

Depletion of Mitochondrial DNA in Liver Under Antiretroviral Therapy With Didanosine, Stavudine, or Zalcitabine

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The “D drug” HIV reverse-transcriptase inhibitors zalcitabine, didanosine, and stavudine are relatively strong inhibitors of polymerase-gamma compared with the “non-D drugs” zidovudine, lamivudine, and abacavir. D drugs deplete mitochondrial DNA (mtDNA) in cultured hepatocytes. This mtDNA depletion is associated with an increased *in vitro* production of lactate. To investigate the origin of hyperlactatemia in HIV-infected patients and the effects of antiretroviral therapy on liver mtDNA, we biopsied liver tissue from 94 individuals with chronic hepatitis C virus (HCV) infection. Eighty subjects were coinfecting with HIV. Serum lactate was measured at the time of biopsy. Hepatic mtDNA and liver histology were centrally assessed. Liver mtDNA content of HIV-infected patients receiving D drugs at the time of biopsy ($n = 34$) was decreased by 47% ($P < .0001$) compared with those without D drugs ($n = 35$). Aside from a possible association between HCV genotype I status and mtDNA depletion in multivariate analysis, there were no other virologic, immunologic, histologic, demographic or treatment-related variables that could explain the mtDNA depletion. Lactate was above the upper limit of normal in only three patients, all of whom were treated with D drugs. The mtDNA in each of them was lower than in any non-D drug patient and significantly ($P = .017$) depleted compared with D drug patients with normal lactate. In conclusion, D drug treatment is associated with decreased hepatic mtDNA in HIV-infected patients with chronic HCV infection. Moderate mtDNA depletion in liver does not necessarily lead to hyperlactatemia, but more pronounced decreases in hepatic mtDNA may be an important contributor to lactate elevation. (HEPATOLOGY 2004;39:311–317.)

Antiretroviral therapy (ART) has significantly decreased the HIV-associated morbidity and mortality in industrialized countries.¹ ART usually consists of a combination of two nucleoside analogue reverse transcriptase inhibitors (NRTIs) with either pro-

tease inhibitors (PIs) or a nonnucleoside reverse transcriptase inhibitor (NNRTI), or of three NRTIs.² With prolonged exposure to antiretroviral drugs, clinicians became aware of long-term side effects of individual ART components. Many adverse effects of the NRTI class of anti-HIV drugs are now related to the fact that NRTIs undergo intracellular triphosphorylation, then inhibit the replication of mitochondrial DNA (mtDNA) by interacting with gamma-polymerase.³ *In vitro* studies point toward differences between the potencies of the individual NRTIs in depleting mtDNA, with the so-called “D drugs” zalcitabine (ddC), didanosine (ddI), and stavudine (d4T) being relatively strong inhibitors of polymerase-gamma compared with the other currently licensed nucleoside analogues (so-called “non-D drugs”).^{4,5}

Studies performed *in vitro* and in animals suggest that depletion of mtDNA may represent an underlying mechanism of NRTI-related hepatic side effects in HIV patients.^{5–7} Cell models and animal data, however, have limitations in predicting clinical toxicities, partly because

Abbreviations: ART, antiretroviral therapy; NRTI, nucleoside analogue reverse transcriptase inhibitor; PI, protease inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; mtDNA, mitochondrial DNA; ddC, zalcitabine; ddI, didanosine; d4T, stavudine; HCV, hepatitis C virus; nDNA, nuclear DNA; ULN, upper limit of normal; ALT, alanine aminotransferase.

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of pharmacokinetic differences between species and partly because of variations in the uptake and phosphorylation of nucleosides into tissues, cells, and mitochondria. To date, only limited observational mtDNA data of HIV patients receiving ART are available,^{8,9} and a systematic study has not been conducted. Furthermore, the available data conflict with regard to zidovudine, because mtDNA depletion has also been observed despite the fact that this NRTI is not a strong inhibitor of gamma-polymerase at clinically relevant concentrations.^{4,5,9}

Slight and asymptomatic elevations of lactate are frequently associated with the prolonged use of NRTIs and may also be related to mtDNA depletion in tissues.^{3,10} Such mild hyperlactatemia has to be distinguished from lactic acidosis or symptomatic hyperlactatemia, which have been reported in association with liver pathology.¹¹ The contribution of liver impairment (particularly of hepatic mtDNA depletion) to both forms of lactate elevation is not clear, although results from an exogenous lactate challenge test have suggested that asymptomatic hyperlactatemia may result from a loss of lactate clearance (which is likely to involve the liver) and increased lactate production (from unknown tissues).¹²

Our aim was to investigate if NRTIs and especially D drugs deplete mtDNA in hepatic tissue of HIV patients and if there is a link between mtDNA depletion in liver and lactate elevation. Because of ethical difficulties in obtaining repeat liver biopsies, we chose a cross-sectional design and selected HIV patients with chronic hepatitis C virus (HCV) coinfection as the principal study population.

Experimental Procedures

Human Subjects. After ethics committee approval, patients were recruited in the Hospital Clínic, Barcelona, and the HIV Centre, Düsseldorf, from February 2001 until October 2002. Consecutive patients were enrolled if they granted informed consent and met the inclusion and exclusion criteria for one of the following study groups.

Group 1 consisted of HIV-1 patients with chronic HCV coinfection, as confirmed by a positive HCV-antibody ELISA and positive HCV-RNA measurements (>500 IU/mL). Patients in this group had to meet further inclusion criteria such as no interferon and ribavirin pretreatment and absence of *HbsAg* and other liver diseases (e.g., autoimmune hepatitis, primary biliary cirrhosis, alpha-1 antitrypsin deficiency, hepatocellular carcinoma, haemochromatosis, and Wilson's disease). Pregnant or lactating females were excluded, as were patients who consumed intravenous or inhaled drugs, drank alcohol excessively (>80 g ethanol/d), or suffered from poorly

controlled diabetes mellitus, end-stage renal disease, or severe respiratory disease. Group 1 was subdivided into three subgroups based on the antiretroviral regimen at the time of biopsy: patients receiving no antiretroviral therapy (subgroup 1A), no D drug at all (subgroup 1B), or at least one D drug (subgroup 1C).

Group 2 subjects had to meet all the entry criteria of group 1, except for the fact that they were HIV-negative. They were recruited to assess possible unspecific effects of HIV infection on mtDNA levels in liver.

Liver Histology. Percutaneous liver biopsies were performed using a Mengini (16-gauge) needle. The largest aliquot of each biopsy cylinder (≥ 20 mm) was embedded in paraffin and centrally assessed by a single pathologist, who was blinded to the clinical and laboratory information. The degrees of liver fibrosis and necroinflammatory activity were scored on Haematoxylin-Eosin and Masson's trichrome stains according to Scheuer's classification.¹³ The percentage of hepatocytes displaying signs of macro- or microvesicular steatosis was also scored.

Quantification of mtDNA. The second aliquot of the liver biopsy (4 mm) was immediately frozen and stored at -70°C until shipment on dry ice for centralized and blinded mtDNA measurements by quantitative Southern blot analysis as described previously.^{5,14,15} mtDNA was probed with a 12.9-kb pair, random-prime digoxigenin-labeled fragment, spanning nucleotide positions 3470 and 16379 of human mtDNA; nuclear DNA (nDNA) was simultaneously detected with a second probe, directed against the multicopy 18S ribosomal DNA gene. The intensities of the mtDNA and nDNA signals were densitometrically quantified using Scion-image (Scion Corporation, Frederick, MD), and mtDNA was normalized for nDNA-content by calculating the mtDNA/nDNA ratio. Two DNA standards extracted from HepG2 hepatoma cells (ATCC HB-8065) and human fibroblasts were run in parallel on every blot to assess assay variations. The mtDNA/nDNA measurements were reliable with an interrun variation of 20%; large variations in the amount of DNA loaded onto the gel do not influence the result.^{14,15} Southern blot analysis was also used to screen for large-scale mtDNA deletions.

Other Measurements. At the time of liver biopsy, a forearm vein was cannulated. After at least one hour, blood was drawn from rested patients without the use of a tourniquet. Patients were instructed to avoid fist clenching and hand pumping. Blood was collected in sodium fluoride/potassium oxalate tubes and placed on ice. Lactate was immediately tested enzymatically in an automated analyzer (Roche/Hitachi 917; Roche, Basel, Switzerland) according to the manufacturer's instructions. Both laboratories' normal reference range was 50 to

220 mg/L. Values were calculated as the upper limit of normal (ULN).

Further evaluation included quantification of serum alanine aminotransferase (ALT), HCV, and HIV viral loads (COBAS AMPLICOR HCV-MONITOR version 2.0 and COBAS AMPLICOR HIV-MONITOR version 1.5, Roche Diagnostics, Basel, Switzerland) and of CD4 lymphocyte counts at the time of biopsy. HCV genotype was determined as described elsewhere.¹⁶

Statistics. The laboratory results and demographic and clinical parameters among groups were univariately compared using χ^2 tests or Fisher exact tests for categorical variables and unpaired t-tests, Wilcoxon Mann-Whitney tests or Kruskal-Wallis ANOVA on the ranks for continuous variables, as appropriate. Those statistical analyses were performed using the Sigma Stat for Windows software version 1.0 (Jandel Corporation, San Rafael, CA). Both univariate and multivariate linear regression analyses were done using SPSS for Windows software Release 11.0.0 (SPSS Inc., Chicago, IL). Trends in mtDNA over time on therapy were estimated using an exponential model that allows variation in the rate of initial decline and subsequent long-term level (asymptote). The nonlinear model was fitted by least-squares in SPSS. The flexibility in the rate of decline allows an approximately linear trend should the data suggest this.

Results

Demographics, Virology, and Immunology. Out of a total of 94 patients biopsied, 80 subjects had HIV and chronic HCV infection (group 1) and 14 patients had chronic HCV infection but no HIV infection (group 2). Eleven patients had no antiretroviral therapy at the time of biopsy (group 1A). Within group 1A, nine patients were naïve to antiretroviral treatment, one female had a 3-month exposure to zidovudine during pregnancy 18 months prior to biopsy, and one male had interrupted a PI-containing first line therapy of 42 months duration for reasons of revised treatment guidelines; his treatment was stopped 9 months prior to biopsy.

The remaining 69 HIV- and HCV-coinfected patients had uninterrupted antiretroviral therapy. At the time of biopsy, 35 were treated without D drugs with various combinations of zidovudine, lamivudine, and abacavir (group 1B) and 34 were treated with one or two D drugs (*i.e.*, zalcitabine, didanosine, and stavudine) (group 1C).

There were no statistical differences between the group 1 subgroups with respect to age, sex, HCV genotype, HCV viral load, time of known HIV infection, and CD4 count (Table 1). The D drug-treated individuals had a slightly shorter mean time of known HCV infection than

those not given D drugs (14.5 *vs.* 18.1 years $P = .04$) and a slightly lower percentage of undetectable HIV-RNA (71% *vs.* 94%, $P = .03$). Both antiretrovirally treated groups did not differ with respect to NNRTI or current PI use; however, patients on D drugs had a longer cumulative exposure to total ART and to PIs than their non-D drug counterparts.

The mean age (43.7 ± 10.8 years) and sex (79% male) of group 2 did not statistically differ from group 1.

Liver mtDNA. For easier comparison of the quantitative mtDNA results, the mean mtDNA/nDNA ratio of group 1A was set as 100%. mtDNA levels (Fig. 1) among HIV-negative patients with chronic HCV infection (group 2: mean mtDNA/nDNA ratio = $114\% \pm 54\%$) did not differ from group 1A, as did those of HIV-infected patients receiving ART without D drugs (group 1B: mean mtDNA/nDNA ratio = $114\% \pm 46\%$). However, when D drugs were used as part of the antiretroviral treatment, mtDNA levels were reduced by 47% compared with biopsies from individuals treated without D drugs (group 1C: mean mtDNA/nDNA ratio = $60\% \pm 27\%$, $P < .0001$) and reduced by 40% when compared with group 1A ($P = .0001$).

The comparison of mtDNA levels among individual NRTIs (Table 2) revealed reduced amounts of mtDNA in livers of patients receiving stavudine, didanosine, or zalcitabine (mtDNA/nDNA ratios of 62%, $P = .0004$; 44%, $P < .0001$ and 51%, $P = .04$ of group 1A-levels, respectively). In contrast, mtDNA levels among patients receiving zidovudine, lamivudine, or abacavir were not reduced (mean mtDNA/nDNA ratios of 111%, $P = .79$; 96%, $P = .41$; 77%, $P = .07$, respectively). There was no statistical association between the use or nonuse of PIs or NNRTIs (at the time of biopsy, or ever) and mtDNA depletion. Furthermore, there were no significant correlations of mtDNA levels with total or current time on ART or PI.

Six patients were treated with two D drugs at the time of biopsy; all were receiving the combination of stavudine and didanosine (Fig. 1). The mean mtDNA/nDNA ratio in these patients was only $41\% \pm 10\%$, which represents 36% of the ratio in D drug-negative patients ($P < .0001$). Compared with the 24 patients receiving stavudine as the only D drug, the mean mtDNA/nDNA ratio among subjects receiving a combination of stavudine and didanosine was decreased by 53% ($P < .0001$).

To further characterize treatment and disease-related factors that may influence mtDNA levels in patients receiving treatment (group 1B and 1C, $n = 69$), multivariate regression analyses were performed in which mtDNA levels were considered as the dependent variable. Each of the factors presented in Table 1 was considered as a co-

Table 1. Demographic, Virologic, and Immunologic Characteristics Among Patients With HIV/HCV Coinfection

HIV and HCV Coinfection	1A (no ART; n = 11)	1B (D drug-negative; n = 35)	1C (D drug-positive; n = 34)	P Value		
				A-B	A-C	B-C
<i>Demographics, virology, and immunology</i>						
Age (y)	39.4 ± 5.8	40.0 ± 5.1	40.4 ± 5.8	NS	NS	NS
Men (%)	73	69	79	NS	NS	NS
Time of known HCV infection (y)	17.2 ± 6.9	18.1 ± 3.8	14.5 ± 5.0	NS	NS	.04
HCV-RNA (log copies/mL)	5.6 ± 0.9	5.9 ± 0.5	5.8 ± 0.5	NS	NS	NS
HCV genotype 1 (%)	36	55	55	NS	NS	NS
Time of known HIV infection (y)	9.4 ± 5.4	11.4 ± 4.4	11.2 ± 2.7	NS	NS	NS
HIV-RNA (log copies/mL)	3.2 ± 0.9	2.4 ± 0.3	2.5 ± 0.4	<.001	.01	NS
HIV-RNA below detection limit (%)	27	94	71	<.001	.02	.03
CD4 lymphocytes/ μ l	622 ± 109	546 ± 240	634 ± 356	NS	NS	NS
<i>Antiretroviral therapy</i>						
Time of current ART (mo)	0	23 ± 18	26 ± 18	NA	NA	NS
Currently NNRTI-treated (%)	0	54	41	NA	NA	NS
Time on current NNRTI (mo)	0	11 ± 13	8 ± 12	NA	NA	NS
On current nevirapine (%)	0	34	29	NA	NA	NS
Currently PI-treated (%)	0	31	47	NA	NA	NS
Time on current PI (mo)	0	7 ± 14	13 ± 19	NA	NA	NS
Cumulative time of ART (mo)	*	60 ± 38	80 ± 33	NA	NA	.008
NNRTI treatment ever (%)	0	66	65	NA	NA	NS
Cumulative time on NNRTI (mo)	0	14 ± 15	13 ± 13	NA	NA	NS
PI treatment ever (%)	*	77	91	NA	NA	NS
Cumulative time on PI (mo)	*	22 ± 19	36 ± 21	NA	NA	.004
Grade of liver fibrosis	1.4 ± 1.0	1.6 ± 1.0	1.9 ± 1.3	NS	NS	NS
mtDNA/nDNA ratio (mean of group 1A: 100%)	100 ± 27	114 ± 46	60 ± 27	NS	.0001	<.0001
Lactate (% of upper limit of normal)	35 ± 16	50 ± 19	60 ± 28	.04	.02	NS

Group variability is calculated as standard deviation.

Abbreviations: NS, not significant; NA, not applicable.

*Two patients had received ART prior to but not at the time of biopsy. Their treatment is discussed in the text.

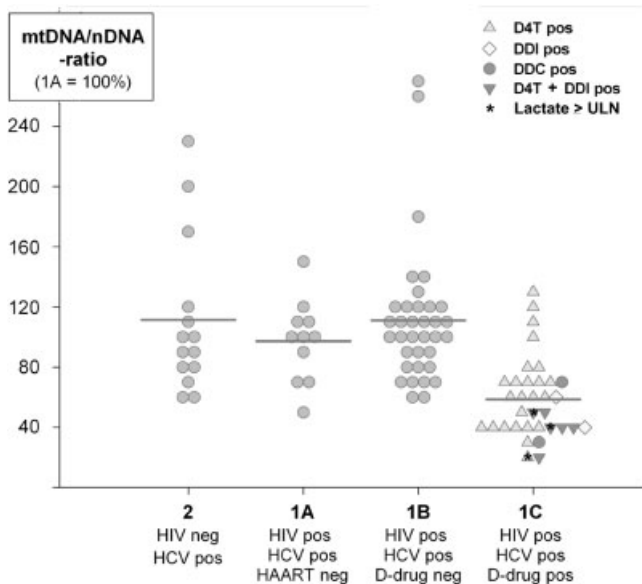


Fig. 1. mtDNA/nDNA ratio in liver among all subjects (mean of group 1A: 100%). The horizontal bar represents group means. The patients with lactate above or equal to the ULN are marked with a star.

variate in both univariate and multivariate regression analyses. Current use of D drugs was significantly associated with mtDNA levels in a univariate analysis ($P < .001$), while no significant associations were detected for use or duration of current PI therapy ($P = .41$, $P = .47$) or NNRTI therapy ($P = .12$, $P = .12$). In addition, the effect of D drug treatment on mtDNA levels was independent of the duration of current ART ($P = .72$). Furthermore, neither the cumulative time on ART ($P = .24$) nor the cumulative time on PI ($P = .20$) or NNRTI ($P = .55$) had any influence. With regard to demographic and HIV disease-related effects, no significant associations were detected between mtDNA levels and age ($P = .49$), gender ($P = .73$), CD4 T cell count ($P = .62$), or undetectable HIV viral load ($P = .60$). Similarly, HCV viral load ($P = .96$), duration of HCV infection ($P = .72$), evidence of hepatic inflammatory activity ($P = .11$), and fibrosis ($P = .46$) were not significantly associated with mtDNA levels. However, there was a trend toward an association between HCV genotype 1 and mtDNA levels ($P = .09$) that was found to be significant after adjusting for the effect of D drug use in multivariate regression analysis ($P = .04$). Further assessment of D drug use and HCV genotype status in a general linear model analysis revealed no evidence for an interaction between these

Table 2. Characteristics of Patients With HIV/HCV Coinfection With Regard to the Use of Particular NRTIs at the Time of Biopsy

HIV and HCV Coinfection	Zidovudine (n = 34)	Lamivudine (n = 55)	Abacavir (n = 14)	Stavudine (n = 30)	Didanosine (n = 8)	No NRTI (n = 11)	P Value
<i>Demographics, virology, and immunology</i>							
Age (y)	39.9 ± 5.2	40.7 ± 5.8	40.0 ± 3.3	40.6 ± 6.2	38.3 ± 4.0	39.4 ± 5.8	NS
Men (%)	68	76	64	80	63	73	NS
Time of known HCV infection (y)	17.5 ± 3.6	15.4 ± 4.9	20.3 ± 2.1	13.8 ± 5.0	14.9 ± 5.1	17.2 ± 6.9	NS
HCV-RNA (log copies/mL)	5.9 ± 0.5	5.8 ± 0.5	5.8 ± 0.7	5.8 ± 0.6	5.9 ± 0.5	5.6 ± 0.9	NS
HCV genotype 1 (%)	53	50	64	55	63	36	NS
Time of known HIV infection (y)	10.6 ± 4.7	10.7 ± 3.8	13.9 ± 2.1	10.7 ± 2.2	11.3 ± 3.9	9.4 ± 5.4	NS
HIV-RNA (log copies/mL)	2.0 ± 0.7	2.1 ± 0.6	2.1 ± 0.5	2.3 ± 0.6	2.3 ± 0.7	3.2 ± 0.9	NS*
HIV-RNA below detection limit (%)	88	87	86	73	75	27	NS*
CD4 lymphocytes/ μ l	548 ± 257	601 ± 314	618 ± 227	632 ± 365	462 ± 188	622 ± 109	NS
<i>Antiretroviral therapy</i>							
Time of current ART (mo)	26 ± 20	23 ± 15	12 ± 10	25 ± 16	19 ± 13	0	NS*
Currently NNRTI-treated (%)	50	53	21	40	50	0	NS*
Time on current NNRTI (mo)	11 ± 13	11 ± 13	2 ± 5	8 ± 12	8 ± 12	0	NS*
Currently PI-treated (%)	29	31	36	47	63	0	NS*
Time on current PI (mo)	9 ± 16	9 ± 16	6 ± 9	13 ± 18	11 ± 15	0	NS*
Cumulative time of ART (mo)	60 ± 39	65 ± 38	60 ± 29	80 ± 34	93 ± 25	†	.02*
NNRTI treatment ever (%)	62	65	57	67	88	0	NS*
Cumulative time on NNRTI (mo)	14 ± 15	14 ± 14	9 ± 13	13 ± 12	15 ± 15	0	NS*
PI treatment ever (%)	76	84	71	93	88	†	NS*
Cumulative time on PI (mo)	21 ± 17	25 ± 19	34 ± 26	36 ± 21	38 ± 19	†	.01*
Grade of liver fibrosis	1.7 ± 1.0	1.8 ± 1.2	1.5 ± 0.9	1.9 ± 1.4	1.6 ± 1.4	1.4 ± 1.0	NS
<i>MtDNA/nDNA ratio (mean of group 1A: 100%)</i>							
1A: 100%	111 ± 49	96 ± 46	77 ± 33	62 ± 28	44 ± 11	100 ± 27	<.001
Lactate (% of upper limit of normal)	50 ± 19	52 ± 22	56 ± 25	59 ± 29	71 ± 35	35 ± 16	NS

Group variability is calculated as standard deviation. Patients receiving zalcitabine (n = 2) are discussed in the text.

Abbreviation: NS, not significant.

*The "no NRTI" group was excluded in the statistical comparison (Kruskal-Wallis ANOVA on the ranks).

†Two patients had received ART prior to but not at the time of biopsy. Their treatment is discussed in the text.

variables ($P = .71$), suggesting that these factors contribute independently to mtDNA depletion in the liver. No other tested variable contributed significantly to mtDNA depletion in multivariate regression analyses, while the association between use of D drugs and mtDNA levels remained highly significant after adjusting for all covariates ($P < .0001$).

We found a significant decline in mtDNA over time on current treatment with D drugs (Fig. 2), dropping from an initial mean of 99.4% (SE, 8.0; group 1A mean, 100%) to a long-term value estimated as 57.5% (SE, 5.3; $P < .00004$). However, most of this decline occurs in the first 6 to 7 months, with no evidence of decline beyond this time ($P = .86$).

Qualitative mtDNA alterations (e.g., mtDNA deletions) were not observed.

Serum Lactate. Only three individuals had a serum lactate above or equal to the ULN. Among the three subgroups of HIV/HCV coinfection, lactate was highest under D drug treatment (group 1C: $0.60 \times \text{ULN} \pm 0.28$), followed by D drug-negative subjects (group 1B: $0.50 \times \text{ULN} \pm 0.19$) and by patients without antiretroviral treatment at the time of biopsy (group 1A: $0.35 \times$

$\text{ULN} \pm 0.16$). Lactate was higher in both treatment groups (i.e., in patients receiving D drugs and in patients without D drugs) compared with subjects without ART at the time of biopsy ($P = .019$ and $P = .042$, respectively). However, there was no statistical difference of lactate with respect to the D drug status in patients on antiretroviral treatment. The lactate of HIV-negative pa-

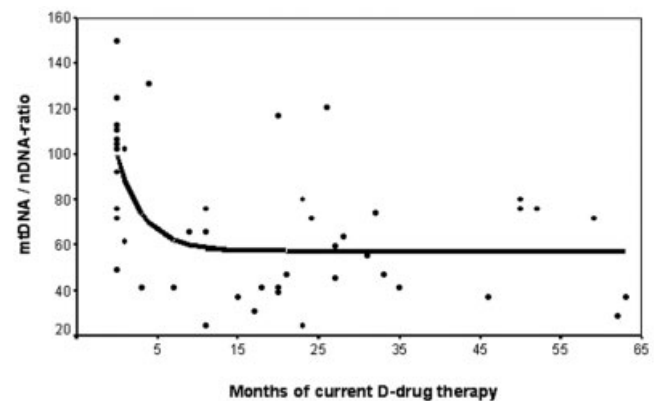


Fig. 2. Trends in mtDNA/nDNA ratio over time on D drug therapy (mean of group 1A: 100%).

tients with chronic HCV infection (group 2: $0.43 \times \text{ULN} \pm 0.12$) did not statistically differ from its HIV positive counterpart (group 1A).

Compared with patients without ART, the mean lactate was elevated in subjects using didanosine ($0.71 \times \text{ULN}$, $P = .016$) and stavudine ($0.59 \times \text{ULN}$, $P = .027$), but also in those receiving zidovudine ($0.50 \times \text{ULN}$, $P = .04$), lamivudine ($0.52 \times \text{ULN}$, $P = .02$) and abacavir ($0.56 \times \text{ULN}$, $P = .046$). In summary, lactate levels tended to be elevated among subjects receiving D drugs, but this was not statistically significant (Table 2).

There was no correlation between mtDNA levels and lactate in the group of all 94 HCV-infected individuals or its subgroups. Stratification of the mtDNA measurements into quartiles revealed only a nonsignificant trend toward lactate elevation with mtDNA depletion among patients coinfecting with HCV and HIV.

The subjects with lactate above or equal to the ULN were all HIV-positive and treated with stavudine at the time of biopsy; two of the three were given additional didanosine. The mean mtDNA/nDNA ratio in the liver of these three patients was $38\% \pm 11\%$ of untreated HIV- and HCV-coinfecting controls ($P = .003$). Compared with the remaining patients on D drugs ($67\% \pm 28\%$), mtDNA levels were significantly diminished ($P = .017$). Furthermore, the mtDNA/nDNA values of patients with serum lactate above or equal to the ULN (range: 25%–47%) were outside the range of mtDNA measurements in individuals without antiretroviral therapy (group 1A range: 49%–150%) or D drugs (group 1B range: 59%–274%).

Other Measurements. Between the subgroups of group 1 (groups 1A, 1B, and 1C), there were no differences with regard to the histologic degrees of liver fibrosis, inflammatory activity, macro- or microvesicular steatosis, or the serum levels of ALT (not shown). There was also no association between macro- or microvesicular steatosis and mtDNA or lactate levels.

Discussion

This study analyzed the hepatic mtDNA content in patients with chronic HIV and HCV coinfection. The HIV patients were divided into three subgroups according to their antiretroviral regimen at the time of biopsy. The major finding is that antiviral therapy with at least one of the D drugs (didanosine, stavudine, and zalcitabine) is associated with mtDNA depletion in liver, whereas no such relation was detected between mtDNA levels and treatment with other antiretroviral drugs.

Two individuals within group 1A were not naïve to antiretroviral treatment. We chose to include these patients in our analysis because we also did not select for a specific du-

ration of the treatment in groups 1B and 1C. However, if we had excluded the two patients with prior ART from group 1A, the significance levels of our results would not have changed, and the association between mtDNA depletion and D drug treatment at the time of biopsy in particular would have still been detectable ($P = .0001$).

We detected a trend ($P = .09$) toward an association between HCV genotype I for mtDNA depletion that was found to be significant after adjusting for the effects of D drug use. Interestingly, a similar association has been documented previously with HCV genotype 1b.¹⁷ Such HCV-associated mitochondrial injury could be explained by increased oxidative stress or by several other mechanisms.^{17,18}

No further virologic, immunologic, histologic, demographic, or treatment-related variables contributed significantly to mtDNA depletion in the multivariate analysis.

The conclusions with regard to the association between the use of individual D drugs and mtDNA depletion are limited by the cross-sectional design of the study, in which the treatment with individual NRTIs is not independent from each other and is not randomized. This may be illustrated by the fact that most patients treated with didanosine were also receiving stavudine. However, the higher mtDNA levels in individuals receiving stavudine as the only D drug, compared with those receiving stavudine plus didanosine, supports an independent effect of didanosine. The coadministration of two NRTIs was previously observed to have additive or synergistic mitochondrial toxicity *in vitro*.⁵

Our model of mtDNA trends over time (Fig. 2) suggests an mtDNA decline during the initial 6 months of D drug therapy, with no further decline beyond this time. The kinetics of mtDNA loss are initially influenced by the degree of gamma-polymerase inhibition and presumably also by the rates of cell division and mtDNA turnover.⁵ Eventually, stable mtDNA levels arise in accordance with the gamma-polymerase hypothesis.

The mean mtDNA/nDNA ratio in the D drug-treated HIV patients was 53% lower, compared with patients receiving anti-HIV treatment without D drugs. The question then arises as to whether or not this relatively moderate mtDNA depletion may be functionally relevant, given the fact that wild type mtDNA levels in the order of 20% can maintain almost normal cell function *in vitro*.¹⁹ Several observations indicate that the *in vitro* threshold of mtDNA levels within cells may differ from the *in vivo* situation in a tissue. For example, anaerobic ATP production by ample glucose supply in the medium may allow a relatively long cell survival *in vitro* despite severe mtDNA depletion. Fibrotic tissue may also be more resistant to mtDNA depletion and thus maintain some residual mtDNA. Indeed, *in situ* hybridization

studies in patients with proven mitochondrial cytopathies failed to establish an *in vivo* threshold necessary for mtDNA mutations to trigger a biochemical dysfunction in clinically affected tissue.²⁰ It is also interesting to note that the magnitude of mtDNA depression was similar in adipose tissue of patients suffering from HIV-associated lipoatrophy¹⁴ and in hepatic tissue of inherited mtDNA replication defects with liver failure.²¹

Our investigations do not demonstrate a clear relationship between mtDNA depletion in hepatic tissue and an increase in serum lactate, but it is important to note that lactate levels were normal in virtually all patients. In contrast to previous reports,²² there was no lactate elevation in individuals receiving stavudine compared with zidovudine, although lactate was somewhat higher with D drug treatment in our study. We are unable to determine the exact reasons for this discrepancy, but possible explanations include the HCV status of our principal study population,¹⁷ effects on mitochondria or hepatic tissue unrelated to mtDNA depletion (as demonstrated for zidovudine^{4,5,23}), and additional mitochondrial toxicity in extra-hepatic tissues.^{9,24} Our study was also not powered to examine associations between hyperlactatemia and NRTI therapy. The findings therefore do not exclude an important contribution of mtDNA levels in liver on serum lactate. This is also supported by the observation that among the D drug-treated subjects, those with a serum lactate above or equal to the ULN had a significant mtDNA depletion compared with those with a normal lactate.

We found that antiviral treatment with D drugs—but not with non-D drugs—is associated with hepatic mtDNA depletion in HCV- and HIV-coinfected subjects. We have evidence that a moderate mtDNA depletion in hepatic tissue alone is not sufficient to cause mild hyperlactatemia, but that more pronounced mtDNA depletion may represent an important factor contributing to lactic acidosis.

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