

DISEASE- AND TREATMENT-RELATED PREDICTORS OF HEPATIC MITOCHONDRIAL DYSFUNCTION IN CHRONIC HIV INFECTION ASSESSED BY NON-INVASIVE ¹³C-METHIONINE BREATH TEST DIAGNOSTIC

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Abstract

Objectives: An increasing proportion of deaths among human immunodeficiency virus (HIV)-infected persons are due to hepatic complications. Hepatitis coinfection, antiretroviral treatment and co-occurrence of metabolic risk factors contribute to hepatic mitochondrial damage manifesting in hepatic steatosis and steatohepatitis. The aim was to assess disease- and treatment-related predictors on hepatic mitochondrial dysfunction in HIV infection by means of a new ¹³C-methionine breath test (MeBT).

Patients and methods: 148 HIV positive individuals with and without antiretroviral treatment (ART) [44 therapy-naïves; 89 patients on combination ART and 15 patients on structured treatment interruption (STI)] and 20 HIV-negative controls were studied prospectively by MeBT.

Results: A decay of ¹³C-methionine metabolism, expressed as cumulated percentage dose recovered over 1.5h (cPDR_{1.5h}), in the subgroups of treatment-naïves and patients on STI compared to controls was detected (cPDR_{1.5h}: 3.4 ± 1.3% and 4.0 ± 2.4% vs. 6.3 ± 1.2%; p<0.01). Multivariate analyses including metabolic, treatment- and disease-related variables showed that antiretroviral treatment with stavudine, didanosine or zalcitabine and treatment-naïvety were best predictors of a reduced MeBT result (cPDR_{1.5h}) (β = -0.56 and -0.50, p<0.05). CD4 count had only a minor association (β = 0.15, p<0.05). No other variable including disease and treatment duration was associated with MeBT outcome. These factors explained 39% of the variance of MeBT results (p<0.05).

Conclusions: Therapy naïvety and treatment with d-drugs were the best predictors of poor MeBT outcome. MeBT may be proposed as a feasible, noninvasive diagnostic instrument for clinical assessment of hepatic mitochondrial function and early detection of drug-induced mitochondriotoxicity in chronic HIV infection.

Key words: ¹³C-methionine breath test, HIV, mitochondrial toxicity, drug toxicity

INTRODUCTION

Hepatic steatosis and elevated liver enzymes are frequent findings in HIV-infected individuals.[1-3] Interim analysis of the DAD study cohort revealed liver related mortality as the most frequent cause of non-AIDS-related death.[4] Hepatitis co-infection and immunodeficiency itself are independent risk factors of hepatic morbidity in HIV-infected individuals.

The long-term effects of combination antiretroviral therapy (cART) on liver function are controversial. By improving the immune system, cART reduces the risk of liver failure in coinfecting and in mono-infected individuals compared to non-treated individuals.[5] Nevertheless nearly 6% of the patients with a median follow-up of two years will experience grade 3 ALT-elevation on antiretroviral treatment. A recent study found a 12% increase in liver-related deaths per year of cART [5-7]. The increasing prevalence of metabolic syndrome (in particular of type-2-diabetes and obesity), of non-alcoholic steatohepatitis in the normal population and multiple potential hepatotoxicities in the setting of HIV infection suggest an increase of liver related morbidity and mortality in future decades [8-13].

Nucleoside inhibitors of reverse transcriptase (NRTI) and protease inhibitors (PI) independently confer hepatotoxic properties, that may lead to liver alterations with many similarities to non-alcoholic steatohepatitis (NASH) in the HIV negative population. Nucleoside analogues, especially the commonly known "d-drugs" d4T (stavudine), ddI (didanosine) and ddC (zalcitabine) are strong inhibitors of DNA-polymerase-γ, a key enzyme involved in mitochondrial DNA replication. Decreased mitochondrial DNA content has been found in several clinical affected tissues (liver, muscle, subcutaneous adipose tissue) and is thought to be a key mediator of NRTI-related mitochondrial toxicity [14-16]. Other mechanisms, like accumulation of mitochondrial DNA mutations and AZT-mediated inhibition of thymidine phosphorylation may be also involved in mitochondrial damaging

[17, 18]. In vitro, protease inhibitors may inhibit adipocyte differentiation and expression of peroxisome proliferator activated receptor gamma (PPAR- γ), which may result in increased (hepatic) fatty acid and cholesterol synthesis. They also inhibit insulin mediated glucose uptake via GLUT 4, inducing insulin resistance and thereby mimicking central pathogenic pathways for development of hepatic steatosis in HIV negative individuals [19, 20]. Intrahepatic lipid accumulation in turn may damage (mitochondrial) membranes and respiratory chain function.

Detection of (hepatic) mitochondrial toxicity, especially in subclinical state is difficult and not standardized. Hepatic ultrasound and MRI can not differ between benign steatosis and steatohepatitis, liver elastography may detect advanced stages of NASH with fibrotic conversions, but visceral obesity often coincided with hepatic steatosis is a relevant technical limiting factor for this new technique [21]. Liver biopsy so far represents the golden standard for detection of hepatic injury but it is limited by its invasive character and therefore not suitable for regular screening and monitoring of hepatic mitochondrial function in clinical practice. This condition might be fulfilled by the recently introduced ^{13}C -methionine breath test (MeBT) a new technique for non-invasive in-vivo assessment of hepatic mitochondrial function [22-24]. Results from our group indicate that individuals with long term antiretroviral (NRTI) treatment and duration of HIV disease, exhibit significant impairment of hepatic mitochondrial function, and therefore lower $^{13}\text{CO}_2$ exhalation, despite normal liver function tests and serum lactate [22, 25]. However, a detailed characterisation of the diagnostic performance of the MeBT in a larger group of HIV-infected individuals is still lacking.

Hence, the aims of the presented study were (i) to determine hepatic mitochondrial function by MeBT in different metabolically and clinically defined HIV-infected patient groups, (ii) to evaluate the influence of specific pharmacological treatment modalities on MeBT outcomes and (iii) to assess potential disease and non-disease related predictors of MeBT outcomes.

MATERIAL AND METHODS

STUDY DESIGN AND STUDY GROUPS

The study was carried out according to Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained from all participants and the protocol was approved by the local Ethics Committee. 150 patients with HIV infection were screened and 148 patients with chronic HIV infection from our outpatient unit and 20 healthy controls were enrolled in this prospective cross-sectional clinical trial. Controls were age and BMI "matched" to those of a patient. Subject inclusion was primarily determined by subject availability with no a priori selection biases. Exclusion criteria were: Evidence of liver cirrhosis, excessive alcohol consumption (>40 g/d in men, >20 g/d in females), severe respiratory dysfunction, anaemia (Hb < 7.4 mmol/l, pregnancy and breast

feeding. Detailed subject characteristics are shown in Table 1 and 2.

The patient group was divided into subgroups depending on individual treatment experience:

- (i) patients with no prior antiretroviral therapy (**HIV+, cART-naives**; n = 44).
- (ii) patients on antiretroviral therapy (**HIV+, cART+**; n = 89). This subgroup was further divided with regard to the NRTI-"partner" [**NNRTI** (n = 47) vs. **PI** (n = 34) based treatment regimens] and the nucleoside analogues itself. Eight patients received one class ART (NRTI or PI only).
- (iii) patients on structured treatment interruption (**HIV+, STI**; n = 15) at time of study.

Based on reported specific mitochondrial toxicity three major NRTI-subgroups were designed:

- (iv) a subgroup with AZT-based regimens (**AZT; AZT/3TC; AZT/3TC/ABC**; n = 43)
- (v) a group with d-drug-containing regimens (**ddI; d4T; ddC** with any other NRTI; n = 28)
- (vi) a non-d-drug, non thymidine-analogues based subgroup with TDF or ABC in combination with either 3TC or FTC (**TDF; ABC**; n = 16).

^{13}C -METHIONINE BREATH TEST

The detailed test procedure is described elsewhere [25]. Briefly, each patient received 2 mg/kg body weight [methyl- ^{13}C]-labelled methionine (Cambridge Isotope, Andover, MA, USA) dissolved in 100 ml water. Breath samples were obtained before substrate administration and at 10 minute intervals for 90 minutes. The $^{13}\text{C}/^{12}\text{C}$ isotope ratio of the breath samples were analyzed by non-dispersive isotope selective infrared spectroscopy (IRIS, Wagner Analysen Technik, Bremen, Germany). Primary results were expressed as the delta (δ) $^{13}\text{C}/^{12}\text{C}$ isotope ratio over baseline (DOB). To measure the proportion of the metabolized substrate the results were expressed as percentage dose of ^{13}C recovered (PDR) over time for each time interval, maximal PDR (PDR_{max}) and cumulative PDR (cPDR) after 90 min test time.

DEMOGRAPHIC, LABORATORY AND ULTRASOUND DETERMINATIONS

Venous blood was drawn with the use of a normal tourniquet. Patients were instructed to avoid fist clenching or hand pumping. Samples were collected in sodium fluoride/potassium oxalate tubes and kept on ice. Lactate was immediately tested enzymatically in an automated analyser (Roche/ Hitachi 917) according to the manufacturer's instructions (laboratories reference range 4.5-19.8 mg/dl). Further evaluation included quantification of ALT (<34 U/l), cholesterol (< 220 mg/dl), triglycerides (< 200 mg/dl), all by Roche/ Hitachi 917. HIV viral load (COBAS AMPLICOR HIV Monitor, Roche Diagnostics, Basel, Switzerland), CD4-count (Coulter counter, Beckmann Coulter, Krefeld, Germany; reference range 300-1400/ μl), he-

Table 1. Basic data of 20 healthy controls and 148 HIV positive patients classified according to treatment modalities. Data are presented as mean (SD), unless otherwise indicated.

Abbreviations: **ART**, antiretroviral therapy; **cART**, combination ART; **STI**, structured treatment interruption; **ALT**; alanine amino-transferase; **HBV**, hepatitis B virus; **HCV**, hepatitis C virus; **HIV**, human immunodeficiency virus.

Variables	HIV+, cART- naives	HIV+ STI	HIV+ cART+	HIV- controls
N	44	15	89	20
Gender, No (%)				
Female‡	7 (16)	2 (13)	9 (10)	9 (45)
Male	37 (84)	13 (87)	80 (90)	11 (55)
Age, yr	35 (10)**	39 (7)	45 (10)	42 (17)
Height, m	1.76 (0.08)	1.77 (0.07)	1.77 (0.08)	1.76 (0.12)
Weight, kg	72.4 (14.7)	71.1 (12.6)	73.0 (11.1)	76.4 (12.6)
Body mass index, kg/m²	23.2 (4.1)	22.7 (4.2)	23.3 (3.1)	24.7 (2.9)
ALT, U/l	33 (29)	53 (56)§	38 (24)	26 (7)
Cholesterol, mg/dl	171 (38)**	184 (32)*	222 (57)	190 (47)†
Triglycerides, mg/dl	134 (90)**	163 (79)*	265 (300)	132 (94)**
Lactate, mg/dl	10.7 (4.4)	11.4 (5.4)	12.4 (5.2)	10.5(5.3)
Disease duration, yr	1.3 (2.2)	8.2 (5.2)++	8.4 (5.4)++	-
CD4 cell count, /µl	394 (242)*	484 (232)	510 (260)	-
HCV status,				
Positive, No (%)‡	3 (7)	4 (27)	5 (6)	0(0)
Negative, No (%)	40 (91)	10 (67)	83 (93)	20(100)
Not tested, No (%)	1 (2)	1 (7)	1 (1)	0(0)
HBV status,				
Positive, No (%)	4 (9)	0 (0)	5 (6)	0(0)
Negative, No (%)	39 (89)	15 (100)	83 (93)	20(100)
Not tested, No (%)	1 (2)	0 (0)	1 (1)	0(0)
Steatosis hepatis, No (%)‡	16 (36)	7 (47)	55 (62)	3(15)

‡ p<0.05 by Chi square test

p<0.001 for all ANOVA tests:

†p = 0.05; *p<0.05; **p<0.001 vs. **HIV+,cART+** by Tukey-HSD or Games-Howell post hoc test

§p<0.05 vs. **HIV- controls** by Tukey-HSD post hoc test

++p<0.001

vs. **HIV+,cART-naives** by Tukey-HSD post hoc test

Table 2. Clinical and laboratory characteristics of 148 HIV positive patients classified according to specific antiretroviral drug therapies.

Abbreviations: **TDF**, tenofovir; **ABC**, abacavir; **AZT**, zidovudine; **3TC**, lamivudine; **ddI**, didanosine; **d4T**, stavudine; **ddC**, zalcitabine; **NNRTI based**, non nucleoside reverse transcriptase inhibitor based combination therapies; **PI based**, protease inhibitor based combination therapies; **ALT**; alanine amino-transferase; **HIV**, human immunodeficiency virus.

Variables	TDF;ABC	AZT;AZT/3TC; AZT/3TC/ABC	ddI; d4T; ddC	NNRTI based	PI based
n	16	43	28	47	34
Age, yr	48(11)	45(10)	42(10)	42(10)	47(10)+
Height, m	1.77(0.10)	1.78(0.07)	1.76(0.08)	1.79(0.08)	1.78(0.08)
Weight, kg	79(12)**	74(12)	68(7)	73(10)	71(11)
Body mass index, kg/m²	25.0(2.6)**	23.2(3.3)	22.3(2.9)	23.6(2.9)	22.3(2.9)
ALT, U/l	43(28)	36(25)	39(22)	38(19)	34(25)
Cholesterol, mg/dl	223(57)	212(55)	227 (53)	219(54)	218(57)
Triglycerides, mg/dl	217(150)	230(210)	253(226)	219(234)	251(166)
Lactate, mg/dl	10.6(3.7)	12.1(5.0)	13.9(6.0)	12.0(5.1)	12.2(5.3)
disease duration, yr	5.5(5.1)**	7.8(5.5)	10.7(4.7)	7.6(5.3)	9.7(5.6)
therapy duration, yr§	4.4(4.4)†	5.0(3.8)	7.0(3.4)	5.1(3.7)	6.3(4.2)
CD4 cell count, /µl	444(197)	540(271)	493(281)	529(228)	475(314)
Steatosis hepatis, No (%)‡	5(31)	24(56)	25(89)	27(57)	22(65)

†p = 0.06 vs. **ddI;d4T;ddC** by Games-Howell post hoc test; §p = 0.04 by ANOVA

p<0.01 vs. **ddI;d4T;ddC by Tukey-HSD or Games-Howell post hoc test; p<0.05 by ANOVA

+p<0.05 by unpaired t-test

‡p<0.001 by Chi square test

patitis B surface antigen (HbsAG) and anti-HCV-antibody at time of study were also measured. Hepatic steatosis was assessed by abdominal ultrasound. Demographic and disease specific parameters (disease and treatment duration) were obtained from patient data charts and standardized questionnaires.

STATISTICS

Statistical analysis was first carried out as a descriptive evaluation of δ (%), PDR (%/h), cPDR (%) and clinical characteristics of patients and control subjects. All data are presented as mean \pm SD unless otherwise specified. For these parameters normality of distribution was shown by one-sample Kolmogorov-Smirnov test and frequency distribution histograms. Chi square test was used for analysis of categorical data. Significance between two groups was tested by independent samples *t*-tests and for comparison of four (three) groups by analysis of variance (ANOVA). Depending on the homogeneity of variance, tested by Levene's test, for pair comparisons a series of Tukey-HSD or Games-Howell *post-hoc* tests were run.

To define the relation between MeBT results as expressed by cPDR_{1.5h} and a set of surrogate observations of the HIV positive patient group a multiple linear regression model using the procedure for general linear models with cPDR_{1.5h} as the dependent variable and a set of explanatory variables [gender, age, BMI, CD4 count, disease duration, therapy duration and treatment groups (i-vi)] was applied. In this model, all covariates have been included based on an *a priori* decision dictated by scientific knowledge and biologic sensibility. In preliminary analysis other study parameters did not improve the quality of the model. Regarding gender and treatment groups the subjects were coded using the technique of dummy variables. To allow a clinical interpretation of the importance of each independent variable partial correlation analysis was used to "quantify" the amount of variance accounted for by each explanatory variable uniquely. The results were regarded as significant when the error probability was less than 0.05. Statistical analysis and graphics were done by commercial software programs (SPSS 11.5.1, SPSS inc., Chicago, IL and Graph PAD Prism, version 4.01, San Diego, CA).

RESULTS

All measurements were completed without complications or adverse events.

Effects of disease and treatment on MeBT outcomes and clinical parameters

Mitochondrial decarboxylation function as assessed by MeBT was reduced for therapy naïve patients and patients on structured treatment interruption compared to the control group (cPDR_{1.5h}: 3.4 \pm 1.3 % and 4.0 \pm 2.4 % vs. 6.3 \pm 1.2 %; $p < 0.01$ for each comparison). In addition therapy naïve patients showed a decreased cumulated ¹³CO₂ exhalation compared to ART-treated patients (cPDR_{1.5h}: 3.4 \pm 1.3% vs. 5.2 \pm 2.3%, $p < 0.001$), whereas controls and ART-treated patients showed no difference ($p = 0.1$) in ¹³CO₂-exhalation (Fig. 1).

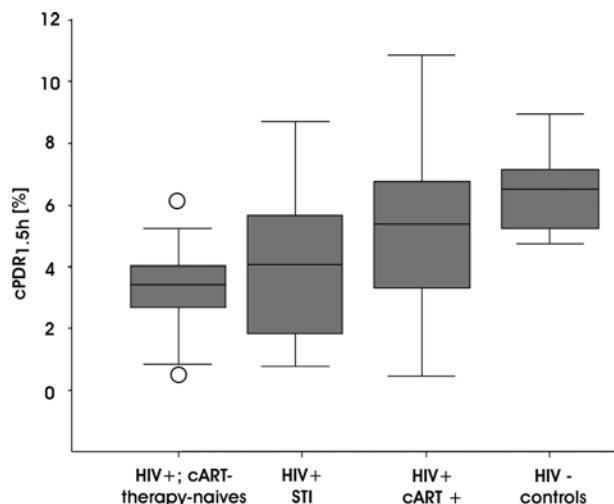


Fig. 1. Cumulative percentage dose rates (cPDR) after oral administration of 2mg/kg body weight [methyl-¹³C]-labelled methionine after 1.5 h test time in 148 HIV-infected patients and 20 healthy controls classified according to treatment modalities.

Abbreviations: cPDR_{1.5h}, cumulative percentage dose rate after 90 minutes test time; ART, antiretroviral therapy; cART, combination ART; STI, structured treatment interruption; HIV, human immunodeficiency virus.

$p < 0.001$ by one-way ANOVA

$p < 0.01$ for HIV- controls vs. HIV+, STI

$p < 0.001$ for HIV+ therapy-naïves vs. HIV+, cART+ and HIV- controls

by Tukey-HSD post hoc test

As expected, the triglyceride- and cholesterol-values were higher in ART-treated subjects compared to other patient groups ($p < 0.05$). This group also had the highest prevalence of hepatic steatosis ($p < 0.05$) and the longest disease duration ($p < 0.001$). CD4 cell count was lower in therapy naïve patients compared to ART-treated subjects ($p < 0.05$). ALT values were slightly elevated in patients on structured treatment interruption compared to control subjects ($p < 0.05$), which might be associated with the slightly higher prevalence of HCV coinfection in this group ($p < 0.05$). The remaining clinical parameters of the patient groups were similar (Table 1).

Effects of specific antiretroviral drug regimens on MeBT outcomes and clinical parameters

There was no difference in MeBT outcomes between NNRTI and PI based combination treatment regimens (cPDR_{1.5h}: 5.0 \pm 2.0 % vs. 5.5 \pm 2.7 %, $p = 0.4$; Fig. 2 A). Other clinical parameters were similar despite a small difference of 5 years in age between both groups ($p = 0.04$; Table 2).

Patients with d-drug-containing regimens showed a distinct decrease in mitochondrial decarboxylation function compared to patients treated either with zidovudine based regimens or tenofovir or abacavir (cPDR_{1.5h}: 3.1 \pm 1.5 % vs. 5.8 \pm 2.2 and 7.0 \pm 1.3 %, $p < 0.001$ for each comparison; Fig. 2 B) In addition, maximal ¹³CO₂ excretion (PDR_{max}) separated all three treatment groups (PDR_{max}: 3.7 \pm 1.7 %/h vs. 7.5 \pm 3.1 %/h vs. 9.6 \pm 1.9 %/h, $p < 0.05$ for each comparison).

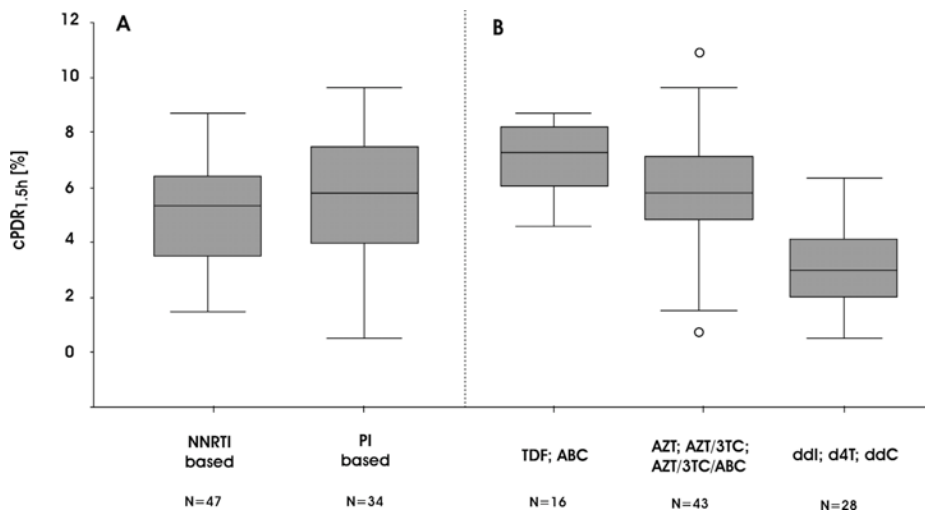


Fig. 2. Effect of non nucleoside reverse transcriptase inhibitor (NNRTI) and protease inhibitor (PI) based combination therapies (A) and of nucleoside- and nucleotide-analogue HIV reverse-transcriptase inhibitors (B) on non-invasive [methyl-¹³C]-methionine breath test results.

Abbreviations: **cPDR_{1.5h}**, cumulative percentage dose rate after 90 minutes test time; **HIV**, human immunodeficiency virus; **TDF**, tenofovir; **ABC**, abacavir; **AZT**, zidovudine; **3TC**, lamivudine; **ddl**, didanosine; **d4T**, stavudine; **ddC**, zalcitabine

(A) $p = 0.4$ for NNRTI vs. PI based combination therapies

(B) $p < 0.001$ by one-way ANOVA

$p < 0.001$ for **ddl; d4T; ddC** vs. **TDF; ABC** and **AZT; AZT/3TC; AZT/3TC/ABC** by Games-Howell *post hoc* test

As expected, patients with d-drug-containing regimens had the lowest body mass index, the longest disease duration, and a trend to the longest duration of therapy compared to patients treated with tenofovir or abacavir based regimens ($p < 0.05$). In addition, hepatic steatosis was most frequently detected in this group compared to both other treatment groups ($p < 0.01$; Table 2).

Influence of surrogate observations on MeBT outcomes

Multiple linear regression using the procedure for gen-

eral linear models showed that biologically important explanatory variables demonstrated in part above [age, gender, BMI, CD4 count, disease and therapy duration and treatment modality (group **i**, **iii**, **iv**, **v**, **vi**)] accounted for 39% of the variance of MeBT outcome ($p < 0.05$). BMI, CD4 cell count were independently and positively, therapy naivety and treatment with d-drug-containing regimens were independently and negatively associated with MeBT outcome (Table 3).

To “quantify” the amount of variance accounted for by each significant explanatory variable uniquely,

Table 3. Coefficients calculated from multiple linear regression models with the ¹³C-methionine breath test outcome parameter cPDR_{1.5h} as the dependent variable and different disease and therapy related explanatory variables in 148 HIV positive patients. The non standardized coefficients are presented with 95% confidence intervals.

Variables	Non standardized coefficients	Standardized coefficients
Intercept*	3.834 (0.058...7.610)	-
Age, yr	-0.038 (-0.949...0.874)	-0.006
BMI [†] , kg/m ²	0.001 (-0.032...0.033)	0.003
Gender [‡]	0.074 (-0.011...0.160)	0.115
Disease duration, yr	0.001 (-0.007...0.009)	0.031
Therapy duration, yr	-0.003 (-0.015...0.009)	-0.069
CD4 cell count, /μl*	0.001 (0.000...0.003)	0.151
HIV+ cART- naives*	-2.731 (-5.455...-0.006)	-0.556
HIV+ STI	-2.083 (-4.789 ...0.623)	-0.280
TDF/ABC	0.862 (-1.824 ...3.548)	0.119
AZT;AZT/3TC; AZT/3TC/ABC	-0.348 (-2.925 ...2.229)	-0.070
ddl; d4T;ddC*	-2.877 (-5.464...-0.290)	-0.502

For all dummy variables 1 = yes; 0 = no; [‡] 1 = male; 2 = female

Corrected R² = 0.39 for the overall model

* $p < 0.05$; [†] $p = 0.08$

partial correlation analysis showed that treatment with d-drug-containing regimens or therapy naivety had a stronger correlation than BMI or CD 4 cell count (**ddI;d4T;ddC**: partial $r = -0.45$, $p < 0.001$; **HIV+cART-naives**: $r = -0.46$, $p < 0.001$; **BMI**: $r = 0.19$, $p < 0.05$; **CD4 cell count**: $r = 0.17$, $p < 0.05$).

DISCUSSION

After decades of viral control the management of metabolic complications, notably liver related morbidity, emerges as a growing challenge for future HIV treatment. It is much more surprising that metabolic liver disease in chronic HIV infection has been largely neglected. General difficulties in the differentiation of metabolic liver damage from other more obvious reasons (e.g. viral hepatitis), the broad spectrum of possible triggers (drug and alcohol abuse, metabolic syndromes) and the lack of appropriate diagnostic tools might be some of the main reasons. Mitochondrial damaging plays a central role in the developing cascade of metabolic liver disease irrespective of the specific trigger.

In previous studies we have shown that the non-invasive ^{13}C -methionine breath test is a valuable, "easy to perform" diagnostic instrument for monitoring hepatic mitochondrial integrity and its modulation by therapeutic intervention in HIV-infected patients [25-28]. In the present study we could show in a larger cohort of metabolically and clinically well defined HIV-infected patients that specific pharmacological treatment modalities are the main determinants of methionine breath test outcomes. D-drug containing cART-regimens and no antiretroviral treatment (treatment naivety) were the main predictors of poor breath test results in HIV-infected individuals (Table 3).

The systemic toxic effects of d-drugs on mitochondria are generally accepted and supported by many clinical and preclinical studies, although the prevalence of steatosis and steatohepatitis, especially in mono-infected individuals is unknown. Most of the histopathological data are derived from HCV-coinfected individuals in those d-drugs like stavudine and didanosine may enhance hepatic inflammation, risk of acute hepatic failure and probably fibrosis progression [29-31].

The poor MeBT outcome of treatment naive individuals supports the findings of our first pilot reports [25, 28]. However, the mechanisms of HIV-related hepatic mitochondrial toxicity have not been explored sufficiently. Although the virus itself cannot enter hepatocytes due to the absence of CD4 receptors, the gp120 protein may bind to CCR5 and CXCR4 receptors expressed by hepatocytes and macrophages and alter cell signalling without direct infection of cells. In hepatocytes this may result in increased production of pro-inflammatory, pro-fibrotic factors like TGF- β 1. The changes of intracellular cytokine environment are thought to be at least in part responsible for increased HCV viral load in coinfecting individuals [32]. Data from our previous pilot study indicate that introduction of cART in treatment naive patients with consecutive suppression of viral load restores hepatic mitochondrial decarboxylation function at least temporarily [28].

In general, treatment interruption also adversely influenced hepatic mitochondrial function in our study. The smaller size, and especially the higher percentage of HCV-coinfected individuals (4/15) and a high proportion of d-drug experienced patients (6/15) does not allow to reach firm conclusions regarding a potential negative impact of treatment interruption on liver function. The recently published SMART study supports the hypothesis, that even CD4 guided treatment interruptions increase the overall mortality not only due to opportunistic infections but also increase the risk of liver related deaths in HIV-infected individuals [33].

The choice of the NRTI partner (NNRTI vs. PI) had no significant effect on breath test results (Fig. 2). This observation may reflect the general impression in clinical practice. It is noteworthy that none of our patients treated with nevirapine had experienced noticeable liver enzyme elevation at time of study. In vitro, protease inhibitors have been shown to impair insulin-mediated glucose uptake in the liver. Recent studies demonstrated an increase of different features of the metabolic syndrome after long term PI treatment. However, a direct effect on liver comorbidity is still under debate and might be overtopped by the "natural" occurrence of metabolic risk factors [11-13]. Furthermore, the metabolic effects of PIs seem to be more drug-than class-specific, suggesting a lower metabolic risk with the use of modern protease inhibitors like atazanavir or lopinavir [34].

Interestingly neither treatment nor disease duration affected hepatic mitochondrial function (Table 2). This observation supports the hypothesis that mitochondrial function does not necessarily diminish over years of HIV infection, unless other hepatotoxic conditions (HCV coinfection, alcohol abuse etc.) lead to an irreversible liver damage and a restoration after (re)initiation or switch of antiretroviral treatment may be still possible. This suggestion is supported by the observation that 6 of 16 patients in the TDF/ABC treatment group had long lasting d-drug experience in the past, and showed a normal breath test results in this study. To confirm this hypothesis, studies with long-term follow-up monitoring in special patient groups have been initiated by our group.

The negative correlation of $\text{cPDR}_{1.5\text{h}}$ with CD4 cell count can be primarily contributed to treatment naive patients who had on average 100 CD4 cells less than cART-treated individuals. After exclusion of these patients in statistical analysis, we no longer found this correlation in our study.

Finally, some important limitations of our study have to be pointed out: As a dynamic function test the MeBT is designed for quantifying the overall hepatic mitochondrial capacity. It may not differentiate the effects of cART from other metabolic factors (alcohol, diabetes mellitus) or from the hepatotoxic effect of hepatitis B or C co-infection. Some of these main factors were tested in our analyses and were not associated with MeBT outcomes.

The overall model with its integrated disease and treatment related factors explains about 40% of the variance of MeBT results, which reflects a good performance for a biologic (metabolic) test. It is of note

that general demographic parameters like age and gender had no effects on hepatic mitochondrial function. The obvious small influence of BMI might be compensated by adjusting the weight dependent administration of substrate or a correcting coefficient. However, compared to the dominating treatment variables (d-drugs and no treatment) the overall influence of BMI is negligible in our study.

A major limitation for general clinical recommendation of this new test might be seen in the lack of validation of the main endpoint i.e. the clinical characterisation of mitochondrial dysfunction with an accepted golden standard. Further comparative studies evaluating histomorphological changes in liver tissue or other surrogate measures of mitochondrial function (e.g. results of invasive function tests) in HIV-infected patients may be appropriate to clarify its role as a relevant metabolic test. Preliminary results from our own group suggests a correlation with both functional (subcellular) and morphological aspects (steatosis, inflammation and fibrosis) of liver damaging. In contrast to conventional techniques such as histopathology, imaging or elastography the ^{13}C -methionine breath test is a dynamic, functional diagnostic tool, that may also detect disturbances of hepatic mitochondrial integrity in subclinical, presteatotic stages. Furthermore, to improve the feasibility of the MeBT, bootstrap analyses in large cohorts are required to reduce the breath sampling size e.g. with 2 or 3 test time points similar to ^{13}C -urea breath test for diagnosis of helicobacter pylori infection.

With regard to these limitations we showed that specific therapy modalities in chronic HIV disease significantly influence hepatic mitochondrial function that can be monitored adequately by the ^{13}C -methionine breath test. The ^{13}C -methionine breath test could be a valuable diagnostic tool for clinical practice, which meets the need for non-invasive functional metabolic tests as the phenotype of chronic HIV infection has changed in recent decades from a primarily infectious to a more metabolic disease.

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