The Forum for Collaborative HIV Research, a project of the Center for Health Services Research and Policy at the George Washington University School of Public Health and Health Services, was founded in 1997. The goal of the Forum is to facilitate discussion regarding emerging issues in HIV clinical research and the transfer of research results into care.

The Forum is a coalition of government agencies, clinical researchers, health care providers, pharmaceutical companies, and patient advocates. The Forum is governed by an Executive Committee made up of representatives from each of the above named constituency groups. The Executive Committee determines the subject and scope of the Forum projects. The Forum brings these constituencies together to identify gaps and impediments in the understanding of the medical management of HIV disease and develops recommendations to fill those gaps. The Forum is a public/private partnership, which receives financial support from its governmental and industry members and with in-kind support from its membership within the academic research, patient care, and advocacy communities.

For more information about the Forum, visit the web site at www.hivforum.org
Many people assisted in making this meeting possible and provided invaluable input. The Forum first wants to thank the Scientific Co-Chairs, Kees Brinkman, MD and William Copeland, PhD, for their time, effort and input.

Several other people provided significant input regarding the agenda and suggestions for speakers. These include Mariana Gerschenson, PhD, Fulvia Veronese, PhD, and Carl Grunfeld, MD.

Mark Mascolini did a brilliant job of writing up the notes from the meeting and prepared this report. Forum staff members Helen Nuamah and Paul Oh handled all the meeting logistics with grace and patience.

We also want to thank Bristol Myers Squibb for covering the costs of the conference dinner.

David Barr – Executive Director
June Bray, Ph.D – Deputy Director
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Mitochondrial Toxicity and HIV Disease
Meeting Report
Washington, DC
June 5-6, 2000

SUMMARY

Mitochondrial toxicity associated with anti-retroviral drugs is an emerging issue in HIV disease, and could pose a major threat to the long-term success of HIV therapy. Many aspects of this toxicity are not well understood. The Forum for HIV Collaborative Research held a meeting on June 5 and 6 in Washington, DC, to develop a better understanding of mitochondrial toxicity and HIV disease.

Invited representatives from Europe, Australia, and the United States included basic scientists with expertise in mitochondrial toxicities and clinical investigators with expertise in HIV disease. They were joined by representatives from regulatory agencies, the pharmaceutical industry, and the patient advocate community to discuss issues of mitochondrial dysfunction in HIV disease. Presentations and discussions included the pathophysiology, prevalence, clinical manifestations, and management of mitochondrial toxicity associated with nucleoside reverse transcriptase inhibitors (NRTIs) used to treat HIV infection. The following questions were addressed:

• What is the role of nucleoside analogue reverse transcriptase inhibitors and/or HIV in the pathogenesis of mitochondrial toxicity?

• What are the clinical and pathological indicators of mitochondrial dysfunction in HIV-infected individuals?

• Is mitochondrial toxicity linked to the lipodystrophy syndrome seen in patients receiving HIV therapy?

• What is the relationship between lactic acidosis and mitochondrial toxicity?

• What is the impact of lactic acidosis from mitochondrial toxicity to morbidity and mortality in HIV disease?
• What is the impact of in utero exposure to antiretroviral agents on both infected and uninfected infants?

• What are the appropriate diagnostic measures (clinical, biochemical and molecular) for mitochondrial dysfunction in HIV infected individuals?

• What, if any, therapeutic options are available for treatment of mitochondrial toxicity in HIV disease?

This meeting continues the Forum’s investigation of metabolic abnormalities on HIV disease and treatment that began with a September 1997 meeting at which many of the now well-described metabolic and fat distribution abnormalities were first discussed. In October 1998, the Forum sponsored a meeting on the prevalence of these symptoms, which led to the development of the FRAM study, a NIH-sponsored state-of-the-art, cross-sectional analysis of these signs and symptoms. In November 1999, the Forum sponsored a meeting on the pathogenesis of metabolic abnormalities associated with HIV disease and treatment. The follow-up from that discussion resulted in this meeting on mitochondrial toxicity held June 2000, and two additional meetings, one on adipocyte biology and another on bone metabolism meeting, to be held August 2000. These meetings will include both basic scientists and clinical investigators, allowing for more specific and in-depth discussions about further research needed to understand the pathogenesis of mitochondrial dysfunction, adipocyte biology, and bone metabolism. The Forum will present the findings from these three meetings at the 2nd International Workshop on HIV-related Lipodystrophy, September 14-15, 2000 in Toronto, Canada.

Key Recommendations

Participants discussed the current data regarding HIV-related mitochondrial toxicity, identified gaps in the research, and made recommendations for further investigation. The key recommendations include the following:

1. Conduct a review of the literature including clinical, animal, and in-vitro studies, and proposed hypotheses on NRTI-associated mitochondrial toxicity. Develop a report that assesses the strength of the evidence, rated on a scale of 1 (poor), 2 (some), 3 (fair) to 4 (strong), for establishing a link between the mitochondrial dysfunction and medical conditions or symptoms, and explore alternative explanatory hypotheses. (The Forum is currently working on this report).
The following table provides a framework for this report: it includes the medical symptoms and conditions that should be evaluated (Column 1) and the types of studies for which evidence of a mitochondrial link should be considered (Columns 2 - 5).

**Table: A framework for reviewing the evidence of a relationship between mitochondrial toxicity and antiretroviral therapies**

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
<th>Column 4</th>
<th>Column 5</th>
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<tr>
<td>Medical condition or symptom</td>
<td>Type of study</td>
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<tr>
<td>Skeletal muscle myopathy</td>
<td>Theoretical</td>
<td>In vitro</td>
<td>Animal</td>
<td>Human*</td>
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<td>Cardiomyopathy</td>
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<td>Peripheral neuropathy</td>
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<td>Lactic acidosis</td>
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<td>Hepatic steatosis</td>
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<td>Pancreatitis</td>
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<td>Nephropathy</td>
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<tr>
<td>Lipodystrophy</td>
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<tr>
<td>Bone marrow toxicity</td>
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<tr>
<td>Ototoxicity</td>
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</table>

*Human evidence of mitochondrial toxicity will depend on development of a case definition, followed by epidemiologic and intervention studies.

2. Consider conducting a pilot study to explore whether screening tests such as lactate levels can be collected and performed consistently at a wide range of research sites and are clinically relevant in detecting mitochondrial dysfunction. Determine the appropriate diagnostic markers for mitochondrial toxicity in HIV disease.

3. Develop a case definition for mitochondrial toxicity and conduct epidemiological studies to determine the prevalence.

4. With cooperation from industry, initiate a registry of mitochondrial toxicities in HIV-positive and HIV-negative pediatric populations exposed to antiretrovirals in utero. This registry would contribute to data on mitochondrial toxicities in infants and children, however, a formal epidemiologic study would be needed to develop prevalence estimates. International cooperation may be necessary for including HIV-positive children in the registry since perinatal prophylaxis now prevents most HIV transmission in the developed world.
Other Recommendations

On the second day of the Forum meeting, panelists split into three working groups to condense data and discussions from the first day and a half of sessions into outlines summarizing key issues and proposing areas for future research and analysis. The breakout groups focused on:

- Basic science of mitochondrial dysfunction
- Adult mitochondrial dysfunction
- Pediatric mitochondrial dysfunction

In addition to the principal recommendations, each group proposed other suggestions for future research and summaries of the issues discussed in their group.

Recommendations of the basic science breakout group

1. Drug development: Studies of new antiretrovirals should include in vitro analysis of human DNA polymerases and animal studies, that focus on mitochondrial pathophysiology.

2. Standardized animal models: Research should try to answer two questions: Is there an ideal animal model for mitochondrial toxicity? Because mitochondrial toxicities can take a long time to emerge, how long should animals be observed? Animal studies should focus on (1) combination therapy, (2) regimens with or without protease inhibitors, and (3) regimens with or without nucleoside analogs. A variety of tissues should be examined of the course of the experiment.

3. Standardized assays: Standardized assays with controls are needed for mitochondrial DNA and lactic acidosis. Development of a kit may resolve some problems.

4. Different mechanisms: Research should address whether antiviral therapy contributes to mitochondrial toxicities by mechanisms other than inhibition of polymerase gamma. Such factors may include oxidative stress, mutations, and altered glycosylation by zidovudine. It should be noted that these events are not mutually exclusive.

5. Other contributing factors: Genetic and environmental factors that may contribute to or prevent mitochondrial toxicity should be assessed, for example, nutrition, antioxidant supplementation, genetic susceptibilities, gender, and age. A microchip analysis of patient databases may be one approach to genetic questions. Such studies have not been done, even though powerful technologies are now available.

6. Compound availability: Researchers need easy access to new and existing nucleoside analogs.
**Recommendations of the adult mitochondrial dysfunction breakout group:**

1. Further investigate the histological features of mitochondrial dysfunction and establish a standard for tissue diagnosis.

2. Conduct a sub-study of HIV-positive patients with suspected mitochondrial toxicity to include an evaluation of 24-hour urine organic acid determinations. Quantitative urine organic acid determination by gas chromatography-mass spectrometry (GC-MS) is a mainstay in the diagnosis of mitochondrial disease and the exclusion of other metabolic disorders of non-mitochondrial origin of inherited mitochondrial and metabolic disorders. However, the proof of its usefulness in diagnosing mitochondrial toxicity from NRTI’s remains to be proven.

3. Include fasting blood samples to enable consistent measurements of new agents’ effects on lipid and glucose levels. Some participants recommended collecting fasting samples only in some trials or sub-studies of trials, to ensure that sample collection requirements would not dissuade a broad range of clinicians from participating in clinical trials.

4. Investigate ways of assessing the effects of interventions for mitochondrial toxicity.

**Recommendations of the pediatric breakout group**

Studies of mitochondrial toxicities in children should focus both on HIV-infected children and on uninfected children exposed to antiretrovirals in utero.

**HIV Infected child analysis**

1. Definitions of mitochondrial dysfunction should probably be unique to pediatrics.

2. Adult screening tools, such as echocardiography, should conform to pediatric standards. Echocardiograms should be interpreted by board certified pediatric cardiologists. Prevalence of toxicity in children should be determined. For example, what is the risk of myocardial involvement with individual drugs and drug combos?

3. Investigators should develop guidelines for pediatric surveillance and treatment.

**HIV Uninfected child analysis**

1. The prevalence of mitochondrial dysfunction and damage should be determined in early infancy, optimally at birth.

2. Definitions of transient and persistent mitochondrial dysfunction in children should be established. The clinical relevance of transient and persistent dysfunction should be established through follow-up, for example, at 2, 4, and 6 years after birth.
3. Uniform collection of risk factors could begin with the following framework:
   - History of maternal/infant antiretrovirals:
     - Dosing
     - Compliance
     - Duration
   - Route of administration
   - Environmental factors
   - Smoking
   - Illicit drug use
   - Drugs other than antiretrovirals
   - Nonmitochondrial risks

4. Studies of mitochondrial dysfunction should include controls, for example, children with hepatitis C virus infection or healthy controls.

5. Screening tools should be assessed in a pilot study that examines children at birth and 12 or more weeks later. These assessments should include standardized clinical exams, urine-organic acids (at 2 weeks) and amino acids, and lactate levels.

6. Persistent mitochondrial dysfunction in uninfected children should be studied by adapting new assays to evaluate children with persistent or recurrent manifestations. Such studies should also determine maternal risk of mitochondrial dysfunction.

**Pediatric Cardiomyopathy**

Although there is clear evidence for mitochondrial toxicity of nucleoside analogs at the basic science level and in animal experiments, the clinical relevance of this evidence with regard to cardiomyopathy is much less clear and these several key questions need to be addressed:

1. Is there a cumulative dose of zidovudine (AZT) or other NRTI's that puts the myocardium at risk?
2. Are there specific combinations of medications that may put the myocardium at risk?
3. Are children more susceptible to these effects than adults?
4. What is the ideal way to study this problem?
   a. Should guidelines be proposed for cardiac surveillance?
   b. Should a cardiac database be established that would standardize the way echocardiograms are performed? Many of these echocardiograms are performed by
adult cardiologists and do not meet pediatric standards. More stringent guidelines would yield better data.

c. Would funding be available to support cardiac surveillance?

5. What treatment guidelines should be developed to manage pediatric patients discovered to have cardiomyopathy while taking nucleoside analogs?
PRESENTATIONS

INTRODUCTION

What’s known and where should research focus?
Kees Brinkman, MD, and William Copeland, PhD, Co-chairs

Dr. Brinkman began the meeting by describing a case that first provoked him to study mitochondrial toxicity and, in so doing, he reminded meeting participants that a principal goal of these sessions is to propose ways to improve prevention, diagnosis, and management of these drug side effects, and so to improve patient care.

He began treating a newly-diagnosed HIV-infected woman with zidovudine (AZT), zalcitabine (ddC), ritonavir, and saquinavir. After 2 months, that regimen was changed to stavudine (d4T), lamivudine (3TC), and saquinavir because of AZT-induced anemia, ddC-induced neuropathy, and ritonavir-induced diarrhea. She responded well virologically and immunologically, but experienced acute nausea and vomiting 7 months after treatment began. Her triglycerides were elevated (8.1 mmol/L) and severe lactic acidosis and ketoacidosis developed, followed by arrhythmias and liver failure. The patient died 8 months after beginning her first antiretroviral regimen.

When discussion with colleagues yielded little insight into this patient’s unexpected course, Dr. Brinkman turned to the literature to pursue the clue provided by the diagnosis of lactic acidosis. His findings led to a published review of nucleoside-related mitochondrial toxicity (Brinkman K, et.al. AIDS 1998; 12(14):1735-44^) and finally a hypothesis suggesting a role for mitochondrial toxicity in the etiology of lipodystrophy. (Brinkman K, et al. Lancet 1999;354:1112-1115). Dr. Brinkman summarized the pathway of mitochondrial toxicity for participants.

- Nucleoside reverse transcriptase inhibitors (NRTIs) deplete mitochondrial DNA by inhibiting DNA polymerase gamma, which regulates replication of mitochondrial DNA.
- Mitochondrial dysfunction leads, for instance, to impaired energy production, impaired neutralization of free radicals, and oxidation of free fatty acids.

^References referred to by speakers in their presentations are either cited in the text or compiled in a list of key references. References for key publications and numbered footnotes may be found at the end of the report.
• Clinical symptoms that may be linked to mitochondrial toxicity include many already-identified NRTI side effects: polyneuropathy, myopathy, cardiomyopathy, hepatic steatosis, lactic acidosis, and pancreatitis.
• Different nucleosides may cause different mitochondrial toxicities because of differing tissue specificities. It is not known, for example, whether all NRTIs penetrate all cells equally well.

Several clinical and research questions about mitochondrial toxicity must be addressed:

• How should mitochondrial toxicity be studied?
• What is the incidence of mitochondrial toxicity in persons taking NRTIs?
• What risk factors predispose persons to mitochondrial toxicities?
• Are symptoms of mitochondrial toxicity reversible? (Evidence already suggests that some are not.)
• Are there effective treatments for mitochondrial toxicities?
• Can mitochondrial toxicity be prevented?

Dr. Copeland continued the introduction by spelling out five requirements for NRTI-induced mitochondrial toxicity:

1. The NRTI must penetrate susceptible cells.  
2. The NRTI must be triphosphorylated within the cell.  
3. The triphosphorylated NRTI must be transported to cellular mitochondria.  
4. The triphosphorylated NRTI must be incorporated into mitochondrial DNA.  
5. The triphosphorylated NRTI must persist in mitochondrial DNA.

Dr. Copeland proposed several goals for studies of mechanisms involved in mitochondrial toxicity:

• What is the level of cellular uptake and absorption of NRTIs?
• How much of the nucleoside analog gets converted into the active metabolite (that is, the triphosphorylated form)?
• How does the nucleoside analog get imported or transported into mitochondria?
• How much of the active metabolite of an NRTI is incorporated into mitochondrial DNA?
• Once incorporated, can the metabolite be removed from mitochondria by exonucleolytic proofreading?
• Which nucleoside analogs show the most or least inhibition of mitochondrial DNA in vitro?
• Which nucleoside analogs show the most or least inhibition of mitochondrial DNA in animal models?
• Which nucleoside analogs show the most or least inhibition of mitochondrial DNA in patients?
• How can animal model systems enhance the understanding of mitochondrial toxicity, and how do animal model findings relate to the clinical system?
• How do in vitro systems relate to the clinical system?
• What other mechanisms may cause or contribute to mitochondrial toxicity?
Among other research hurdles, Dr. Copeland listed limits on how much is known about intracellular and intramuscular concentrations of NRTI metabolites. He underlined the need for better systems to study effects of future NRTIs on nuclear as well as mitochondrial polymerases. And he called for continued development of animal models that can address these questions.
BASIC ASPECTS OF MITOCHONDRIAL DYSFUNCTION

Historical perspective on nucleoside analogs

Yung-Chi Cheng, PhD

NRTIs have a long history of use for cancer and viral diseases. Their primary mechanism of action is inhibition of DNA synthesis in target cells. Their selectivity for cancer cells or virally infected cells depends on subtle differences in deoxynucleotide or DNA metabolism in normal cells and target cells.

Trying to determine mechanisms of NRTI toxicity, Dr. Cheng and colleagues found that DNA polymerase gamma has high affinity for compounds such as zalcitabine (ddC). Further work showed that the nucleus of a cell contains one copy of DNA whereas mitochondria contain 300 to 1000 DNA copies. Whereas alpha, beta, delta, and epsilon polymerases can be found in nucleus, only polymerase gamma can be found in mitochondrial. In nuclei these polymerases are active only in proliferating tissues, whereas mitochondrial polymerase gamma is active in all tissues.

Work in Dr. Cheng’s laboratory showed that 0.2 µmol of ddC completely inhibits mitochondrial DNA synthesis. The NRTIs didanosine (ddI), stavudine (d4T), and zidovudine (AZT) also proved to inhibit mitochondrial DNA synthesis, but AraC did not. When mitochondrial DNA synthesis is inhibited, glycolysis provides the cell with energy. Lactic acid production is a marker of glycolysis and does not correlate with mitochondrial DNA depletion in the same way for every NRTI. This finding suggests that NRTIs exert toxic effects through mechanisms other than inhibiting mitochondrial DNA synthesis.

Dr. Cheng next addressed whether NRTI effects on mitochondrial DNA synthesis are reversible. To find out, he determined whether mitochondrial DNA doubling time—which decreases in the presence of NRTIs—returns to normal after an NRTI is removed from the culture system. Doubling time did return to normal levels when NRTI exposure stopped.

Further work sought to determine whether certain compounds can ameliorate the toxic effects of NRTIs on mitochondrial DNA. Among the compounds he studied was (-)SddCTP, later labeled 3TC. Dr. Cheng showed that (-)SddCTP does not inhibit mitochondrial DNA synthesis because it is not efficiently transported into mitochondria. That finding led him to suggest that (-)SddCTP (3TC) may reduce the mitochondrial toxicities of other NRTIs.
Dr. Cheng concluded that inhibition of mitochondrial DNA synthesis appears to be one of the key mechanisms associated with NRTI side effects. Whereas dideoxynucleoside analog action on mitochondrial DNA synthesis may be reversible, the same action by deoxynucleoside analogs may not be. However, deoxynucleoside or dideoxynucleoside analogs may exert an action against the mitochondrial DNA via some mechanism other than the inhibition of mitochondrial DNA synthesis.

**Effects of antiretroviral nucleoside analogs on DNA polymerase gamma activity**

*William Copeland, PhD*

Human mitochondrial DNA polymerase gamma is a two-subunit complex composed of 140 kDa and 55 kDa polypeptides. The first polypeptide (the catalytic subunit) is responsible for DNA polymerase activity, 3’-5’ exonuclease activity, and 5’-dRP lyase activity, whereas the second polypeptide (an accessory subunit) is a processivity factor and a DNA binding factor (1-3).

Why is polymerase gamma the most sensitive DNA polymerase in human cells? DNA polymerase gamma is the only replicative DNA polymerase that is strongly sensitive to most all of the anti-HIV nucleoside analogs such as dideoxynucleoside triphosphates and AZT triphosphate, whereas the other replicative DNA polymerases (alpha, beta, delta, and epsilon) show only weak inhibition by AZT triphosphate. Polymerase beta is strongly inhibited by dideoxynucleoside triphosphates but is a repair DNA polymerase and not a replicative one. (Table 1).

**Table 1. Eukaryotic DNA polymerases (4-7)**

<table>
<thead>
<tr>
<th>Replication</th>
<th>Alpha</th>
<th>Beta</th>
<th>Gamma</th>
<th>Delta</th>
<th>Epsilon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Nucleus</td>
<td>Nucleus</td>
<td>Mitochondria</td>
<td>Nucleus</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Inhibitors:</td>
<td>NaCL</td>
<td>Strong</td>
<td>None</td>
<td>None</td>
<td>Strong</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>Strong</td>
<td>None</td>
<td>None</td>
<td>Strong</td>
<td>Strong</td>
</tr>
<tr>
<td>NEM</td>
<td>Strong</td>
<td>None</td>
<td>Strong</td>
<td>Strong</td>
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</tr>
<tr>
<td>ddNTPs</td>
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<td>STRONG</td>
<td>Weak</td>
<td>Weak</td>
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<tr>
<td>AZT-TP</td>
<td>Weak</td>
<td>Strong</td>
<td>STRONG</td>
<td>Weak</td>
<td>Weak</td>
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</tbody>
</table>

Source: William Copeland, PhD

Dr. Copeland and colleagues cloned the human DNA polymerase gamma to assess the mitochondrial activity of NRTIs (8). He found that ddC and d4T triphosphates are strong inhibitors, and AZT, 3TC, and carbovir triphosphates are moderate inhibitors. Although 3TC triphosphate appears to
be as strong an inhibitor as AZT triphosphate in this system, it may not be efficiently transported into mitochondrial DNA.

Dr. Copeland also calculated a parameter for these nucleoside analogs called a discrimination factor, that is, a measure of how many nucleoside triphosphates are incorporated into DNA template for every normal nucleotide incorporated. The discrimination factors for ddC triphosphate and d4T triphosphate were low, 7 and 9, respectively. For carbovir triphosphate the factor was 2080; for 3TC triphosphate, 11,300; and for AZT triphosphate, 12,700.

Further work in Dr. Copeland’s laboratory sought to isolate key residues on polymerase gamma that recognize ribose structures. He discovered three such residues, including one that discriminated ribose from deoxyribose and one implicated in sensitivity to dideoxynucleosides. A single tyrosine residue allows incorporation of dideoxynucleosides into mitochondrial DNA. But when Dr. Copeland replaced that tyrosine with a phenylalanine, polymerase gamma discriminated against dideoxynucleosides (2).

Dr. Copeland also studied whether the 3’-5’ exonuclease of DNA polymerase gamma could remove nucleoside analogs from the 3’ terminus of DNA. The efficiency of removal by that mechanism proved to be poor for anti-HIV nucleoside analogs. Dr. Copeland believes that DNA polymerase gamma is the primary cellular target of NRTIs. Although incorporation of triphosphorylated NRTIs into mitochondrial DNA by polymerase gamma mediates mitochondrial toxicity, there are probably other routes to inhibition of mitochondrial DNA synthesis.

Clinical aspects of DNA polymerase gamma activity
Robert Naviaux, MD, PhD

The striking overlap between the symptoms of an inherited mitochondrial disease (i.e. Alpers syndrome) and the toxicities related to the nucleoside analogs zidovudine (AZT) and FIAU suggest that they are all mediated by the same mechanism—mitochondrial DNA polymerase gamma deficiency. All polymerases can be measured on different substrates: RNA, single-stranded DNA, and double-stranded DNA. Polymerase gamma is unique in using homopolymeric RNA and single-stranded, heteropolymeric DNA nearly equally well.
Dr. Naviaux and colleagues developed an in vitro assay that can detect mitochondrial polymerase from different tissue samples. He illustrated the use of this assay in a case report of a boy who developed normally until the age of 19 months, when a viral infection was followed by ataxia, encephalopathy, a hypotonic state, lethargy, poor feeding, and hypoglycemia. The assay showed that mitochondrial DNA content was 30% of normal in skeletal muscle and 25% of normal in liver.

Despite treatment for neurodegenerative disorders, the boy suffered six more setbacks, culminating in rotaviral gastroenteritis, liver failure, cortical blindness, and death. Liver biopsy showed steatosis and fibrosis, findings indicating chronic liver failure that had remained occult until the boy’s final illness. Magnetic resonance imaging of the brain had been completely normal despite profound seizures and encephalopathy. But autopsy revealed significant loss of neuronal and cerebellar Purkinje cells.

This case illustrates apparent developmental shutdown of polymerase gamma activity. Mitochondrial DNA depletion syndrome resembles Alpers syndrome in that both may be characterized by developmental regression, encephalopathy, hepatopathy, and sharp decreases in tissue levels of mitochondrial DNA.

In a roundtable discussion, Dr. Naviaux strongly recommended including 24-hour urine organic acid determinations in at least a substudy of HIV-positive patients with suspected mitochondrial toxicity. Quantitative urine organic acid determination by gas chromatography-mass spectrometry (GC-MS), he explained, is a mainstay in the diagnosis of mitochondrial disease and the exclusion of other metabolic disorders of nonmitochondrial origin at medical centers specialized in the diagnosis and treatment of inherited mitochondrial and metabolic disorders. When mitochondria fail, a number of metabolic intermediates appear that are the result of redox disturbances in carbohydrate metabolism, the Krebs cycle, and fatty acid and branched-chain amino acid metabolism. These signature metabolites may include: lactate, 3-methylglutaconic acid, fumarate, malate, 2-oxoglutarate, glutaric acid, adipic acid, suberic acid, 2-methylsuccinate, 2-methyl-3-hydroxy-isobutyric acid, ethylmalonic acid, 3-hydroxyisobutyric acid, tiglylglycine, transcinnamoylglycine, butyrylglycine, and isovalerylglycine. These metabolites accumulate as end products that are naturally concentrated in the urine by the kidneys. The concentration of these metabolites in the urine is frequently two orders of magnitude greater than concentrations observed in the plasma. For this reason organic acid determinations in urine have a much greater sensitivity than those from blood. Moreover, by obtaining 24-hour collections of urine for mass
spectrometric analysis, the natural circadian variations observed in the blood can be avoided, and an accurate picture of intermediary metabolism can be obtained. Dr. Naviaux volunteered to discuss providing this type of analysis for a clinical trial substudy.

In further discussion, one participant asked what research has determined about varying polymerase gamma levels in populations not taking NRTIs. He suggested that interindividual levels must vary substantially because some patients treated with NRTIs will have no evidence of mitochondrial toxicity, whereas others will suffer such toxicities no matter what NRTIs they take. Dr. Naviaux confirmed that polymerase gamma levels in muscle vary slightly from person to person, and that DNA sequence polymorphisms in the catalytic subunit do occur. However, the role of these factors in determining NRTI-sensitivity is unknown. In addition, pharmacogenetic variation in other gene products, such as those regulating tissue-selective drug import, phosphorylation and catabolism are expected to play important roles in determining individual susceptibility.
ANIMAL MODELS IN MITOCHONDRIAL RESEARCH

HIV-1 transgenic mice and AZT
William Lewis, PhD

Nucleoside analog reverse transcriptase inhibitors (NRTIs) serve as cornerstones of AIDS therapy. Early in the AIDS epidemic, NRTI complications appeared to be uncommon. The observation of zidovudine (AZT) mitochondrial myopathy in humans, and its recapitulation in vitro and in vivo, established mitochondrial toxicity as a bona fide side effect of NRTIs.

Factors that influence NRTI toxicity were articulated in the DNA polymerase gamma hypothesis. They include subcellular availability and abundance, the ability of the NRTI to be phosphorylated, the ability of its triphosphate moiety to inhibit mitochondrial DNA polymerase-gamma, and tissue requirements for oxidative phosphorylation. Mechanisms of NRTI toxicity may include mitochondrial DNA depletion or oxidative stress. Accordingly, research may disclose opportunities to treat these side effects.

Dr. Lewis described a series of hypotheses and animal experiments concerning the effects of AZT on mitochondrial DNA to define mechanisms for cardiomyopathy from AZT. He first hypothesized that long-term treatment with AZT caused or contributed to the pathogenesis of dilated cardiomyopathy in AIDS. In his initial experiments, electron microscopy showed disruption of mitochondria in hearts of rats treated with AZT, but the rest of the ultrastructural architecture in these animals remained intact. Mitochondrial RNA was depleted in AZT-treated rats, while levels of other RNAs from nuclear-encoded genes were similar to those of controls.

Dr. Lewis hypothesized that AZT triphosphate inhibits DNA polymerase gamma in skeletal muscle mitochondria and this results in defective replication of mitochondrial DNA. That process could be the subcellular mechanism causing myopathy associated with AZT therapy in people with HIV infection. Work by Dr. Lewis confirmed decreased mitochondrial DNA and RNA in skeletal muscle of rats treated for 35 days with AZT in water compared with control rats receiving water without AZT. Alterations in mitochondrial ultrastructure appeared after 5 weeks of treatment. These findings led to the hypothesis that AZT inhibits mitochondrial DNA replication in two ways based on his biochemical
studies: by competing with thymidine triphosphate binding to DNA polymerase gamma, and by terminating nascent mitochondrial DNA.

On a clinical basis, Dr. Lewis argued, it seems reasonable to suggest that AZT induces cardiomyopathy in AIDS by depleting mitochondrial DNA, but the mechanism has not been proven. Studies of didanosine (ddI) in Dr. Lewis’s laboratory detected no changes in cardiac mitochondrial ultrastructure in rats treated for 35 days with 500 mg/kg of ddI daily, or less.

FIAU is a thymidine nucleoside analog that was being developed to treat hepatitis B infection until it caused the deaths of several patients in early clinical trials. Dr. Lewis hypothesized that FIAU triphosphate inhibits polymerase gamma. FIAU administered to woodchucks, a model of hepatitis B virus infection, caused accumulation of lipid droplets and mitochondrial damage in cardiac, skeletal muscle, and liver cells, whereas control animals did not show those changes. “Lakes of lipids” in these FIAU treated cells were accompanied by mitochondrial ultrastructural changes. The animals treated with FIAU appeared sick.

In an attempt to distinguish the pharmacologic effects of AZT on cardiomyopathy from the effects of HIV itself, Dr. Lewis pursued studies in transgenic mice in which AIDS symptoms such as wasting and nephropathy develop. A “two-by-two” study design allowed him to compare AZT treatment versus no treatment in transgenic mice versus normal wild type mice. He detected early changes associated with heart failure in AZT-treated animals and to a lesser extent in transgenic untreated mice.

Ongoing work also shows altered aortic contractility of both transgenic mice and mice treated with AZT. This change appears to be principally related to alterations in relaxation. If confirmed, these findings would have apparent implications in the study of cardiovascular disease in humans being treated with antiretrovirals.

**Mitochondrial toxicity of antiretroviral nucleoside analogs in monkeys after transplacental exposure**

*Mariana Gerschenson, PhD*

Treatment of women with antiretroviral nucleoside analogs during pregnancy reduces levels of HIV and leads to decreased rates of HIV transmission to the fetus and to neonates during birth.
Dr. Gerschenson’s work addresses the genotoxic and functional consequences to the fetus after antiretroviral nucleoside analog exposure. The following evidence points to mitochondrial genotoxicity:

- AZT is incorporated into mitochondrial DNA
- AZT incorporation causes chain termination of replicating mitochondrial DNA, leading to depletion and/or degradation of mitochondrial DNA

Functional consequences of these events could include:

- Mitochondrial morphological abnormalities
- Oxidative phosphorylation enzyme activity alterations

In Dr. Gerschenson’s animal models, one group of pregnant *Erythrocebus patas* monkeys were treated with AZT at a dose of 6 mg/kg body weight (equivalent to human dosing during pregnancy) for the last 10 weeks (50%) of gestation. A second group of monkeys was treated with the same amount of AZT and additionally dosed with lamivudine (3TC) at 3.6 mg/kg body weight for the last 4 weeks of gestation. Fetuses were delivered by cesarian section, all major organs were obtained, and mitochondria were isolated from tissue and analyzed for morphological, biochemical, and genetic alterations.

Dr. Gerschenson’s presentation included data from fetal heart and skeletal muscle. Transmission electron microscopy indicated the presence of normal, intact mitochondria in heart and skeletal muscle not exposed to drugs *in utero*. Hearts of AZT-exposed fetuses had more mitochondria than those of control fetuses, and the mitochondria were structurally normal. Mitochondria in hearts of monkeys exposed to AZT and 3TC were more abundant than those of AZT-exposed fetuses and had an abnormal appearance. Skeletal muscle of AZT-exposed fetuses had disrupted myofibers, and those exposed to AZT and 3TC had disrupted inner and outer mitochondrial membranes. Exposure to AZT had no apparent effects on cerebellum, cerebrum, or placenta morphologies.

Complex I oxidative phosphorylation enzyme-specific activities in heart and skeletal muscle were significantly decreased in AZT-exposed fetuses compared with control animals and even more decreased in animals exposed to both AZT and 3TC (*P* ≤ 0.05 for both groups of exposed fetuses versus controls). Complex II and IV oxidative phosphorylation enzyme-specific
activities in heart were significantly increased in AZT-exposed fetuses compared with control animals ($P \leq 0.05$), but not in AZT plus 3TC-exposed fetuses.

Mitochondrial DNA levels in the heart and skeletal muscle of AZT-exposed monkeys decreased as doses of AZT increased; this inverse correlation was not seen in cerebrum or cerebellum. Exposure to 3TC plus AZT further decreased mitochondrial DNA levels in skeletal muscle, heart, cerebellum, and cerebrum.

Mitochondrial DNA degradation and depletion were demonstrated by Southern and slot blot in the heart, skeletal muscle, and cerebrum of AZT- and AZT plus 3TC-exposed fetuses. Lesser effects were seen in the cerebellum.

Dr. Gerschenson questioned whether enough AZT penetrates mitochondria to account for these changes. Although AZT levels varied greatly from animal to animal, she determined by radioimmune assays measurements that showed enough drug reaches mitochondria to cause depletion of mitochondrial DNA. She concluded that in utero exposure to AZT and 3TC, in doses and protocols similar to those used by pregnant women, is genotoxic and has functional consequences. The genotoxicity is evidenced by AZT incorporation into mitochondrial DNA and resulting depletion of mitochondrial DNA. Functional consequences include disruption of oxidative phosphorylation enzyme activity. These findings are consistent with earlier observations of AZT incorporation into mitochondrial DNA (Olivero et al. *J Natl Cancer Inst* 1997;86:1602) and suggest that incorporation truncates the DNA template in critical fetal target tissues.

Discussion following Dr. Gerschenson’s presentation raised three points:

1. A critical question is whether depletion of mitochondrial DNA is the ultimate cause of the defects detected.

2. It is possible that the defects documented by Dr. Gerschenson are species specific.

3. Future animal studies focused on mitochondria should examine adipose tissue to help determine whether mitochondrial toxicity has a role in lipodystrophy.
Background on Antiretroviral Nucleoside Analogues in Humans: History of Lactic Acidosis Reports

Barbara Styrt, MD, MPH

(Views are those of the speaker and do not necessarily represent the US FDA or US government)

Six antiretroviral nucleoside analogue reverse transcriptase inhibitors (NRTIs) are approved for marketing in the United States, zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), and abacavir. These approvals took place over more that a decade so the amount of experience with the different drugs varies widely. In 1993, Freiman and colleagues (Freiman JP, Helfert KE, Hamrell MR, Stein DS, “Hepatomegaly with severe steatosis in HIV-seropositive patients,” AIDS 1993;7:379-385) described 8 cases of hepatomegaly with severe steatosis in individuals who had received at least 6 months of NRTI therapy. Several had concomitant metabolic acidosis, pancreatitis, or myopathy. Six of them died. Most were women described as being mildly to moderately overweight.

Around the same time, fialuridine (FIAU), a nucleoside analogue being studied for treatment of hepatitis B infection, was associated with liver failure, lactic acidosis, pancreatitis, neuropathy, and myopathy in patients enrolled in pilot studies. Seven individuals died or needed liver transplants, and development of FIAU stopped.

An advisory committee of the US Food & Drug Administration (FDA) convened in September 1993 to consider the side effects of FIAU and related findings with zidovudine and other agents (Transcript, Antiviral Drugs Advisory Committee Meeting, “Mitochondrial damage associated with nucleoside analogues,” Food and Drug Administration, Rockville, MD, September 21, 1993). The committee concluded that quantitative and organ-specific relationships of mitochondrial DNA polymerase inhibition by these drugs remained unclear. Distinctive features of FIAU compared with other NRTIs included the findings that it is not a DNA chain terminator and that its elimination from tissue is prolonged. Initial attempts to estimate the incidence of events such as liver damage and lactic acidosis among people taking NRTIs were hampered by inadequate evidence for quantitative comparisons.
Measures to alert physicians to these potential complications of NRTI therapy, in addition to the public meeting discussion, included a June 1993 “Dear Doctor” letter and the addition of a boxed warning concerning hepatomegaly/steatosis and lactic acidosis to the zidovudine label.

A search of the FDA’s adverse event reporting system in 1995 yielded 15 to 17 additional reports of fatty liver with lactic acidosis among patients taking NRTIs (Styrt BA, Piazza-Hepp TD, Chikami GK, “Clinical toxicity of antiretroviral nucleoside analogs,” Antiviral Research 1996;31:121-135). As NRTI combinations became increasingly standard in the treatment of HIV infection over the next several years reports of lactic acidosis and hepatic steatosis continued. Boxed warnings describing these events—including possible risk factors and discussing suspension of therapy—were extended to the labeling of all approved antiretroviral NRTIs, and these label changes were announced in JAMA in 1998 (New Safety Information Added to Antiretroviral Package Inserts. In Nightingale SL, “From the Food and Drug Administration,” JAMA 1998;280:1128).

Additional information from adverse events entered into the FDA’s database of spontaneous adverse event reports was presented. Dr. Styrt noted that spontaneous reporting systems for drug side effects have certain limitations:

- Under-reporting, biased reporting, duplication, and time dependence
- Incomplete data, variable descriptions, complex cases
- Lack of controls, difficulty in distinguishing between drug and disease effects
- Causality and incidence difficult or impossible to determine

However, spontaneous reporting is valuable because it can bring rare events to attention and it can generate hypotheses for further study.

Reports of lactic acidosis in patients receiving antiretroviral NRTIs from the FDA databases of spontaneous adverse event reports were summarized in a poster at the 1999 Interscience Conference on Antimicrobial Agents and Chemotherapy (Boxwell DE, Styrt BA, “Lactic acidosis (LA) in patients receiving nucleoside reverse transcriptase inhibitors (NRTIs),” Abstract 1284, 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, California, 1999) (Table 2).
Table 2. **Clinical characteristics of lactic acidosis reported with combination NRTIs via spontaneous reporting**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical symptoms</td>
<td>1 to 6 weeks of nausea, vomiting, abdominal pain, weight loss,</td>
</tr>
<tr>
<td></td>
<td>malaise, dyspnea</td>
</tr>
<tr>
<td>Other diagnoses</td>
<td>Fatty liver, pancreatitis, creatine kinase elevation, triglyceride</td>
</tr>
<tr>
<td></td>
<td>elevation</td>
</tr>
<tr>
<td>NRTI use</td>
<td>Usually more than 6 months; specific NRTIs involved appeared to</td>
</tr>
<tr>
<td></td>
<td>parallel relative trends of use</td>
</tr>
<tr>
<td>Gender and weight</td>
<td>Predominantly female gender and high body weight not characteristic</td>
</tr>
<tr>
<td></td>
<td>or expected in NRTI-treated populations</td>
</tr>
<tr>
<td>Fatalities</td>
<td>Most reported cases were fatal; nonfatal cases included some with</td>
</tr>
<tr>
<td></td>
<td>the characteristic syndrome who recovered after stopping therapy,</td>
</tr>
<tr>
<td></td>
<td>and some with elevated lactates who lacked other manifestations</td>
</tr>
<tr>
<td></td>
<td>but received the same diagnostic code and were therefore</td>
</tr>
<tr>
<td></td>
<td>retrieved in the search</td>
</tr>
</tbody>
</table>

Source: Boxwell DE, Styrt BA. 1999 ICAAC poster 1284.

Dr. Styrt proposed the following conclusions concerning spontaneous reports of lactic acidosis with NRTIs:

- A characteristic syndrome is recognized in certain patients on long-term NRTIs (similar if searched as hepatomegaly, fatty liver, or lactic acidosis).
- The clinical presentation may be nonspecific, then catastrophic.
- Some patients die, some get better, and some have laboratory abnormalities without evidence of a clinical syndrome.
- The proportion of women, and of patients with elevated body weights, among reported cases is striking relative to expected use of NRTIs.
- Similar events are reported with different NRTIs.
- The syndrome overlaps with FIAU toxicity and with various organ system disorders that can be associated with mitochondrial dysfunction. But no marketed NRTI has a demonstrated risk/benefit ratio even remotely comparable to that of FIAU.

The quality and quantity of information from these spontaneous reports, Dr. Styrt suggested, do not permit:

- Calculation of incidence or relative risk
- Distinction between specific drug relationships and reporting bias
- Distinction between events of similar nature but varying severity and disparate clinical/laboratory events with differing implications
- Determination of mechanism
- Evaluation of effects of intervention

Dr. Styrt said further study would be needed to quantify possible associations with specific drugs and risk factors and to identify any interventions that might improve early recognition and outcome.
Metabolic and morphologic changes in HIV infection, disease, and therapy: Is there an association with mitochondrial toxicity?

Andrew Carr, MD

Dr. Carr summarized reports of lipodystrophy and lactic acidemia in a dozen cohorts, and specifically in the Australian cohort he studies. Among patients taking protease inhibitors (PIs), the lipodystrophy syndrome may be characterized by peripheral fat loss (lipoatrophy), central fat gain, dyslipidemia, insulin resistance, and, rarely, type 2 diabetes. But individuals who have been treated only with nucleoside reverse transcriptase inhibitors (NRTIs) often manifest related signs and symptoms, typically peripheral lipoatrophy with or without (1) abdominal distention, (2) lactic acidemia (>2.0 mmol/L), or (3) liver dysfunction.

In 10 cohorts of patients taking NRTIs with or without PIs, the prevalence of lipodystrophy ranged from 25% to 84%. In three cohorts of patients treated only with NRTIs, prevalence ranged from 17% (in a cohort of women) to 53%. In the Australian NRTI-only cohort, the prevalence was 32%. Table 3 shows the cumulative findings.

Table 3. Prevalence of lipodystrophy and lactic acidemia in 13 European, US, and Australian cohorts

<table>
<thead>
<tr>
<th>Therapy</th>
<th>n</th>
<th>Percent with lipodystrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any</td>
<td>6192</td>
<td>56</td>
</tr>
<tr>
<td>NRTI + PI</td>
<td>3972</td>
<td>62</td>
</tr>
<tr>
<td>NRTI ± PI</td>
<td>1552</td>
<td>49</td>
</tr>
<tr>
<td>NRTI</td>
<td>526</td>
<td>35</td>
</tr>
</tbody>
</table>

Source: Andrew Carr, MD, based on 13 cohorts reported in 1999.

In the Australian cohort, Dr. Carr compared physical and laboratory findings in five groups: 14 treated only with NRTIs and with lipodystrophy syndrome (NRTI+LD+), 28 treated only with NRTIs without lipodystrophy (NRTI+LD-), 102 treated with NRTIs and PIs with lipodystrophy (NRTI/PI+LD+), 44 treated with NRTIs and PIs without lipodystrophy (NRTI/PI+LD-), and 32 antiretroviral-naive persons without lipodystrophy (naive).

Among the 14 NRTI+LD+ individuals, 10 stopped taking their NRTIs, three were hospitalized, and one died with liver failure. Nausea and fatigue improved in some but not all individuals who discontinued NRTIs. Mean weight increased 2.5 kg after NRTIs were stopped but had not returned to
pretreatment levels at the time of Dr. Carr’s report at the Forum meeting.

In the NRTI groups, more patients with lipodystrophy than without lipodystrophy had more than a 3-kg loss in body weight (79% vs. 4%, \( P < 0.0001 \)), fatigue (79% vs. 14%, \( P < 0.0001 \)), and nausea (71% vs. 7%, \( P < 0.0001 \)). In the NRTI/PI groups, those with and without lipodystrophy did not differ significantly in these measures, and rates of these measures were substantially lower than in the NRTI+LD+ group. Clinical findings in the NRTI+LD+ group included hepatomegaly, ascites, peripheral edema, peripheral neuropathy, dyspnea, breast enlargement, and encephalopathy. None had myopathy, which is usually considered a mitochondrial toxicity.

Nearly all cohort members had average pretreatment weights. Unlike the FDA finding that women were predominantly affected by NRTI-associated lactic acidosis, these Australian patients with lactic acidemia were all men, a finding reflecting the distribution of HIV infection in Australia.

Current treatment with stavudine (d4T) (\( P = 0.003 \)) and duration of d4T use (\( P = 0.02 \)) correlated with lipodystrophy and lactic acidemia in this cohort, whereas current treatment with zidovudine (AZT) had what seemed a protective effect (\( P = 0.01 \)).

Average serum lactates were normal (<2.0 mmol/L) in all the groups studied except the NRTI+LD+ group (4.6 mmol/L, \( P < 0.0001 \)). Significantly more people in the NRTI+LD+ group than in the other groups had lactates greater than 2.0 mmol/L (86%, \( P < 0.0001 \)). Lactate levels have not returned to normal in all those who stopped taking their NRTIs. Levels of triglycerides and cholesterol were substantially higher in the NRTI/PI+LD+ group (4.0 and 6.0 mmol/L, respectively) than in the NRTI+LD+ group (2.0 and 5.2 mmol/L, respectively). Dr. Carr was unable to distinguish any biochemical marker change that preceded symptoms of lipodystrophy.

In a multivariate analysis comparing the NRTI+LD+ and NRTI+LD- groups, older age, higher CD4-cell count, current d4T use, and total duration of NRTI use correlated with lipodystrophy and lactic acidemia (Table 4). That last finding, Dr. Carr said, suggests that all NRTIs ever taken—not just NRTIs currently being taken—influence the emergence of this syndrome.

Table 4. Possible risk factors for lipodystrophy and lactic acidemia in patients taking NRTIs but not PIs (n = 42) *

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*Mitochondrial Toxicity and HIV Disease* 24
In the Western Australian cohort, factors that correlated with increased risk of lipodystrophy and lactic acidemia were older age, white race, time on dual NRTI therapy before HAART, and total time on d4T. Total time on nevirapine, a nonnucleoside reverse transcriptase inhibitor, correlated with a reduced risk of this syndrome.

Dr. Carr compared multiple symmetric lipomatosis (MSL) type 1 (a lipodystrophy-like syndrome in persons not taking antiretrovirals) with lipodystrophy related to highly active antiretroviral therapy (HAART). This comparison seen in Table 5 highlighted several distinctions between these conditions. Participants pointed out that this comparison raises serious question regarding a link between MSL type 1 and lipodystrophy.

Table 5. **HAART-related lipodystrophy compared with multiple symmetric lipomatosis type 1**

<table>
<thead>
<tr>
<th></th>
<th><strong>HAART lipodystrophy</strong></th>
<th><strong>MSL type 1</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol abuse</td>
<td>No</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Body mass index</td>
<td>Normal</td>
<td>Normal/low</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>No</td>
<td>90%</td>
</tr>
<tr>
<td>Intra-abdominal fat deposition</td>
<td>Common</td>
<td>No</td>
</tr>
<tr>
<td>Peripheral lipoatrophy</td>
<td>Common</td>
<td>Possible</td>
</tr>
<tr>
<td>Lipomata</td>
<td>5% to 10%</td>
<td>100%</td>
</tr>
<tr>
<td>Thoracic lipomata</td>
<td>No</td>
<td>Occasional</td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>Common</td>
<td>Common</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>Common</td>
<td>Less common</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>Common</td>
<td>Uncommon</td>
</tr>
<tr>
<td>Lactic acidemia</td>
<td>Occasional</td>
<td>Not known</td>
</tr>
</tbody>
</table>

Source: Andrew Carr, MD.

Considering the role of individual NRTIs in lipodystrophy and lactic acidemia, Dr. Carr noted a lack or correlation between in vitro and in vivo effects. Long-term exposure tends to implicate drugs currently being taken. Concomitant use of hydroxyurea selects for preferential exposure to certain NRTIs. Clinical studies suggesting preferential toxicity by certain NRTIs have been nonrandomized, but

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* Factors that did not correlate with lipodystrophy/lactic acidemia were duration of HIV infection, current use of didanosine (ddI) or lamivudine (3TC), or duration of d4T or AZT use.

Source: Andrew Carr, MD.
randomized studies have begun.

**HIV lipodystrophy and mitochondrial toxicity: An open question**

*Richard Gregg, MD*

Dr. Gregg’s presentation underscored the complexity of lipodystrophy syndromes and mitochondrial dysfunction, and the consequent difficulty in linking the two. The differentiation and morphogenesis of fat is complex, he said, as are the diverse metabolic syndromes. Such syndromes, including HIV-associated lipodystrophy, may have many causes. In addition, mitochondrial dysfunction has multiple phenotypes.

Dr. Gregg began his analysis of these issues by discussing adipogenesis, the formation of fat cells. Preadipocyte precursor cells exist in adipose depots. They mature into adipocytes through complex patterns of gene activation. Maintenance of adipose depots involves continual recruitment of preadipocytes, and hormonal influences are critical in that process. Regulation of regional differences in adiposity is not understood.

There are several potential sites of interference in preadipocyte conversion: (1) initial proliferation, followed by growth arrest, (2) hormonal induction/clonal expansion, and (3) final mitosis and terminal differentiation.

Dysmetabolic syndromes in general are characterized by dyslipidemia (elevated triglycerides and small dense LDL, low levels of HDL), insulin resistance (leading to hyperinsulinemia and possibly diabetes), abdominal obesity, hepatic lipid accumulation, and hypertension. These factors all increase the risk of atherosclerosis.

Lipodystrophy can be categorized as follows:

- Congenital forms
- Acquired forms
- Cachectic/catabolic states (resulting from cancer, major trauma, surgery, or sepsis)

The basic features of lipodystrophy syndromes are a paucity of subcutaneous and other adipose tissue depots and impaired energy storage leading to metabolic alterations. These alterations may lead to hyperinsulinemia, hypertriglyceridemia, and increased appetite.
Genetic lipodystrophy syndromes, such as congenital generalized lipodystrophy and familial partial lipodystrophy, are rare autosomal-recessive disorders. They manifest themselves in childhood, adolescence, or midlife. About 100 patients with congenital generalized lipodystrophy have been described. This condition is marked by nearly complete absence of metabolically active adipose tissue, preservation of mechanical adipose tissue, muscle hypertrophy, elevated triglycerides, and severe insulin resistance. The molecular cause remains unknown.

Familial partial lipodystrophy (also call Dunnigan type; FPLD) is characterized by gradual, extreme loss of subcutaneous adipose tissue in the extremities, excess fat on the face and neck, reduced truncal fat (with some abdominal depots preserved), and preserved intermuscular mechanical adipose tissue. The molecular defect leading to FPLD has only recently been described (Cao and Hegele, Hum. Mol. Gen. 9:109(2000)). This was the first report of a mutation underlying a human degenerative disorder of adipose tissue.

The molecular defect in PLD is a missense mutation in the gene for nuclear lamin A/C, a structural component of the nuclear envelope (Shackleton et al., Nat. Gen. 24: 153 (2000)). However, the known functions of lamins A and C do not yet provide a clear understanding of the molecular mechanisms for the tissue specificity and metabolic dysregulation in FPLD.

Acquired lipodystrophy syndromes are progressive partial lipodystrophy and acquired partial lipodystrophy. Progressive partial lipodystrophy is often preceded by infection, autoimmune disease, or stress. Its pathogenesis involves cytotoxic lymphocytes and autoantibodies. Acquired partial lipodystrophy, more common than progressive partial lipodystrophy, is marked by progressive loss of facial and neck adipose tissue and sparing of distal subcutaneous fat depots. Other features are mesangiocapillary glomerulonephritis and dysfunction of the complement system.

Researchers have proposed mitochondrial dysfunction as the cause of multiple symmetric lipomatosis (Klopstock et al. Mol Cell Biochem 1997), a rare lipodystrophy syndrome also known as Madelung’s disease. This syndrome is characterized by large subcutaneous fat masses on the neck, and sometimes on the shoulders and other parts of the trunk. Neurologic complications, including peripheral neuropathy, are also seen in people with the syndrome. Multiple symmetric lipomatosis mostly affects men, often alcoholics, and it has a midlife onset. Factors that suggest a mitochondrial etiology are (1)
ragged red fibers in muscle biopsies, (2) reduced cytochrome C oxidase activity, and (3) mitochondrial DNA deletions or mutations.

Several cytokines are implicated in cachexia and insulin resistance. Tumor necrosis factor (TNF)-alpha, interleukin (IL)-1, and interferon (IFN)-gamma can cause anorexia and have catabolic effects on adipocytes. TNF-alpha has several direct effect on culture adipocytes: (1) induction of lipolysis and depletion of adipocyte triglyceride pools, (2) downregulation of lipogenic enzymes, (3) loss of transport activity, and (4) impaired insulin signaling. These changes have not been replicated in isolated adipocytes and only partially replicated in vivo.

The roles of these cytokines in cachexia and insulin resistance are important because HIV induces proinflammatory cytokines. HIV infection can cause 10-fold elevations in IL-1a, IL-2, IL-12, and IFN-gamma. Levels of IL-10 and TNF-alpha also rise during infection. Treatment with highly active antiretroviral therapy can lower, but not normalize, levels of certain cytokines.

IL-4 is another link between adipose function and the immune system. Recent work detected IL-4 and a novel, resident pericapillary T lymphocyte in human adipose tissue, where IL-4 promotes adipocyte differentiation (Smith and Hausman, *Int J Obesity*;24:S27(2000). IL-4 colocalized with CD43, a T-cell marker. These provocative findings suggest that T cell-adipocyte interactions may be closely linked to the development of lipodystrophy in HIV-AIDS.

Table 6 summarizes the etiology of lipodystrophy syndromes.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Etiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital generalized lipodystrophy</td>
<td>Genetic, molecular cause unknown</td>
</tr>
<tr>
<td>Familial partial lipodystrophy</td>
<td>Genetic, lamin A/C R482Q mutation</td>
</tr>
<tr>
<td>Madelung’s disease</td>
<td>Alcoholism, mitochondrial dysfunction</td>
</tr>
<tr>
<td>Acquired partial lipodystrophy</td>
<td>C3-NeF, autoantibodies</td>
</tr>
</tbody>
</table>

Source: Richard Gregg, MD

Dr. Gregg outlined three theories of metabolic imbalances in HIV infection:

- Protease inhibitors and/or nucleoside reverse transcriptase inhibitors → mitochondrial toxicity → lipodystrophy → metabolic imbalances

- HIV infection → immune dysfunction → cytokine imbalances → adipose tissue dysregulation → lipodystrophy → metabolic imbalances
In summary, Dr. Gregg stressed four points:

1. There are several forms of genetic and acquired lipodystrophy.
   - Molecular defects and etiologies vary.
   - Mitochondrial involvement is not a general feature.

2. Mitochondrial diseases are diverse.
   - Encephalopathies, neuropathies, myopathies
   - Adipose dysfunction atypical

3. Regional regulation of fat depots is very complex.
   - Multiple levels of regulation are not understood.

4. Adipose biology and immune function are interrelated.
   - Complement factors have a role in energy balance.
   - Cytokines profoundly affect adipocytes.

Tissue specificity and other clinical aspects of mitochondrial toxicity of antiviral nucleosides

Patrick Chariot, MD

Dr. Chariot began his presentation by outlining the early history of mitochondrial toxicity associated with nucleoside analog reverse transcriptase inhibitors (NRTIs). Mitochondrial toxicity of NRTIs was first recognized in 1990 when Marinos Dalakas reported in the New England Journal of Medicine a series of patients with zidovudine (AZT)-induced mitochondrial myopathy. At that time, AZT was the only NRTI available for antiretroviral therapy and the first example of a drug that could induce a mitochondrial disorder.

The next NRTIs to be licensed were didanosine (ddI) and zalcitabine (ddC). ddC is responsible for a peripheral neuropathy associated with accumulation of mitochondria; ddI can induce rhabdomyolysis and a liver disease manifested histologically by microvacuolar steatosis, which is the most typical aspect of mitochondrial hepatic dysfunction. Stavudine (d4T), the next NRTI to become available, was responsible for a number of cases of lactic acidosis.

Now, most HIV-infected patients receive combination therapies, which have dramatically
improved the prognosis of the disease. But from a toxicologic point of view, combination regimens have made the effects of individual drugs difficult to understand, since almost all organs or tissues can be involved. One can easily imagine that combination therapies potentiate toxicities of individual agents, but a clear demonstration of that hypothesis is still lacking. Because HIV infection has now become a chronic disease, another important recent issue concerns the long-term side effects of antiretroviral treatments.

Dr. Chariot summarized the most typical manifestations of NRTI toxicity that may involve mitochondrial dysfunction (Table 7).

Table 7. NRTI toxicities that may involve mitochondrial dysfunction

<table>
<thead>
<tr>
<th>Target organ or tissue</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulatory system</td>
<td>Anemia, neutropenia</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>Myalgia, proximal weakness, rhabdomyolysis</td>
</tr>
<tr>
<td>Liver</td>
<td>Micro- or macrovacuolar steatosis, with or without liver failure</td>
</tr>
<tr>
<td>Heart</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>Peripheral nerves</td>
<td>Axonal polyneuropathy</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Acute pancreatitis</td>
</tr>
<tr>
<td>Metabolic system</td>
<td>Lactic acidosis</td>
</tr>
</tbody>
</table>

Source: Patrick Chariot, MD

The toxic manifestations of NRTIs are tissue- or organ-specific. In genetically-induced mitochondrial disorders, clinical involvement of a given organ grossly parallels the percentage of mutant molecules of mitochondrial DNA found in the organ, and this percentage varies widely from one organ to another in a given individual, and from one individual to another, even within the same family. In toxic mitochondrial disorders due to NRTIs, there is also some evidence that clinical involvement of a given organ parallels mitochondrial DNA depletion (J Hepatol 1999; 30:156-160). Toxic manifestations also vary from one compound to another. For AZT, the main target organs or tissues are blood, skeletal muscle, myocardium, and liver; for ddI, pancreas, nerve, and liver; for ddC, peripheral nerves and pancreas; for d4T, peripheral nerves and liver. Lamivudine (3TC) seems to be far less toxic than other compounds, but some cases of blood and liver involvement have been reported.

FIAU, an investigational NRTI, proved to be toxic to liver, skeletal muscle, pancreas, and nerves. Some lessons can be drawn from the FIAU trials, in which five patients with hepatitis B virus infection died. Retrospective analysis of FIAU pilot studies revealed that the underlying chronic hepatitis
B and HIV infection may have obscured FIAU-induced hepatotoxicity. Dr. Chariot warned that all events in the course of a treated chronic disease should now be considered possible manifestations of drug toxicity.

Because AZT was the first NRTI used to treat HIV infection and is still widely used in current combination therapies, most clinical studies and most reports on NRTI-associated mitochondrial toxicity focus on this drug. AZT is known to be responsible for a mitochondrial myopathy manifested as myalgia, proximal weakness or fatigue, elevated blood creatine kinase, ragged red fibers on muscle biopsy, myofilamentous abnormalities, lipid droplet accumulation, cytochrome C oxidase (COX) deficiency, elevated blood lactate/pyruvate ratios, and mitochondrial DNA depletion in muscle. COX is complex IV of the mitochondrial respiratory chain and is encoded by both mitochondrial DNA and nuclear DNA. SDH is part of complex II and is entirely encoded by nuclear DNA, which could explain why an overexpression is observed in most cases and not a deficiency.

Electron microscopy confirms the existence of abnormal mitochondria. During 10 years in the hospital in Creteil, Dr. Chariot and colleagues found that AZT myopathy was by far the most frequent cause of myopathy in HIV-positive patients. The most sensitive test for detecting AZT-associated mitochondrial toxicity is histochemical analysis for COX (Ann Neurol 1993;34:561-565). Normal type I or type 2 fibers are brown, while abnormal deficient fibers are not colored in this analysis. In this way AZT-associated myopathy can easily be distinguished from muscle symptoms in HIV-positive patients not taking AZT. Histochemical analysis for COX is also useful in demonstrating that mitochondrial dysfunction involves both skeletal and smooth muscle fibers (Neuropathol Appl Neurobiol 1995;21:540-547). A consequent hypothesis of these findings regarding vessels is that some ischemic manifestations in the central nervous system could be explained by NRTI-induced mitochondrial toxicity.

When muscle biopsy is impossible to perform, either for technical reasons such as the absence of an adequate myopathology unit or because of patient refusal, evaluation of blood lactate/pyruvate ratios can be useful, since most patients with AZT myopathy have an impaired redox status and an elevated lactate/pyruvate ratio in blood (Arthritis Rheum 1994;37:583-586).

From a practical point of view, blood lactate and pyruvate determinations have a bad
reputation. They are purportedly unreliable and difficult to use in clinical studies. Dr. Chariot has found that immediate deproteinization and control of pH in pyruvate assays are two critical points in determining the blood lactate/pyruvate ratio, while absence of venostasis was less important. The lactate/pyruvate ratio increases significantly when adequate deproteinization is not immediately performed (Arch Pathol Lab Med 1994;118:695-697).

The tissue specificity of NRTI toxicity has seldom been studied in humans. Dr. Chariot and coworkers reported the case of an HIV-infected patient treated with AZT who had liver steatosis, lactic acidosis, and typical features of AZT myopathy. Southern blot analysis demonstrated mitochondrial DNA depletion in liver and muscle tissue, and not in myocardium and kidney, which means that clinical and histologic involvement paralleled molecular findings regarding mitochondrial DNA (J Hepatol 1999,30:156-160).

To address the question of muscle tissue specificity, Dr. Chariot and colleagues compared the in vitro toxicity of AZT, ddI, and ddC in cultured human muscle cells (J Neurol Sci 1997;149:19-25). They found that all three drugs led to decreased proliferation and differentiation of muscle cells, increased lactate production, and decreased COX and SDH activity. Dr. Chariot observed only mild differences between the three NRTIs, despite obviously different clinical toxicity characteristics in skeletal muscle. Such negative studies illustrate the difficulty in characterizing the mechanisms of tissue specificity of NRTI toxicity.

Dr. Chariot listed the following unanswered questions about mitochondrial toxicity:

- Is mitochondrial dysfunction involved in the HIV lipodystrophy syndrome?
- Is mitochondrial damage biologically reversible?
- How should clinicians characterize patients with different treatment-related complications? (According to risk factors such as oxidative stress and viral coinfections?)
- In combination therapies, what is the respective role of each agent in a toxic disorder?

To evaluate some risk factors for mitochondrial dysfunction, such as oxidative stress, Dr. Chariot and coworkers evaluated the selenium and vitamin E status of patients with or without muscle symptoms (Muscle Nerve 1997;20:386-389). They found that plasma selenium was significantly lower in patients with muscle involvement than in patients without muscle symptoms, while vitamin E status was similar in both groups.
Dr. Chariot concluded with suggestions for further studies:

- Evaluation of blood lactate/pyruvate ratios in patients taking various NRTI combinations
- Quantification of mitochondrial DNA in various tissue samples, including adipose tissue, obtained from biopsied organs or at autopsy.
- Evaluation of lactate production and mitochondrial function in cells cultured with combinations of antiviral drugs.

**Mitochondrial dysfunction and perinatal exposure to nucleosides: an epidemiologic evaluation**

*Stephane Blanche, MD*

The deaths of two children participating in a study in France evaluating tolerance to zidovudine (AZT) plus lamivudine (3TC) to prevent mother-to-child transmission of HIV led Dr. Blanche and colleagues to hypothesize that perinatal exposure to nucleoside analogs may cause mitochondrial toxicity. These two children were clearly suffering from mitochondrial disease. The known mitochondrial toxicity of nucleosides, the rarity of genetic mitochondrial diseases, and preliminary results in an animal model all argued that these cases resulted from drug toxicity to mitochondria.

Dr. Blanche noted that this hypothesis generated much criticism and incredulity, and this incredulity persists, for a variety of reasons. Some suggest that the observations do not correspond to mitochondrial diseases. The clinical, radiological and biological data were coherent and similar to what is observed in inherited mitochondrial disease. Others believe that these mitochondrial diseases are genetic in origin, pointing out that the frequency of these conditions in the general population is not known. In addition, some have expressed doubts that these children suffered persistent toxicity, since no mitochondrial DNA depletion was observed. Such DNA depletion is the hallmark of muscular mitochondrial toxicity, as described in adults treated with AZT. Finally, critics noted that this phenomenon does not seem to have been observed elsewhere, at least as assessed from mortality rates.

Without making *a priori* judgments about the possible pathophysiologic basis of this phenomenon, one can employ epidemiologic methods to substantiate the hypothesis. This approach, Dr. Blanche explained, involves evaluating the frequency of mitochondrial diseases in a group of children.
with a known number of members followed prospectively. Prospective follow-up studies of infants born to HIV-positive mothers being conducted in industrialized countries allow this type of analysis, provided that the follow-up study is not based on a register, because the recruitment bias of register-based studies is considerable. In addition, the sample must be large enough to facilitate analysis of the putative toxicity at low frequency. A highly detailed analysis of each suspected file is required, with complementary evaluation for the children concerned, because the information collected for HIV surveillance and contained in the database are never sufficient to confirm or exclude the diagnosis of mitochondrial disease.

The French pediatric survey that includes more than 5000 mother-child pairs recruited since 1986 with both infected and uninfected children, enabled Dr. Blanche and colleagues to perform such an epidemiologic study on over 4000 uninfected children. However, the clinical and biologic heterogeneity of mitochondrial diseases creates substantial diagnostic problems. Bearing in mind these difficulties, the French investigators systematically screened the French cohort for clinical and/or biologic symptoms of mitochondrial dysfunction. Every patient file was individually analyzed in detail in collaboration with the center following the child. The cases were classified on the basis of their files by reviewers blinded to the each child’s exposure to antiretroviral agents. In practice, this meant not knowing the child’s date of birth.

As would be expected for pediatric mitochondrial disease, the list of symptoms used for screening was extremely long. Because no such screening had ever been performed, Dr. Blanche noted, it is difficult to assess the efficacy of this approach.

The French investigators used the database of the French perinatal survey for March 1, 1999 and considered only children not infected by HIV. Among all children in the survey at that date, 1842 had not received perinatal antiretroviral treatment. Most of them entered the register between 1994 and 1996. Another 2208 children received at least one antiretroviral. For 33 children, data concerning antiretroviral treatment were missing, contradictory, or incomplete.

This analysis identified 124 cases with one or more symptoms that could indicate mitochondrial toxicity. Ninety had neurologic symptoms, although neurologic symptoms are undoubtedly the least specific. Twenty-three of the children identified had not received antiretroviral treatment, and 100 had been treated during the perinatal period. Treatment information for one child was missing.
Dr. Blanche and colleagues classified these 124 cases into three groups.

1. In 43 children, symptoms spontaneously disappeared, so no conclusion could be drawn about possible mitochondrial toxicity.
2. In 19 children, the symptoms could be explained by a clearly identified nonmitochondrial disease, and in 37 children, the corresponding data could be obtained or is not yet available.
3. In the remaining 25 children, mitochondrial dysfunction was judged possible, with three degrees of probability: weakly plausible, strongly suspected, or proven by enzymology or histology.

All but one of 25 children with possible mitochondrial dysfunction had been exposed to antiretroviral agents. Data for the one exception were conflicting, and therefore the child was considered not to have been exposed. Diagnostic difficulties and clinical heterogeneity led Dr. Blanche and colleagues to classify the cases into three groups: weak suspicion (9 children), strong suspicion (6 children), and proven (10 children).

Some of this information was published in *The Lancet* in 1999 (Blanche S, et al. *Lancet* 1999;354:1084-1089). Dr. Blanche noted, however, that one of the *Lancet* cases is not included in the current analysis because the child was not a member of the same study population: He was transferred to a center participating in the study shortly after birth, but he had not been born in the participating maternity unit. Therefore this case was excluded to ensure epidemiologic rigor.

Since this publication, 3 further children in the survey proved to have mitochondrial disease. Two of these children have severe neurologic disorders, and one has high blood lactate levels. Dr. Blanche added that the brother of one of the cases reported in *The Lancet* is also affected, suffering a form of persistent high blood lactate at the age of 2 years and significant histologic abnormalities.

Table 8 lists the enzymatic anomalies found in 9 of the 10 children with proven mitochondrial disease. The results are expressed as ratios to one of the other complexes, complex III. The anomalies all involve complex I, complex IV, or both. None of these children showed significant mitochondrial DNA depletion.
Table 8. Respiratory chain defects in 9 antiretroviral-exposed (but untreated) children with mitochondrial disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>Complex III/Complex IV (% of control ratio)</th>
<th>Complex III/Complex I (% of control ratio)</th>
<th>Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 liver</td>
<td>93</td>
<td>211</td>
<td>Complex I</td>
</tr>
<tr>
<td>2 liver</td>
<td>199</td>
<td>110</td>
<td>Complex IV</td>
</tr>
<tr>
<td>3 muscle</td>
<td>240</td>
<td>272</td>
<td>Complexes I and IV</td>
</tr>
<tr>
<td>4 muscle</td>
<td>133</td>
<td>279</td>
<td>Complex I</td>
</tr>
<tr>
<td>5 muscle</td>
<td>185</td>
<td>80</td>
<td>Complex IV</td>
</tr>
<tr>
<td>6 muscle</td>
<td>201</td>
<td>78</td>
<td>Complex IV</td>
</tr>
<tr>
<td>7 muscle</td>
<td>181</td>
<td>90</td>
<td>Complex IV</td>
</tr>
<tr>
<td>8 muscle</td>
<td>175</td>
<td>130</td>
<td>Complex IV</td>
</tr>
<tr>
<td>9 muscle</td>
<td>295</td>
<td>206</td>
<td>Complexes I and IV</td>
</tr>
</tbody>
</table>

Prepared by Pierre Rustin.

Besides these 10 proven cases, Dr. Blanche added, the overall biologic and clinical picture for six further children was compatible with mitochondrial disease. Four suffered neurologic problems of diverse severity and persistent, substantial high blood lactates.

In total, then, the French investigators counted 16 cases of proven or strongly suspected mitochondrial disease among 2208 exposed children. There was only 1 such case among the children not exposed to antiretrovirals or for whom information concerning the mother’s treatment was insufficient. The difference is highly statistically significant ($P < 0.004$).

Among the limitations of this type of study, Dr. Blanche said the most important is that the exposed and nonexposed children are not evenly or randomly distributed in time, because treatment to prevent perinatal transmission was introduced in 1994.

Dr. Blanche proposed that persistent inhibition of polymerase gamma is not a likely cause of mitochondrial disease in these children. Such inhibition is believed to be transitory, reversible after treatment, and probably associated with depletion of mitochondrial DNA, which was not observed in the French pediatric cases. Dr. Blanche speculated that there might be secondary alteration of the mitochondrial DNA, or a deleterious effect of the integration of the nucleosides into the DNA.

Other issues remain unresolved, such as the role of nucleoside pharmacokinetics during this period of metabolic immaturity. There may also be a genetic susceptibility to this toxicity, which may involve pharmacokinetic parameters or mitochondrial function, or either mitochondrial or nuclear DNA.
In parallel with this analysis, Dr. Blanche and colleagues have undertaken a systematic prospective study of lactate levels in these children’s blood at various times during and after treatment. The aim is to assess the incidence of high lactate concentrations. Again, the French team is aware of the major methodologic difficulties in interpreting such assays, which are subject to numerous artifacts. To minimize this problem as much as possible, the French pediatric network is using a standardized protocol for sample collection and analysis. Dr. Blanche described some of the preliminary findings of this effort.

At 1 month of age, and thus during antiretroviral treatment, 33% (n=144) percent of the children have up to 3 mmol/L lactate concentrations, whether before or after feeding. The percentage is 18% (n=288) at 6 months but still significant. Although it difficult to assess the contribution of artifacts to these values, Dr. Blanche pointed out a significant correlation between high lactate levels at 6 months and other signs of toxicity. For example, children with high blood lactates have lower hemoglobin counts at various time points. A relational analysis with the mother’s treatment is currently underway.

**Neurologic symptomatology in the French Pediatric Cohort**

*Marc Tardieu, MD*

In 8 children with mitochondrial dysfunction after in utero exposure to zidovudine (AZT) or AZT plus lamivudine (3TC) (Blanche S, et al *Lancet* 1999;354:1084-1089), most symptoms involved neurologic lesions. Dr. Tardieu reported three patterns:

1. Brain stem dysfunction (similar to Leigh disease)
2. Necrosis of brain parenchyma, leading to intractable seizures and coma
3. White matter lesions, sometimes associated with visual impairment

Among these 8 children, 4 had been exposed only to AZT and 4 to AZT/3TC. Three remain asymptomatic but have severe biological or neurologic abnormalities; 2 have died.

Dr. Tardieu described the cases of 2 of 3 additional children with mitochondrial dysfunction who had been exposed to antiretrovirals in utero. One had elevated serum lactates 1 month after birth (3 mmol/L). At 17 months both blood and cerebrospinal fluid lactates were elevated. Left hemiparesis
developed at 18 months, with nystagmus, poor fixation, drooling, and ataxia. Large lesions were
detected in white matter, even in the cerebellum.

Elevated serum lactates (2.6 and 3 mmol/L) were also measured in the second infant. Muscle
biopsy showed ragged red fibers. The child has remained asymptomatic. She had been evaluated
because her brother was among those in the first report who had severe neurologic disease.

**Retrospective analysis of children in the United States exposed to antiretroviral nucleoside
analogs with death as an endpoint**

*Betsy Smith, MD*

To determine the prevalence of mitochondrial dysfunction among children exposed to antiretrovirals
in utero, Dr. Smith and colleagues analyzed five separate US cohorts of children

born to mothers taking antiretrovirals. The cohort review had three goals:

- Gather evidence to verify US safety experience
- Determine whether evidence indicated that antiretroviral treatment recommendations for pregnant
  women and infants should be changed
- Test an approach that could be used for prospective safety review in large-scale perinatal trials of
  new treatments

This analysis was limited to *deaths* among children up to 60 months of age born to HIV-infected
women before December 1998. Data were abstracted and summarized on standard tables. All deaths
were categorized into one of five classes:

1. No evidence for mitochondrial disease
2. Limited signs, symptoms, and/or laboratory data consistent with mitochondrial dysfunction, but
   mitochondrial disease unlikely
3. Signs, symptoms, and/or laboratory data based on which a mitochondrial disorder might reasonably
   be included in the differential diagnosis
4. Signs, symptoms, and/or laboratory data strongly suggesting mitochondrial dysfunction, or proved
   to be caused by mitochondrial dysfunction
5. Sudden infant death syndrome (SIDS)

The cohorts studied included two from the National Institutes of Health—the Women and Infants
Transmission Study (WITS) and children treated in Pediatric AIDS Clinical Trials Group (PACTG)
studies—and three from the Centers for Disease Control (CDC)—the Perinatal AIDS Collaborative Transmission Study Group (PACTS), the Pediatric Spectrum of Disease (PSD) cohort, and infants and children in linked birth-death records from HIV-reporting states.

The database consisted of 23,758 infants and children. Among those, 3120 had not been exposed to antiretrovirals, 10,476 were exposed to zidovudine (AZT) alone, 1376 were exposed to AZT plus lamivudine (3TC), 501 were exposed to other antiretroviral combinations, and 8284 had an unknown exposure status. Many in this last group were born before 1994, so most of them were probably not exposed to antiretrovirals.

In the PACTG cohort, the working group evaluated 1898 records. Of the 29 children who had died, 23 were ranked in class 1, 4 in class 3, and 2 in class 5 (SIDS). Of the 4 class 3 cases, all were HIV positive; 2 had been exposed to AZT and 2 had no antiretroviral exposure. The working group concluded that AZT was not clearly associated with a risk of mitochondrial symptoms in this cohort. Four HIV-negative children in the PACTG cohort had died, 3 ranked as class 1 and 1 as class 5 (SIDS). Symptoms before this SIDS death and the autopsy report did not suggest mitochondrial dysfunction.

In the CDC pediatric surveillance group, investigators counted 9 deaths among 5579 live births. They ranked 8 deaths as class 1 and 1 as class 5 (SIDS). Six of the children who died had been exposed only to AZT, and the antiretroviral exposure status of the other 2 children could not be established.

The CDC Pediatric Spectrum of Disease (PSD) cohort includes 17 deaths among 3798 HIV-uninfected children, 792 deaths among 4363 HIV-infected children, and 68 deaths among 4964 with undetermined infection status. Of the 17 deaths among the uninfected children, the working group rated 10 as class 1, 3 as class 5 (SIDS), and 3 as unclassifiable.

The Perinatal AIDS Collaborative Transmission Study (PACTS) cohort includes 1954 uninfected children, only one of whom was classified as class 3. This infant was born in 1991 and had not been exposed to antiretrovirals before or after birth.

The Women and Infants Transmission Study (WITS) includes 1280 HIV-uninfected children, 178 infected children, and 179 with indeterminate infection status. Among the uninfected children, 5 of 7
deaths were classified as class 1 and 2 as class 5 (SIDS). Among those with indeterminate infection status, 7 of 10 were classified as class 1 and 3 as class 5. Among 34 infected children who died, 8 were class 1, 7 class 2, and 19 class 3.

Dr. Smith also reviewed findings from the Bangkok short-course AZT trial and PACTG 076/219. Among 170 children exposed to antepartum AZT from 36 weeks of gestation in the Bangkok trial, there were “no unusual clinical findings or increased risk of nonspecific diagnoses” compared with the placebo group.

ACTG 219 is a long-term follow-up of children enrolled in the first perinatal AZT trial, ACTG 076. Analysis of this cohort showed “no evidence of increased mitochondrial symptoms” in AZT-exposed versus unexposed children. Loss to follow-up was comparable in the exposed and unexposed group.

Dr. Smith reported that the working group has reached the following conclusions about deaths related to mitochondrial toxicity in antiretroviral-exposed children:

• Based on evaluation of deaths in PACTG clinical trials and long-term follow-up study, prospective cohorts studies (WITS and PACTS) and US surveillance databases (PSD, HIV Surveillance), we have not observed any deaths we can attribute primarily to mitochondrial disease.
• Specific treatment did not correlate with increased risk.
• Rare and weakly linked effects may be missed in a large retrospective cohort review with losses to follow-up.
• Large numbers in the cohorts provide some assurance of no missed large effects.

In discussions after the presentations by Drs. Stephane Blanche, Marc Tardieu, and Betsy Smith, panelists observed that the French and US analyses cannot be compared because they assessed different endpoints. The French Pediatric Cohort investigators documented signs, symptoms, and laboratory values suggesting mitochondrial dysfunction among living HIV-negative children who had been exposed to antiretrovirals in utero. A few of those children later died. The US analysis tried to find evidence of mitochondrial disease only among children who had died.

Dr. Blanche argued that evaluating living children allows a better appraisal of possible mitochondrial dysfunction. Another participant agreed that death is a “hard” endpoint that does not necessarily exclude the possibility of mitochondrial dysfunction. He knows of children in the Netherlands who could be ranked as class 3 or 4 by the US system who had signs of mitochondrial dysfunction that
disappeared when NRTIs were discontinued. Dr. Smith proposed that the best way to characterize the US cohort analysis may be as an estimate of the number of antiretroviral-exposed children who suffered a “severe hit” of mitochondrial disease that could have been established after death. None of the US children appeared to suffer such a “hit.”

Abnormal echocardiograms in children with long-term exposure to AZT
Craig Sable, MD

Cardiac complications are common in pediatric patients with HIV infection. A recent prospective study reported a 10% to 15% cumulative incidence of cardiac dysfunction in HIV-infected children during 2 years of follow-up. The cause is unknown but likely to be multifactorial, involving the immune system, infection (with both HIV and opportunists), and drug toxicity.

Evidence concerning the cardiotoxicity of zidovudine (AZT) has been contradictory. AZT has been implicated in skeletal muscle myopathy as a result of damage to mitochondrial DNA. Myocardial damage has also been observed in animals exposed to AZT.

Dr. Sable proposed the following hypothesis: Dose-dependent AZT-induced left ventricular (LV) dysfunction occurs in pediatric patients with vertically transmitted HIV infection and is reversible upon discontinuation of AZT. To test this hypothesis, he conducted a case-control study involving 6 AZT-treated children who presented with LV dysfunction that normalized after AZT was withdrawn. Each case patient was matched with 2 or 3 controls by CDC classification, age, gender, and body surface area.

A pediatric cardiologist reviewed echocardiograms of the 6 case patients at baseline, at the time of LV dysfunction, and after recovery. Echocardiograms of 15 controls were evaluated at baseline and when they were matched to cases. Cardiac dysfunction was defined as an ejection fraction less than 55% or a shortening fraction less than 28%. Dr. Sable and colleagues also measured levels of plasma troponin T and I.

The mean age of cases and controls was 11 years, and they had similar CD4-cell counts (means between 550 and 600 cells/mm³) and viral loads (means around 50,000 HIV RNA copies/mL). Antiretroviral regimens did not differ significantly between cases and controls. All case patients were
taking AZT when LV dysfunction was noted, and all the controls had been exposed to AZT. Thirteen of the 15 cases and controls were taking additional nucleoside reverse transcriptase inhibitors (NRTIs), and 11 of 15 were taking a protease inhibitor.

Table 9 shows mean ejection fractions for the case and control patients.

Table 9. Mean ejection fractions for 6 pediatric patients with left ventricular (LV) dysfunction while taking AZT\(^*\)

<table>
<thead>
<tr>
<th>Time</th>
<th>Ejection fraction</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>62% ± 5%</td>
</tr>
<tr>
<td>Time of LV dysfunction</td>
<td>43% ± 2%</td>
</tr>
<tr>
<td>Recovery</td>
<td>59% ± 4%</td>
</tr>
</tbody>
</table>

\(^*\)Ejection fractions in 15 matched controls were normal (≥55%) at baseline and when they were matched to cases.

Source: Craig Sable, MD.

Mean time to normalization of LV function in case patients was 4.5 ± 3.2 weeks after discontinuation of AZT, but that may be an overestimate.

Case patients had a longer mean AZT treatment duration than controls (64 vs. 52 months) and higher cumulative doses per kilogram (35.7 g/kg vs. 26.6 g/kg), but those differences were not statistically significant. Nor did cases and controls differ significantly in cumulative dose or cumulative dose per body surface area. Troponin T and I levels—specific markers of myocyte injury and death—were normal in cases at baseline and at the time of cardiac dysfunction.

Dr. Sable listed several limitations of this study: (1) small study group, (2) multiple drug therapies and disease stages, (3) retrospective design, and (4) complete echocardiographic data not available. He proposed the following conclusions:

- Cardiac dysfunction, presenting as left ventricular dysfunction, was observed in a cohort of HIV-infected children treated with AZT.
- Left ventricular dysfunction was reversed when AZT was discontinued.
- Cardiac dysfunction in these children may represent a myocardial toxicity of AZT because the duration of AZT treatment and the cumulative dose of AZT were higher in cases than in matched controls.
• Cardiac dysfunction did not correlate with CDC stage, CD4-cell count, or viral load in these children.
• Direct cellular injury and necrosis are unlikely in these children because they had normal troponin levels and left ventricular dysfunction normalized after AZT was stopped.

Dr. Sable suggested that a large prospective multicenter study of AZT cardiotoxicity in pediatric patients with HIV infection is warranted. Mitochondrial DNA analysis of myocardial and/or skeletal myocytes at the time of LV dysfunction, he maintained, may aid in understanding the pathophysiologic basis of AZT-induced cardiotoxicity.

In a discussion after Dr. Sable’s presentation, Lynne Mofenson, MD, briefly summarized the results of a study by Steven Lipshultz, MD. His comparison of echocardiographic findings in 382 AZT-exposed and unexposed children uncovered no differences between the groups in shortening fraction, contractility, or mass. Dr. Sable suggested that those results correlate with his belief that AZT treatment is only one factor in cardiac dysfunction among HIV-positive children.
Dr. Gropman offered a wide-ranging summary of the manifestations and diagnosis of human mitochondrial diseases. Table 10 summarizes the principal clinical features.

Table 10. **Clinical features of human mitochondrial diseases**

<table>
<thead>
<tr>
<th>Encephalopathy and central nervous system features</th>
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<tbody>
<tr>
<td>Seizures</td>
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<tr>
<td>Strokes</td>
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<tr>
<td>Dementia</td>
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<tr>
<td>Mental retardation, developmental delay</td>
</tr>
<tr>
<td>Ataxia</td>
</tr>
<tr>
<td>Brainstem involvement</td>
</tr>
<tr>
<td>Neurosensory involvement</td>
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<tr>
<td>Deafness</td>
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<td>Visual loss</td>
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<table>
<thead>
<tr>
<th>Neuromuscular manifestations</th>
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</thead>
<tbody>
<tr>
<td>Myopathy</td>
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<tr>
<td>Muscle weakness</td>
</tr>
<tr>
<td>Elevated CPK</td>
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<tr>
<td>Myalgia</td>
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<tr>
<td>Fatigue</td>
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<td>Neuropathy</td>
</tr>
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<td>Axonal</td>
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<tr>
<td>Poor temperature regulation</td>
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<th>Gastrointestinal</th>
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<tbody>
<tr>
<td>Pseudo-obstruction</td>
</tr>
<tr>
<td>Reflux</td>
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<td>Failure to thrive</td>
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| Exocrine pancreas failure                         |

**Mitochondrial Toxicity and HIV Disease**
<table>
<thead>
<tr>
<th>Endocrine pancreas dysfunction</th>
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<tbody>
<tr>
<td>Growth failure</td>
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<td>Thyroid dysfunction</td>
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<td>Hypoparathyroidism</td>
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<td>Multiple endocrine dysfunction</td>
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<td>Rashes</td>
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<td>Hair anomalies</td>
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<th>Psychiatric</th>
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<th>Lactic acidosis</th>
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<td>Fatigue</td>
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<td>Cramps, muscle pain</td>
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<td>Respiratory distress</td>
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<td>Gastrointestinal distress</td>
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<td>Arrhythmias, dysrhythmias</td>
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<td>Dizziness, syncope</td>
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<td>Temperature instability</td>
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Source: Andrea Gropman, MD

Dr. Gropman reviewed non-HIV-related mitochondrial dysfunction syndromes, beginning with *benign or multiple symmetric lipomatosis type I*. That syndrome is characterized by low body mass index; symmetric accumulation of nonencapsulated fat masses, especially in the subcutaneous regions of shoulders, neck, an mediastinum; atrophy of subcutaneous fat in the extremities; and peripheral neuropathy. Hallmarks of *benign or multiple symmetric lipomatosis type II* are high weight, diffuse lipomatosis, and peripheral neuropathy.

Types I and II share several features:

- Elevated triglycerides and insulin resistance
- Lipomatous tissue indicating a defective lipolytic response to adrenergic stimulation
- Possible triglyceride storage defects
- Etiologic relation to alcohol consumption
- Point mutations at np 8344 or deletions of mitochondrial DNA

Diagnosis of human mitochondrial cytopathies is based on clinical features, suggestive pedigree, and laboratory evaluation. Lab abnormalities common in individuals with these syndromes are (1) elevated serum or cerebrospinal fluid lactate (>2 mmol/L), elevated pyruvate, and a lactate/pyruvate ratio greater than 20. Defects of intermediary metabolism include elevated alanine, tiglylglycine,
methylglutaconic acid, and other amino acids; organic acid abnormalities such as dicarboxylic aciduria; and abnormal acylcarnitine profiles.

EEG, evoked responses, or EMG can detect abnormal brainstem transmission, seizures, or sensorimotor neuropathies and myopathies. Signal changes in the basal ganglia, thalamus, white matter, and cerebellum can be detected by magnetic resonance imaging (MRI). \(^3\)H MRS can provide a noninvasive measurement of lactate. The following abnormalities can be revealed by muscle biopsy:

- Ragged red fibers
- Lipid inclusions
- Mitochondrial clustering under the subcarcolemma
- Pleomorphic mitochondria
- Fiber type disproportion
- Swollen, enlarged mitochondria
- Disruption of cristae and paracrystalline inclusions
- Electron transport chain uncoupling
- By DNA analysis—mitochondrial DNA depletion, point mutations, and deletions

Numerous therapeutic interventions have been proposed for mitochondrial dysfunction syndromes, including L-carnitine, coenzyme Q10/Idebenone, and supplementation of vitamins K, C, and E, thiamine, riboflavin, selenium, DCA, and uridine. But these interventions have not been systematically studied in large groups of patients.

Dr. Gropman then reviewed mitochondrial toxicities related to nucleoside analogs. She referred to Dr. Blanche’s Lancet report of mitochondrial dysfunction detected in 8 HIV-negative children who had been exposed to nucleoside reverse transcriptase inhibitors (NRTIs) in utero. Dr. Blanche’s finding represented 0.46% excess in expected risk of mitochondrial disease in the general population. Dr. Gropman said prospective studies are ongoing to determine whether in utero exposure to NRTIs carries a substantial risk of mitochondrial toxicity.

Evidence of mitochondrial toxicity resulting from in utero exposure to NRTIs includes:

- Leigh syndrome
- Seizures, developmental delay, and apnea
- Elevated lactate/pyruvate ratio
- Elevated tansaminases and CPK
- Decreased activity of complexes I and IV
- White matter signal abnormalities (leukodystrophy on MRI)
- Abnormal muscle biopsies (ragged red fibers, deformed mitochondria, and aggregates)
In adults, recognized side effects of NRTIs that may reflect mitochondrial toxicity include:

- Lactic acidosis
- Skeletal muscle myopathy
- Cardiotoxicity
- Exocrine pancreas failure
- Bone marrow failure
- Lipodystrophy

Cardiotoxicity may affect both children and adults taking NRTIs. Dr. Gropman reported that cardiotoxicity has been associated not only with zidovudine (AZT), but also with didanosine (ddI) and zalcitabine (ddC). Left ventricular dilation, decreased ejection fraction, and congestive heart failure may occur after prolonged NRTI therapy. Endomyocardial biopsy shows intramyocytic vacuoles, myofibrillar loss, dilated sarcoplasmic reticulum, and disruption of cristae. The cardiotoxic effects of NRTIs have been reversible upon cessation of therapy.

Hepatic toxicity has been documented in patients taking AZT, ddI, or ddC. Fatal hepatomegaly has occurred in patients with intracellular fat accumulation, lactic acidosis, and adult Reye’s syndrome. Pathologic features are micro- and macrovesicular steatosis and abnormal structural features of mitochondria. Molecular features may include mitochondrial DNA depletion.

Dr. Kees Brinkman has hypothesized that the HIV lipodystrophy syndrome may be a mitochondrial toxicity. Lipodystrophy was first noticed in patients taking protease inhibitors (PIs) and NRTIs and was marked by peripheral lipoatrophy, central fat accumulation, dyslipidemia, and insulin resistance. Several investigators have proposed that PIs inhibit host cell proteins required in carbohydrate and lipid metabolism. PIs may also induce apoptosis (programmed cell death) of peripheral adipocytes by cytoplasmic retinoic-acid binding protein-1, a molecule mediating cis-9-retinoic acid stimulation of the retinoic X receptor.

Clinical monitoring for mitochondrial dysfunction in patients taking antiretrovirals may include serum lactate levels, screening for other metabolic markers such as organic acids and amino acids, screening for mitochondrial DNA depletion in blood and tissue, liver function tests, echocardiography, muscle biopsy, and magnetic resonance scanning of muscle and brain.
Neurologic disorders associated with HIV infection and antiretroviral therapy: The unproven role of mitochondrial toxicity

David Simpson, MD

Dr. Simpson characterized his presentation as a “cautionary tale” about distinguishing between toxicities due to antiretrovirals and those due to HIV itself. From 40% to 70% of patients with HIV infection have central or peripheral nervous system involvement, which is frequently misdiagnosed. To complicate matters, varied nervous system syndromes in HIV-infected people often coexist, and many studies of these syndromes attributed to antiretroviral toxicity have had inadequate untreated HIV/positive controls.

The primary central nervous system disorders in this population are HIV dementia in adults and encephalitis in children, but the pathophysiology of both is poorly understood. Distal symmetric polyneuropathy is the most common neurologic disorder. It is detected in one third of patients with AIDS and becomes more common as HIV infection becomes more advanced. Nearly all persons who die from AIDS have autopsy evidence of distal symmetric polyneuropathy. But determining the prevalence of this condition during life is difficult because of differing definitions, variable skills of examiners, and the confounding effects of antiretrovirals.

Distal symmetric polyneuropathy is associated with diabetes mellitus, alcoholism, the D-drug nucleoside reverse transcriptase inhibitors (NRTIs)—didanosine (ddI), zalcitabine (ddC), and stavudine (d4T)—and, rarely, vitamin B\textsubscript{12} deficiency. In a prospective cohort study of primary HIV neuropathy, about half of the cohort had diabetes, suffered from alcoholism, or were taking NRTIs that could account for the neuropathy. Other risk factors for HIV neuropathy are older age, poor nutritional status, pre-existing neuropathy, or concomitant treatment with other neurotoxic agents including certain antibacterial and antineoplastic agents, phenytoin, and thalidomide.

Early dose-ranging studies of patients treated with d4T correlated a higher risk of neuropathy with higher doses of the drug. A recent analysis of the Johns Hopkins cohort in Baltimore found the highest crude incidence of neuropathy among patients taking ddI, d4T, plus hydroxyurea, followed by those taking ddI/hydroxyurea, ddI/d4T, d4T, or ddI.
The Start I and Start II trials studied different NRTI combinations plus the protease inhibitor (PI) indinavir: d4T/lamivudine (3TC), zidovudine (AZT)/3TC, and d4T/ddI. The incidence of neuropathy proved low in all treatment arms, perhaps because study participants began therapy with relatively high CD4-cell counts.

Dr. Simpson and colleagues analyzed neuropathy among patients enrolled in ACTG 175, a large placebo-controlled comparison of NRTI monotherapy and dual NRTIs (Simpson D, et al. *AIDS* 1998;12:2425-2432). They documented a 9% rate of peripheral neuropathy in this large population with moderately advanced HIV infection (CD4-cell counts between 200 and 500 cells/mm³ at baseline). Drs. Simpson and Katzenstein’s review of diagnoses of distal symmetric polyneuropathy established by clinicians at the ACTG study sites documented both underdiagnosis and overdiagnosis of this condition. The rate of neuropathy was highest among patients taking AZT/ddC compared with those taking AZT/ddI or single NRTIs, but these differences were not statistically significant. Predictors of neuropathy were older age and lower Karnofsky score.

The pathogenesis of HIV-associated distal symmetric polyneuropathy remains unknown. Axonal degeneration is a universal consequence of aging which increases the risk of neuropathy in older patients with HIV infection. Prominent macrophage infiltration, expression of tumor necrosis factor alpha and interleukin 1, and possibly inhibition of nerve growth factors may contribute to the pathogenesis of neuropathy. Dr. Simpson said that mitochondrial dysfunction could play a role in D-drug neuropathy based on in vitro and animal data, but further evidence to confirm that possibility in humans is required.

Management of distal symmetric polyneuropathy should begin with an attempt to identify and correct metabolic or nutritional abnormalities, and to detect potential neurotoxins. If a neurotoxic NRTI is implicated, clinicians should consider lowering the dose or replacing it with another agent, but only after weighing such modifications against the possibility of losing control of HIV replication. A prospective analysis of patients exposed to ddC in ACTG 155 indicated that symptoms of D-drug neuropathy will usually remit within 4-8 weeks following drug withdrawal. However, it is unknown whether objective signs of neuropathy are completely reversible following long-term D-drug therapy.

Dr. Simpson believes skeletal muscle myopathy is now a rare complication of HIV infection. It may occur at any stage of HIV disease and has been associated with AZT. However, HIV myopathy
and AZT-associated myopathy are very difficult to distinguish. Ragged red fibers are the hallmark of mitochondrial dysfunction in muscle biopsies. Evaluating this marker in studies of myopathy among people taking or not taking AZT raises questions about the AZT’s role in myopathy. Drs. Simpson and Morgello studied muscle biopsies from 25 patients with myopathy. Scattered myofiber degeneration was apparent in all 25, as was denervation in 11. But ragged red fibers were extremely rare. Mitochondrial abnormalities detected by electronmicroscopy were common regardless of whether patients were taking AZT.

Improvement in myopathy after withdrawal of AZT has also been inconsistent from study to study. The following criteria have been used to rank the diagnosis of myopathy as definitive, probable, or possible:

- Progressive proximal muscle weakness
- Elevated creatine kinase
- EMG evidence of myopathy
- Biopsy evidence of myopathy

Epidemiological studies do not provide strong support for a major role of AZT in HIV myopathy. Among patients enrolled in ACTG 016, a placebo-controlled trial of AZT monotherapy, the two groups differed little in myalgia, limb weakness, or creatine kinase elevation. Investigators noted no change in muscle strength among people who stopped taking AZT. In ACTG 175, rates of myopathy did not differ between D-drug-treated patients and AZT-treated patients.

Current treatment proposed for mitochondrial toxicities, while theoretically promising, have not demonstrated efficacy in controlled trials, and have had varying efficacy in anecdotal case series. Agents proposed for treatment include coenzyme Q, riboflavin, and L-carnitine. A study by Dr. Simpson failed to confirm an earlier report of carnitine deficiency in HIV-positive patients with neuropathy. Clinicians should try to avoid combining agents with overlapping neurologic toxicities.

Dr. Simpson concluded with these points:

- Neurologic diseases are common in people infected with HIV.

Mitochondrial Toxicity and HIV Disease
• Antiretroviral-associated mitochondrial toxicities have been studied in vitro and in animal models, but the clinical significance of these toxicities in humans is unknown.
• Research should work toward identifying a diagnostic gold standard for mitochondrial toxicities.
• Advances in this field will depend on rigorous epidemiologic studies and clinical trials that have adequate control groups.

Developing tools for investigation of mitochondrial toxicity in the clinical setting
David Nolan, MD

Dr. Nolan discussed mitochondrial toxicity research in the context of the clinical setting, where clinical data from a well-characterised observational cohort can be utilized to inform basic science research. The clinical aspects discussed were the lipodystrophy syndrome and lactic acidosis/hyperlactataemia, while the discussion of mitochondrial toxicity research focused on the development of investigational methods of appropriate sensitivity and reproducibility.

In examining the possibility that lipodystrophy represents a mitochondrial toxicity, Dr Nolan proposed that clinical data could contribute through addressing a number of questions:
• Do NRTIs contribute to the syndrome, and are these drugs sufficient to cause the syndrome?
• Are there differences between NRTIs in relation to the risk of developing the syndrome?
• Does the risk of developing lipodystrophy correlate with risk of developing other mitochondrial toxicities, particularly hyperlactataemia.

Dr. Nolan outlined the history of the lipodystrophy syndrome, and its temporal relationship to the introduction of protease inhibitors as well as to stavudine. He distinguished two components of lipodystrophy: (1) changes in fat distribution, including subcutaneous fat wasting and intra-abdominal or localized fat accumulation, and (2) metabolic complications including dyslipidemia, insulin resistance, and (rarely) diabetes, indicating that there is increasing evidence that these endpoints should be considered separately. Fat distribution abnormalities have been associated with nucleoside reverse transcriptase inhibitors (NRTIs), and it has been demonstrated that NRTIs are sufficient to cause these changes in the absence of PIs. Metabolic complications, on the other hand, have been more strongly associated with protease inhibitors (PIs), and occur in the absence of body composition changes.

While NRTIs are sufficient to cause subcutaneous fat wasting, the addition of PI therapy accelerates subcutaneous fat wasting compared with patients taking only NRTIs, and in the clinical
setting it is PIs that are ‘dominant’ in contributing to fat wasting.

In addition, results from the Western Australian cohort indicate that the risk of subcutaneous wasting among people taking stavudine (d4T) was about twice that of people taking zidovudine (AZT), a finding confirmed in other cohort studies. These analyses have also confirmed a link between increased risk of lipodystrophy with host factors such as older age and white race.

The contribution of NRTIs to subcutaneous fat wasting is also supported by the results of studies in which a PI has been replaced with a nonnucleoside reverse transcriptase inhibitor (NNRTI) or with the NRTI abacavir. In general, a switch to the NNRTI efavirenz had modest effects on metabolic profiles and little or no effect on lipoatrophy. Switching to the NNRTI nevirapine, or to abacavir, had a greater impact on metabolic profiles than did switching to efavirenz, but again there was little change in lipoatrophy. A study by Thierry Saint-Marc in which AZT replaced d4T found improvements in lipoatrophy after the switch, but the study was nonrandomized. A randomized study is now being done to test Saint-Marc’s results.

Dr Nolan concluded that there is an established role for NRTIs in the pathogenesis of subcutaneous fat wasting and fat composition changes, and that this effect was most apparent with stavudine use. NRTIs are also associated with hyperlactataemia, a marker of mitochondrial dysfunction. While protease inhibitors are strongly and independently associated with the metabolic syndrome, they interact with NRTIs to compound subcutaneous fat wasting.

Dr. Nolan proposed that the synergistic effect of NRTIs and PIs on adipose tissue could be explained either by a divergent model, in which NRTIs and PIs independently affect fat, or by a convergent model, in which NRTIs and PIs affect fat through a common pathway. Given the possibility that PIs may enhance mitochondrial toxicity as a ‘final common pathway’ of pathogenesis prompted further clinical studies examining the role of PI use in hyperlactataemia, a recognized marker of systemic mitochondrial dysfunction.

### Convergent and divergent models of NRTI and PI effects on adipose tissue

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*Mitochondrial Toxicity and HIV Disease*  52
Dr. Nolan described a 33-month study comparing lactate levels in 349 persons taking either d4T or AZT in a HAART regimen. Levels clustered between 0.8 and 1.3 mmol/L, but were about 0.5 mmol/L higher in the d4T group. In both groups, levels plateaued after the first 12 months of therapy and remained essentially flat afterwards. These results suggest a re-equilibration of lactate that remains steady over the long term. Use of d4T correlated with higher lactate levels after 9 months ($P = 0.003$). Lactates did not differ significantly when investigators compared study participants taking lamivudine (3TC) versus didanosine (ddI), a PI versus an NNRTI, or indinavir versus nelfinavir. And lactate levels did not correlate with age, sex, race, or baseline ALT.

Dr. Nolan proposed three conclusions: Acute severe symptomatic NRTI lactic acidosis is rare, while low-grade, chronic asymptomatic raised lactates are common among people taking NRTIs. Treatment with d4T is a predominant risk factor for lactic acidemia and for subcutaneous fat wasting. PIs contribute to fat wasting but have no demonstrable effect on lactate levels. This favors the assertion that PIs and NRTIs contribute independently to the pathogenesis of lipodystrophy, rather than through a synergistic effect which increases systemic mitochondrial toxicity.

Dr Nolan then moved to a discussion of mitochondrial toxicity and its investigation. Mitochondrial disease is characterized by tissue specificity and a loose genotypic/phenotypic correlation. These characteristics reflect:

- Metabolic requirements of affected tissues
- Dependence on oxidative phosphorylation
- Accumulation of mutant mitochondrial DNA, especially in postmitotic tissue
- A threshold effect—clinical effects generally require a mutation load greater than 80% (or mitochondrial DNA depletion).

Dr. Nolan suggested three implications:

- Diagnosis of mitochondrial disease may require sampling of involved tissue.
- Phenotype may become apparent with age.
• Hyperlactatemia is not an invariant feature of these toxicities.

The systemic (versus tissue-specific) manifestations of mitochondrial toxicity can be addressed by buffy-coat mitochondrial DNA analysis, which is currently being used in an Australian analysis of samples collected before and after treatment in patients with symptomatic lactic acidosis. Tissue-specific studies include subcutaneous fat biopsies, histologic assessment, and mitochondrial DNA analysis. Dr Nolan emphasized the role of quality assurance in basic science research into mitochondrial toxicity, particularly with regard to techniques such as mtDNA depletion assays and heteroplasmy detection, and suggested that collaboration in QA programs is paramount.

Data was presented from subcutaneous fat biopsies in patients affected by lipodystrophy as well as HIV-positive untreated controls, where morphologic assessment was made using light and electron microscopy. Electron microscopy revealed distinct changes in affected patients, characterised by cytoplasmic expansion and mitochondrial proliferation, accompanied by morphologic changes in adipocyte mitochondria with elongated mitochondrial forms and disoriented crystal architecture. This is consistent with an involvement of mitochondria in the process of lipodystrophy. At the light microscopy level, subcutaneous fat in affected patients showed considerable adipocyte cell loss with lipogranulomata formation, consistent with other descriptions of adipocyte apoptosis.

Establishing that NRTIs, particularly d4T, contributes to lipodystrophy by inducing mitochondrial toxicity will require careful study, and attention should be directed at elucidating the pharmacological differences between the thymidine analogues that may account for their different risk profiles, in the context of the ‘pol-gamma hypothesis’. Cellular handling of d4T will have to be clarified, specifically, the intracellular activation of the drug by thymidine kinase. Also, because it is unlikely that d4T accumulates in adipose tissue, Dr. Nolan said, research will have to determine if adipose tissue is the target organ, or whether disordered energy metabolism is responsible for fat wasting.

Treatment of lactic acidosis/hyperlactatemia

Kees Brinkman, MD
The clinical features of lactic acidosis and hyperlactatemia include nausea, vomiting, abdominal pain, hyperventilation, liver failure, and arrhythmias.

Lonergan and colleagues described 10 patients with hyperlactatemia and hepatic steatosis in a cohort of 1245 HIV-positive persons (Lonergan JT, et al. Clin Infect Dis 2000. In press). All were taking stavudine (d4T), 5 with lamivudine (3TC), 3 with didanosine (ddI), 1 with ddI and hydroxyurea, and 1 with ddI, hydroxyurea, and 3TC. Eight had abdominal pain, nausea, and dyspnea, while the other 2 had only elevated transaminases. Three of the 10 had severe hyperlactatemia (>5 mmol/L), but all elevations resolved between 34 and 111 days of stopping the nucleoside reverse transcriptase inhibitors (NRTIs). Five of 6 liver biopsies performed showed microvesicular hepatic steatosis.

Analysis of this case series and others led Dr. Brinkman to the following conclusions:

- Not all cases of hyperlactatemia and lactic acidosis are fatal.
- Recovery is slow. Lactate levels return to normal between 2 to 12 weeks after NRTIs are withdrawn.
- Most cases have evidence of liver pathology (hepatic steatosis)(if studied).
- Initial reports involved patients taking AZT or ddI, whereas the most recent cases involved patients taking d4T.
- These conclusions should be considered with the caveat that all observations reported so far have been uncontrolled.

Before the protease inhibitor (PI) era, Fortgang and colleagues estimated the incidence of lactic acidosis among patients taking NRTIs between 1989 and 1994 (Am J Gastroenterol 1995;90:1433). The cohort included 1896 patients with 1590 person-years of NRTI experience. Of those, 322 had evidence of hepatomegaly or fatty liver. Among that group of 322, 41% had viral hepatitis, 36% alcohol-induced liver disease, 12% mycobacterial disease, 3% malignancy, and 2% cholangiopathy. Two persons had hepatic steatosis with or without lactic acidosis, representing 1.2 cases per 1000 person-years of NRTI treatment. Both individuals were taking AZT monotherapy. Dr. Brinkman noted that this estimate may not be relevant now that NRTIs are routinely administered with other antiretrovirals.

Earlier this year Swiss HIV Cohort investigators offered a cross-sectional analysis involving 988 patients taking antiretrovirals (Boudaker K, et al. 7th Conference on Retroviruses and Opportunistic Infections. 2000. Abstract 57). Normal lactate levels were documented in 881 (89.2%) and elevated
lactates (>1.1 the upper limit of normal) in 107 (10.8%). Fourteen individuals (1.4%) had lactates more than 2.2 times the upper limit of normal. The risk of hyperlactatemia was higher among patients taking d4T, with or without ddI, than among those taking AZT.

A cross-sectional analysis of 221 persons at Dr. Brinkman’s institution in Amsterdam, Onze Lieve Vrouwe Gasthuis (OLVG), included 50 antiretroviral-naive individuals, 60 taking d4T, and 101 taking AZT. All were clinically asymptomatic. Dr. Brinkman and colleagues measured normal lactate levels in 92% of the naive group, 81% of those taking an AZT regimen, and 72% of those taking a d4T combination. Lactates were mildly elevated (2 mmol/L, lactate < 5 mmol/L) in 8% of the naive group, 19% of the AZT group, and 27% of the d4T group. Differences between the d4T and naive groups were statistically significant, but differences between the d4T and AZT groups were not. Serious lactate elevations (>5 mmol/L) were recorded in nobody in the naive or AZT groups and in one person taking d4T, but that high level had returned to normal 2 weeks later and appeared to be related to extensive exercise just before the initial sample was taken.

Therapeutic options* for mitochondrial dysfunction include the following possibilities:

- **Supportive supplementation**
  - Thiamine (vitamin B₁) ±
  - Riboflavin (vitamin B₂) ±
  - Coenzyme Q ±
  - L-carnitine ±
- **Free radical scavengers**
  - Vitamins C, E, K₃ ±
  - Creatine ?
- **Steroids**
- **Dichloroacetate (DCA)** ?

* ± studies published on inherited mitochondrial disease reported both positive effects & no effect on the outcome
? no studies have been conducted to date

Dr. Brinkman noted that appropriate dose finding studies for these agents have not been performed and therefore appropriate doses are not known. Dr. Brinkman proposed the following algorithm for treatment of hyperlactatemia:
The OLVG/Dutch management protocol for lactic acidosis calls for:

- Interruption of NRTIs
- Intravenous fluid support
- Vitamin supplementation
  - Vitamin B complex forte (4 mL bid includes 20 mg of riboflavin bid and 100 mg of thiamin bid)
  - L-carnitine (1000 mg bid)
- Continue treatment until lactate levels are normal

In the past 5 months Dr. Brinkman and colleagues managed 6 patients with lactic acidosis by following this protocol. Three had been taking d4T, ddI, and hydroxyurea, 2 were taking d4T/3TC, and 1 was taking ddI, hydroxyurea, and abacavir. Lactate levels ranged between 5.4 and 18 mmol/L. Three patients had pancreatitis. All 6 survived, but Dr. Brinkman did not know whether to attribute that success to the treatment protocol or to increased alertness leading to earlier detection and management.

Future options for management of mitochondrial toxicity include NRTI-sparing regimens, but Dr. Brinkman wondered how realistic that option is, given the central role NRTIs have assumed in antiretroviral combinations and their central nervous system activity. Other management options include lower NRTI doses, adjunctive therapies such as vitamins, or other, better NRTIs. Whether abacavir or the investigational agent DAPD are free of mitochondrial side effects remains unknown. Early evidence
suggests that another investigational NRTI, Fd4C, increases the antiviral activity of AZT and d4T while blocking mitochondrial toxicity of ddI, ddC, and d4T, in a similar fashion to that already identified for 3TC.
REFERENCES

BASIC ASPECTS OF MITOCHONDRIAL DYSFUNCTION

Effects of Antiretroviral Nucleoside Analogues on DNA Polymerase Gamma Activity

Bill Copeland, Ph.D


ANIMAL MODELS IN MITOCHONDRIAL RESEARCH

HIV1 Transgenic Mice and AZT

William Lewis, Ph.D.


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Mitochondrial toxicity of antiretroviral nucleoside analogs in monkeys after transplacental exposure

Mariana Gerschenson, PhD


CLINICAL ASPECTS – ADULTS AND PEDIATRICS

Tissue Specificity and Other Clinical Aspects on Mitochondrial Toxicity of Antiviral Nucleoside

Patrick Chariot, M.D.


Mitochondrial Toxicity and HIV Disease


Neurological Disorders Associated with HIV infection and ARV Therapy: The Role of Mitochondrial Toxicity.

David Simpson, M.D.


Program Agenda

_Mitochondrial Toxicity Meeting_

Forum for Collaborative HIV Research
Washington, DC
June 5–6, 2000

MONDAY, JUNE 5, 2000

9:00 – 10:00 A.M. Continental Breakfast

INTRODUCTION

10 A.M. Facilitator: June Bray, Ph.D.
Forum Director: David Barr
Co-Chairs: Kees Brinkman, MD.
Bill Copeland, Ph.D.

BASIC ASPECTS OF MITOCHONDRIAL DYSFUNCTION

10:30 A.M. Historical Perspective on Nucleoside Analogues
Yung-Chi Cheng, Ph.D

10:50 A.M. Effects of Antiretroviral Nucleoside Analogues on DNA Polymerase Gamma Activity
Bill Copeland, Ph.D.
11:10 A.M.  
*Clinical Aspects of DNA Polymerase Gamma Activity*

Robert Naviaux, M.D.

11:30-11:45 A.M.  
*Questions*

11:45-12:05 P.M.  
*Coffee Break*

**ANIMAL MODELS IN MITOCHONDRIAL RESEARCH**

12:05 P.M.  
*HIV1 Transgenic Mice and AZT*

William Lewis, Ph.D.

12:25 P.M.  
*Mitochondrial Toxicity of Antiviral Nucleoside Analogues on Monkeys Exposed In-Utero*

Mariana Gerschenson, Ph.D.

12:45-1:00 P.M.  
*Questions*

1:00-2:00 P.M.  
*Lunch*

**CLINICAL ASPECTS – ADULTS AND PEDIATRICS**

2:00 P.M.  
*Background on Antiretroviral Nucleoside Analogues in Humans*

Barbara Styrt, M.D.

2:20 P.M.  
*Metabolic and Morphologic Changes in HIV Infection, Disease, and Therapy. Is There an Association with Mitochondrial Toxicity*

Andrew Carr, M.D.
HIV Lipodystrophy and Mitochondrial Toxicity: An Open Question.
Richard Gregg, MD

Tissue Specificity and Other Clinical Aspects on Mitochondrial Toxicity of Antiviral Nucleoside
Patrick Chariot, M.D.

Questions

Coffee Break

Perinatal Induced Mitochondrial Dysfunction in the French Cohort
Stephane Blanche, M.D.

Neurological Symptomatology in the French Pediatric Cohort
Marc Tardieu

Abnormal Echocardiograms in Children with Long-Term Exposure to AZT
Craig Sable, M.D.

Retrospective Analysis of Children in the United States Exposed to Antiretroviral Nucleoside Analogues
Betsy Smith, M.D.

Discussion

Dinner:
Marcels Restaurant
2401 Pennsylvania Avenue, NW
TUESDAY, JUNE 6, 2000

8:00 – 9:00 A.M.    Continental Breakfast

DIAGNOSIS AND THERAPEUTICS

9:00 A.M.    Clinical Markers for Mitochondrial Disease
              Andrea Gropman, M.D.

9:20 A.M.    Neurological Disorders Associated with HIV infection
              and ARV Therapy: The Role of Mitochondrial Toxicity.
              David Simpson, M.D.

9:40-10:00 A.M.    Coffee Break

10:00 A.M.    Developing the Tools for Investigation of
              Mitochondrial Toxicity in the Clinical Setting
              David Nolan, M.D.

10:20 A.M.    Treatment of Lactic Acidosis / Hyperlactatemia
              Kees Brinkman, M.D.

10:40-11:45 A.M.    Questions

11:45-12:45 P.M.    Lunch

12:45-1:15 P.M.    Identification of Current Issues of Mitochondrial
                    Research and HIV

1:15-2:15 P.M.    Discussion of Priority Issues

2:15-2:30 P.M.    Coffee Break
2:30-3:15 P.M.   Discussion of Priority Issues (continued)

3:15-4:00 P.M.   Identify the Next Steps
FORUM FOR COLLABORATIVE HIV RESEARCH

Mitochondrial Toxicity Meeting

June 5-6, 2000

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