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# Mitochondrial DNA Subhaplogroups L0a2 and L2a Modify Susceptibility to Peripheral Neuropathy in Malawian Adults on Stavudine Containing Highly Active Antiretroviral Therapy

*Elizabeth Kampira, MSc,*† Johnstone Kumwenda, FRCP,‡ Joep J. van Oosterhout, MD, PhD,‡§ and Collet Dandara, PhD**

**Background:** Peripheral neuropathy (PN) is one of the main toxicities associated with stavudine. Genetic variants in mitochondrial DNA (mtDNA) haplogroups have been associated with increased risk of developing PN in European non-Hispanic and black patients on stavudine containing antiretroviral therapy (ART). We investigated mtDNA haplogroups and their role in susceptibility to stavudine-induced peripheral in Malawian patients on ART.

**Method:** Two hundred and fifteen adults on stavudine containing regimens were recruited from the ART clinic at Queen Elizabeth Central Hospital, Blantyre, into a cross-sectional study to investigate the effects of genetic variants in mtDNA of individuals in relation to response to treatment. Patients were categorized according to whether or not they had developed PN after a minimum of 6 months on stavudine containing ART. Whole mtDNA coding regions of each patient were sequenced, and CD4 count, viral load, and creatinine were determined. The mtDNA variation was correlated with clinical characteristics.

**Results:** Fifty-three (25%) of the participants developed PN after starting stavudine containing ART. Mitochondrial DNA subhaplogroup L0a2 was independently associated with increased risk of PN in a multivariate model (odds ratio, 2.23; 95% confidence interval, 1.14 to 4.39; $P = 0.019$), and subhaplogroup L2a was independently associated with reduced risk of PN (odds ratio, 0.39; 95% confidence interval, 0.16 to 0.94; $P = 0.036$).

**Conclusions:** Genetic variation in mtDNA confers differential risk of developing PN in patients on stavudine containing ART among Malawians.

**Key Words:** stavudine, mtDNA, subhaplogroup, peripheral neuropathy, toxicities

(*J Acquir Immune Defic Syndr* 2013;63:647–652)

## INTRODUCTION

Peripheral neuropathy (PN) is one of the most common neurological complication associated with HIV infection, occurring in up to 35% of the patients.[1,2] PN may result from HIV infection itself or from the neurotoxic side effects of antiretroviral drugs, especially nucleoside reverse transcriptase inhibitors (NRTIs).[1,3] Among NRTIs, stavudine and didanosine have the highest propensity to cause PN. Cui et al[4] showed that stavudine inhibits neurite regeneration; however, the mechanism of neurotoxicity is not fully established. Phosphorylated molecules of NRTIs in the mitochondrial matrix inhibit the activity of mitochondrial polymerase gamma by competing withdeoxyribonucleotide triphosphatesfor the binding site and then as they incorporate into the growing mitochondrial DNA (mtDNA) strands by causing inhibition in the synthesis of mtDNA thus prematurely terminating chain elongation.[5,6] In addition to mtDNA polymerase gamma inhibition, mitochondria dysfunction could be the result of increased mtDNA polymorphisms and oxidative stress caused by NRTI that may have adverse effect on mitochondrial structure and function.[7] It has recently been suggested that polymorphisms in mitochondrial genes may explain variations in the response to antiretroviral therapy (ART) between individuals.[8]

The evolution of the human mtDNA is characterized by the emergence of distinct lineages or haplogroups. These haplogroups are characterized by specific sets of haplotypes or single nucleotide polymorphisms (SNPs).[9] For example, the mutations m.5147 G>A, m.5711 A>G, m.6257 G>A, and m.8460 A>G describe subhaplogroup L0a2. Many of the described haplogroups are characteristic of different populations and/or ethnic groups.[10,11] The mtDNA lineage L characteristically

Received for publication January 29, 2013; accepted April 10, 2013.

From the *Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa; and †Department of Pathology, ‡Department of Medicine, and §Malawi-Liverpool Wellcome Trust Clinical Research Programme, College of Medicine, University of Malawi, Malawi.

Presented at the Wellcome Trust 6th Meeting of the Directors of the African Institutions Initiativein Accra, November 28, 2012, Ghana.

Funded by Welcome Trust and Department for International Development (DFID) through Health Research Capacity Strengthening Initiative, Southern Africa Consortium for Research Excellence, Medical Research Council of South Africa, the National Research Foundation of South Africa and University of Cape Town.

The authors have no conflicts of interest to disclose.

Correspondence to: Collet Dandara, PhD, Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Observatory 7995, South Africa (e-mail: collet.dandara@uct.ac.za).

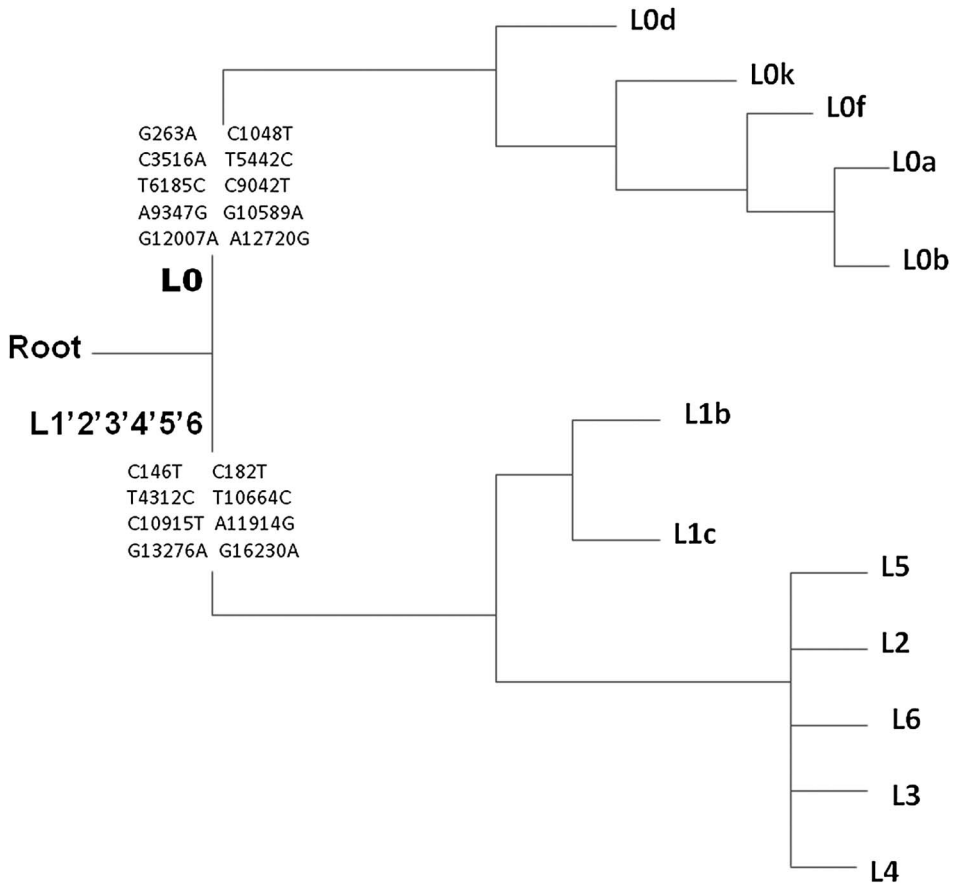


**FIGURE 1.** Phylotree indicating the branching of the mtDNA L major group in Africa populations.

defines African populations and is divided into 2 branches, L0 and L1-6[12] (see Fig. 1). The distribution and frequency of these mtDNA L subhaplogoups within Africa seem to have distinctive patterns in different geographic regions or ethnic groups.[11,13–15] For example, more than 90% of Khoisan group of South Africa carry the haplogroup L0, whereas this haplogroup is found in only 20% of East Africans.[14]

Analysis of mitochondrial subhaplogroups among European patients enrolled in AIDS clinical Trials Group studies showed that individuals in haplogroup T had increased risk of developing PN during ART.[8,16] The distribution and frequencies of L subhaplogroups in Africa varies widely between geographic regions and ethnic groups.[11,15] Although some genetic studies on African have focused on mtDNA, this has mainly been aimed at unraveling demographic phenomena related to the settlement of populations and ethnic groups on the continent, whereas information on the association of subhaplogroups with risk of drug toxicities is very sparse and completely lacking among Malawians and other African populations. Our aim was therefore to investigate the association of mtDNA subhaplogroups with development of PN among adult Malawians on stavudine containing ART.

## MATERIALS AND METHODS

### Participants

Unrelated HIV/AIDS patients from an ART cohort at Queen Elizabeth Central Hospital in Blantyre, Malawi, were recruited into a cross-sectional study. The patients completed a structured questionnaire that collected demographic information, medical history, and ancestry of each participant up to their grandparent's level. Pregnant women, patients on tuberculosis treatment, persons who experienced PN before ART initiation and those who had missed their medication in the past 3 days were excluded from participation. Participants had to be on stavudine containing regimen for at least 6 months. The protocol was approved by the College of Medicine Research Ethics Committee of the University of Malawi and the Human Research Ethics Committee of the University of Cape Town. All participants gave written informed consent. The study conformed to the declarations of Helsinki 2008.

### Clinical and Laboratory Measurements

History and physical examinations were performed at enrollment. Stavudine-associated PN was defined as a history of characteristic symptoms of numbness, dysesthesia, and pain in the feet and legs that had started after initiation of ART.[2] The glomerular filtrate rate was estimated with the US K-DOQI group method.[17] A sample for CD4 cell count (FACSCount flow cytometer; Beckton Dickinson, Franklin Lakes, NJ) and HIV-1 RNA (Amplicor HIV Monitor, version 1.5; Roche Diagnostic Systems, Basel, Switzerland) was collected.

Blood samples for DNA extraction were collected in ethylenediaminetetraacetic acid-coated tubes and were kept at −20°C until DNA extraction. DNA was isolated by use of

a GenElute Blood Genomic DNA Kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's protocol. The mtDNA for each of the 215 samples was amplified in 9 partially overlapping fragments using the primers reported by Ramos et al.[18,19] Each time mtDNA templates were amplified in a total volume of 25 μL in a reaction that consisted of ×1 green GoTaq reaction buffer, 200 μM of deoxyribonucleotide triphosphates, 1.0 mM of $MgCl_2$, 0.4 μmol of each primer, 0.5 U of Taq DNA polymerase, and 20 ng of DNA were performed in GeneAmp PCR System 9700 by Life technologies (New York, NY). The polymerase chain reaction (PCR) programmes each consisted of an initial denaturation step at 94° C for 5 minutes, followed by 35 cycles of denaturation at 94° C for 1 minute, and annealing and extension step at 57–64.4° C, 72°C for 40 seconds, and 2.5 minutes, respectively, with a final extension step of 5 minutes at 72°C.[18] PCR products were purified using the exonuclease and shrimp alkaline phosphatase (ExoSap).

## Sequence Analysis and Quality Control

Instead of targeting confirmed informative SNPs, we sequenced whole mtDNA coding region to search for any novel SNPs in this Malawian population because of the known genetic diversity in African populations. Through the series of 9 PCR fragments and use of forward and reverse primers and additional internal primers all samples (n = 215) were sequenced from nucleotide position 577–15953 of the mtDNA according to the revised Cambridge reference sequence (rCRS) Locus NC_012920.1. Capillary electrophoresis for sequencing reactions was run on an ABI PRISM 3130 × l Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned to the rCRS for the human mitochondrion Locus NC_012920.1 and assembled using Lasergene 10 Core Suite software supplied by DNASTAR package (Madison, WI). After assembly of the sequences, mutations (polymorphisms) were determined as nucleotide differences when compared with the Cambridge reference sequence.

## Subhaplogroup Analysis and Statistical Analysis

We classified mtDNA mutations into haplogroups according to the databases of van Oven and Kayser[12] www.phylotree.org/ and Accetturo et al.[20] Stata for windows software (version SE/11, 4905; Stata Corp, College Station, TX) was used for statistical analysis. Subhaplogroup frequencies were compared between participants presenting with and without PN using the Fisher exact tests.

To assess the relationship between independent variables [sex, body mass index (BMI), age, duration on ART, CD4 count, viral load, estimated glomerular filtrate rate, and subhaplogroups] and the presence of PN, univariate logistic regression model was performed. Variables, which showed a degree of association with PN ($P < 0.1$), were included in multivariate logistic regression models, where 1 subhaplogroup was included in the absence of other subhaplogroups. Odds ratios are reported with 95% confidence intervals and a $P$ value of $<0.05$ was considered significant.

# RESULTS

We enrolled 215 maternally unrelated adult (according to family history information) ART patients. All were Malawian Bantu speakers, 132 (61%) were women and all were on stavudine and lamivudine containing first-line ART for at least 23 months, median duration of 25 months (range, 23–29). Two (1%) patients had severely elevated serum creatinine levels of 5.4 and 6.0 mg/dL with an estimated glomerular filtrate rate of 15.4 mL/min/1.73 $m^2$ and 10.3 mL/min/1.73 $m^2$, respectively. One percent and 3% of patients were current smokers and alcoholics, respectively. Fifty-three (25%) patients had PN. Table 1 provides further details of patient characteristics.

## Mitochondrial Variation and Haplogroup Analysis

All 215 samples were successfully sequenced and 143 positions showed nucleotide differences when compared with bases on the rCRS.[21] Of these 143 mutations, 134 (94%) have been reported in specific L subhaplogroups before; 7 (5%) of the mutations, m.3579 A>G, m.3606 A>G, m.5090 T>C, m.10463 T>C, m.12192 G>A, m.13104 A>G, and m.15038 G>A, with frequencies of 2%, 2%, 6%, 1%, 3%, 7%, and 9%, respectively, are being reported in an African population for the first time but have been observed previously in non-African populations.[12,22,23] Of the few remaining changes, 2 (1%), m. 12769 G>A with a frequency of 2% and m.14612 G>A with a frequency of 4%, were novel and are not on either MITOMAP or Phylotree. The 134 SNPs were then used to construct haplogroups and subhaplogroups according to software provided by van Oven and Kayser.[12] Major L haplogroups (L0–L3) were identified in the study population and were further characterized into 9 subhaplogroups, namely L0a1, L0a2, L0d, L0f, L0k, L1c, L2a, L3d, and L3e (Table 2). Subhaplogroup L0a2 had the highest frequency (28%), whereas L3d (2%) was the least common. We did not observe haplogroups L4, L5, and L6 and subhaplogroup L1b that have been reported in other African populations.[14,24] Two mitochondrial subhaplogroups were associated with the presence of PN (see Table 2). In a multivariate logistic regression model, the L0a2 subhaplogroup was an independent risk factor for PN (odds ratio, 2.23; 95% confidence interval, 1.14 to 4.39; $P = 0.019$) (Table 3). On the other hand, the presence of the L2a subhaplogroup was associated with reduced risk for PN (odds ratio, 0.39; 95% confidence interval, 0.16 to 0.94; $P = 0.036$). The mutations, m.5147 G>A, m.5711 A>G, m.6257 G>A, and m.8460 A>G, described subhaplogroup L0a2.

# DISCUSSION

The study was undertaken to investigate the role of mtDNA subhaplogroups in the susceptibility to stavudine-induced PN in HIV/AIDS patients from Malawi. We observed that there was no relationship between gender and the risk of PN. The role of age on PN is in contrast to our earlier findings that reported there was association between age and risk of PN.[2] Unlike in other studies where height has

**TABLE 1.** Patient Characteristics and PN Diagnosis

| Characteristic | No PN | PN | Total (n = 215) | *P* |
|---|---|---|---|---|
| Gender (female) | 103 (63) | 29 (56) | 132 (61) | 0.251 |
| Median age (IQR) (yrs) | 37 (31–46) | 41 (36–48) | 38 (32–46) | 0.063 |
| Age categories (yrs) | | | | |
| <40 | 99 (61) | 24 (45) | 123 (57) | Reference |
| ≥40 | 63 (38) | 29 (55) | 92 (43) | 0.045 |
| Height (cm) | 160 (155–165) | 159 (154–165) | 159 (154–165) | 0.198 |
| Median BMI (IQR) (kg/m$^2$) | 23 (21–25) | 23 (21–26) | 23 (21–25) | 0.140 |
| BMI categories | | | | |
| Underweight (<18.5 kg/m$^2$) | 5 (3) | 1 (2) | 6 (2.8) | 0.751 |
| Normal | 130 (80) | 37 (70) | 167 (78) | Reference |
| Overweight (>25 kg/m$^2$) | 27 (17) | 15 (28) | 42 (19) | 0.072 |
| Median CD4 (IQR) (cells/μL) | 344 (212–495) | 344 (281–486) | 344 (227–489) | 0.805 |
| CD4 categories (cells/μL) | | | | |
| 0–199 | 35 (22) | 8 (16) | 43 (21) | 0.479 |
| 200–349 | 44 (28) | 17 (35) | 61 (30) | 0.585 |
| ≥350 | 76 (49) | 24 (49) | 100 (49) | Reference |
| Viral load (CPs/mL) | | | | |
| <400 | 135 (87) | 45 (92) | 180 (88) | |
| ≥400 | 20 (13) | 4 (8) | 24 (12) | 0.374 |
| Duration on ART (IQR) (mo) | 25 (23–28) | 25 (23–32) | 25 (23–29) | 0.883 |
| Median eGFR (IQR) (mL/min) | 136 (114–153) | 124 (111–146) | 134 (113–151) | 0.837 |

*Data are expressed as N (%) except for age unless otherwise noted. eGFR calculated using US K-DOQI group method.
IQR, interquartile range; eGFR, estimated glomerular filtrate rate.

been associated with increased risk for PN, this was not the case in the Malawi cohort.[25] In antiretroviral naive patients, PN has been reported to be more common with CD4 counts <200 cells per microliter and HIV-1 RNA >10,000 copies per milliliter.[26,27] In our study, all patients were on ART for at least 23 months and the vast majority showed good control of HIV replication, which likely explains why we did not observe an association of CD4 and HIV-1 RNA with PN.

In some studies, the risk of PN was associated with malnutrition[28,29]; therefore, our finding that high BMI (>25 kg/m$^2$) with borderline significance ($P$ = 0.055) in patients experiencing PN could be remarkable. One possibility is that we overlooked the diagnosis of type II diabetes in many obese patients; however, in a cohort that included many patients from the current study, we found that diabetes mellitus was very uncommon.[2] Another explanation is that the studies that found an association, low BMI with PN mainly included patients who were not on ART.[28,30] After starting ART, the prevalence of malnutrition steadily decreased and the pathogenesis and risk factors of PN are likely to be different. In ART patients, high BMI has been identified as a risk factor for high lactate syndromes and lactic acidosis.[2,30]

**TABLE 2.** The Association of mtDNA Subhaplogroups With PN Among Malawian ART Patients

| Subhaplogroups | Total (%) | No PN (%) | PN (%) | *P* |
|---|---|---|---|---|
| L0a1 | 13 (6.0) | 11 (7.0) | 2 (4.0) | 0.527 |
| L0a2 | **61 (28.0)** | **39 (24.0)** | **22 (42.0)** | **0.022** |
| L0d | 15 (7.0) | 11 (7.0) | 4 (8.0) | 0.766 |
| L0f | 6 (3.0) | 5 (3.0) | 1 (2.0) | 1.00 |
| L0k | 9 (4.0) | 8 (5.0) | 1 (2.0) | 0.458 |
| L1c | 27 (13.0) | 21 (13.0) | 6 (11.0) | 1.00 |
| L2a | **53 (25.0)** | **46 (28.0)** | **7 (13.0)** | **0.028** |
| L3d | 5 (2.0) | 2 (1.0) | 3 (6.0) | 0.097 |
| L3e | 26 (12.0) | 19 (12.0) | 7 (13.0) | 0.809 |
| Total | 215 (100) | 162 (100) | 53 (100) | |

Values given in bold indicate significant differences.

The mtDNA variation has been used in human phylogeography in association with population genealogy and also in studies trying to define the risk mtDNA polymorphisms in human disease.[11,20,31,32] Previous studies have demonstrated that European populations with haplogroup T are more susceptible to developing stavudine-associated PN compared with other haplogroups.[8,33] A study conducted in blacks of African origin showed that subhaplogroup L1c was associated with increased susceptibility to developing stavudine-associated PN,[34] whereas in this Malawi population, 2 subhaplotypes that, L0a2 and L2a, seem to be the important markers.

This is the first study to be carried out within the indigenous Africans with known demographic information. However, our study has several limitations that include a small sample size, which makes it difficult to determine the effects of subhaplogroups with low frequencies (eg, L3d with 2%), a weakness in the objective assessment of PN (eg, clinical

**TABLE 3.** Multivariate Logistic Regression Analyses of Factors Associated With PN

| Covariate | Model 1 Adjusted OR (95% CI), L2a Group | *P* | Model 2 Adjusted OR (95% CI), L0a2 Group | *P* |
|---|---|---|---|---|
| Age (yrs) | | | | |
| ≥40 | 1.84 (0.97–3.49) | 0.064 | 1.76 (0.92–3.34) | 0.087 |
| BMI (kg/m$^2$) | | | | |
| <18.5 vs. 18.5–25 | 0.93 (0.10–8.43) | 0.948 | 0.80 (0.08–7.33) | 0.846 |
| >25 vs. 18.5–25 | 1.98 (0.94–4.18) | 0.074 | 2.09 (0.98–4.48) | 0.055 |
| L2a (vs all L subhaplogroups) | **0.39 (0.16–0.94)** | **0.036** | | |
| L0a2 (vs all L subhaplogroups) | | | **2.23 (1.14–4.39)** | **0.019** |

findings including nerve conduction velocity and/or intra-epidermal nerve fiber density), possibility of undiagnosed pre-ART neuropathy and lack of information on the role of other factors associated with stavudine-induced PN such as polymorphisms in host cytokine genes [35]

## CONCLUSIONS

We report a significant association between L02a with increased risk of PN and a protective effect of L2a in Malawians on stavudine-based ART. Although it is unlikely that in our setting subhaplogroups can be introduced as biomarkers for tailoring antiretroviral drugs to individual patients in the near future, our findings help better understand the mitochondrial toxicopathology of NRTI's and if confirmed by other studies may improve drug selection for standard regimens at population level and lead to better precision medication.

## ACKNOWLEDGMENTS

*We thank the ART clinic team at Queen Elizabeth Central Hospital and all participants in the study and University of Cape Town, Pharmacogenetics group for teamwork.*

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