

CHANGES IN MITOCHONDRIAL DNA AS A MARKER OF NUCLEOSIDE TOXICITY IN HIV-INFECTED PATIENTS

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ABSTRACT

Background Nucleoside analogues can induce toxic effects on mitochondria by inhibiting the human DNA polymerase γ . The toxic effects can range from increased serum lactate levels to potentially fatal lactic acidosis. We studied changes in mitochondrial DNA relative to nuclear DNA in the peripheral-blood cells of patients with symptomatic, nucleoside-induced hyperlactatemia.

Methods Total DNA was extracted from blood cells. A nuclear gene and a mitochondrial gene were quantified by real-time polymerase chain reaction. Three groups were studied: 24 controls not infected with the human immunodeficiency virus (HIV), 47 HIV-infected asymptomatic patients who had never been treated with antiretroviral drugs, and 8 HIV-infected patients who were receiving antiretroviral drugs and had symptomatic hyperlactatemia. The patients in the last group were studied longitudinally before, during, and after antiretroviral therapy.

Results Symptomatic hyperlactatemia was associated with marked reductions in the ratios of mitochondrial to nuclear DNA, which, during therapy, averaged 68 percent lower than those of non-HIV-infected controls and 43 percent lower than those of HIV-infected asymptomatic patients never treated with antiretroviral drugs. After the discontinuation of antiretroviral therapy, there was a statistically significant increase in the ratio of mitochondrial to nuclear DNA ($P=0.02$). In the patients followed longitudinally, the decline in mitochondrial DNA preceded the increase in venous lactate levels.

Conclusions Mitochondrial DNA levels are significantly decreased in patients with symptomatic, nucleoside-related hyperlactatemia, an effect that resolves on the discontinuation of therapy. (N Engl J Med 2002; 346:811-20.)

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NUCLEOSIDE analogues that inhibit reverse transcriptase remain a cornerstone of antiretroviral therapy for human immunodeficiency virus (HIV) infection. These agents inhibit HIV replication but can also inhibit the human DNA polymerase γ ^{1,2} and thereby replication of mitochondrial DNA, leading to depletion of mitochondrial DNA and drug toxicity.³⁻⁶ Mitochondrial toxicity is at least partially responsible for adverse

effects such as lactic acidosis, hepatic steatosis, myopathy, cardiomyopathy, peripheral neuropathy,⁷ pancreatitis, and possibly the lipodystrophy syndrome.^{5,6,8-12} Treatment of other conditions, such as hepatitis or cancer, with nucleoside analogues can also result in mitochondrial toxicity.^{13,14}

Early studies on zidovudine-induced myopathy found a decrease in total mitochondrial DNA in muscle-biopsy specimens.¹⁵⁻¹⁷ In vitro studies of various anti-HIV nucleoside analogues have also shown that they can cause a reduction in the mitochondrial content of human lymphoblastoid cells,¹⁸ CEM cells,¹⁹ and HepG2 cells.²⁰ Mitochondrial toxicity leads to decreased oxidative phosphorylation, intracellular accumulation of lipids, and increases in lactic acid.^{3,21} Patients with hyperlactatemia due to mitochondrial toxicity may present with symptoms such as fatigue, shortness of breath, weight loss, lipid abnormalities, and hepatic steatosis. The syndrome of hyperlactatemia is now well known among HIV-infected patients receiving antiretroviral therapy, particularly in those receiving stavudine (d4T, or Zerit).²²⁻²⁵ Its presentation, severity, and frequency are distinct from those of lactic acidosis, which is a rare, acute, and potentially fatal adverse effect of nucleoside analogues.²⁶⁻²⁹ Whether chronic hyperlactatemia is a risk factor for progressive liver damage²⁹ or lactic acidosis³⁰ remains uncertain.

The gold standard for the diagnosis of nucleoside-related mitochondrial toxic effects is muscle or liver biopsy; however, biopsy is not practical for routine screening and monitoring. Random measurements of venous lactate have been used to monitor for mitochondrial damage,^{31,32} but the clinical usefulness of this method remains unclear. Lack of specificity is a problem, as is technical and physiologic variability.³⁰

We describe a novel polymerase-chain-reaction (PCR) assay for mitochondrial DNA performed on samples of venous blood. We applied it to three groups of subjects: normal volunteers not infected with HIV, HIV-infected asymptomatic patients nev-

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er treated with antiretroviral drugs, and HIV-infected patients with symptomatic, nucleoside-related hyperlactatemia. Finally, in the group of patients with hyperlactatemia, we quantified ratios of mitochondrial to nuclear DNA longitudinally before, during, and after the discontinuation of therapy.

METHODS

Sample Collection and DNA Extraction

Samples consisted of buffy coats of peripheral venous blood that were collected in acid citrate dextrose and stored at -70°C until used. Plasma viral loads were measured by the Amplicor Ultra-Sensitive HIV-1 Monitor assay (Roche Molecular Systems, Branchburg, N.J.). Total DNA was extracted from $200\ \mu\text{l}$ of sample with the QIAamp DNA Blood Mini kit (Qiagen, Mississauga, Ont., Canada). For the standard curves, similar samples were collected from 24 non-HIV-infected male volunteers, and the DNA was extracted and pooled. The nuclear-genome-equivalent content of the HIV-negative DNA pool was determined by calibration with the kit's control human DNA of known nuclear-genome-equivalent concentration (Roche Applied Science, Laval, Que.).

This study was approved by the research-ethics board of the University of British Columbia and Providence Health Care. All patients in the study provided written informed consent.

Quantitative Real-Time PCR

For each DNA extract, the nuclear gene for the human polymerase γ accessory subunit (ASPOLG) and the mitochondrial gene human cytochrome-*c* oxidase subunit I (CCOI) were quantified separately by real-time quantitative PCR (with the use of a Roche LightCycler). For the CCOI gene, the CCOI1F 5'TTCGCCGACCGTTGACTATT3' and CCOI2R 5'AAGATTATTACAAATGCATGGGC3' primers were used for the PCR amplification. The oligonucleotides 3'fluorescein-labeled CCOIP1 5'GCCAGCCAGGCAACCTTCTAGG-F3' and 5'LC Red640-labeled CCOIP2 5'L-AACGACCACATCTACAACGTTATCGTCAC-P3', the 3' end of the latter blocked with a phosphate molecule, were used as hybridization probes. For the nuclear DNA ASPOLG gene, the ASPG3F 5'GAGCTGTTGACGGAAAGGAG3' and ASPG4R 5'CAGAAGAGAATCCCGGCTAAG3' primers were used for the PCR, and the oligonucleotides 3'fluorescein-labeled ASPGP1 5'GAGGCGCTGTTAGAGATCTGTCTAGAGAG-F3' and 5'LC Red640-labeled, 3'phosphate-blocked ASPGP2 5'L-GGCATTT-CCTAAGTGAAGCAAGCA-P3' were used as hybridization probes.

The real-time PCR reactions were performed in duplicate for each gene with the LightCycler FastStart DNA Master Hybridization Probes kit (Roche Applied Science). The PCR reactions contained 5 mM magnesium chloride, $1.0\ \mu\text{M}$ of each primer, $0.2\ \mu\text{M}$ 3'fluorescein probe, $0.4\ \mu\text{M}$ 5'LC Red640 probe, and $4\ \mu\text{l}$ of a 1:10 dilution of the DNA extract in elution buffer. The PCR am-

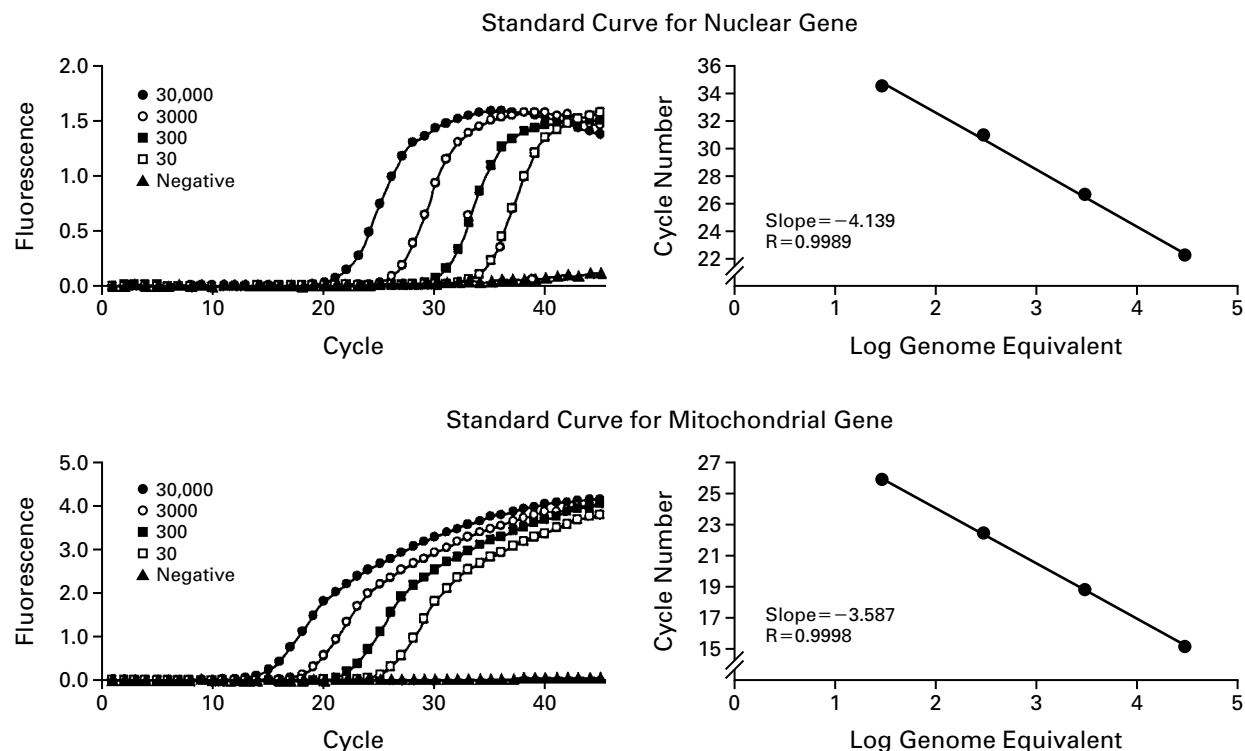


Figure 1. Typical Real-Time Polymerase-Chain-Reaction Standard Curves Generated for the Nuclear Gene for the Human Polymerase γ Accessory Subunit and the Mitochondrial Gene Human Cytochrome-*c* Oxidase Subunit I with Use of Serial Dilutions of the Pooled DNA Extracts from HIV-Negative Male Volunteers.

The numbers (30 to 30,000) shown in the standard curve for the nuclear gene indicate the number of nuclear-genome equivalents included in each run. The same numbers were arbitrarily used in the standard curve for the mitochondrial gene.

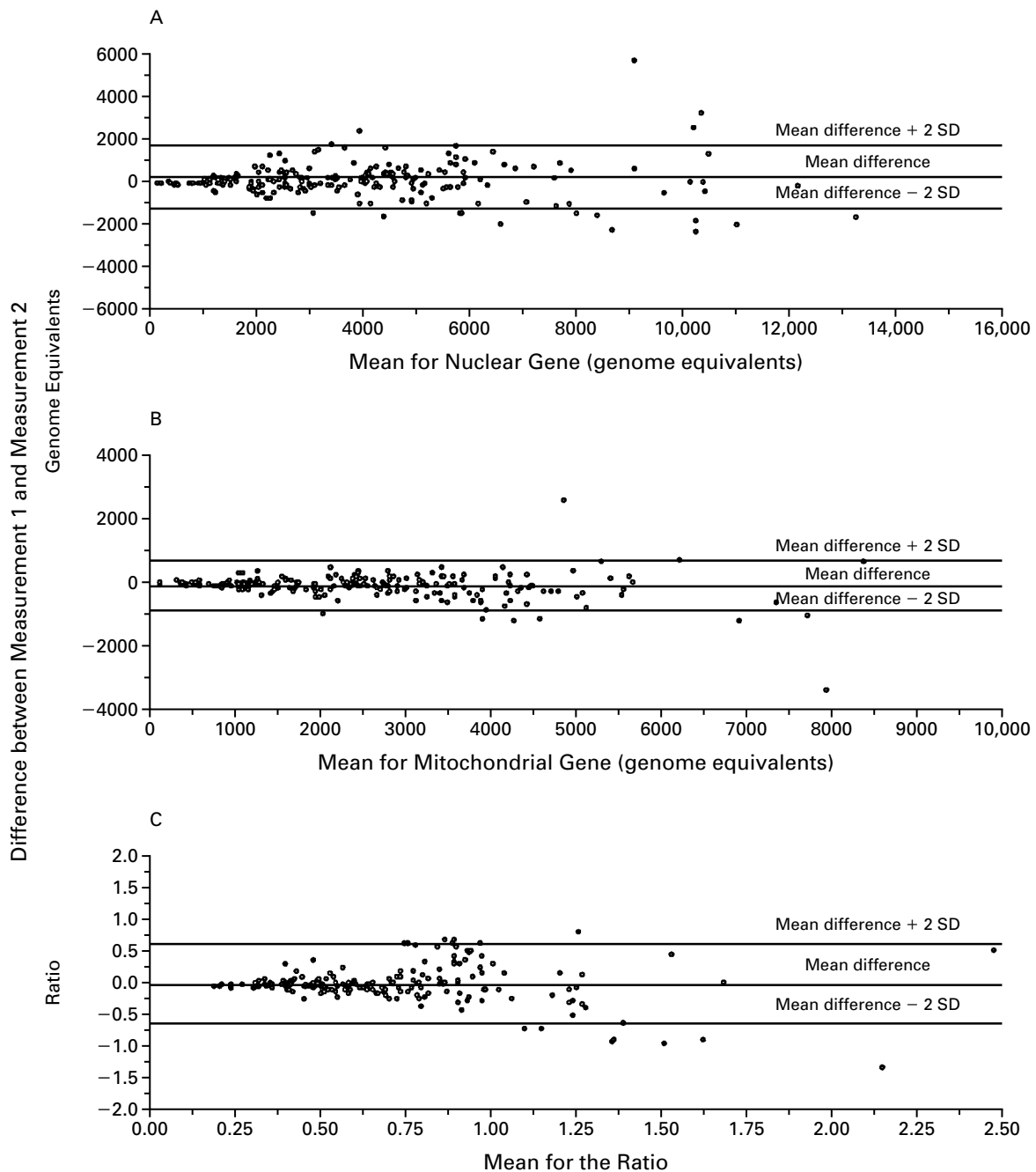


Figure 2. Levels of Agreement between Duplicate Measurements of the Nuclear Gene for the Human Polymerase γ Accessory Subunit (Panel A), the Mitochondrial Gene Human Cytochrome-c Oxidase Subunit I (Panel B), and the Ratio of Mitochondrial to Nuclear DNA (Panel C) Derived from Them.

The standard deviations were calculated by using the differences between the first and second values of the duplicate measurements for each subject.

plification consisted of a single denaturation–enzyme-activation step of 10 minutes at 95°C, followed by 45 cycles of 0 seconds at 95°C, 10 seconds at 60°C, and 5 seconds at 72°C, with a temperature-transition rate of 20°C per second. A single fluorescence acquisition was performed at the end of each annealing step. A standard curve of 30, 300, 3000, and 30,000 nuclear-genome equivalents was included in each run, and the same nuclear-genome equivalent values were used for both the nuclear (ASPOLG) and the mitochondrial (CCOI) gene quantifications.

The data were analyzed by using the second-derivative maximum of each amplification reaction and relating it to its respective standard curve. If a sample had an ASPOLG measurement above 15,000 or below 150 genome equivalents, the assay was repeated at a higher or lower dilution of the extract, respectively, to fall within a DNA concentration range that yielded a constant ratio of mitochondrial to nuclear DNA. The results from the quantitative PCR were expressed as the ratio of the mean mitochondrial DNA value of duplicate measurements to the mean nuclear DNA value of duplicate measurements for a given extract (mtDNA:nDNA). The mtDNA:nDNA ratio was arbitrarily set around 1.0 by the fact that the same nuclear-genome equivalent values were used to generate both standard curves.

Venous Lactate Measurement

Specimens of venous blood for measurement of lactate were collected in sodium fluoride–potassium oxalate tubes, with the use of a normal tourniquet and no specific instructions to the patient other than the avoidance of fist clenching or hand pumping. The laboratory's normal reference range for lactate is 0.7 to 2.1 mmol per liter.

Plasma Drug Levels

The concentrations of protease inhibitors (indinavir, ritonavir, saquinavir, nelfinavir, and lopinavir) and nonnucleoside reverse-transcriptase inhibitors (nevirapine, delavirdine, and efavirenz) were determined in the stored plasma samples that were collected for viral-load testing by high-performance liquid chromatography (HP 1100, Agilent, Palo Alto, Calif.) coupled with tandem mass spectrometry (API-2000 LC/MS/MS System, AB/MDS-Sciex, Foster City, Calif.). In brief, the antiretroviral drugs were extracted with acetonitrile and the precipitated plasma proteins were separated by centrifugation (16,000×g) followed by filtration (Ultrafree-MC Filters, Millipore, Bedford, Mass.). The drugs in the filtrate were partially separated by high-performance liquid chromatography (Zorbax XDB C-18 column, Agilent) and quantified by standard methods on the mass spectrometer. The samples were collected in acid citrate dextrose anticoagulant, which diluted the blood somewhat. The time at which the last doses of antiretroviral drugs were administered was unknown. Qualitative plasma drug levels were used to establish whether the patients were taking antiretroviral drugs.

Longitudinal mtDNA:nDNA Analysis

Blood samples from the eight HIV-infected patients with symptomatic hyperlactatemia who were receiving antiretroviral drugs, which were collected before, during, and after the discontinuation of antiretroviral therapy (between 6 and 17 samples per patient, covering a period of 22 to 28 months), were assayed to determine the mtDNA:nDNA ratios. All samples collected at least one month after the initiation of the antiretroviral regimen but before discontinuation of therapy (three to six samples per patient) were used to calculate the mean mtDNA:nDNA ratio during therapy. All samples collected after the discontinuation of therapy, with no limitations on time but before the initiation of a new regimen, were used to calculate the mean mtDNA:nDNA ratio when the patient was not receiving therapy. Data from Patient 1 were excluded from this analysis because there were no samples available after the discontinuation of treatment and before the start of a new regimen. All samples collected both when the patient was not receiving therapy (zero to three per patient) and after the new stavudine-free regimens were started (zero to nine per patient) were included in the calculation of the mean mtDNA:nDNA ratio when the patient was not receiving stavudine. Group means were calculated by using one mean value for each patient.

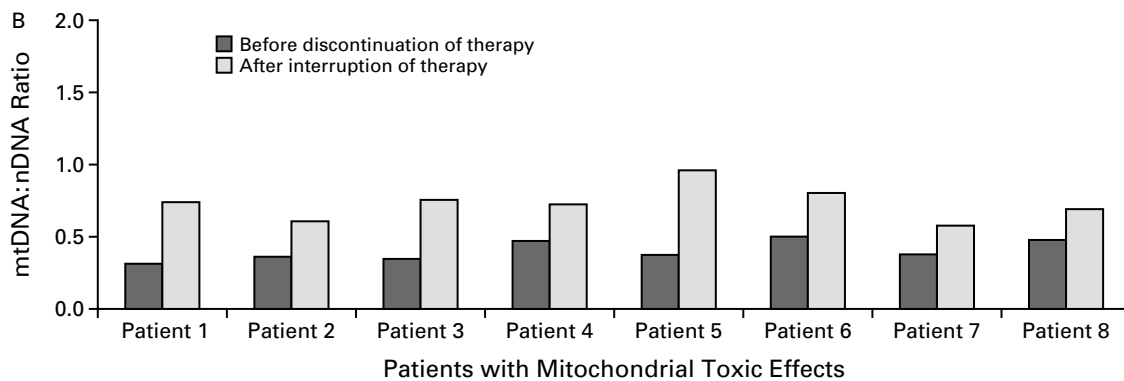
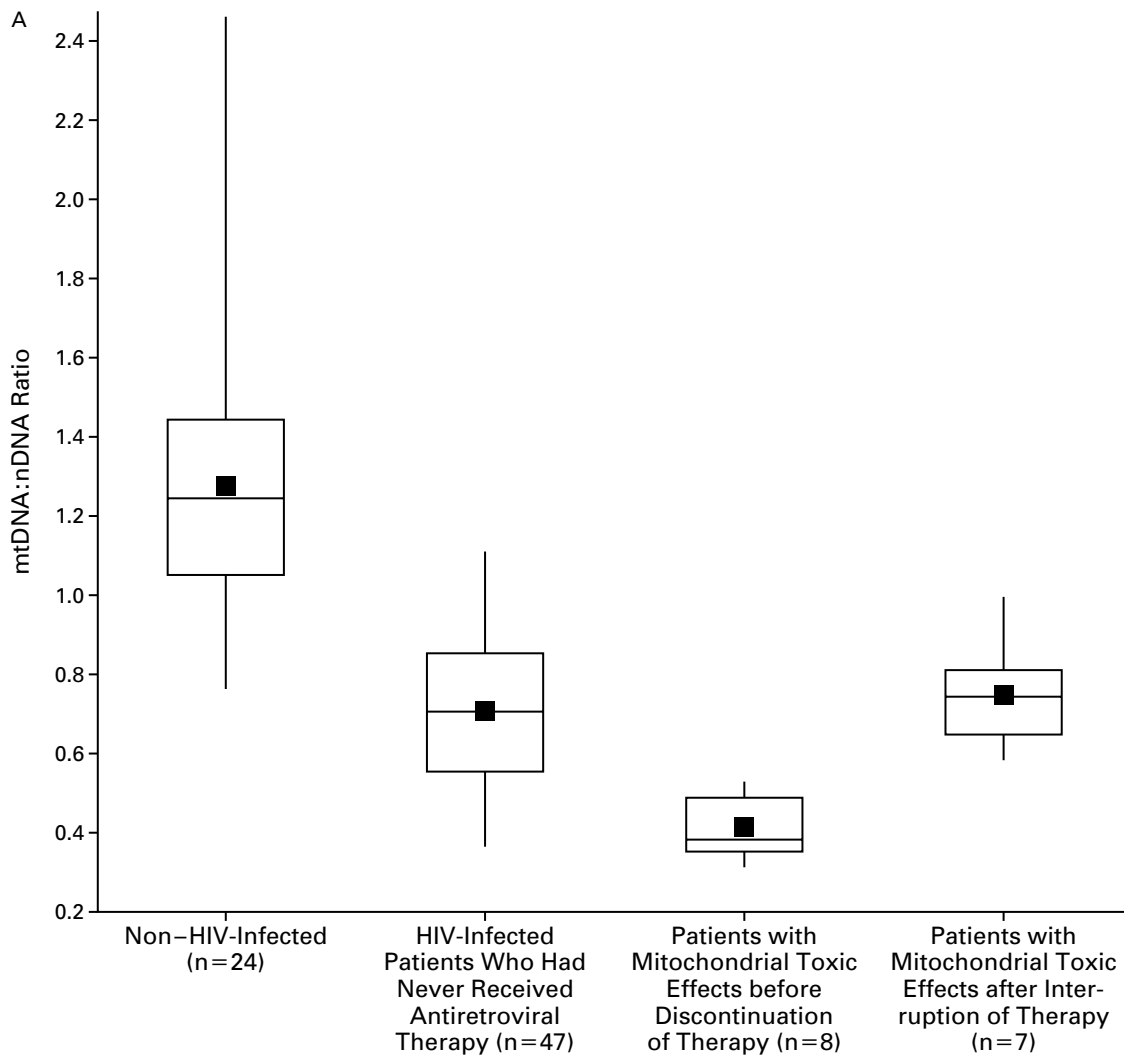
Statistical Analysis

To study the level of agreement between duplicate measurements of mitochondrial and nuclear DNA, scatter diagrams (showing the line of equality) were first constructed and Pearson's correlation coefficients were calculated. The coefficient of determination, as defined by the proportion of the variation in the value of the dependent variable (second measurement) that is explained by its linear relation with the independent variable (first measurement), was also calculated. To assess the degree of agreement between measurements, a plot of the difference between the measurements as compared with their mean value was constructed.³³ This plot allowed us to investigate the relation between the measurement error and the true value. Although the true value is unknown, the mean of the two measurements provides a reasonable estimate. These plots are useful in identifying homoscedastic or heteroscedastic patterns between the differences and their mean. Limits of agreement (mean difference ± 2 SD) were also included in these plots.

For the patients with mitochondrial toxic effects, we assessed the relation between the mtDNA:nDNA ratio and the CD4 cell counts by first calculating the Spearman correlation between the repeated measurements of the mtDNA:nDNA ratio and the CD4 cell count for each of the eight patients, and then testing the hypothesis that the mean of the patient-specific correlations was zero by the Wilcoxon signed-rank test. A similar approach was followed with the other clinical tests. For the comparison of subgroups that contained multiple observations per patient, the mean for each patient was calculated while the patient was receiving therapy, after

Figure 3 (facing page). Ratio of Mitochondrial to Nuclear DNA in the Three Groups of Subjects.

Panel A shows comparative box plots of ratios of mitochondrial DNA to nuclear DNA (mtDNA:nDNA) in 24 non-HIV-infected controls (mean [\pm SD], 1.28 ± 0.38); 47 HIV-infected, asymptomatic patients who had never received antiretroviral therapy (no detectable protease inhibitor or nonnucleoside reverse-transcriptase inhibitor in plasma samples) (0.72 ± 0.19); and 8 HIV-infected patients with symptomatic mitochondrial toxic effects treated with antiretroviral drugs. For the latter, the mtDNA:nDNA ratios when eight patients were receiving therapy (0.41 ± 0.08) and when seven were not receiving therapy (0.74 ± 0.13) are depicted. The vertical lines indicate the maximal and minimal mtDNA:nDNA ratios observed within each group; the top and bottom of the box indicate the 25th and 75th percentiles; the middle line indicates the median; and the black square shows the mean mtDNA:nDNA ratio. In Panel B, for each of the eight patients with mitochondrial toxic effects, the darker bar represents the mean of all mtDNA:nDNA ratios measured while the patient was receiving antiretroviral therapy, and the lighter bar represents the mean of all mtDNA:nDNA ratios measured while the patient was not receiving therapy, after the interruption of antiretroviral therapy, except for Patient 1, for whom the mean ratio while not receiving stavudine is shown, since no sample taken when the patient was not receiving therapy was available.



therapy was discontinued, and while the patient was not receiving therapy or was receiving a new stavudine-free regimen. The means of these values (i.e., the means of the individual patients' means) were then compared by the Wilcoxon signed-rank test.

RESULTS

Mitochondrial DNA Assay

The mitochondrial DNA assay made use of real-time PCR to quantify the mitochondrial DNA content of blood cells in relation to their nuclear DNA content. The standard curves for both the mitochondrial gene (CCOI) and a nuclear gene (ASPOLG) were generated by serial dilution of the male HIV-negative DNA pool and showed linearity over the range studied (Fig. 1). The level of agreement of the duplicate measurements performed for this study is illustrated in Figure 2. For the nuclear and the mitochondrial gene, respectively, 96 percent and 98 percent of the differences between duplicate measurements were less than 2 SD from the mean difference, with Pearson's correlation coefficients of 0.94 and 0.99 and coefficients of determination of 89 percent and 97 percent, respectively. For the mtDNA:nDNA ratio (Fig. 2C), the level of agreement was highest at lower values of the ratio, and overall, 92 percent of duplicate measurements were less than 2 SD from the mean, with a Pearson's correlation coefficient of 0.72 and a coefficient of determination of 52 percent.

Population Comparison

The assay was applied to three groups of men: 24 non-HIV-infected controls, 47 HIV-infected, asymptomatic patients never treated with antiretroviral drugs, and 8 HIV-infected patients with symptomatic hyperlactatemia who were treated with antiretroviral drugs. Figure 3 presents the mtDNA:nDNA ratios obtained for the non-HIV-infected and HIV-infected control groups. The mean mtDNA:nDNA ratio among the non-HIV-infected controls was significantly higher than that among the HIV-infected asymptomatic patients never treated with antiretroviral drugs ($P < 0.001$). Among the eight HIV-infected patients with symptomatic hyperlactatemia, the mean mtDNA:nDNA ratio of the last sample collected before antiretroviral therapy was discontinued was 0.28 ± 0.06 . This value was 22 percent of the value in the non-HIV-infected subjects (1.28 ± 0.38) and 39 percent of the value in the HIV-infected asymptomatic patients never treated with antiretroviral drugs (0.72 ± 0.19), and it was significantly lower than the value in either of these control groups ($P < 0.001$).

Characteristics of the Patients

The eight patients receiving antiretroviral therapy (Table 1) had mitochondrial toxic effects characterized by varying degrees of progressive hyperlactatemia (Fig. 4), fatigue, rapid weight loss, and a reduced anaero-

bic threshold during exercise testing.²⁵ Their antiretroviral regimens varied from first-line to salvage therapy, and all were receiving stavudine. Antiretroviral therapy was interrupted in all eight patients as a result of the development of symptomatic hyperlactatemia. In all cases, lactate decreased and symptoms resolved on the discontinuation of antiretroviral therapy.

Longitudinal mtDNA:nDNA Ratios

Longitudinal measurements of mtDNA:nDNA ratios in samples collected before, during, and after antiretroviral therapy are presented in Figure 4. Among the eight patients with symptomatic hyperlactatemia, the mean mtDNA:nDNA ratio during therapy (0.41 ± 0.08) was significantly lower than that among both the non-HIV-infected subjects ($P < 0.001$) and the HIV-infected, asymptomatic patients who had never received antiretroviral therapy ($P < 0.001$). The mean mtDNA:nDNA ratio during therapy was also significantly lower than both the mean mtDNA:nDNA ratio when the patients were receiving no therapy (0.74 ± 0.13 , $P = 0.02$) and the mean mtDNA:nDNA ratio when the patients were not receiving stavudine (0.69 ± 0.06 , $P = 0.008$). The mean mtDNA:nDNA ratio obtained for the HIV-infected, asymptomatic patients who had never received antiretroviral therapy was not significantly different from the mean mtDNA:nDNA ratio when the patients were receiving no therapy ($P = 0.43$) or the mean mtDNA:nDNA ratio when the patients were not receiving stavudine ($P = 0.26$).

The results of several laboratory tests were investigated to explore their possible relation with the mtDNA:nDNA ratio. Among the HIV-infected, asymptomatic patients who had never received antiretroviral therapy, CD4 cell counts were not significantly correlated with the mtDNA:nDNA ratio (Spearman's $\rho = 0.08$; $P = 0.59$; mean CD4 cell count, 233 ± 162 per cubic millimeter; range, 10 to 830). For the eight HIV-infected patients with symptomatic hyperlactatemia receiving antiretroviral therapy, there were also no significant correlations between the mtDNA:nDNA ratio and CD4 cell count ($P = 1.00$; mean CD4 cell count, 191 ± 146 per cubic millimeter; range, 10 to 620), platelet count ($P = 0.29$), white-cell count ($P = 0.31$), alanine aminotransferase level ($P = 0.25$), aspartate aminotransferase level ($P = 0.08$), albumin level ($P = 0.20$), or the international normalized ratio ($P = 0.24$).

Mitochondrial DNA and Lactate Levels

In Patients 1, 4, and 8 (Fig. 4), the decrease in the mtDNA:nDNA ratio clearly preceded the development of hyperlactatemia (earlier data on lactate levels were unavailable for the other five patients). Similarly, in Patients 4, 6, and 8, the time required for the

MITOCHONDRIAL DNA LEVEL AS A MARKER OF NUCLEOSIDE TOXICITY

TABLE 1. CHARACTERISTICS OF THE EIGHT HIV-INFECTED MALE PATIENTS WITH SYMPTOMATIC MITOCHONDRIAL TOXIC EFFECTS AND THEIR ANTIRETROVIRAL REGIMENS.

PATIENT No.	AGE (YR)	BEFORE STOPPING THERAPY			OFF THERAPY		AFTER RESUMING THERAPY	
		DRUG REGIMEN	WEEKS OF STAVUDINE*	LAST HIV PLASMA VIRAL LOAD copies/ml	WEEKS OFF THERAPY	HIGHEST HIV PLASMA VIRAL LOAD copies/ml	DRUG REGIMEN	WEEKS TO HIV PLASMA VIRAL LOAD <50 COPIES/ml
1	47	Stavudine, didanosine, lamivudine, abacavir, hydroxyurea	175	<50	13	223,000	Saquinavir, ritonavir, nevirapine	12
2	41	Stavudine, didanosine, lamivudine, saquinavir, delavirdine, nelfinavir, nevirapine, abacavir, hydroxyurea	144	<50	≥45	178,000	Not applicable	Not applicable
3	44	Stavudine, didanosine, lamivudine, abacavir	59	90	15	177,000	Lamivudine, abacavir, nevirapine, lopinavir-ritonavir	18
4	48	Stavudine, lamivudine, saquinavir, ritonavir	58	<50	17	584,000	Lamivudine, abacavir, nevirapine, lopinavir-ritonavir	20
5	41	Stavudine, didanosine, efavirenz	33	<50	17	425,000	Lamivudine, saquinavir, ritonavir, efavirenz	17
6	57	Stavudine, didanosine, efavirenz	33	<50	17	750,000	Zidovudine, lamivudine, saquinavir, ritonavir, efavirenz	17
7	44	Stavudine, didanosine, lamivudine, saquinavir, indinavir, nevirapine, abacavir, lopinavir	192	<50	≥28	63,300	Not applicable	Not applicable
8	43	Stavudine, indinavir, delavirdine	143	<50	≥26	138,000	Not applicable	Not applicable

*The time shown is the number of weeks during which the patient had continuously been prescribed stavudine as part of his drug regimen before stopping therapy.

mtDNA:nDNA ratio to rebound was similar to or shorter than that needed for the hyperlactatemia to resolve (i.e., to reach the range of 0.5 to 2.1 mmol per liter). A weak correlation (Spearman's rho = -0.32, P=0.02) was observed between the lactate levels and the mtDNA:nDNA ratio.

On the basis of the limited mtDNA:nDNA data available from the eight HIV-infected patients with symptomatic hyperlactatemia who were receiving antiretroviral therapy, the maximal in vivo half-life of mitochondrial DNA was estimated to range from 4.5 weeks (Patient 3) to 8 weeks (Patient 4). The maximal in vivo doubling time of mitochondrial DNA was estimated to range from 4 weeks (Patient 5) to 16 weeks (Patient 1). Short lapses in therapy (as occurred in the cases of Patients 3, 4, and 7), as well as extremely low circulating drug levels (as in Patients 4, 6, and 7), were also associated with increasing levels of mitochondrial DNA. For these patients, the maximal time before lactate levels returned to the normal range after the discontinuation of antiretroviral treatment varied from 4 weeks (Patient 8) to 28 weeks (Patient 5).

DISCUSSION

The assay we used to determine the mtDNA:nDNA ratio can be performed on peripheral blood collected fresh or previously frozen. The assay was most reliable in the low range of mtDNA:nDNA values (Fig. 2C), which is the clinically relevant range. Mitochondrial DNA was significantly depleted in HIV-infected patients with symptomatic hyperlactatemia who were receiving antiretroviral therapy. The decrease in mitochondrial DNA preceded the rise in venous lactate levels, an observation that is consistent with the view that hyperlactatemia is a consequence of depletion of mitochondrial DNA, and it was reversible in all the patients we studied. Mitochondrial DNA:nDNA ratios were significantly lower in HIV-infected, asymptomatic patients who had never received antiretroviral therapy than in non-HIV-infected controls (P<0.001), a difference that was not explained by the lower CD4 counts in the former group. This finding is consistent with the results of recent in vitro studies in which HIV-infected cells had signs of mitochondrial necrosis.³⁴ Furthermore, a mitochondria-controlled mechanism of cell death has been postulated in HIV infection.³⁵⁻³⁸

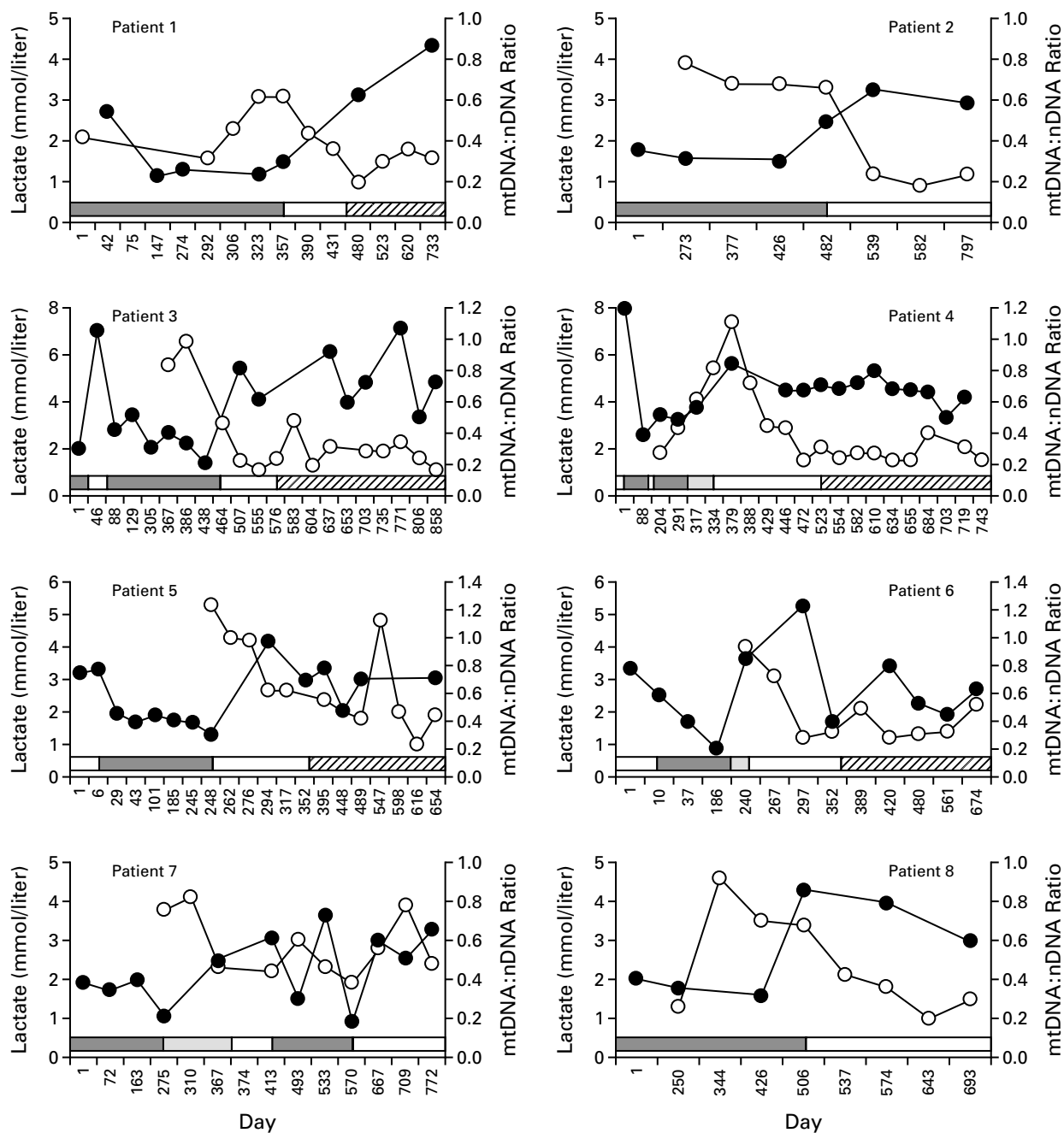


Figure 4. Longitudinal Analysis of Venous Lactate Levels (Open Circles), mtDNA:nDNA Ratios (Solid Circles), and Antiretroviral-Drug Regimens (Bars) over Time in the Patients with Symptoms of Mitochondrial Toxicity.

The bar is dark gray when the patients were receiving the drug regimen that led to mitochondrial toxicity, white when they were not taking any antiretroviral drugs, and hatched when they were receiving a new regimen (Table 1). These antiretroviral-drug data are based on information on medical charts, drug-prescription dates, and plasma drug levels. Light gray regions indicate samples in which the plasma drug levels of protease inhibitor, nonnucleoside reverse-transcriptase inhibitor, or both were more than 2 SD below the average trough concentration (according to the drug manufacturer's monograph). For clarity and simplicity, time is expressed as the days on which the samples were collected.

In inherited mitochondrial diseases, severe symptoms tend to occur when the levels of mitochondrial DNA reach approximately 20 percent of normal, which is similar to the level of depletion observed in HIV-infected patients with symptomatic hyperlactatemia who are receiving antiretroviral therapy. The in vivo doubling times of mitochondrial DNA estimated from this relatively small data set are similar to the time of approximately 35 days observed for cultured cells in which mitochondrial DNA had been depleted by ethidium bromide treatment.³⁹

The fact that the mtDNA:nDNA ratios measured while the patients were not receiving antiretroviral therapy were similar to those observed once the patients resumed nucleoside-containing therapy without stavudine points toward a possible association between stavudine and mitochondrial toxicity.^{22-25,32} Lactate levels remained normal after the resumption of therapy without stavudine. In vitro, the triphosphated form of stavudine is incorporated into DNA more readily than other currently used nucleoside analogues and exerts the greatest inhibition on the human polymerase γ .² This may explain the apparent association between mitochondrial toxicity and stavudine. Further studies are needed to establish the relative mitochondrial toxicity in vivo of various nucleoside analogues.

The frequency of lactic acidosis in patients treated with nucleoside analogues has been retrospectively estimated to lie between 1 and 2 cases per 1000 person-years.²⁶ Another study with a broadened case definition of hyperlactatemia estimated the incidence at 20.9 cases per 1000 person-years of treatment.²² We found that approximately 20 percent of patients receiving antiretroviral therapy had elevated lactate levels in random venous samples.³² A validated quantitative mitochondrial DNA assay could be a useful tool to monitor and evaluate mitochondrial toxicity among HIV-infected patients receiving antiretroviral therapy, as well as among patients with other diseases, such as hepatitis and cancer, which are also treated with nucleoside analogues.

Supported by the British Columbia Centre for Excellence in HIV/AIDS and by a Joint Research Scholarship from the British Columbia Health Research Foundation and St. Paul's Hospital Foundation (to Dr. Côté).

A patent application relating to the assay used in this study has been filed by the University of British Columbia. The patent includes the names of Drs. Côté, O'Shaughnessy, and Montaner.

We are indebted to Shawna Langridge for technical assistance, to the patients, and to the volunteers who donated blood for this study.

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