Improvement of Mitochondrial Toxicity in Patients Receiving a Nucleoside Reverse-Transcriptase Inhibitor–Sparing Strategy: Results from the Multicenter Study with Nevirapine and Kaletra (MULTINEKA)

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Background. Nucleoside reverse-transcriptase inhibitor (NRTI)–related mitochondrial toxicity has been suggested as a key factor in the induction of antiretroviral-related lipoatrophy. This study aimed to evaluate in vivo the effects of NRTI withdrawal on mitochondrial parameters and body fat distribution.

Methods. A multicenter, prospective, randomized trial assessed the efficacy and tolerability of switching to lopinavir-ritonavir plus nevirapine (nevirapine group; n = 34), compared with lopinavir-ritonavir plus 2 NRTIs (control group; n = 33) in a group of human immunodeficiency virus–infected adults with virological suppression. A subset of 35 individuals (20 from the nevirapine group and 15 from the control group) were evaluated for changes in the mitochondrial DNA (mtDNA) to nuclear DNA ratio and cytochrome c oxidase (COX) activity after NRTI withdrawal. Dual-energy X-ray absorptiometry (DEXA) scans were used to objectively quantify fat redistribution over time.

Results. The nevirapine group experienced a progressive increase in mtDNA content (a 40% increase at week 48; P = .039 for comparison between groups) and in the COX activity (26% and 32% at weeks 24 and 48, respectively; P = .01 and P = .09 for comparison between groups, respectively). There were no statistically significant between-group differences in DEXA scans at week 48, although a higher fat increase in extremities was observed in the nevirapine group. No virologic failures occurred in either treatment arm.

Conclusions. Switching to a nucleoside-sparing regimen of nevirapine and lopinavir-ritonavir maintained full antiviral efficacy and led to an improvement in mitochondrial parameters, which suggests a reversion of nucleoside-associated mitochondrial toxicity. Although DEXA scans performed during the study only revealed slight changes in fat redistribution, a longer follow-up period may show a positive correlation between reduced mitochondrial toxicity and a clinical improvement of lipodystrophy.

Lipodystrophy syndrome remains one of the most important and relatively common adverse effects of long-term highly active antiretroviral therapy (HAART) and often leads to treatment withdrawal and psychological and social upset. Studies assessing the relative contribution of pharmacological treatment to fat redistribution have suggested a predominant role for mitochondrial toxicity in lipoatrophy and have identified the use of specific nucleoside analogues as a statistically significant risk factor for peripheral lipoatrophy [1, 2].
These observations have become of paramount importance after the good results observed with viral suppression maintenance for human immunodeficiency virus (HIV)-infected patients using nucleoside reverse-transcriptase inhibitor (NRTI)—sparing regimens, which may be an option to avoid mitochondrial toxicity in these patients [3–5]. In addition, the use of protease inhibitors (PIs) has been frequently associated with insulin resistance, dyslipidemia, and central lipohypertrophy, which is an observation that further supports the use of a nonnucleoside reverse-transcriptase inhibitor (NNRTI), such as nevirapine, which has been shown to have a protective role in cases of lipid abnormalities [6, 7].

The Nevirapine-Kaletra (NEKA) study was a randomized, open-label pilot study that compared the efficacy and safety of a nucleoside sparing simplification approach that used nevirapine at a dosage of 200 mg twice daily in addition to a standard dose of lopinavir-ritonavir with that of a conventional triple-drug HAART regimen of lopinavir-ritonavir and 2 NRTIs in a group of antiretroviral-experienced patients with longstanding viral suppression [8]. The results of this pilot study showed that dual therapy with nevirapine plus lopinavir-ritonavir at standard dosage was as potent and safe as triple standard-of-care HAART at 48 weeks of follow-up [8]. It further supported the assessment of this approach in a larger multicenter, randomized trial evaluating key safety aspects, such as the possible reduction in mitochondrial toxicity with use of an NRTI-sparing approach and consequent improvement in lipodystrophy. Some studies have demonstrated recovery of fat loss in extremities after nucleoside withdrawal [3–5], but to date, few data have been reported assessing changes in mitochondrial parameters with use of this strategy. The study also assessed the improvement in lopinavir-ritonavir–associated metabolic abnormalities as a result of the better lipid profile attributed to nevirapine.

PATIENTS AND METHODS

Study design and participants. The Multicenter Study with Nevirapine and Kaletra (MULTINEKA) was a prospective multicenter study with a randomized, open design and a follow-up period of 48 weeks (ClinicalTrials.gov NCT00335686). The main objective was to determine the changes in mitochondrial parameters among patients who received the NRTI-sparing regimen of nevirapine plus lopinavir-ritonavir.

Eligible patients were adults (≥18 years of age) with long-term HIV infection who had been undergoing treatment with a HAART regimen that included 2 NRTIs combined with a PI or an NNRTI (eg, efavirenz or nevirapine) for at least 6 months. All patients were stable, had presented with an undetectable plasma viral load (<80 copies/mL) for at least 6 months before screening (at least 2 measurements within a 6-month period), and presented with liver function test results (transaminase enzymes) <5 times greater than normal values. Exclusion criteria included opportunistic infection and/or neoplasm within the previous 6 months; clinical suspicion of or documented resistance to study drugs; poor treatment adherence; pregnancy, breast-feeding, and/or refusal of contraception during the study follow-up period; known allergic drug hypersensitivity to study drugs and similar drugs; and enrollment in other clinical trials. Individuals with a known history of mitochondrial disease, as well as those receiving toxic drugs for mitochondria (eg, antiglycosides and statins) were also excluded from the study.

Study participants were recruited from HIV units from 13 Spanish tertiary care hospitals. The study was approved by the ethics committees of each participating hospital and by the Spanish Health Authorities, and all patients gave written informed consent.

Patients enrolled in the study were randomized to either substitute their current PI or NNRTI for lopinavir-ritonavir (lopinavir 400 mg/ritonavir 100 mg capsules administered twice per day) (Kaletra; Abbott) and continue therapy with 2 NRTIs (the control group) or to switch to 200 mg of nevirapine administered twice per day (Viramune; Boehringer Ingelheim) plus twice-daily lopinavir-ritonavir (the nevirapine group). Patients without previous exposure to nevirapine received a daily dose of 200 mg for the first 2 weeks, which was increased to 200 mg of nevirapine twice daily thereafter.

All study patients were included in the clinical (including changes in fat distribution), virologic, and immunologic end points, whereas only the 35 patients from the centers in Catalonia entered the mitochondrial study (main end point), because some of the tests required fresh blood analysis.

End points. The primary end point of the study was the in vivo analysis of changes in mitochondrial DNA (mtDNA) content by means of the ratio of mtDNA to nuclear DNA (nDNA) and changes in mitochondrial respiratory chain complex IV (cytochrome c oxidase [COX] IV) enzyme activity as markers of mitochondrial toxicity after switching from an NRTI to the NNRTI nevirapine.

Secondary end points of the study included the following: (1) evaluation of the ability of the lopinavir-ritonavir plus nevirapine combination to stop lipoatrophy or achieve a reversion of the process, as measured by changes in dual-energy X-ray absorptiometry (DEXA) and self-reported questionnaires of body fat changes; (2) assessment of the hypolipemiant effects of nevirapine on lopinavir-ritonavir–associated dyslipidemia as measured by changes in lipid profile; (3) appraisal of the safety and tolerability of the lopinavir-ritonavir and nevirapine combination throughout the 48-week follow-up period, determined by the percentage of patients with adverse events and interruption of therapy as a result of toxicity or other reasons; and (4) evaluation of the efficacy of the lopinavir-ritonavir plus nevirapine simplified treatment combination in maintaining...
viral suppression (plasma HIV RNA level, <50 copies/mL) and progression towards immunological recovery of patients.

Interventions and follow-up. All patients were assessed at baseline and at weeks 4, 12, 24, 36, and 48 of follow-up. The results of hematological analysis, clinical chemistry, and lipid profiles (total cholesterol levels, high-density lipoprotein [HDL] and low-density lipoprotein [LDL] cholesterol levels, and triglyceride levels, measured by the Friedewald equation) were evaluated at every visit. HIV RNA quantification and CD4+ and CD8+ T cell counts were performed at baseline and every 12 weeks thereafter. Samples were available for genotypic analysis for those patients who experienced virologic failure during the study. DEXA scans and self-reported questionnaires regarding body fat changes were evaluated at baseline and every 24 weeks thereafter during the follow-up period.

Mitochondrial DNA study. Peripheral blood mononuclear cells (PBMCs) were obtained at baseline and at weeks 24 and 48 with use of Ficoll’s density gradient centrifugation [9] from patients who participated in the mitochondrial study. The aforementioned PBMC isolation method removes most of the platelets and reduces the risk of possible sample contamination that may influence the quantitation of mtDNA.

The relative quantification of mtDNA was performed essentially as described elsewhere [11], but small modifications were made to adapt the protocol to the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) [10]. Primer and probe sequences were mtND2-F (5′-CATCTTTGCA-GGCACACTCATC), mtND2-R (5′-TGGTTAGAAACTGGAATAAAAGCTATGCA), and mtND2-P (5′-AGGCTAGTACGTGACGTTTTTTTACCTGA) for the mitochondrial gene and 18S-F (5′-AGTGGAGCTGCGGCTTAAT), 18S-R (5′-ACCCACGG-AATCGAGAAAGAG), and 18S-P (5′-CCGGACACGGAGTACAGATT-GACAGAT) for the nuclear gene. A DNA reference sample was always included in each polymerase chain reaction (PCR) performed throughout the study to assess the interexperiment variation.

Relative quantification of mtDNA content was determined using the comparative cycle threshold (CT) \(2^{-\Delta \Delta CT}\) method after verifying that the efficiency of both PCR amplification products was similar [12]. The \(\Delta CT\) value was obtained by subtracting the mean nDNA reference CT value from the average CT value of mtDNA. The average \(\Delta CT\) of the control (lopinavir-ritonavir plus NRTIs) baseline group was used as the calibrator. This study showed mtDNA content results as fold changes, calculated according to the formula \(2^{-\Delta \Delta CT}\), where \(\Delta \Delta CT\) was the difference between \(\Delta CT\) and the \(\Delta CT\) calibrator value.

Mitochondrial function study. The COX enzyme activity was spectrophotometrically measured according to the methodology previously described by Rustin et al [13] and slightly modified for minute amounts of biological samples [14]. The specific COX activity was expressed in absolute values as nanomoles per minute per milligram of protein.

Statistical analysis. The analyses were done per protocol or using the on-treatment approach for all variables in the study. Intention-to-treat analyses, which considered missing values to equal treatment failure, were also performed for the measurement of efficacy.

Variables with a normal distribution were described as mean (± standard deviation) and compared with use of the independent-sample t test or paired-sample t test; otherwise, median values (interquartile range) and nonparametric tests (Mann-
A total of 67 subjects were recruited and randomly assigned to receive nevirapine plus lopinavir-ritonavir (nevirapine group; ) or to maintain their current treatment with 2 NRTIs, substituting only the PI or NNRTI (control group; ). One patient from the catalonian centers entered the mitochondrial study. There were no statistically significant differences between the treatment arms (table 1).

Mitochondrial toxicity. There were no statistically significant differences in mtDNA content and COX activity between groups at baseline (table 1). The nevirapine group showed a progressive and stable increase in mtDNA content throughout the follow-up period (a 40% increase), which achieved statistical significance versus the control group at week 48 ( ). Regarding the COX activity, the nevirapine group showed an improvement during the follow-up period (26% and 32% increases at weeks 24 and 48, respectively), whereas in the control group, COX activity was impaired. Differences between groups reached statistical significance at week 24 ( ) and week 48 (figure 2).

Lipodystrophy assessment. DEXA scans showed no statistically significant changes in fat distribution in any group at week 48 with respect to baseline values ( for the nevirapine group; for the control group). No differences were seen between groups at week 48 ( ). Despite this, a lasting increase in fat on the upper and lower limbs was observed throughout the entire follow-up period, mostly in the nevirapine group (from 13.2% at baseline to 17.7% at week 48 in the upper limbs and from 11.1% to 14.1% in the lower limbs), whereas the control group mainly showed a fat increase in the trunk area (from 20.6% to 22.6% at week 48), resulting throughout the study in a progressive increase in the trunk-to-extremity fat ratio, compared with the nevirapine group (figure 3). Based on the questionnaire on subjective body evaluation, no significant changes in body fat distribution were seen between groups at week 48 ( ).

Mitochondrial Recovery in Patients with HIV Infection • CID 2009;49 (15 September) • 895

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mitochondrial subgroup</th>
<th>All patients</th>
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<tbody>
<tr>
<td></td>
<td>Nevirapine group (n = 20)</td>
<td>Control group (n = 15)</td>
</tr>
<tr>
<td>Age, median years (IQR)</td>
<td>40 (37.4–36.3)</td>
<td>39 (37–42.8)</td>
</tr>
<tr>
<td>Risk, %</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Male, %</td>
<td>89</td>
<td>92</td>
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<tr>
<td>Female, %</td>
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</tr>
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<td>MSM, %</td>
<td>78.9</td>
<td>30.8</td>
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<tr>
<td>Heterosexual sex, %</td>
<td>10.5</td>
<td>30.8</td>
</tr>
<tr>
<td>Drug use, %</td>
<td>5.3</td>
<td>23.1</td>
</tr>
<tr>
<td>Other, %</td>
<td>5.3</td>
<td>15.4</td>
</tr>
<tr>
<td>Baseline CD4+ T cell count, median cells/mm³ (IQR)</td>
<td>483 (351–559)</td>
<td>457 (405–587)</td>
</tr>
<tr>
<td>Duration of HAART, median years (IQR)</td>
<td>9.16 (4.48–11.64)</td>
<td>6.66 (3.81–9.82)</td>
</tr>
<tr>
<td>NRTI, %</td>
<td>Didanosine</td>
<td>52.6</td>
</tr>
<tr>
<td></td>
<td>Didanosine and stavudine</td>
<td>5.3</td>
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<td></td>
<td>Zidovudine</td>
<td>21.1</td>
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<td>NNRTI, %</td>
<td>PI</td>
<td>57.9</td>
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<tr>
<td></td>
<td>NNRTI</td>
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<tr>
<td></td>
<td>mtDNA relative amount *, mean value (±SD)</td>
<td>−2.357 (0.86)</td>
</tr>
<tr>
<td></td>
<td>COX activity, mean nmol/min/mg protein (±SD)</td>
<td>98.89 (13.16)</td>
</tr>
</tbody>
</table>

NOTE. COX, cytochrome c oxidase; IQR, interquartile range; MSM, men who have sex with men; mtDNA, mitochondrial DNA; NNRTI, nonnucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; PI, protease inhibitor; SD, standard deviation.

* mtDNA relative content at baseline was calculated by subtracting the nuclear DNA measurement from the mtDNA amount.
Figure 2. Changes in mitochondrial parameters with respect to baseline values. $P$ values indicate differences between groups. Full squares represent the nevirapine group, and empty squares represent the control group.

from baseline at week 48, $P = .012$) and a lower increase in triglycerides versus the control group (table 2).

Clinical evolution and safety profile. During the follow-up period, a total of 42 adverse events were detected. Of these, 25 were treatment-related adverse events (12 in the nevirapine group and 13 in the control group); 4 were rated as severe (grades III and IV), including 1 in the nevirapine arm and 3 in the control arm. Gastrointestinal complaints—mainly diarrhea, vomiting, and abdominal disturbances—were the most frequently observed adverse events. They mainly appeared during the first 12 weeks of therapy, were usually mild (grade I or II), and were transient in most patients.

Six (17.6%) of the patients from the nevirapine arm and 13 (39.4%) of the patients from the control arm discontinued treatment ($P = .05$ for comparison between groups). Of these, 3 (50%) and 7 (53.8%), respectively, discontinued the treatment as a result of diarrhea (table 3). Seventy percent of treatment discontinuation occurred within the first 24 weeks of follow-up; the remaining 21% occurred at week 36.

In the group of patients who withdrew from the study, only...

Figure 3. Evolution of fat redistribution. Comparison of median percentage of body fat change for upper limbs, lower limbs, trunk, and total throughout the follow-up period for both study groups. Full squares represent the nevirapine group, and empty squares represent the control group.
Table 2. Lipid Profiles in Both Study Groups through the Study Follow-Up Period

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nevirapine group, median mg/dL (IQR)</th>
<th>Control group, median mg/dL (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol level</td>
<td>205 (185–226)</td>
<td>196 (174–216)</td>
</tr>
<tr>
<td>LDL cholesterol level</td>
<td>129 (110–148)</td>
<td>122 (99–134)</td>
</tr>
<tr>
<td>HDL cholesterol level</td>
<td>47 (39–53)</td>
<td>46 (39–51)</td>
</tr>
<tr>
<td>Triglyceride level</td>
<td>109 (96–221)</td>
<td>136 (85–184)</td>
</tr>
</tbody>
</table>

NOTE. HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein.

Table 3. Total and Treatment-Related Adverse Events

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nevirapine group (n = 33)</th>
<th>Control group (n = 33)</th>
<th>Total (n = 66)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total adverse events</td>
<td>24</td>
<td>18</td>
<td>42</td>
</tr>
<tr>
<td>Treatment-related adverse events</td>
<td>12</td>
<td>13</td>
<td>25</td>
</tr>
</tbody>
</table>

\( ^a \) Dyslipemia.

\( ^b \) Ascites requiring hospital admission, splenectomy requiring hospital admission, and diarrhea.

1 from the nevirapine group and 3 from the control group belonged to the mitochondrial substudy; the rest of participants in this substudy completed the follow-up.

Mean transaminase levels did not show any significant change from baseline, and no patient developed grades III or IV liver toxicity or acute clinical hepatitis during follow-up (data not shown).

Virologic and immunologic assessments. All patients had viral suppression (viral load, <50 copies/mL) at 48 weeks of follow-up. For the intention-to-treat analyses (in which treatment discontinuation was regarded as treatment failure), 27 (81.8%) of the patients from the nevirapine arm and 20 (60.6%) from the control arm remained stable, with an undetectable plasma viral load at the end of the study (\( P = .05 \) for comparison between groups). Median CD4\(^+\) T cell count remained unchanged in both treatment arms (from 452 cells/mm\(^3\) [range, 303–596 cells/mm\(^3\)] to 476 cells/mm\(^3\) [335–607 cells/mm\(^3\)] in the nevirapine group and from 471 cells/mm\(^3\) [range, 385–722 cells/mm\(^3\)] to 556 cells/mm\(^3\) [range, 338–850 cells/mm\(^3\)] in the control group; \( P = .082 \) and \( P = .088 \), respectively) with no statistically significant differences at week 48 between groups.

DISCUSSION

The results of this larger multicenter study reinforce our previous results from the NEKA study [8] and show dual antiretroviral therapy with nevirapine plus standard dose lopinavir-ritonavir to be as potent and safe as standard care regimens after 48 weeks of treatment in antiretroviral-experienced subjects with long-lasting viral suppression. The current study also shows that substituting NRTI drugs with the NNRTI nevirapine leads to a progressive and maintained increase in mtDNA content throughout the entire follow-up period and, thus, an improvement in mitochondrial function, as well as an increase in HDL-cholesterol, which are effects that were not observed in the control group.

The specific etiology of peripheral lipoatrophy and some other clinical and metabolic consequences of antiretroviral therapy are now clearer. Regarding the possible etiology of lipoatrophy, current evidence supports a multifactorial mechanism with a predominantly drug-related adverse effect, mainly described as NRTI-related mitochondrial DNA polymerase gamma inhibition; it is also a possible immune reconstitution or proinflammatory cytokine-mediated phenomenon [15]. The suggestion that NRTI-induced mitochondrial toxicity is a key factor in the induction of HAART-related lipoatrophy, remains one of the strongest, as it is supported by increasing evidence showing that NRTIs strongly affect mitochondria in the adipose tissue [16]. In fact, it has been suggested that the toxic effects of NRTIs on human adipose cells could not only limit themselves to a lipoatrophic effect but also extend to an opposite lipohypertrophic effect depending on the oxygen availability. Furthermore, the lipoatrophic and lipohypertrophic phenotype characteristic of lipodystrophy could be a differential consequence of NRTI effects, depending on the metabolic status of the targeted adipose tissues [17]. Although our study design does not allow us to fully identify the causes of mitochondrial dysfunction in patients with HIV infection, these results suggest that NRTIs and their affinity for uptake by mitochondrial DNA polymerase gamma could play an important role in the process and that, despite other possible causes of mitochondrial dysfunction, the mitochondrial function is improved in patients...
Mitochondrial toxicity becomes apparent particularly during medium-term to long-term therapy with NRTIs, and in addition to lipodystrophy, it may also lead to a wide range of severe adverse events in HIV-infected patients, including lactic acidosis, hepatic steatosis, neuropathy, myopathy, cardiomyopathy, and pancreatitis [18]. The measurement of mitochondrial DNA and its function in PBMCs may be of value for the early prevention of these toxicities in treated patients with HIV infection. A significant number of studies have shown the association between the use of NRTIs and mtDNA depletion, as well as the association between low mtDNA levels and subsequent development of lipodystrophy in patients with HIV infection [2, 19–33]. On the other hand, it has also been suggested that mtDNA content in peripheral blood cells may not be an accurate biomarker of mitochondrial toxicity in lipodystrophic adipose tissue because of a poor correlation between this parameter and any other mitochondrial parameter, as well as because of the preservation of mtDNA-dependent mitochondrial functions despite severe mtDNA depletion [34–37]. All of these discrepancies between studies further underline the complexities involved in the relationship between the lipodystrophic syndrome and mitochondrial function and show some of the difficulties involved in their evaluation [38]. Thus, the current study includes not only the mtDNA determination but also the study of the COX enzyme activity to evaluate the mitochondrial function.

Although there was a trend toward positive fat redistribution in nevirapine-treated patients who were evaluated by DEXA scan that showed a progressive fat increase in upper and lower limbs over time, our results did not achieve statistical significance for these parameters, and these patients did not eventually reveal any evident fat change. Most probably, this lack of statistical significance could be attributable to some study limitations, such as the small number of patients or the limited period of follow-up. Our data does not support slow recovery of adipose tissue in spite of NRTI withdrawal and mitochondrial lesion improvement. This could be attributable to a variety of factors. First, there could be a differential capacity to revert mitochondrial toxicity in adipose tissue (the target tissue for lipodystrophy, which could be still damaged) with respect to the mononuclear cells that we used as a study model (which usually have a faster turnover). Second, it could be attributable to a general mitochondrial improvement in all body cells and mitochondria, but fat recovery could take longer. A longer follow-up of the patients who underwent the treatment switch would have helped us to address this question. Finally, the effects of HIV infection per se on mitochondrial function and lipodystrophy development cannot be forgotten. HIV has been reported to induce mitochondrial damage in the absence of antiretroviral treatment by modulating inflammatory and/or apoptotic cellular mechanisms [39–41]. This could lead to chronic depletion of mtDNA content and deterioration of mitochondrial function, despite the fact that all of the patients maintained virologic suppression throughout the study. In addition to the effect of HIV on mitochondria, the immune response to HIV infection would increase proinflammatory cytokines, such as tumor necrosis factor-α, all of which would contribute to lipodystrophy syndrome, independently of antiretroviral therapy and/or NRTI withdrawal.

In conclusion, in antiretroviral-experienced subjects with long-lasting viral suppression treated with NRTIs, switching to a nucleoside-sparring regimen using nevirapine and lopinavir-ritonavir retained the entire antiviral efficacy and led to an improvement in mtDNA/nDNA ratio and COX activity, suggesting a reversion of nucleoside-associated mitochondrial toxicity. Although this improvement in mitochondrial function did not translate into significant changes in fat redistribution during the study, a longer follow-up may show a positive correlation between this reduced mitochondrial toxicity and a clinical improvement of lipodystrophy.

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References