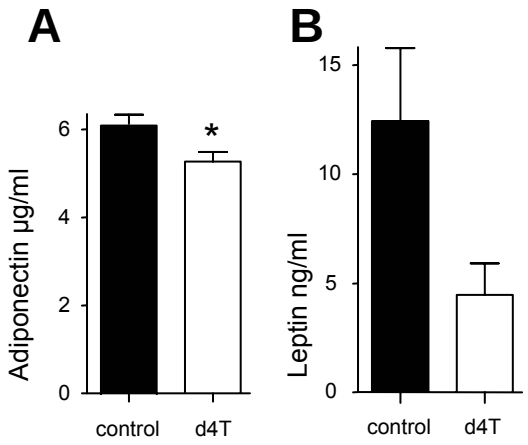



**Figure 28. Effect of a d4T on plasma leptin and adiponectin levels in C57BL/6 mice**
We measured plasma leptin and adiponectin in control and treated mice. Plasma adiponectin was significantly decreased by d4T (A). Leptin levels showed a decrease as well although statistically insignificant (B). Values are the mean ± SEM of 4 mice per group. * $p<0.05$, ** $p<0.01$.versus control.

**5.4.6 Effect of AZT and d4T on mouse insulin sensitivity.** As disturbances in glucose homeostasis often accompany fat redistribution in HAART treated patients (149;365) we next wanted to assess whether decreased subcutaneous fat in our murine model (Figure 24) is accompanied by alteration in insulin sensitivity. After 15 weeks of NRTI treatment the GTT in mice did not demonstrate significant differences in plasma glucose levels of treated and control animals after the glucose challenge (Figure 29).

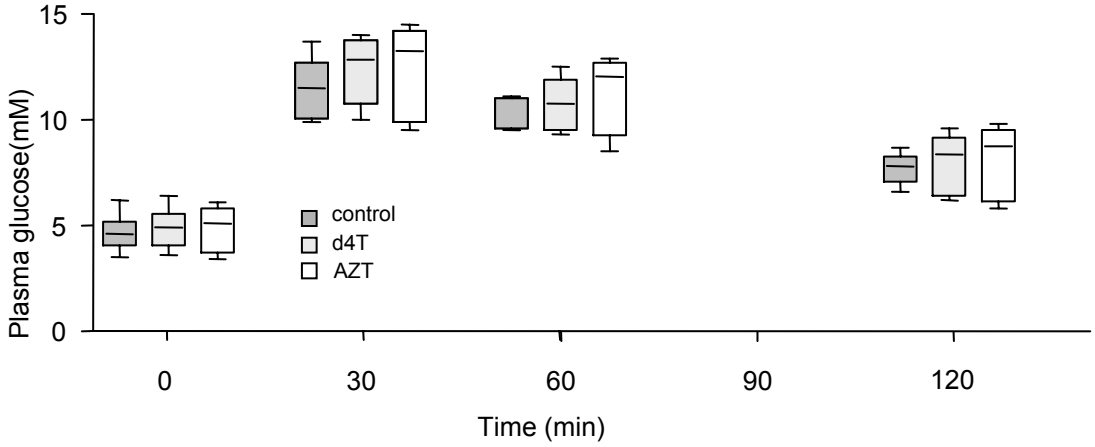



**Figure 29. Glucose tolerance test**
Mice were fasted for 18 hours with free access to drinking water. Then mice were challenged intra-peritoneally with a glucose load of 1.5 g glucose/kg body weight. Blood glucose measurements were performed before injection and at 30, 60 and 120 minute after the injection. Values are the mean ± SEM and quartiles of 10 mice per group.

# 6 DISCUSSION

Mitochondrial involvement has been suspected in the pathogenesis of HIV-associated lipodystrophy. Several studies have shown decrease in mtDNA levels in subcutaneous adipose tissue in lipoatrophic patients taking NRTI (85;408). This depletion has been proposed to contribute to the pathogenesis of HIV lipodystrophy, although extent and specificity of this effect remain unknown. If indeed mtDNA depletion was a crucial pathogenetic factor we would expect the activity of complexes I, III, IV and V of the respiratory chain to be significantly reduced since they are partially encoded by mitochondrial genes.

Adipose tissue has a very high plasticity potential. Its mass depends on the rate of preadipocyte proliferation and differentiation, adipocyte loss, and adipocyte size. Alterations in any of these processes can result in adipose tissue dys-/atrophy or hypertrophy (74;188;193;409). Using the 3T3-L1 cell line to study the effects of NRTI on the various differentiation forms of adipose tissue cells, we were able to show that mitochondrial toxicity as determined by mtDNA depletion depends on cell proliferation and cell differentiation. AZT and d4T were chosen as they have been most closely related to lipoatrophy and ddC has been shown to be one of the most powerful drugs leading to mtDNA depletion in cell culture systems (25;410). Significant mtDNA reduction occurred only if adipocytes were exposed to d4T or ddC before and during the differentiation process. Fully matured adipocytes, however, were resistant to mtDNA depletion showing even increased values for mtDNA content. The latter could reflect a compensatory responses in order to offset eventual cellular dysfunction (50;88). Similarly, growth arrested confluent preadipocytes showed no alteration in mtDNA after exposure to the NRTI tested. Our data imply that factors potentially promoting preadipocyte differentiation including insulin, cortisol or adipocyte cell death (observed *in vitro* and *in vivo* in association with protease inhibitor exposure (18;146;351;411) would render adipose tissue more susceptible to mtDNA depletion. We suspect that the mitochondrial proliferation during cell division and differentiation combined with an increased demand for energy supply during cell proliferation leads to a raised sensitivity for mtDNA depletion induced by d4T and ddC. Considering this, our data may have implications for HIV-therapy during pregnancy where proliferation-dependent NRTI-toxicity could be of particular relevance (412;413). On the other hand, AZT had some effect on preadipocyte differentiation leading to decreased numbers of differentiated

cells, reduced lipid droplet accumulation and premature cell detachment as described by others (349). Our results using AZT concentration at around $C_{max}$ are in line with a recent study revealing no obvious morphological changes of fully mature 3T3-L1 cells, only a trend towards reduced markers of adipogenesis (CEBPβ, PPARγ), and no alteration in gene expression after differentiation in the presence of d4T (10μM) or ddC (0.2μM) (403). Given a known antiproliferative effect of AZT and the requirement of clonal expansion of preadipocytes for effective differentiation (338) we speculate that this mechanism contributes to impaired adipogenesis associated with AZT treatment.

Although, to our knowledge, our study is the first to analyze mtDNA depletion in cultured adipocytes at different developmental stages, we were unable to quantitate any functional impairment of cell function solely due to mtDNA depletion. Several reports have indicated ddC to be a potent inhibitor of mitochondrial DNA polymerase-γ and mtDNA replication in other cell lines including hepatocytes (353;410;414). Surprisingly, we found ddC leading to mtDNA depletion only in proliferating preadipocytes but not differentiating adipocytes even at concentrations 30 times above $C_{max}$ values. In contrast, a recent study revealed a significant effect of ddC on mitochondrial mass and membrane potential measurements in the 3T3-F442A adipocytes cell line *in vitro* (349). Since mtDNA was not assessed in that study, this discrepancy may be due to differences in origins of cell lines or indicate mitochondrial dysfunction independent of mtDNA depletion.

Efficient uptake and metabolisation of d4T and AZT within 3T3-L1 adipocytes have been shown as both are phosphorylated to their mono-, di-, and triphosphate anabolites with phosphate anabolites being the major intracellular component of AZT compared to the parent being the main component of d4T (350). Using therapeutic drug concentrations these authors did not detect any impairment in adipocyte function or viability, partially confirming our results.

More importantly, however, our data argue that despite the significant mtDNA depletion after d4T incubation, 3T3-L1 adipocytes are able to differentiate and maintain central functions like fat storage. MtDNA depletion was not associated with impairment of the respiratory chain function as measured by individual enzymatic complex activities. Instead, there was even some evidence for an increased activity of complexes I and IV in AZT treated cell which could reflect a compensatory response to other mechanisms of mitochondrial toxicity or impaired energy production (89)**.** Although there are numerous studies demonstrating an effect of NRTI therapy on mtDNA content

in various tissues, there are less data demonstrating this depletion as a relevant cause for mitochondrial and adipocyte dysfunction. This association, however, is relevant for the understanding of the pathogenesis of lipoatrophy as already pointed out by Brinkman et al (49) and is also crucial for therapeutic consequences. For example, interventions aimed to increase mtDNA levels might fail to improve subcutaneous fat loss, others, like uridine that improve peripheral fat content, may have no significant effect on mtDNA content**.** Thus, using a sensitive functional assay, our data provide evidence that severe mtDNA depletion might coexist with normal mitochondrial function and macroscopic integrity of adipocytes *in vitro,* at least for the time studied here. It is, however, reasonable to speculate that *in vivo* additional factors like the HIV-infection itself (59;415) or hepatitis C infection (57) could potentially exacerbate any effect on mitochondria and further decrease the mtDNA content. In HIV-patients other organs that are heavily depending on mitochondrial integrity including the liver and heart could be more susceptible to mtDNA depletion and resulting toxicity.

The question arises what other effects of NRTI could lead to adipocyte dysfunction? Although we cannot exclude other mechanisms than mtDNA depletion leading to mitochondrial toxicity, our studies also emphasize the critical influence of AZT and d4T on adiponectin production. These effects were observed already at concentrations around therapeutic $C_{max}$ levels where no mtDNA depletion was detectable. Both NRTI have been often associated with the development of lipoatrophy. Impairment of pyruvatedehydrogenase activity as well as compromised fatty acid oxidation may be other factors contributing to cell dysfunction. Some studies reported decreased serum adiponectin in a murine model of lipoatrophy (407) or demonstrated that adiponectin levels are significantly lower in a sample of HIV-infected subjects with fat redistribution caused by HAART (416). In our experiments, adipocytes differentiated in the presence of around $C_{max}$ concentrations for d4T and AZT secreted significant less adiponectin while ddC had no effect. These data extend studies with 3T3-L1 cells that did not address adiponectin production but revealed no effect of NRTI on adipogenesis, lipolysis, or protein synthesis (350). More recently, similar results have been published in a study using microarray analysis that revealed reduced adiponectin expression after treatment of 3T3-L1 cells with AZT+3TC (403). We speculate that loss off adiponectin could be a significant step in the development of lipoatrophy associated with NRTI treatment. Consequently, administration of

adiponectin might be a therapeutic option for improving IR and fat redistribution in HIV-infected patients in the future (169;407;417;418).

Some important limitations of our study have to be considered. Employing a culture system aims at the principle evaluation of cellular mechanisms. Another benefit is to study cell differentiation under well characterized and standardized experimental conditions. Direct extrapolation to the human *in vivo* situation, however, is limited because of significant differences in time course of drug concentration (plasma levels of NRTI show a short half live time), time of drug exposure, cellular uptake and phosphorylation, and lack of secondary factors like cytokines and viral infection. Confirming the main results in primary preadipocyte culture give us a chance to exclude possible species-specific differences in enzymatic activity for drug-phosphorylation or dependency of adipocyte function on mtDNA content. This is relevant considering earlier observations that failed to find mitochondrial toxicity in animal models prior to clinical trail that later revealed fatal liver failure due to mitochondrial damage in humans (419). On the other hand, human therapeutic drug concentration may not be sufficient to generate a phenotype *in vitro* an therefore we chose drug levels of about $C_{max}$ values observed in HIV-patients and 30 time higher concentrations to allow for phenotypic changes during culture. Other systems like the murine 3T3-F442A adipocytes require even reduction (e.g. AZT) to enable experiments with NRTI because of cellular toxicity (349). Finally, although we extended the cell culture time up to 2 months some long term toxicities might remain undetectable *in vitro*.

In summary, our results suggest that adipocyte proliferation and/or differentiation is required for mtDNA depletion. Individual drugs may have distinct impact on preadipocytes and mature adipocytes and mtDNA depletion appears to be a poor predictor for mitochondrial dysfunction in our adipocyte cell model. However, the half life of respiratory chain complexes may be longer and reduced activities of respiratory chain complexes may develop later in cell life. Finally, we provide evidence that NRTI directly impair adipocyte function by reducing adiponectin production. These data have important implication of the understanding and treatment of the HIV-therapy associated lipodystrophy syndrome.

The present study suggests that AZT may contribute to alteration in several of the processes determining adipocyte mass. In our fat ‘tissue’ model AZT contributes to atrophy through reduction of preadipocyte proliferation rate and inhibition of clonal expansion rate ultimately resulting in inhibition of the overall differentiation capacity.

It is believed that in both primary preadipocytes and preadipose cell lines, differentiation requires growth arrest (232). Preadipose cell lines undergo one or two rounds of DNA replication and cell division after growth arrest (232). It has been shown that inhibition of DNA synthesis at this stage inhibits the differentiation into fat cells (236-238). The drug rapamycin is able to inhibit the process of clonal expansion (402). This inhibition interferes with the subsequent adipocyte differentiation confirming the critical role of clonal expansion in 3T3-L1 differentiation model. Although primary human preadipocytes do enter the differentiation process after being isolated even without cell division (239) it is considered that these cells may have already undergone this critical cell division *in vivo*. A detailed analysis of the anti-adipogenic effects of AZT was performed in an attempt to identify potential mechanisms. Two families of transcription factors, C/EBPs and PPARs, involved in terminal differentiation by transactivation of adipocyte-specific genes, are induced early during adipocyte differentiation. After hormonal induction of differentiation expression of PPARγ becomes detectable around the second day, and peaks in mature adipocytes (246;250). PPARγ expression is preceded by a transient increase in the expression of C/EBPβ and C/EBPδ isoforms (246-248). C/EPBα is the next key player in adipocyte differentiation (242;246;248;251). Constitutive expression of C/EPBα alone induces 3T3-L1 cells differentiation (420;421). AZT appears to be able to inhibit preadipocyte differentiation at the point of mitotic clonal expansion most likely through inhibition of the early S phase, with subsequent effects on the cascade of differentiation markers C/EBPα, PPARγ expression. C/EBPβ activates the expression of both the C/EBPα and PPARγ genes during preadipocyte differentiation through the C/EBP regulatory elements in their promoter regions (422-424). Although C/EBPβ expression was not affected, we wish to emphasize the fact that C/EBPβ expression starts within 4h after the induction of adipocyte differentiation, but at this point this transcription factor is unable to bind to the C/EBP regulatory element in the C/EBPα promoter. Only when preadipocytes enter S phase at the beginning of mitotic clonal expansion, C/EBPβ begin to acquire the capacity to bind to the C/EBP regulatory element and concomitantly become centromere associated (425). Eventual effect on the above mentioned processes could account for the observed decrease in the expression of C/EBPα and PPARγ without detectable decrease in the C/EBPβ expression. Therefore, most likely a mechanism where AZT prevents the normal differentiation dependent expression of C/EBPα and PPARγ by

antagonizing the clonal expansion in connection with/or without C/EBPβ could be suggested. Although differentiation was impaired microscopically (acquisition of adipocyte morphology) and estimated through TG accumulation a mechanism where AZT may perturb molecular pathways, independent of but additional mechanisms to C/EBPβ, C/EBPα and PPARγ expression cannot be excluded.

C/EPBα is expressed just before the transcription of most adipocyte-specific genes initiate. The promoters of adipocyte genes, like GLUT-4, leptin, adiponectin and the insulin receptor bind C/EPBα in order to be transactivated. In this case down-regulation in the expression of adipocyte secreted factor adiponectin is additional confirmation for impaired and incomplete differentiation. Adiponectin levels in HIV-infected men with lipodystrophy are approximately 50% of those without lipodystrophy, and 25% of those in uninfected controls (369). Adiponectin correlates with insulin sensitivity (369;416;426;427) and dystrophic fat demonstrates reduced expression of this adipocyte specific marker (151).

Cell viability was slightly affected and only after long incubation and very high drug concentrations. Annexin V/ Propidium iodide staining demonstrated insignificant contribution of AZT treatment to level of apoptosis observed in control cultures. The molecular mechanism responsible for the anti-adipogenic effects of AZT is not known. MtDNA depletion has been suggested but in this model such depletion has not been observed even at highest concentrations and such an explanation would not explain the normal differentiation in presence of drugs like d4T and ddC, which are much stronger mtDNA depleters. Recent studies demonstrated that the presence of AZT inhibits the phosphorylation of dThd by TK2 suggesting that mitochondrial toxicity of some NRTI in adipocytes may be due to the depletion of normal mitochondrial dNTP pools (428). Whether or not such a mechanism can account for the observed inhibition remains to be shown. We conclude from our studies that AZT has strong influence on the proliferation capacity of primary human and 3T3-L1 fibroblasts as well as on human preadipocytes (Figure 14). In 3T3-L1 model it affects the differentiation process through inhibition of the critical clonal expansion. *In vivo* drug effect will be also determined by differential drug concentrations and drug penetration the site of action.

In order to evaluate how PI affect preadipocyte proliferation, differentiation and the rate of adipocyte loss and adipocyte size, we examined the effect of IDV. Many *in vitro* studies prior to ours suggested that IDV inhibits differentiation process (38;347). The present study suggests that IDV may contribute to alteration in the mass of adipose

tissue. Data obtained from our murine adipocyte *in vitro* model, however, would not supprot that IDV contributes to atrophy by reducing preadipocyte proliferation rate or inhibiting clonal expansion rate with ultimate inhibition of the overall differentiation capacity. Our investigation into the mechanism of IDV contribution to the development of lipodystrophy syndrome suggests that IDV may promote adipocyte cell death and disturbance of the process of replacement of lost adipocytes through reduction of the viability of preadipocytes undertaking differentiation. Evidence for these effects were observe already at physiological concentrations and at concentration higher than those observed in plasma from patients treated with therapeutic doses of IDV. Considering the much shorter effect on adipocyte depletion in our model, it is possible that the effects of IDV on the 3T3-L1 cell line observed *in vitro* may also occur *in vivo* in a longer period. Our attempt to determine the eventual antiadipogenic effects of IDV, as confirmed by other *in vitro* studies (347), lead to the observation that IDV does not impair preadipocyte differentiation, the expression of the cascade of differentiation markers (C/EBPβ, C/EBPα, PPARγ) and mitotic clonal expansion. These results are in accordance with recently reported lack of effect of IDV on adipogenic transcription factors expression in healthy HIV-negative volunteers after four weeks IDV treatment (48).

As we have already mentioned, C/EBPβ is known to activate the expression of both the C/EBPα and PPARγ in the process of preadipocyte differentiation through C/EBP regulatory elements in their promoter regions (342;423;424). In this regard we exclude a potential mechanism where IDV prevents the standard differentiation dependent expression of C/EBPα and PPARγ by eventual interference with C/EBPβ function.

Differentiating 3T3-L1 preadipocytes exhibited a continuous loss in cell number (Figure 19) and demonstrated increased trypan blue staining after treatment with IDV. Surprisingly such an effect was restricted to differentiating 3T3-L1 preadipocytes, and in the absence of differentiation stimulus these precursors proliferated normally in the presence of IDV and showed no signs of cell death even when drug exposure was extended to up to 15 days. It is tempting to speculate that some cellular or molecular changes occur during differentiation and sensitize adipocytes to IDV-induced cell death. Down-regulation in the expression of adipokines such as adiponectin could be interpreted as either impaired adipocyte function or eventual induction of processes

leading to dedifferentiation. However, we believe that dedifferentiation alone is an unlikely scenario as there was a substantial percent of dead or dying adipocytes after 20 days of IDV exposure. Although some degree of dedifferentiation can not be definitely excluded, the primary response of adipocytes to IDV appears to be their loss of cell viability and function (as hinted by the decrease in adiponectin expression and secretion (Figure 22). Hoechst/ Propidium iodide staining was in accordance with the results from trypan blue staining, and both methods demonstrated a clear loss of adipocyte viability in response to IDV. When considered together, there is a clear difference in the effects of IDV on proliferating and differentiating 3T3-L1 preadipocytes as well as on mature adipocytes. Although hypothesised by others (347) the precise molecular mechanism of IDV antiadipogenic effects is currently unknown. Inhibition of glucose transport has been demonstrated to cause apoptosis in some cell lines in *in vitro* experiments (429). In this regard it is important to note that IDV and RTV were found to inhibit insulin-stimulated glucose uptake in 3T3-L1 adipocytes and the proposed mechanism was interference with the function of GLUT4 (342). Another interesting fact is the indirect inhibition of glucose uptake in obese (*ob/ob*) mice following antibody-mediated insulin depletion and the consequent adipose-specific cell death (195). Thus, IDV-dependent impairment of GLUT4 function and inhibition of insulin sensitizing by adiponectin in 3T3-L1 adipocytes may provide a mechanistic explanation for the cell death. With this mechanism, it would be easy to explain the observation that IDV had significant effect on 3T3-L1 adipocyte viability under our experimental conditions. However, recent new results surprisingly demonstrated increased glucose uptake from subcutaneous adipose tissue (SAT) from lipoathrophic patients on a PI based regimen (367). This observation contradicts hypotheses in which lipoatrophy is induced through PI blocked insulin-stimulated glucose uptake in adipose tissue. Alternatively, this increased glucose uptake in subcutaneous fat of lipoatrophic patients may even compensate for the fat loss and may reflect compensatory use of other glucose transporter (e.g. GLUT-1). Whatever the mechanism of IDV-induced inhibition of 3T3-L1 cell viability, common mechanism for PI-associated adipose tissue abnormalities could be applied to some other PI as a simplest explanation. Our initial studies with other PI (obtained form tablets) revealed some effects similar to those elicited by IDV. For example, RTV (30μM) significantly reduced the amount of cytoplasmic TG in 3T3-L1 adipocytes as measured by Oil Red O staining, with an effect on cell viability even stronger than that of IDV. Future studies are necessary in order to address the particular mechanisms and to examine the

hypothesis that PI-induced antagonism of adiponectin production is responsible for some of the symptoms of lipodystrophy syndrome. We conclude from our studies that IDV and perhaps other PI are able to affect cell viability of the 3T3-L1 cell line model of differentiation. Although we determined only the effect of IDV, different reports associate almost all PI with lipodystrophy syndrome in treated patients (430). *In vivo* drug effect will depend on differential drug concentrations and on the eventual rate of drug penetration at the site of action. The aetiology of lipodystrophy syndrome appears to be mutlifactorial, reflecting the effect of drug treatment to numerous biochemical pathways. The *in vivo* relevance concerning the *in vitro* effect of IDV on adipocyte differentiation has been recently confirmed by a study reporting lack of effect of IDV on adipogenic transcription factors expression in healthy HIV-negative volunteers after four weeks IDV treatment (48). Our findings do not exclude eventual atrophic effects of IDV on subcutaneous adipose tissue in relation to metabolic abnormalities. Additional *in vitro* and *in vivo* research will be necessary to test hypothetical mechanisms and to acquire a more complete understanding of the factors and mechanisms leading to the development of lipodystrophy syndrome.

In HIV-associated lipodystrophy, the group of NRTI has been predominantly associated with the development of lipoatrophy (3;28). Several studies have shown decreased mtDNA levels in subcutaneous adipose tissue in lipoatrophic patients taking NRTI (85;408). This depletion has been proposed to contribute to the pathogenesis of HIV lipoathrophy, although extent and specificity of this effect remain unknown and definite prove for a unique role of mtDNA depletion in lipoatrophy is lacking (348)**.** If indeed mtDNA depletion were a crucial pathogenetic factor we would expect this depletion to accompany the atrophy of adipose tissue. Our results demonstrated that both d4T and AZT are able to attenuate the acquisition of body fat mass in C57BL/6 mice despite of similar water and food consumption in treated and control mice (Figure 25 and 26). This effect was clearly observable after 15 weeks treatment with a daily dose corresponding to the human therapeutic dose and after macroscopic examination was predominantly contributed to atrophy of fat tissue. Interestingly these alterations occurred without significant depletion of mtDNA content in subcutaneous fat (Figure 27A).

Insulin resistance and impaired glucose tolerance are among the major metabolic abnormalities in HAART-lipodystrophy syndrome (362;363). Even diabetes has been reported in association with the therapy (149). Disturbances in glucose homeostasis very

often accompany fat redistribution in HAART-treated patients (149;365). Although a recent study demonstrated increased glucose uptake by subcutaneous adipose tissue (SAT) from lipoathrophic patients on a PI based regimen (367), it is generally believed that in situations of HAART-induced lipodystrophy, insulin resistance correlates with decrease in subcutaneous and increase in intra abdominal fat (368;369), and there are plenty of studies confirming such correlation (149;368;370;372;431). Even genetic forms of lipoatrophy (Beradinelli-Seip syndrome) present with insulin resistance (348). Under our experimental conditions the NRTI-induced atrophy of peripheral fat was not associated with altered insulin sensitivity (Figure 29) and would thus argue for only minimal contribution of NRTI to peripheral insulin resistance. It remains possible that this could be different in humans if one considers a more variable genetic background and different caloric intake, which are known factors to have an influence on peripheral insulin sensitivity.

Plasma adiponectin, has been shown to inversely correlate with insulin sensitivity (432)**.** Some studies reported decreased serum adiponectin in a murine model of lipoatrophy (407) or demonstrated that adiponectin levels are significantly lower in a sample of HIV-infected subjects with fat redistribution caused by HAART (416). Our results demonstrated that d4T is able to decrease adiponectin levels in C57BL/6 mice despite the similar insulin sensitivity in treated and control mice. We speculate that under these experimental conditions, NRTI reduce adiponectin production either directly through impairment of adipocyte function or indirectly through reduction of adipokine producing fat. It is highly probable that the effect on adipokine production comes from pure lack of adipocytes as we detected decrease in blood leptin which normally correlates well with the mass of adipose tissue (406). In the settings of HIV-associated lipodystrophy, plasma levels of leptin have been found to be decreased as well (433). In summary, our murine model of lipodystrophy demonstrates that 15 weeks treatment with a therapeutic dose of AZT and d4T attenuates the acquisition of body fat mass, without significant effect on adipocyte mtDNA content and without alteration in insulin sensitivity. These results complement earlier study by Fromenty *et al.* where the authors used a much sorter period of treatment (6 week) and were unable to detect differences in body weight acquisition in d4T treated mice (405). These data have important implication of the understanding of the mechanism of HIV-therapy associated lipodystrophy syndrome suggesting that, besides mtDNA depletion in subcutaneous fat, other mechanisms could play a role in the development of this lipoatrophy.

In summary we propose that NRTI deplete with different potency mtDNA *in vitro* in our adipocyte models and *in vivo* in our murine model. NRTI induced mtDNA depletion is an unspecific event during treatment with thymidine-analogues as it occurred in almost all the cell lines and tissues analysed. The level of NRTI induced mtDNA depletion depends on the adipocyte developmental stage. Functionally, mtDNA depletion even with an extend up to 80% from the original does not correlate with detectable impairment of the respiratory chain enzyme activity and mitochondrial potential, neither does it correlate with detectable alterations in processes such as cell proliferation and differentiation in our adipocyte cell lines and primary cultures. In a search for the mechanism of NRTI antiadipogenic effects, AZT has been found to inhibit adipogenic differentiation of 3T3-L1 cells through inhibition of the critical step of clonal expansion. On the other hand IDV has been found to affect adipocyte viability and function without any detectable effect on preadipocyte differentiation. Some NRTI and PI are capable of affecting adiponectin expression and secretion *in vitro* and *in vivo* (patients and mice).

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**RESEARCH CONTRIBUTIONS**
**Results of this thesis have been reported in the following articles and abstracts:**

**Manuscripts**

Metodi V. Stankov, Thomas Lücke, Anibh M. Das, Reinhold E. Schmidt, and Georg M.N. Behrens. **Relationship of mitochondrial DNA depletion and respiratory chain activity in preadipocytes treated with nucleoside-analogue reverse transcriptase inhibitors. Antiviral Therapy** 2007; **12**:205–216

Behrens G, Stankov M, Schmidt RE. Mitochondrienfunktion muriner und humaner Zellen unter dem Einfluss von NRTI. 2006. In: **HIV/AIDS Wunschwelt – Heilung: Evidenz für Fortschritt oder Stillstand?** C. Hoffmann, H. Jäger (Hrsg), MIC-Management Information Center, Landsberg/Lech, 167-168.

**Manuscripts in preparation**

Metodi V. Stankov, Reinhold E. Schmidt, and Georg M.N. Behrens. **Zidovudine inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells.**

Metodi V. Stankov, Reinhold E. Schmidt, and Georg M.N. Behrens. **Adipocyte viability and function but not preadipocyte differentiation is compromised by indinavir.**

Metodi V. Stankov, Thomas Lücke, Anibh M. Das, Reinhold E. Schmidt, and Georg M.N. Behrens. ***Ex vivo*** **analyses of the relationship of mitochondrial DNA depletion and respiratory chain activity in human preadipocytes treated with nucleoside-analogue reverse transcriptase inhibitors.**

Metodi V. Stankov, Thomas Lücke, Anibh M. Das, Reinhold E. Schmidt, and Georg M.N. Behrens. **Lipoatrophy and ubiquitous mtDNA depletion in mice following long-term stavudine treatment.**

**Abstracts**

Metodi Stankov, RE Schmidt, and G Behrens. **Lipoatrophy and ubiquitous mtDNA depletion in mice following long-term stavudine treatment.** 14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, California, USA, 2007, Paper # N-136*(travel award)*

Metodi V. Stankov, Thomas Lücke, Anibh M. Das, Reinhold E. Schmidt, and Georg M.N. Behrens. **Nucleoside reverse transcriptase inhibitors in murine and human adipocytes: impact on mitochondrial DNA depletion and respiratory chain activity.** 8th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV. **Antiviral Therapy** 2006; **11**:L32

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M Stankov, R E Schmidt, G M N Behrens. **HAART modulates liver lipid metabolism through direct effect on hepatocytes. Antiviral Therapy.** The XV International AIDS Conference, 2004. **MedGenMed**. 2004 Jul 11; **6**(3):WePeB5894.

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| 1984-1989 | National High School of Mathematic and Natural Science 'Acad. L. Chakalov', Sofia, Bulgaria. |

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| 1991-l997 | B. Sc and M. Sc. Molecular Biology 'St. Kliment Ohridski' University of Sofia, Bulgaria. (GPA: 3,93 out of 4) |
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**Declaration on the dissertation**

I hereby declare that this Dissertation ’Molecular analysis of the HIV-therapy associated lipodystrophy-syndrome’ is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Hannover, 28.11.2006

Metodi Stankov