Mitochondrial Toxicity in HAART

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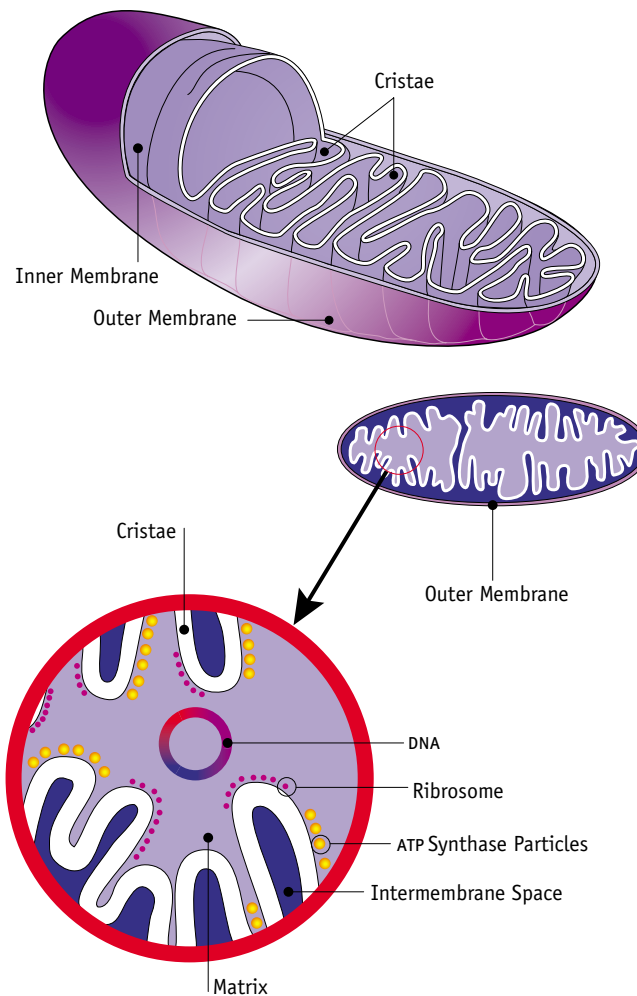available online, visit http://www.PRN.org All rights reserved. © SEPTEMBER 2002.

DAMAGE TO CELLULAR MITOchondria has snowballed into one of the most feared toxicities of antiretroviral therapy. Yet its incidence and prevalence among patients being treated with various nucleoside reverse transcriptase inhibitors (NRTIs)—believed to be the most likely culprits—have not been elucidated and the precise role, if any, of drug-induced mitochondrial damage in the development of hallmark NRTI-related side effects remains unclear. Needless to say, a great deal of research is still needed to better understand the link between antiretroviral treatment and mitochondrial damage and the ways this toxicity can be monitored, managed, and if possible, avoided completely.

Gaining insights into mitochondria damage ultimately requires the validation of tools that can be used in both the research setting and in the clinic to accommodate the diagnostic and prognostic needs of health-care providers. And this is precisely what Dr. Hélène Côté and her colleagues have been up to at the B.C. Centre for Excellence in HIV/AIDS, located in Vancouver, British Columbia. They have developed a PCR-based mitochondrial DNA assay that has, in effect, yielded key information regarding the possible causes and consequences of mitochondrial damage in the setting of HIV.

Dr. Côté introduced PRN members to the B.C. Centre team's work at the May PRN meeting, which was surely just a sample of the important findings to come.

FIGURE 1. A Mitochondrion: The Anatomy of a Powerhouse

Each mitochondrion contains an outer and inner membrane, separated by an intermembrane space. The inner membrane is thrown into curtain-like folds that project inward, dubbed the cristae. Within the walls of the cristae is the matrix that harbors the mitochondrial DNA (mtDNA), ribosomes, and ATP synthase particles. Most cells in the human body, with the exception of erythrocytes, contain hundreds of mitochondria. Each mitochondrion contains approximately ten mtDNA strands.

Source: Brinkman, 1998. Adapted with permission of AIDS and Lippincott Williams & Wilkins

The Mighty Mitochondria

ALL CELLS IN THE BODY, WITH THE exception of erythrocytes, contain hundreds of mitochondria (see Figure 1). Found within the ribbon-like structure of these energy powerhouses are the enzyme complexes and mitochondrial DNA (mtDNA) needed to help carry out oxidative phosphorylation—the aerobic process of forming high-energy bonds, primarily ATP, that can be broken down and used by cells to generate energy (see Figure 2).

The mitochondrial enzyme complexes, which make up the oxidative phosphorylation system (OXPHOS), are composed of subunits, several of which are encoded by mtDNA. Mitochondrial DNA—there are between two and ten copies of mtDNA inside each mitochondria—consist of a double-stranded circular DNA molecule that is prone to errors during replication. It contains no protective histones and has a mutation rate 17 times higher than that of the nuclear genome.

A number of enzymes—polymerases ($\alpha, \beta, \gamma, \delta$, and ϵ)—are required to catalyze the formation of new nuclear or mitochondrial DNA. Of these, only polymerase-γ is responsible for the replication of mtDNA.

During cellular division, there is an even distribution of cellular DNA but not mtDNA in each daughter cell. Both mutated and wildtype forms of mtDNA are randomly segregated into the cellular progeny. The severity of a defect caused by mtDNA mutation depends on the nature of the genetic error and on the proportion of

mutant mtDNA within the mitochondria and the cell. For example, a cell that contains approximately 80% dysfunctional mitochondria is likely to result in a down regulation of the energy-producing OXPHOS. And, depending on the proportion of cells with dysfunctional mitochondria in each organ system—along with the energy demands of each organ system—symptomatic complications can occur.
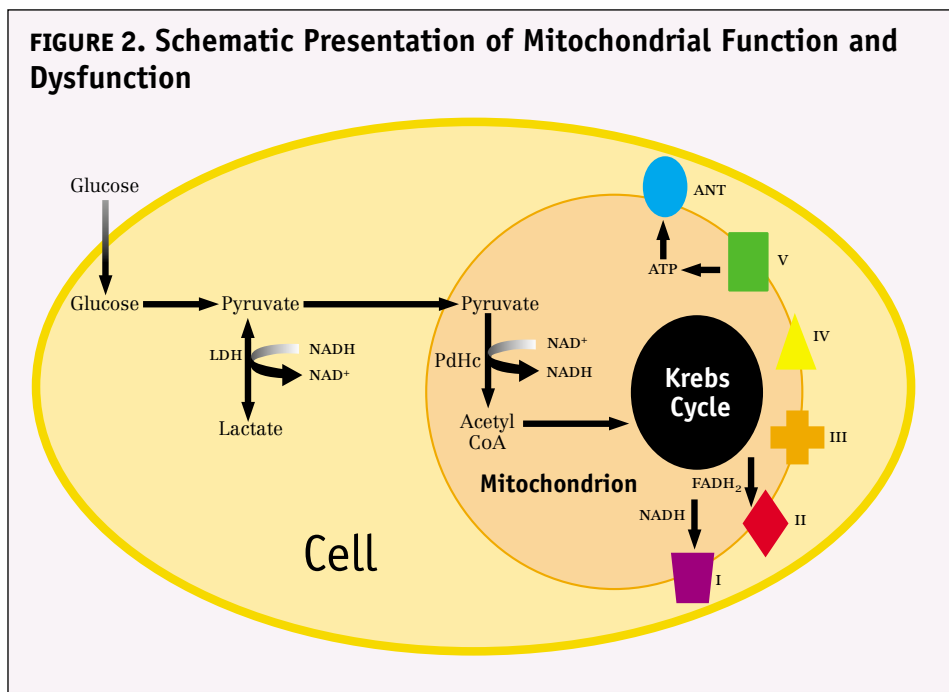
Impaired mitochondrial function—resulting from key hereditary mutations, duplications, or deletions in mtDNA—has been associated with a number of clinical manifestations (Johns, 1996, 1995; Wallace, 1992; Munnich, 1992; Chinnery, 1999). These include complications associated with structural mitochondrial gene mutations (e.g., Leber's hereditary optic neuropathy and neurogenic muscle weakness, ataxia, and retinitis pigmentosa); tRNA and rRNA mutations (e.g., mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes [MELAS] and myoclonic epilepsy with ragged red fibres [MERRF]); and large-scale mtDNA deletions (e.g., Kearns-Sayre syndrome and chronic progressive external opthalmoplegia).

Nonhereditary factors that have long been known to impair mitochondrial DNA synthesis include alcohol and recreational drug usage, obesity, and aging (the accumulation of mtDNA mutations above a threshold level). And more recently, there has been a significant amount of research into mitochondrial toxicity associated with the use of nucleoside reverse transcriptase inhibitors—the backbones of virtually all antiretroviral drug regimens used in the treatment of HIV today.

NRTIs and Mitochondrial Toxicity

IN HIV-INFECTED PATIENTS, REPORTS OF THE specific mitochondrial diseases detailed above have been few and far between. However, a number of manifestations related to these diseases overlap with hallmark side effects of NRTI therapy for HIV (as well as viral hepatitis and cancer), particularly: neurological disorders, such as peripheral neuropathy and dementia; muscular complications including hypotonia, myopathy, and cardiomyopathy; hepatocellular manifestations such as steatosis and lactic acidosis; pancreatitis; pancytopenias; and various renal problems (Brinkman, 1998).

Like cellular DNA polymerase-γ, HIV con-

FIGURE 2. Schematic Presentation of Mitochondrial Function and Dysfunction

Once transported inside the cell, glucose is metabolized to produce adenosine triphosphate (ATP) via two pathways. The anaerobic metabolic pathway, glycolysis, occurs in the cytoplasm outside the mitochondrion. During this initial metabolic pathway, glucose is converted into pyruvate. Pyruvate is then transported into the mitochondria, where oxidative phosphorylation is completed and ATP is produced. When oxidative phosphorylation function is interrupted by way of mitochondrial toxicity, ATP production will decline and the NADH/NAD+ ratio will rise, followed by impairment of the flux through the Krebs cycle, channeling of acetyl-coenzyme A (CoA) toward ketogenesis, lactic acidemia, and an increased lactate/pyruvate ratio. The Roman-numbered blocks represent the four respiratory chain complexes (I–IV), ATP synthase (V), and the adenine nucleotide translocator. Each complex is composed of several subunits—ranging from four subunits in complex II to approximately 43 complexes in complex I—each of which is encoded by either nuclear or mitochondrial DNA. LDH, lactate dehydrogenase; PDHC pyruvate dehydrogenase complex; FADH2, reduced form of flavin adenine dinucleotide; ANT, adenine nucleotide translocator.

Source: Brinkman, 1998. Adapted with permission of *AIDS* and Lippincott Williams & Wilkins.
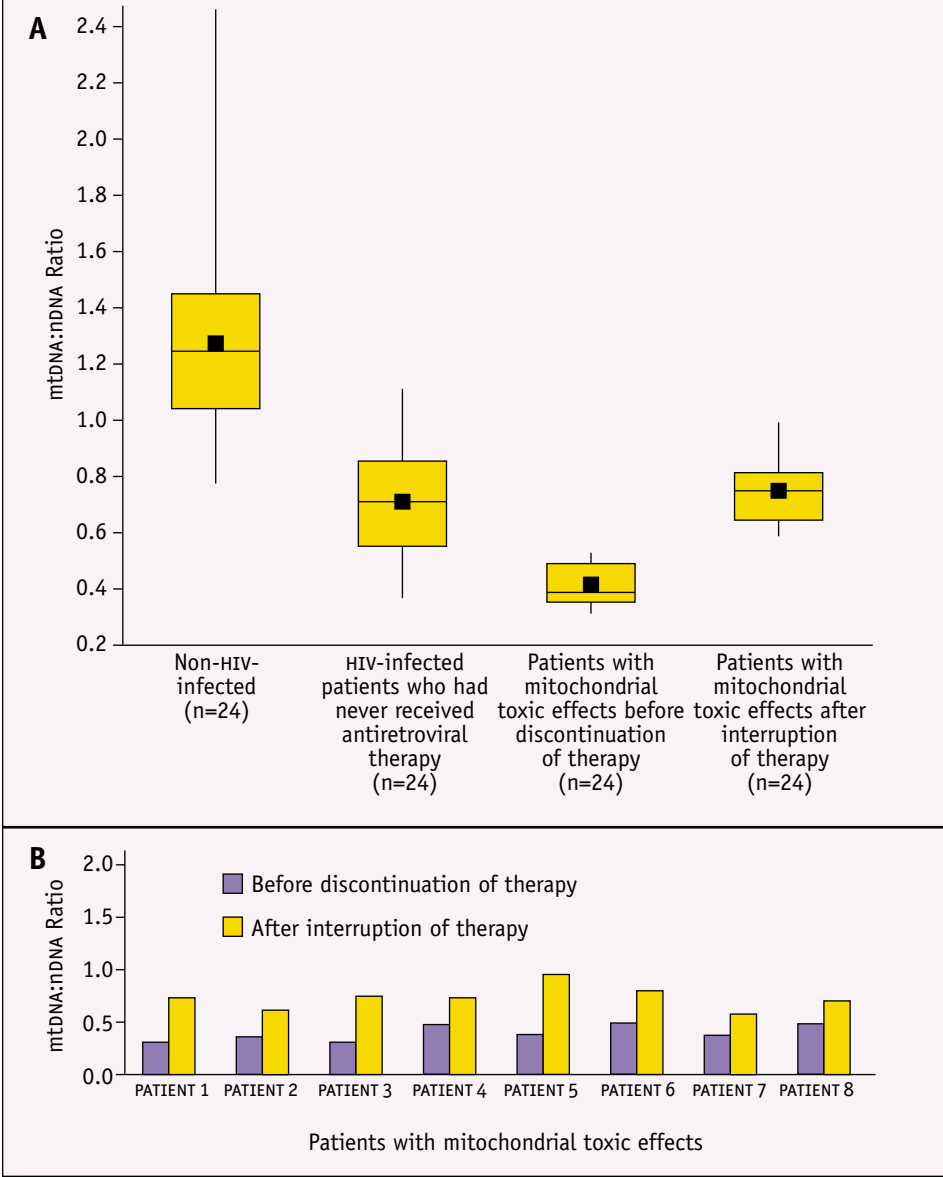
tains its own polymerase: reverse transcriptase. After NRTIs are triphosphorylated intracellularly to nucleotides, they are incorporated into the growing DNA chain by the HIV reverse transcriptase. Because they lack the hydroxyl group needed for further chain elongation, the NRTIs terminate completion of the HIV-DNA chain. However, these nucleotides can also be mistaken for natural substrates by polymerase-γ and, in turn, may deplete or damage mtDNA during replication.

In vitro, these effects have been clearly demonstrated, although the different cell-lines used for the experiments can lead to different results. In recent experiments, all available NRTIs have been studied in the same assay, allowing comparison of the individual toxicities among these NRTIs (Birkus, 2002).

Clinical evidence of NRTI-associated mitochondrial toxicities dates back several years, including a handful of reports linking zidovudine-associated myopathy and mitochondrial damage (Arnaudo, 1990; Dalakas, 1990; Chariot, 1995; Peters, 1993; Casademont, 1996). In addition, at least two reports have demonstrated a clear association between neuropathy and mitochondrial toxicity in rabbits receiving zalcitabine (Hivid) (Anderson, 1994; Feldman, 1994). There was also a case report published in a 1999 issue of the *Journal of Hepatology*, in which a patient who experienced severe liver steatosis and lactic acidosis while receiving zidovudine was found to have seriously depleted mtDNA in his skeletal muscle and in liver tissue samples (Chariot, 1999).

Unfortunately, few clinical studies have

FIGURE 3. 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 [±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.

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examined the possible associations between other NRTIs, mitochondrial toxicity, and specific adverse events. While the widening interest in mitochondrial toxicity—particularly as it might relate to lipodystrophy—may lead to the development and implementation of additional studies, a primary concern is the absence of a reliable, noninvasive test. As explained by Dr. Côté, the gold standard for the diagnosis of NRTI-related toxic effects—whether in the brain, the heart, skeletal muscle, the pancreas, or the liver—is a tissue biopsy. "However, biopsies embody a certain amount of risk for patients involved and, thus, are not practical for routine screening and monitoring."

Hyperlactatemia and Venous Lactate Measurements

ELEVATED LACTATE LEVELS ARE NOT A NEW observation in the setting of HIV care, although they seem to be becoming more prevalent in the setting of HAART. There is both lactic acidosis, a relatively uncommon but potentially lethal complication, and mild to moderate asymptomatic hyperlactatemia, which is seemingly innocuous but develops in a sizeable percentage of HIV-positive patients. Of particular interest has been the role of mitochondrial damage as it relates to either decreased hepatic clearance of lactate or increased lactate production by cells throughout the body.

Because of the likely relationship between hyperlactatemia and mitochondrial toxicity, there has been some interest in venous lactate measurements as a surrogate marker for underlying mitochondrial dysfunction. However, conclusions regarding lactate measurements and its potential role in clinical practice have not yet been established. There has been much consternation among researchers and other experts regarding the specificity of lactate measurements, given physiologic factors that can affect lactate levels, including physical exertion prior to blood draws and the use of tourniquets or fist clenching at the time blood is taken.

To help make heads or tails of lactate measurements, Dr. Marianne Harris, Dr. Julio Montaner and their colleagues initiated a study to explore random venous lactate (RVLA) among 331 HIV-positive patients receiving antiretroviral therapy (Harris, 2000). At least two blood draws for each patient

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 d4T*	Last HIV-RNA	Weeks Off Tx	Highest HIV-RNA	Drug Regimen	Weeks to HIV-RNA <50 copies/mL
1	47	D4T, ddI, 3TC, ABC, HDX	175	<50	13	223,000	SQV, RTV, NVP	12
2	41	D4T, ddI, 3TC, SQV, DLV, NFV, NVP, ABC, HDX	144	<50	≥45	178,000	N/A	N/A
3	44	D4T, ddI, 3TC, ABC	59	90	15	177,000	3TC, ABC, NCP, LPR/r	18
4	48	D4T, 3TC, SQV, RTV	58	<50	17	584,000	3TC, ABV, NVP, LPR/r	20
5	41	D4T, ddI, EFV	33	<50	17	425,000	3TC, SQV, RTV, EFV	17
6	57	D4T, ddI, EFV	33	<50	17	750,000	ZDV, 3TC, SQV, RTV, EFV	17
7	44	D4T, ddI, 3TC, SQV, IDV, NVP, ABC, LPR/r	192	<50	≥28	63,300	N/A	N/A
8	43	D4T, IDV, DLV	143	<50	≥26	138,000	N/A	N/A

* The time shown is the number of weeks during which the patient had continuously been prescribed D4T as part of his drug regimen before stopping therapy.

ABC, abacavir (Ziagen); ddI, didanosine (Videx); d4T, stavudine (Zerit); HDX, hydroxyurea (Hydrea); 3TC lamivudine (Epivir); ADV, zidovudine (Retrovir); EFV, efavirenz (Sustiva); NVP, nevirapine (Viramune); DLV, delavirdine (Rescriptor); IDV, indinavir (Crixivan); LPV/r, lopinavir/ritonavir (Kaletra); RTV, ritonavir (Norvir); SQV, saquinavir (Invirase/Fortovase).

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were included in the analysis. Lactate levels were >2.1 mmol/L in 68/331 (20.5%), and >3.0 mmol/l in 27/331 (8.1%) patients. RVLA levels were consistently normal in 105 patients (<2.1 mmol/L), and consistently abnormal (>2.1 mmol/l) in 33 patients.

Dr. Montaner's team also looked at factors associated with abnormal RVLA levels. In the univariate analysis, the following factors were significantly associated with elevated lactate levels: current stavudine use, longer time on stavudine, current hydroxyurea use, current didanosine use, current lamivudine use, current protease inhibitor use, currently taking more than two NRTIs, currently taking more than three antiretrovirals in general, and lower HIV-RNA levels. In the multivariate analysis, abnormal RVLA levels were independently associated with current stavudine use (odds ratio 4.1), time on d4T (odds ratio 1.1 per three months), and current treatment with hydroxyurea (odds ratio 4.1).

Dr. Côté also discussed hyperlactatemia data coming out of the University of Cali-

fornia, San Diego (UCSD), presented at the 9th Conference on Retroviruses and Opportunistic Infections earlier this year in Seattle (Lonergan, 2002). Studied was the incidence of symptomatic hyperlactatemia—defined as at least one of the following clinical manifestations: nausea, abdominal pain, abdominal bloating, anorexia, fatigue, or elevated alanine aminotransferase (ALT), along with confirmed hyperlactatemia—and its relationship to specific antiretroviral regimens.

Of 2,144 HIV-positive patients receiving at least one NRTI as a component of their treatment through the UCSD clinic, 81 cases of hyperlactatemia were documented. The absolute values of lactate were only mildly elevated in the majority of the subjects (2 to 4 mmol/L). The risk of symptomatic hyperlactatemia rose with the number of NRTIs used, and was highest among patients who were taking stavudine and didanosine combined (59.4/1,000 person-years) and lowest among patients taking zidovudine and lamivudine combined

(3.0/1,000 person-years). Among patients who combined three NRTIs, the risk was highest among those who combined stavudine, didanosine, and lamivudine (119.0/1,000 person-years) and lowest among patients combining abacavir, zidovudine, and lamivudine (12.7/1,000 person-years). Interestingly, lactic acidosis was very uncommon with any regimen, with an incidence of roughly 1/1000 patient-years.

With the conclusion of these and other studies, it is still not clear what role lactate measurements may have in the routine monitoring of HIV-positive patients' health, including those receiving NRTIs. While there have been reports indicating that mild hyperlactatemia is much more common in the setting of HIV than was once thought—estimated incidence rates have ranged from 5% to 35% of patients being treated with NRTIs—its clinical significance is not known. For example, it has yet to be determined if mild hyperlactatemia is progressive. Mild elevations in lactate levels often sponta-

neously stabilize in HIV-positive patients, and there are no data at this time to suggest that mild hyperlactatemia is a risk factor for either lactic acidosis or liver disease. As for the role of routine lactate measurements in ferreting out patients who are experiencing mitochondrial toxicity, more prospective data are needed to prove its utility as a valid surrogate marker. However, both Drs Côté and Montaner believe that physicians may want to consider incorporating routine venous lactate determinations in the monitoring of patients on NRTI-containing antiretroviral therapy.

A Direct Assay for Mitochondrial Toxicity?

IF MITOCHONDRIAL TOXICITY IS PROVEN TO be the root source of NRTI-associated side effects—including hyperlactatemia and lactic acidosis—then the most logical approach in the routine monitoring of HIV-positive patients would be to look directly for changes in mitochondrial DNA. In the March 14, 2002, issue of the *New England Journal of Medicine*, Dr. Côté and her colleagues described a novel approach by which total DNA is extracted from blood cells and both a nuclear gene (ASPOLγ) and a mitochondrial gene (CCOI) are quantified by real-time PCR (Côté, 2002). This assay was then applied to three groups of volunteers: individuals not infected with HIV (n=24), HIV-infected asymptomatic patients naïve to antiretroviral treatment (n=47), and HIV-infected patients with symptomatic, NRTI-related hyperlactatemia (n=8). In the group of patients with hyperlactatemia, the research team quantified ratios of mitochondrial to nuclear DNA longitudinally before, during, and after the discontinuation of therapy, which, incidentally, were all stavudine-containing regimens. A reduction in this ratio might be indicative of mtDNA depletion.

First up, the standard curves for both CCOI and ASPOLγ were generated by serial dilution of the male HIV-negative DNA pool and showed linearity over the range studied. The level of agreement of the duplicate measurements performed for this study was also determined. For the nuclear and the mitochondrial gene, respectively, 96% and 98% of the differences between duplicate measurements were less than 2 SD from the mean difference. As for the mtDNA:nDNA ratio, the level of agreement was highest at lower values of the ratio,

and overall, 92% of duplicate measurements were less than 2 SD from the mean.

Next Dr. Côté and her colleagues compared the mtDNA:nDNA ratios for the three groups (see Figure 3). Among the eight HIV-infected patients with symptomatic hyperlactatemia, the mean mtDNA:nDNA ratio prior to treatment cessation was 0.28±0.06. This value was 22% of the value in the HIV-negative controls (1.28±0.38) and 39% of the value in the treatment-naive HIV-positive controls (0.72±0.19); the comparisons between these two groups and the group of NRTI-treated patients with hyperlactatemia were statistically significant.

Interestingly, the mean mtDNA:nDNA ratio among the non–HIV-infected controls was significantly higher than that among the HIV-positive treatment-naive patients. "These HIV-positive patients were not receiving treatment during the study and had not received any treatments in the past. Still, the mean ratio was noticeably lower in this group. One possible explanation might be found in the results of *in vitro* studies showing mitochondrial necrosis in HIV-infected cell lines," she said, referring to a paper published in a 1999 issue of *AIDS* (Plymale, 1999).

As for the longitudinal mtDNA:nDNA ratios among the eight NRTI-treated patients with hyperlactatemia, Dr. Côté reported that the mean ratio during therapy was significantly lower than both the mean ratio when the patients were receiving no therapy and the mean ratio when the patients were on therapy but not receiving stavudine. Similarly, the mean mtDNA:nDNA ratio obtained for the treatment-naive HIV-positive patients was not significantly different from the mean ratio when these eight hyperlactatemic patients were receiving no therapy or the mean ratio when the patients were not receiving stavudine—only in the setting of NRTI treatment and hyperlactatemia did these eight patients have significantly lower mtDNA:nDNA ratios compared to the latter groups.

Dr. Côté also pointed out that, in three of the NRTI-treated patients, 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, improvements in the mtDNA:nDNA ratio preceded normalization of lactate levels in three patients who ceased NRTI treatment.

With respect to mitochondrial DNA "repair" associated with NRTI cessation, the

maximal *in vivo* half-life of mtDNA ranged from 4.5 weeks to eight weeks in the eight patients who stopped treatment, and the maximal *in vivo* doubling time of mtDNA ranged from four weeks to 16 weeks. It was also noted that lactate levels returned to normal levels in all patients, in as little as four weeks following treatment cessation to as much as 28 weeks.

"Perhaps the most important conclusion that we can draw from this study is the potential importance of a validated quantitative mitochondrial DNA assay," Dr. Côté inferred. "It will be important to evaluate its usefulness for evaluating and monitoring mitochondrial toxicity in HIV-positive patients receiving NRTIs, as well as in other diseases treated with these drugs."

Comparing Drug Regimens

AS DISCUSSED ABOVE, HAART REGIMENS CONsisting of stavudine and didanosine appear much more likely to be associated with hyperlactatemia than regimens containing both zidovudine and lamivudine. To see how these NRTI combinations affect mtDNA:nDNA ratios in patients initiating therapy for the first time, Dr. Côté's group, in collaboration with Drs. Lydia Ruiz and Bonaventura Clotet in Barcelona, analyzed blood samples collected from a subset of volunteers participating in the international SWATCH study—a 162-patient evaluation of two HAART combinations, didanosine/stavudine/efavirenz (arm A) and lamivudine/zidovudine/nelfinavir (arm B), versus alternating the two regimens every three months (arm C) (Côté, 2002a).

Thirty-six patients were included in the mitochondrial DNA substudy. For patients in arms A and B, total DNA was extracted from PBMC samples collected at weeks 0, 12, and 48. For patients in arm C, samples collected at weeks 0, 12, 24, 36, and 48 were analyzed. The mtDNA:nDNA ratio within each extract was determined in the same manner described in the study discussed above.

At week 12, changes in the mtDNA:nDNA ratio were not statistically significant for any of the arms, although a decrease was observed in arms A and C. By week 48, however, the ratio was significantly decreased for arms A and B, with arm C showing a strong trend in the same direction. In arm A, 11/12 patients experienced a decrease in their mtDNA:nDNA ratio, 11 of them below 30% of their baseline value.

Eleven of 12 patients in arm A and 9/12 patients in arm B also experienced a decrease in their mtDNA:nDNA ratio, with one patient in each arm experiencing a ratio decrease <30% of their baseline value. It was also interesting to note that, within arm C, after 24 weeks of therapy, switching to stavudine/didanosine/efavirenz for 12 weeks was accompanied by a significant decrease in the mtDNA:nDNA ratio. Switching back to zidovudine/lamivudine/nelfinavir was accompanied by an increase in the mtDNA:nDNA ratio, although this did not reach statistical significance.

In her review of this study, Dr. Côté pointed out that there was substantial inter-patient variability, both in terms of the baseline mtDNA:nDNA ratio and in terms of the effect of therapy on this ratio, especially early on. Moreover, while the mean decrease in the mtDNA:nDNA ratio over 48 weeks of treatment was greater among patients treated with the didanosine/stavudine-containing regimen, the magnitude of decline was not statistically significant from that of arms B and C.

The Word from Barcelona

TO BETTER UNDERSTAND THE EFFECT OF stavudine-containing regimens on mtNDA:nDNA ratios, in relation to other selected antiretroviral drug regimens, Dr. Côté and her group conducted a cross-sectional study on a sample of patients receiving care through the B.C. Centre Drug Treatment Program (Côté, 2002b). Eligible patients had continuously received saquinavir plus ritonavir with either nevirapine (N=20), lamivudine (N=15), stavudine (N=53), or lamivudine plus stavudine (N=69), for four to 30 months.

According to preliminary results, reported at the XIV International AIDS Conference in Barcelona, stavudine-sparing regimens were associated with a higher median mtDNA:nDNA ratio than stavudine-containing regimens (1.16 versus 0.95); these data were statistically significant. The ratios were skewed toward lower values for the stavudine-containing regimens but more normally distributed for the stavudine-sparing combinations.

As summarized by Dr. Côté during her oral presentation of these data, "these results represent a conservative estimate of the magnitude of the effect of stavudine-containing regimens on mtDNA:nDNA ratios, due to survivor bias effect. Further

work is currently under way to evaluate other antiretroviral regimens such as those containing didanosine, zalcitabine, or hydroxyurea, which may further enhance the effect here described."

Conclusions

IN RECAPPING HER LECTURE, DR. CÔTÉ concluded that patients' mtDNA:nDNA ratio is relatively easy to determine using materials, including blood and tissues, that are easily obtained. "Fresh samples work well, but so do frozen samples, which is good news for research purposes." With respect to the mtDNA:nDNA ratio, it correlates well with the development of NRTI-related side effect symptoms such as symptomatic hyperlactatemia, with notable decreases before the onset of hyperlactatemia and increases prior to the recovery of hyperlactatemia upon discontinuing drug treatment. "We're very excited about these findings and the potential clinical role of the mtDNA:nDNA ratio," she said. "It's still being studied for further validation." *prn*

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