PHARMACOGENETICS OF STAVUDINE: ROLE OF GENETIC VARIATION IN MITOCHONDRIAL DNA AND POLYMERASE GAMMA AMONG ADULT MALAWIAN HIV/AIDS PATIENTS

by

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DECLARATION

I declare that this thesis is a presentation of my original research work and where collaborations were involved it has been clearly indicated. It is submitted for the Degreee of Doctor of Philosophy in Human Genetics at the University of Cape Town. I have used the PLoS Medicine convention for citation and referencing for chapters 1,2,3, 8 and 9 whilst chapters 4,5, 6 and 7 citations are according the respective journal format. I retain all ownership rights to the copyright of the thesis therefore; I have not allowed and will not allow anyone to use this thesis or dissertation with the intention of passing it off as his or her own work. I declare that this work was approved by the College of Medicine Ethics Committee (COMREC) of University of Malawi protocol number P.02/10/861 and the Human Research Ethics Committee at the University of Cape Town (UCT) protocol number REF: 103/2009

Elizabeth L. Kampira Date

ABSTRACT

Introduction: Infectious diseases are endemic in Africa, especially tuberculosis (TB), malaria and human immunodefiency virus (HIV)/acquired immunodeficiency syndrome (AIDS). Genomics research has the potential to improve the health of Africans through identification of genetic markers associated with either disease susceptibility or therapeutic drug response. This project was set to investigate the genetic correlates for drugs associated with mitochondrial toxicity that are used as part of HIV therapy, especially nucleoside reverse transcriptase inhibitors (NRTIs). Toxicity from NRTIs manifests through metabolic diseases such as peripheral neuropathy, lipodystrophy, lactic acidosis and hyperlactatemia but show interpatient variability. Studying African populations is likely to open the door for the population to benefit from novel diagnostic tools and drugs developed on the basis of pharmacogenomics knowledge. In an effort to contribute to this knowledge, the role of variation in mitochondrial DNA (mtDNA) and polymerase gamma (POL-γ) on how patients respond to stavudine-containing antiretroviral therapy (ART) among adult Malawian HIV/AIDS patients was investigated.

Methodology: Two hundred and fifteen (n = 215) adult HIV/AIDS patients on stavudine containing ART regimens were recruited from antiretroviral therapy clinic at Queen Elizabeth Central Hospital, Blantyre, into a cross sectional study to investigate the effects of genetic variants in mtDNA and polymerase-γ. The patients were required to have been on stavudine (d4T) containing antiretroviral therapy (ART) for at least six months. The HIV/AIDS patients were categorised into their respective clinical manifestation groups as follows: those presenting with 1) peripheral neuropathy, 2) lipodystrophy, 3) hyperlactatemia, and 4) patients presenting with no signs and symptoms of stavudine associated toxicity. The whole mitochondrial DNA coding region for each patient was sequenced while a section of mtDNA POL –γ was sequenced. In addition, mtDNA levels were quantified, CD4 count, viral load and creatinine levels were determined. Different statistical methods were used to analyze the data and these included network analysis, distance based phylogenetic analysis, principal component analysis, manova, and logistic regression.

Results: With respect to mtDNA characterisation, 184 haplotypes were observed which belong to 9 mtDNA L lineage subhaplogroups and placing Malawian ethnic groups with other groups from Southern and Southeast Africa**.** Mitochondrial DNA subhaplogroup L0a2 was independently associated with increased risk of peripheral neuropathy (OR 2.23; 95% CI, 1.14 – 4.39; p = 0.019) and subhaplogroup L2a was independently associated with reduced risk of peripheral neuropathy (OR, 0.39; 95% CI, 0.16 – 0.94; P = 0.036). Mitochondrial DNA subhaplogroup L3e appeared to be protective against lipodystrophy as none of the individuals with this subhaplogroup presented with lipodystrophy. No mutations in POLG-γ were associated with stavudine toxicities. It was observed that mitochondrial levels were lower in HIV/AIDS patients (P = 0.05) when compared to health controls, patients with peripheral neuropathy (P = 0.039) and patients presenting with hyperlactatemia (P = 0.024) when compared to those without. Significantly low mitochondrial levels (P = 0.01) were observed in the subhaplogroup L0a2 compared with L2a.

Conclusion: Variation in clinical response and susceptibility to drug associated side effects observed with respect to mtDNA subhaplogroups could point to effects of subhaplogroup specific mutations on the structure of mitochondria and associated proteins. Our result shows that the Malawian population, although related to other African populations, exhibit a unique mtDNA genetic pattern. Our data also shows that genetic predisposition may play an important role in the pathophysiology of stavudine-associated mitochondrial disorders in patients on NRTIs. We suggest that the current efforts for genomics, proteomics, metabolomics science and personalized medicine applications in Africa should to be expanded in scope to include mitochondrial individual variations, as illustrated in this study.

Preface

This thesis includes written chapters and manuscripts for journals in accordance with general provision 6.7 in the General Rules for the degree of Doctor of Philosophy (PhD) of the University of Cape Town, with the approval of 2013 of the university Doctoral Degrees Board. Chapter 1 gives an introduction to the thesis; chapter 2 presents the comprehensive literature review giving the necessary background to the thesis. Chapter 3 provides detailed information on participants' recruitment, clinical parameters. Chapters 4-7 comprises of the journal manuscripts (published, accepted, in revision, ready for submission), while chapter 8 reports on polymerase gamma variation and its role in stavudine-associated toxicities. The list of manuscripts (chapters 4-8) is as follows:

- **Chapter 4: Diversity of mtDNA variation in Malawian ethnic groups**
  Kampira E, Kumwenda K, Oosterhout J.J, Mbiyavanga M, Elson J, Dandara C. Diverse mtDNA subhaplogroups in seven Malawian ethnic groups. *PLos ONE (**Revision resubmitted)***
- **Chapter 5: mtDNA variation and susceptibility to peripheral neuropathy**
  Kampira E, Kumwenda J, Oosterhout J.J, Dandara C. Mitochondrial DNA subhaplogroups L0a2 and L2a modify susceptibility to peripheral neuropathy in Malawian adults on stavudine containing highly active antiretroviral therapy. *J Acquir Immune Def Syndr 2013; 63(5) 647-652 (**Published**)*
- **Chapter 6: mtDNA variation and susceptibility to lipodystrophy**
  Kampira E, Kumwenda J, Oosterhout J.J, Dandara C. Mitochondria subhaplogroups and differential risk of stavudine induced lipodystrophy in Malawi HIV/AIDS patients. *Pharmacogenetics Journal (**Accepted for publication***)
- **Chapter 7: Effects of stavudeine containing ART on mitochondria levels**
  Kampira E, Dzobo K, Kumwenda J, van Oosterhout JJ, Parker IM, Dandara C. Alteration of mitochondrial DNA levels in HIV/AIDS patients presenting with stavudine associated adverse events. *OMICS Journal **(For Submission)***
- **Chpater 8: Polymerase gamma variation and stavudine associated toxicity**

Lastly, **Chapter 9** is a comprehensive discussion bringing the findings from the different publications together and synthesizing their meaning. Highlighting what this thesis is contributing in terms of new knowledge as well as outlining possible gaps in knowledge that still need to be covered that is likely to form part of future projects. Conclusions that are informed by findings from all the publications.

My contribution to the co-authored work: Together with my supervisors, as the PhD candidate, I was involved in the conception of the study, its design and protocol set-up. I was in charge of sample collection, performed 100% of the experimental work, analysed the data with the assistance from supervisors, and statistical experts. I am the lead author and drafted all the first versions of the articles and corrected all the subsequent versions. All co-authors commented on all manuscripts and approved the final versions of the manuscripts for submission.

DEDICATION

I dedicate this work to my children

Hendrick and Heather

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First and foremost, glory be to God almighty for taking me through this journey. It has been by his grace that I have come this far. I would like to express my sincere gratitude to my supervisors Associate Professor Collet Dandara and Professor Johnstone Kumwenda for their tireless support during my PhD study and research, especially their patience, motivation, skills, enthusiasm, immense knowledge and guidance throughout the PhD journey. Furthermore I would like to thank our collaborator Professor Joep van Oosterhout for the stimulating discussion, clinical support and advice. Their ability and team work made my entire time of research and writing of this thesis very interesting and I have great respect for these people.

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My love and gratitude goes to my Dad and late mum who passed away whilst I was studying. You were wonderful and such loving and caring mum may your soul rest in peace.

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List of abbreviations

acetyl-CoA	Acetyl Coenzyme A
adPEO	Autosomal dominant ophthalmoplegia
AIDS	Acquired immunodeficiency syndrome
arPEO	Recessive ophthalmoplegia
ART	Antiretroviral therapy
ARV	Antiretroviral
ATP	Adenosine-5´-triphosphate
ATPase 6	Adenosine-5´-triphosphate synthase 6
ATPase 8	Adenosine-5´-triphosphate synthase 8
AZT	Zidovudine
β-oxidation	Beta oxidation
CCR5	Chemokine receptor type 5
CoA	Coenzyme A
CoQ	Coenzyme Q
COX I-III	Complex I-III
CPEO	Chronic progressive external ophthalmoplegia
CPEO	Chronic progressive external ophthalmoplegia
Cu^{2+}	Copper (II) ion
CYB	Cytochrome-b
Cyt c	Cytochrome c
d4T- DP	Stavudine diphosphate
d4T	Stavudine
d4T-MP	Stavudine monophosphate
d4T-TP	Stavudine triphosphates
ddC	Zalcitabine
ddI	Didanosine
D-Loop	Displacement loop
DNA	Deoxyribonucleic acid
DNC	Deoxynucleotide carriers
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
Exo	Exonuclease
FAD	Flavin adenine dinucleotide
FADH	Flavin adenine dinucleotide hydrogenase
Fe^{2+}	Iron (II) ion
Fe-S	Iron sulfur
Gly	Glycine
H_2O_2	Hydrogen peroxide
HAART	high active antiretroviral therapy
HALS	HAART associated lipodystrophy syndrome
HC	Healthy controls
HCO_3	Bicarbonate
HDL	High density lipoprotein

HIV	Human immunodeficiency virus
HT	Hypertension
IL-1β	Interleukin-1beta
KSS	Kearns-Sayre syndrome
LAC	Lactate
LD	Lipodystrophy
LDL	Low density lipoprotein
Leu	Leucine
LHON	Leber hereditary optic neuropathy
LS	Leigh syndrome
MELAS	Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episode
MERRF	Myoclonic epilepsy with ragged-red fibers
MILS	Maternally inherited Leigh syndrome
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
Mn-SOD	Superoxide dismutase
mtDNA	Mitochondrial deoxyribonucleic acid
mtSSB	Mitochondrial single stranded DNA-binding protein
NaAc	Sodium acetate
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogenase
NARP	Neurogenic weakness with ataxia and retinitis pigmentosa
ND1-6	Nicotinamide adenine dinucleotide dehydrogenase
nDNA	Nuclear DNA
NNRTIs	Non nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
O_2^-	Superoxide
OH	Hydroxyl
OXPHOS	Oxidative phosphorylation
$^{\bullet}OH$	Hydroxyl free radical
PDC	Pyruvate dehydrogenase complex
PIs	Protease inhibitors (PIs)
PN	Peripheral neuropathy
POL-γ	Polymerase gamma
QS	Quantitation Standard
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SANDO	Sensory ataxia neuropathy
SCAE	Spinocerebellar ataxia with epilepsy
SNPs	Single nucleotide polymorphisms
tRNA	Transfer ribonucleic acid
WHO	World Health Organisation

1. CHAPTER 1: INTRODUCTION

1.1. Overview

Human immunodeficiency virus/Acquired immunodeficiency syndrome (HIV/AIDS) is one of the major health problems in the world with 34 million people infected and sixty eight percent (23.5 million) of them residing in Sub-Saharan Africa [1]. As a result of the WHO "3 by 5" call to action, over 6 million individuals have been started on highly active antiretroviral therapy (HAART) in resource poor areas [1]. There are several classes of antiretroviral (ARV) drugs that have been approved for the treatment of HIV (Table 1.1). The ARV drugs are classified according to their mechanisms of action. They include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase and fusion inhibitors, blocking viral activities at different stages of the HIV life cycle as shown in Figure 1.1. The World Health Organisation (WHO) recommends a combination of two NRTIs and one NNRTI as first-line therapy for the treatment of HIV. The idea behind combination ARV drug therapy of is based on the synergistic or additive potential of two or more drugs with independent modes of action and different biochemical targets (Figure 1.1) in order to improve therapeutic efficacy and prevent or delay the development of resistance of the virus to the individual components of the combination.

Table 1.1: List of FDA approved antiretroviral drugs and their classification

Drug classification	Specific drugs
Nucleoside reverse transcriptase inhibitors (NRTIs)	stavudine (d4T), zidovudine (ZDV), didanosine (ddi), zalcitabine (ddc), lamivudine (3TC), abacavir (ABC), tenofovir (TDF), emtricitabine (FTC)
Non-nucleoside Reverse transcriptase inhibitors (NNRTIs)	ripivirine (RPV), etravirine (ETR), delavirdine (DLV), nevirapine (NVP), efavirenz (EFV)
Protease inhibitors (PIs)	tipranavir (TPV), indinavir (IDV), saquinavir (SQV), lopinavir (LPV), ritonavir (LRTV), fosamprenavir (FOS), darunavir (DRV), atazanavir (ATV), nelfinavir (NFV), Lopinavir/ritonavir (LPV/RTV)
Integrase Inhibitors	raltegravir (RAL), dolutegravir (TAF)
Fusion inhibitors	Maraviroc (MVC), enfuvirtide (T-20)

Approved drugs according to US Food and Drug Administration in 2013

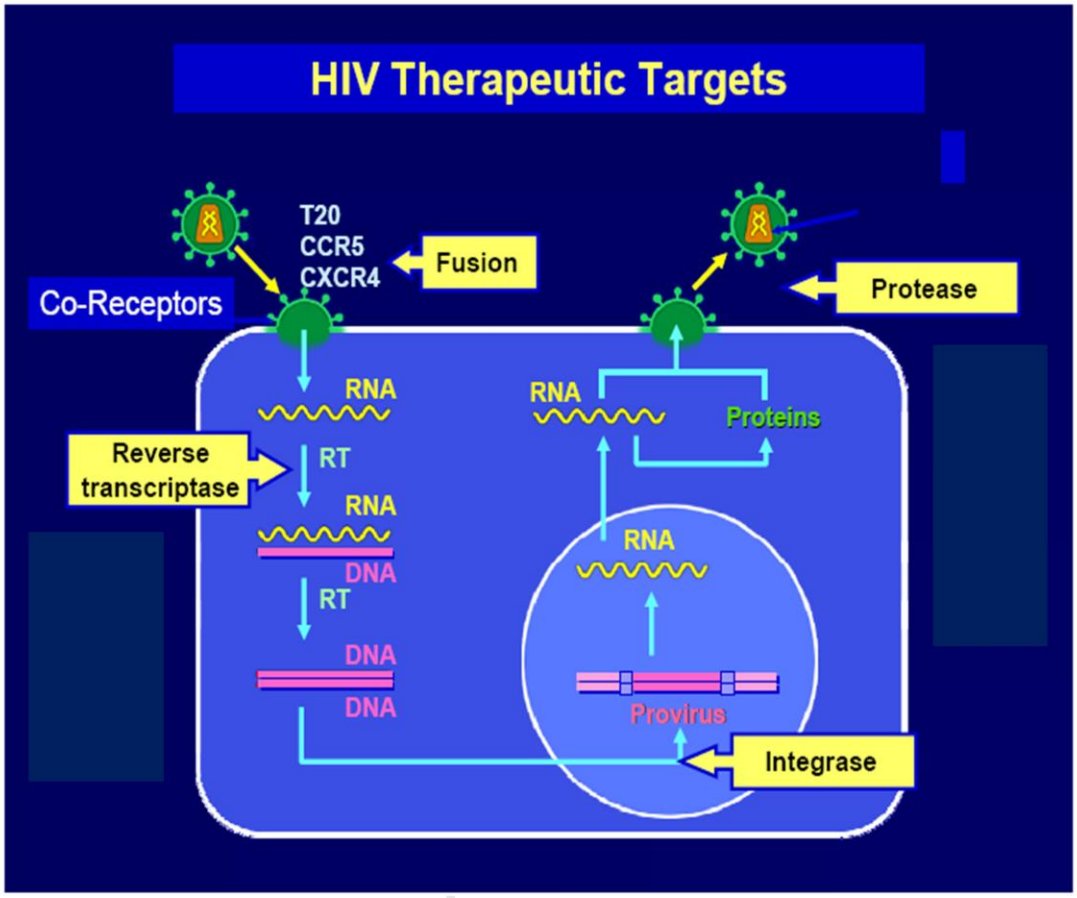

Figure 1.1: HIV life cycle showing the different stages targeted by antiretroviral drugs (yellow arrows)
Diagram adapted from Sohn HA, and Ananworanich J [2]

Amongst adult Malawians, HIV/AIDS is the leading cause of death and a major contributing factor to the country's low life expectancy of 52 years. According to WHO report, the country has an HIV prevalence of 11% among the 15 – 49 year olds with high prevalence among women (13%) compared to men (8%) [1]. At the end of September 2011, the Malawi National antiretroviral therapy (ART) programme reported nearly 500, 000 people had been initiated on ART since 2004. The first-line regimen that was chosen by the Malawi ART program is a generic combination of stavudine (d4T), lamivudine (3TC) and nevirapine (NVP) [3]. This combination therapy is effective against the virus; however, one of the limitations of ART in general is the occurrence of adverse drug events. Stavudine, a very potent antiretroviral drug, has

been the most associated with adverse drug events in many studies [4,5,6]. Common adverse events include peripheral neuropathy and lipodystrophy [5]. Much less common but equally important adverse drug effects include lactic acidosis, dyslipidemia, pancreatitis and glucose intolerance [7,8]. As a result of these adverse drug events, WHO in 2010 recommended the phasing out of use of stavudine replacing it with alternative NRTIs with better safety profiles such as tenofovir or zidovudine [9]. However, many low and middle income countries are still using stavudine in their standard first-line ART regimen due to lack of resources [10].

Toxicities related to stavudine (d4T) use are thought to occur as a result of defective mitochondrial DNA (mtDNA) replication [11]. Phosphorylated d4T has structural resemblance to natural dNTPs and inhibits mitochondrial DNA polymerase gamma activities by competing for the binding site (POL-γ) [12] . Phenotype characteristics of stavudine associated toxicities in HIV infected patients receiving antiretroviral therapy are similar to diseases associated with mtDNA dysfunction. Researchers have identified some mitochondrial DNA mutations (haplotypes) that are associated with increased risk of stavudine toxicities, but these haplotypes have been described mainly in studies among patients of European origin [13].

1.2. References

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2. CHAPTER 2: LITERATURE REVIEW

2.1. Mitochondrial Structure and Functions

2.1.1. Mitochondrial structure

Mitochondria are small intracellular organelles that live, divide and fuse in an endosymbiotic relationship in the cytosol of all human cells except red blood cells. The human mitochondrion is a circular, double stranded molecule and has 16,569 base pairs coding for 37 genes. These genes encode 13 polypeptides that are essential for ATP production as well as 22 transfer RNAs (tRNA) and 2 ribosomal RNAs (rRNA), which form part of the organelle's internal protein synthesis machinery [1] (Figure 2.1). The polypeptides encoded in the mitochondrial genome are subunits of the respiratory chain of complexes I - III, cytochrome c oxidase and ATP synthase [2] as indicated in Table 2.1. Mitochondrial (mtDNA) has a continuous coding region without introns and a non-coding region called the displacement loop (D-Loop) [3]. The D-Loop region is 1121 base pair long and it contains the origin of replication [3,4].

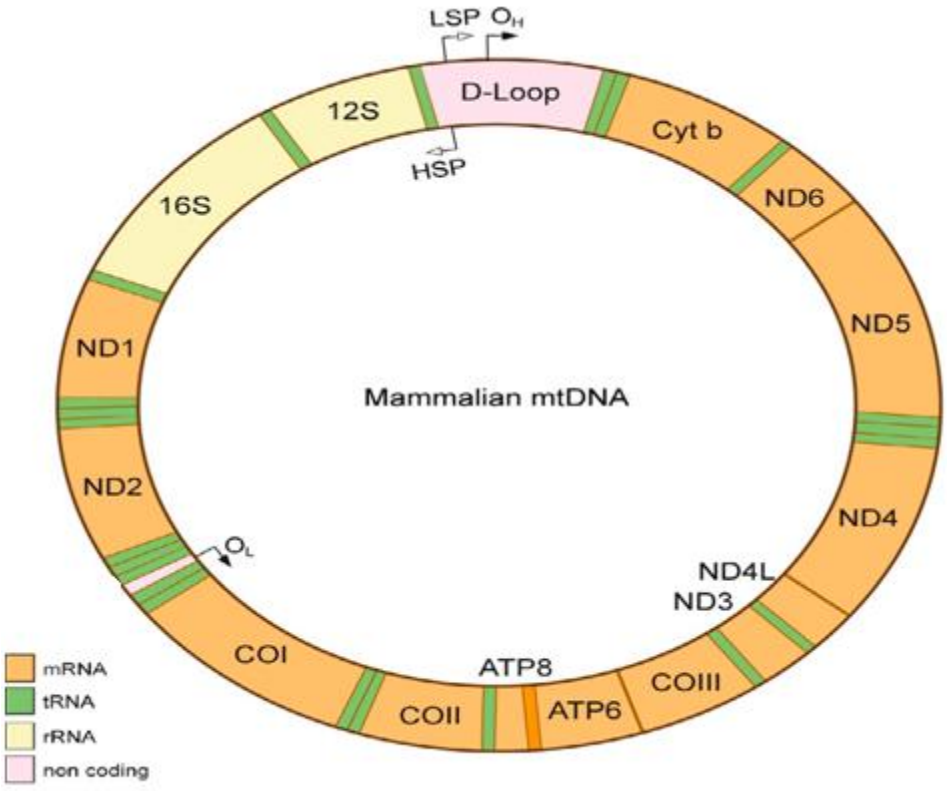


Figure 2.1: A diagrammatical representation of human mtDNA molecule
mtDNA consist of the displacement loop (D loop) (non-coding) region anchors promoters for transcription of light strand promoter (LSP) and heavy strand promoter (HSP) and the origin of leading strand replication (OH). The coding region has 13 mtDNA genes (ND1–6, Cyt b, COI–III, ATP6, and ATP8) in orange, genes for the two rRNAs (in yellow), and 22 tRNAs (in green) are indicated by boxes. The origin of lagging strand replication (OL) is embedded in a cluster of tRNA genes. Modified from Park and Larson 2011 [3]

Mitochondria play an important role in cellular respiration and are regarded as the powerhouse of the cells. In stepwise processes, which are controlled by genes encoded within mtDNA and a number of oxidative enzymes, nutrients and oxygen are processed into adenosine triphosphates (ATP). Cellular respiration can be aerobic or anaerobic and mitochondrion is involved in aerobic respiration which is divided into three main categories; glycolysis, Kreb's cycle (citric acid cycle) and electron transport chain. Glycolysis and the Krebs cycle are suppliers of the electrons that are necessary for energy production within the electron transport chain.

Glycolysis occurs in the cytosol independent of the mitochondria where glucose is partially oxidized to yield only two ATP molecules, two pyruvate and 2 NADH (Nicotinamide Adenine Dinucleotide) [5,6]. Pyruvate is oxidized by the pyruvate dehydrogenase to form acetyl-CoA

[20] which is also metabolized by citrate synthase to acetate which enters the Krebs cycle where a number of oxidative reactions take place that regenerate oxaloacetate at the end of the cycle. Apart from pyruvate, fatty acid and proteins are degraded to form Acetyl-CoA as well, which enters the Krebs cycle. Fatty acids are transported across the outer mitochondrial membrane and once inside the mitochondrial matrix, fatty acids undergo β-oxidation to form Acetyl-CoA. Proteins are broken down by proteases into their constituent amino acids and their carbon backbones become a source of energy by being converted to acetyl-CoA. The high energy electrons generated during this process reduce oxidized nicotinamide adenine dinucleotide (NAD^+), along with another electron carrier, flavin adenine dinucleotide (FAD) to form NADH and $FADH_2$ [5,7]. However during this process less energy is generated via substrate-level phosphorylation. In the final stage of energy metabolism, the high-energy electrons within NADH and $FADH_2$ are passed to a set of mitochondria-membrane-bound enzymes in a process referred to as the respiratory chain (Electron Transport Chain) or oxidative phosphorylation [5].

2.1.2. Electron transport chain

The electron transport chain is the primary site for ATP synthesis, which occurs within the mitochondrion, consists of five multimeric protein complexes (Table 2.1 and two electron carrier molecules called coenzyme Q (CoQ) and cytochrome c (Cyt c) [8]. The process involves transfer of electrons in a sequence from NADH and FADH by complex 1 through II, III and IV respectively, to molecular oxygen as shown in Figure 2.2 [5,9]. At the same time hydrogen ions are pumped out of the mitochondrial matrix into the intermembrane space, whilst an electrochemical gradient is generated in the matrix. The flow back of hydrogen ions into the matrix is enhanced by ATP synthase protein complex (Complex V) and these protons release free energy which is used to produce ATP by ATP synthase protein complex [5,9].

Table 2.1: Respiratory chain complexes and their composition

Complex	Nuclear subunits	Mitochondrial subunits	Total subunits
I	39	7 (ND1 – 6, ND4L)	46
II	4	0	4
III	10	1 (CYB)	11
IV	10	3 (COX I –III)	13
V	14	2 (ATPase 6 and 8)	16

ND: NADH-dehydrogenase, CYB: cytochrome-b, COX: cytochrome oxidase. ND 1-6 are genes from the leading strand replicated from the heavy strand and ND4L from the lagging strand replicated from the light strand.

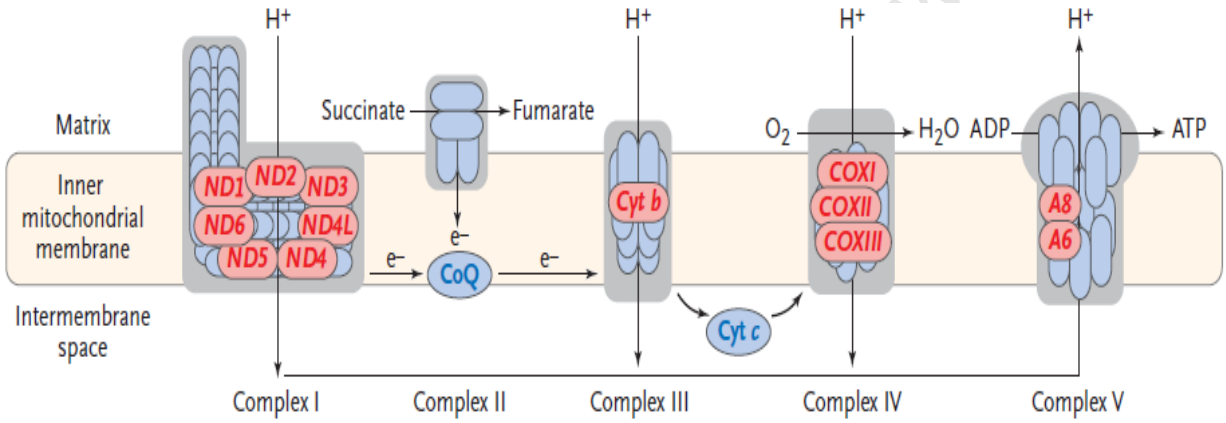


Figure 2.2 Schematic presentation of the electron transport chain.
NADH and FADH convey its electrons to complex I and II respectively, in the genes involved in electron transport chain. Coenzyme Q (CoQ) and cytochrome *c* carries electrons from between complexes I and IV and free energy is generated as electrons are transferred which is used to pump protons from the matrix to the intermembrane space. A proton gradient across the inner membrane is established and the energy stored is used to drive ATP synthesis as protons flow back to the matrix through complex V. Adapted from DiMauro and Schon 2003 [8]

Most of the genes involved in the mitochondrial complex are coded for by mitochondrial DNA. Mitochondrial DNA has no protective histones, and is exposed to harmful reactive oxygen species (ROS) that are generated as by-product of oxidative phosphorylation (OXPHOS) during ATP production. Mitochondrial DNA therefore has a higher rate of spontaneous mutations than nuclear DNA [10,11]. The reactive oxygen species include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl free radical ($^{\bullet}OH$) [12]. During these processes electrons from complex

I and II may leak into the matrix and react with oxygen to form superoxide which is disproportionated by the mitochondrial superoxide dismutase (Mn-SOD) to hydrogen peroxide [11,12]. The hydrogen peroxide is further reduced to water by glutathione peroxidase. In the presence of transition metals, iron (II) ion (Fe^{2+}) and copper (II) ion (Cu^{2+}), hydrogen peroxide can be converted to reactive hydroxyl radical ($^{\bullet}OH$) [10,12]. Accumulation of these reactive oxygen species in the cell may cause damage to mtDNA, cellular proteins, lipids, nucleic acids and also may inhibit iron sulfur (Fe-S) centres of Electron Transport Chain (ETC) complexes I, II, and III, and Krebs cycle aconitase, resulting in a shutdown of mitochondrial energy production [10,12]. The reactive oxygen species peroxidizes lipids in the mitochondrial and cellular membranes increasing their permeability [10,11]. Damage to mitochondrial proteins, for example polymerase gamma, may result in deletions, rearrangements, and other mutations in mtDNA [10,11].

In addition to energy production, mitochondrial are also involved in other biochemical processes including calcium storage and mobilization, ammonia detoxification via the urea cycle, apoptosis, recycling and decomposition of carbohydrates and fats [13]. Unlike nuclear DNA, all mtDNA is maternally inherited.

2.2. Mitochondrial genetics and inheritance

In Mendelian genetics, the emerging single cell embryo from the union of egg and sperm contains equal matching sets of chromosomes from mother and father. During replication the cell duplicates its chromosomes, so that each daughter cell receives equal numbers of paternal and maternal chromosomes. The result is that each cell in the body will carry identical genes and identical mutations. In contrast, the mtDNA is maternally inherited, the mtDNA in sperm cells are present at the base of the tail which is usually degraded and lost during fertilization. Therefore a woman carrying mtDNA mutations will pass these on to all her children and only her daughters will transmit them to their offspring [14,15]. The result is that each cell in the body will carry identical mtDNA genes and identical mutations. However mtDNA may exhibit both different nucleotides at the same position in the same tissue [8].

2.3. Heteroplasmy

Most of the diseases caused by mtDNA mutations that are inherited have a wide tissue distribution [16]. However further mutations can accumulate either in mitochondrial molecules in a single cell or particular tissue during a person's life time [8]. Accumulation of mutations may have detrimental effects over time, affecting some of the processes catalyzed by mitochondrial derived genes [10]. Unlike nuclear DNA (nDNA), mtDNA may exhibit different genotypes (mutant gene coexists with wild type) in the same cell, a term referred to as heteroplasmy [8,17]. The condition in which only wild type mtDNA is observed in the cells, is referred to as homoplasmy [8].

Mitochondrial DNA has been used to associate population genealogies as well as determining risk of human disease because of its unique characteristics such as maternal inheritance, lack of recombination, high mutation rate and high copy numbers within the cell [2,18,19]. Studies of the human mtDNA genome have been carried out by analyzing single nucleotide polymorphisms (SNPs). These studies have demonstrated distinct geographical distributions of mtDNA and that SNPs patterns can be classified into groups of haplotypes [2,8,18].

2.4. Genetic variation and human population profiling: role of mtDNA

Mitochondrial and Y-chromosome DNA variation has been used to define population relatedness for a long time. Unlike mtDNA, Y-chromosome DNA variations are morphologically male specific. Therefore the world population has been characterised on the basis of specific haplotypes or sets of single nucleotide polymorphisms (SNPs) in the mtDNA called haplogroups [18]. There are many haplogroups including A, C, H, J, T, M and V representing different human populations [19]. Mitochondrial DNA lineage termed L geographically characterises African populations [18]. Haplogroup L has been divided into two branches, L0 and L1-6 [20]. Furthermore these haplogroups are differentiated into subhaplogroups, [19,21] and L0 comprises of five branches of L0a, L0b, L0d, L0f and L0k. The distribution and frequencies of these L subhaplogoups within Africa is structured around geographic areas and ethnic groups [19,21]. According to Anderson-Mann [22] the distribution frequencies of subhaplogroups across Africa varies (Figure 2.3). For example, the frequency of haplogroup L0 is more than 90% in the Khoisan population of South Africa compared to the 20% observed in East African populations

who have similar origin [23]. Subhaplogroup L0a is believed to have originated in Eastern Africa and is common in eastern, central, and southeastern Africa, but is hardly found in northern, western, and southern Africa [24]. L0d and L0k are found almost universally among southern African Khoisan (SAK) speakers, although a recent study indicates that L0k is also found in the Southeast African population while haplogroup L0d is also present among the Turkana of Kenya and populations in Mozambique [24,25,26]. Despite being confined to the Khoisan, studies have shown that there is no phylogenetic relationship between L0d and L0K haplogroups [27,28]. In contrast L0f is relatively rare and is geographically confined to East Africa, mainly in Tanzania and Uganda [23,24].

The L1-6 branches are widespread all over the African continent with L2 being dominant in West Africa [24]. However, recent work has shown that Mozambique in Southeastern Africa has 42% prevalence of subhaplogroup L2a, compared to 15% among the Tanzania, Kenyan and Ugandan Bantu speakers while L2a has a frequency of 38% among non-Bantu speakers in these countries [22,26]. This shows that within and between populations subhaplogroup distribution can be highly variable, probably due to population dispersal, migration and intermarriages. Haplogroup L1 is divided into two subhaplogroups L1b and L1c with L1b being most frequent in West Africa while L1c is common in Central Africa with some presence in Southeastern Africa [22,24]. Although L3 is the most frequent haplogroup (34%) in Africa and is the most prolific haplogroup in Ethiopia its subhaplogroup L3e is equally distributed between the west and east Africa [22,24]. Furthermore L3 haplogroup also gives rise to M and N haplogroups sometimes referred to as haplogroups L3M and L3N, respectively. Thus, studies on mtDNA have been used to investigate genetic relatedness of sometimes linguistically distinct populations. Malawi is one geographical area that has remained understudied with respect to the genetic relatedness of its different ethnic groups.

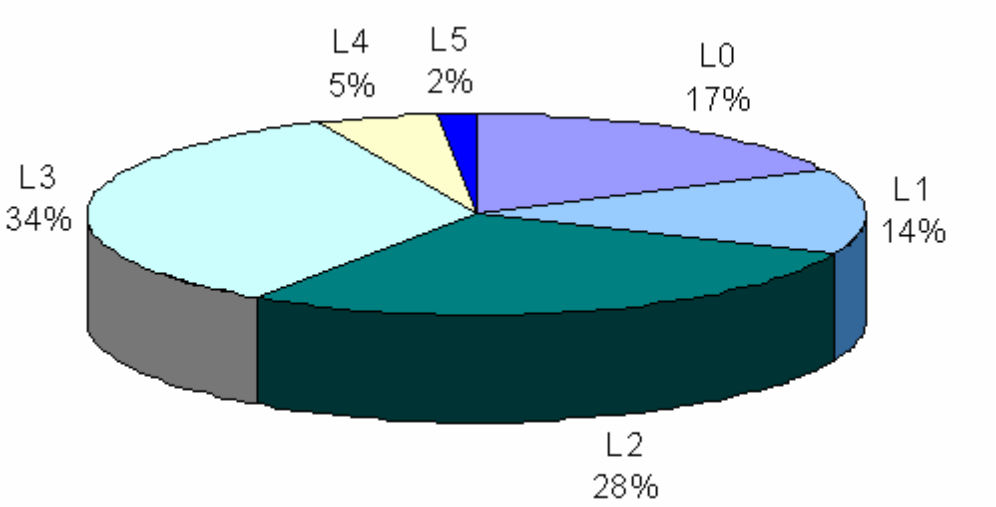


Figure 2.3: The mtDNA haplogroup L distribution in African populations.
The haplogroups are not equally distributed with majority of the population haplogroup L3 followed by L2, L0 and L1 [22]. L3 derivatives are present in nearly all the African populations mostly in western and eastern/southeastern sub-Saharan Africa, particularly among speakers of the Bantu language and is thought to have originated in eastern Africa [24]

2.4.1. Bantu migration and the people of Malawi

Mitochondrial DNA genetic differences between different populations are thought to have come mainly as result migration patterns followed by different groups; an example is the migration of the Bantu speaking people. It is believed that Bantu-speaking people came from an area around northwestern Cameroon/southern Nigeria and spread throughout sub-Saharan Africa [29,30,31]. However, before the Bantu expansion, the region is thought to have been inhabited by hunting and iron foraging peoples, such as Pygmies from central Africa, proto-Khoisan around the Kalahari Desert, Hadza and Sandawe from East Africa and Khoe and San from South Africa and Namibia. The people participating in the Bantu migration split into two routes; one group followed the western route travelling south and roughly following the Atlantic coastline through the Democratic Republic of Congo, while the second group moved eastwards and settled near the Great Lakes of East Africa (see Figure 2.4) [32,33]. Those following the Eastern Bantu migration split into two more groups, with one group travelling along the Ruvuma River toward the coast, reaching present-day Natal by the end of the third century A.D., and the other travelling along the shores of Lake Malawi, through what is now Eastern Zimbabwe, reaching the Northern Transvaal around A.D. 500.

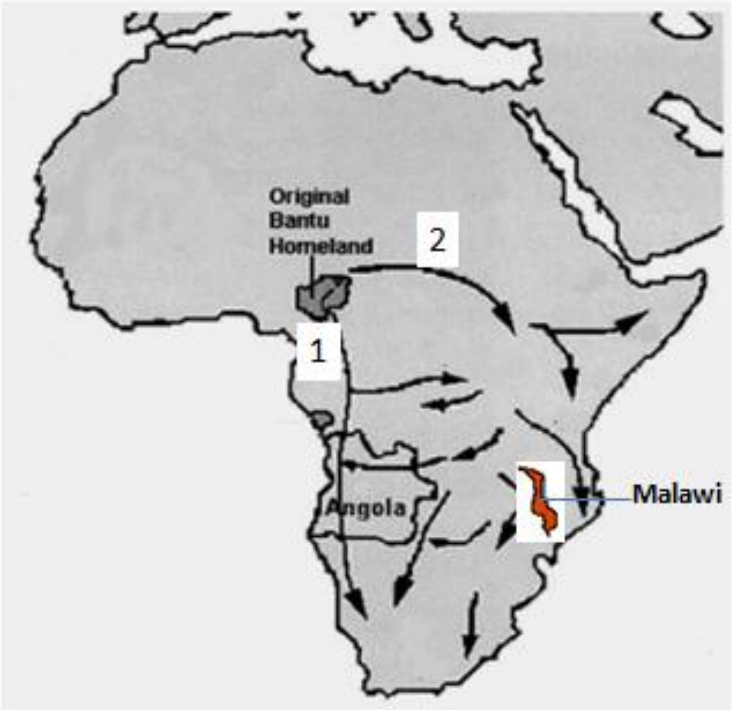


Figure 2.4: Map of Africa showing the two proposed Bantu speakers migration routes from their homeland.

Adapted from Beleza et al 2005 [32]

Following this migration, the Malawian population is thought to have emerged. The Republic of Malawi is located in the Southeast of Africa and its 16 million populations is made up of eight major ethnic groups comprised of the following: Chewa/Nyanja (38%, ~ 6 million); Lhomwe (18%, ~ 3 million) Yao (13%, ~ 2 million); Ngoni (12%, ~ 2 million); Tumbuka (9%, ~ 1.5 million); Sena (3.5%, ~ 700 000); Tonga (2%, ~ 300 000); and Ngonde (1%, ~ 160 000) and other small ethnic groups of Bantu speakers as well as a few Asians and Europeans (3.5%, ~ 700 000) [34,35]. The Chewa tribe (Phiri and Banda clans) of Malawi also known as Nyanja in other countries such as Zambia, Mozambique, Namibia, Tanzania, and Zimbabwe, migrated from the

Luba area known as Malambo in what is now called the Democratic Republic of Congo and is one of the tribes which is highly concentrated in the central region [36,37]. Earlier settlement shows that the Chewa were the first Bantu speakers to settle in Malawi and established the Maravi kingdom in the 16th century [38]. They are concentrated in the central region but also show their presence in Chikwawa and Nkhata-Bay districts in southern and northern Malawi respectively, which are dominated by other ethnic groups. Generally people identify themselves by languages of major tribes in their area of geographical residence [36]. They follow matrilineal system and their language (called Chichewa or Chinyanja) is the official language of Malawi.

The Lhomwe people were the last group to have migrated into Malawi from Mozambique in the 1930s with the majority settling in Thyolo, Mulanje and Phalombe. They are thought to be descendants of the Bantu speakers who migrated East from Central Africa [24]. On arrival mixed with people whom they found already there, for example Mang'anja and practice small scale farming. They share with the Chewa the culture or practice of matrilineal kinship. Although the Lhomwe ethnic group constitutes the second largest ethnic group in Malawi (18% of the total population), their language is spoken by a mere 2.4% of the population.

Ngoni is one of the tribes whose distribution is highly dispersed throughout Malawi as they are found in all three regions of Malawi and lead to the decline of the Maravi Kingdom. The Malawi Ngoni (also referred to as Nguni), are offshoots of the Zulu-Swazi who emigrated from Natal and Swaziland in Southern Africa [39,40] and their migration was referred to as mfecane. They mainly migrated in two groups; one group moving towards the north and settling in Mzimba (northern Malawi) later a group proceeded to Tanzania, while the other group moved southwest and settled in Ntcheu (central Malawi) [40]. Later a number of offshoots arose from these groups in the nineteenth and early twentieth century and settled in various parts of the country (including Dedza, Mchinji, Mwanza, Neno and Thyolo) [41].

Another ethnic group found in Southern Malawi is the Sena whose name derives from a Mozambican town of Sena where they are thought to have migrated from. Sena are thought to be a group of languages with dialects being Sena, with sub ethnic groups Makua, Shona, Rabu and Phodzo and other Southeast Bantu [41]. The majority of the Sena are found in Nsanje and

Chikwawa districts and to a lesser extent in parts of Mwanza and Thyolo highlands which are dominated by the current the Chewa, Ngoni and Lhomwe [42].

The Tumbuka people are part of Bantu speakers of Malawi and are mainly found in the northern part of Malawi [43] but also in neighboring countries such as Zambia and Tanzania [37]. Tumbuka is spoken in Zambia by the Senga, Fungwe and Yombe ethnic groups [44]. They form the major ethnic groups of the Northern region of Malawi and there are an increasing number of Tumbuka women within Ngoni society because of intermarriages. The Tonga is another tribe which is found in the Northern part of Malawi and their language is known as Chitonga. While traditionally the Tumbuka are agriculturists, the Tonga are fishermen. Like the northern Ngoni and Sena, Tumbuka and Tonga practice patrilineal descent.

Yao is the third largest ethnic group and are widely distributed in the southern part of Malawi, originating from northern Mozambique and parts of Tanzania [37,45]. They migrated in two different groups, the Mangochi Yao who settled in Chiradzulu, Blantyre, Zomba and Mulanje districts, and the Machinga Yao settled in the Mangochi, Machinga and Liwonde and they hardly mixed [45]. The latter group established themselves as fishermen due to their proximity to the lake and was the first people to be agents of Arabs during slave trade. They assimilated the Chewa practice of matrilineal lineage. Despite political boundaries, it has proven difficult to distinguish the Malawian population by language. Considering the main groups of African languages Afro-asiatic, Niger-Congo, Nilo-Saharan, and Khoisan, Malawian Bantus are related to other groups within the African populations found in Southern Africa. Despite language relatedness with other African populations, their lineage based on the mtDNA haplogroup is not known.

Mitochondrial DNA haplogroup and sub-haplogroup distribution in Africa has been used for unraveling demographic phenomena that contributed to the settlement of ethnic groups over the continent. However, little is known about population and ethnic characteristics in relation to haplogroups of Southeast African countries, for instance Malawi, Zambia and Zimbabwe [25,46]. It is important to describe the phylogeography of the Malawian population in the context of African mtDNA haplogroup variations, in order to search for possible subhaplogroup matches

within Southeastern Africa. Furthermore, researchers have also embarked on human genetics studies in order to unveil population mtDNA haplogroup dispersions with the aim of understanding haplogroups or subhaplogroups that may be associated with differential susceptibility to disease or poor response to treatment at individual as well as population levels, therefore more data is required, especially from Africa.

2.5. Mitochondrial Mutations and Diseases

Mitochondrial diseases have been related to molecular defects in human mtDNA determined by a set of factors including mutations acquired or inherited, either in mitochondrial DNA or in nuclear genes that code for mitochondrial components [47,48]. In addition, adverse effects of drugs, infections and other or environmental factors may result in mitochondrial dysfunction [47,48]. Disorders may either affect only a single organ (e.g. the eye in Leber hereditary optic neuropathy [LHON]) or may involve multiple organ systems and present with prominent neurologic and myopathic features irrespective of age. Disease prevalence ranges between 1:8 500 – 1:39 000 in Europeans [49]. The more common clinical syndromes associated with mitochondrial disorders, include the Kearns-Sayre syndrome (KSS), chronic progressive external ophthalmoplegia (CPEO), mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibers (MERRF), neurogenic weakness with ataxia and retinitis pigmentosa (NARP), and Leigh syndrome (LS) with 11.5:100 000 prevalence of all mitochondrial diseases in both adults and children [8,15,50]. Furthermore, the characteristic features of the disorders could be categorized as either neurological or systemic [51]. An example of a systemic manifestation is lactic acidosis and of a neurological disorder is Kearns-Sayre syndromes both of which are associated with dysfunction of the mitochondrial respiratory chain [51]. A unique characteristic of mitochondrial diseases is that the same mtDNA mutation can produce quite different phenotypes (genocopies of mitochondrial disease), and different mutations can produce similar phenotypes (phenocopies of mitochondria disease) hence patients are classified by mtDNA genetic defect rather than by clinical manifestation [2,48].

Phenotypic expression in relation to mitochondrial disorders depends on the molecular and genetic features of the mutation, its tissue distribution and the relative dependence of each organ system on the mitochondrial energy supply [52]. An example is the mutation m.A3243G in the

tRNA (Leu(uur)) gene which is associated with either maternally inherited diabetes and deafness (MIDD) or chronic progressive external opthalmoplegia (CPEO) characterised by a limitation of eye movement, ptosis, and muscle weakness [53,54]. It has been reported that the tRNA (Leu(uur)) gene variant m.A3243G causes dysfunction of β cells in the pancreas that impairs insulin secretion in response to an increase in the glucose level, a situation which may lead to development of hyperglycemia [55,56]. In another study, high mutation load of the same variant in the skeletal muscle compared to blood was found to be associated with myopathy [57].

Leigh syndrome is a mitochondria disease associated with symmetrical magnetic resonance imaging (MRI) abnormalities in the brain stem, cerebellum, basal ganglia mostly accompanied with increased lactic acid level either in the blood or cerebrospinal fluid. It is thought to be caused by mutations in the NADH dehydrogenase but similar signs and symptoms may also occur with mutations in COX III, ATPase 6, and certain tRNA genes [58]. An example is the ATPase 6 T8993G mutation, which may present maternally inherited Leigh syndrome (MILS) or as neuropathy, ataxia and retinitis pigmentosa (NARP) depending on whether the proportion of mutated mtDNA is more than 90% or 70-90% of the total cell mtDNA, respectively, [59]. The mutation leads to the change in the amino acid leucine to proline which decreases the flow of hydrogen protons through ATP synthase protein complex by 30% [60]. Another relatively common mitochondrial disease is the Leber's hereditary optic neuropathy (LHON) which is characterised by the bilateral loss of central vision, caused by atrophy of the optic nerve. It normally only affects vision, but in some instances there are also cardiac conduction complications, peripheral neuropathy and cerebellar ataxia. The disease is associated with the mutations m.G3460A, m.G11778A and m.T14484C found in ND2, ND4 and ND6 genes, respectively, which make up complex I [61,62,63,64]. A mutation at position m.G11778A in the NADH dehydrogenase gene is considered as the primary pathogenic and is responsible for 50% of cases and also causes the most severe form of the disease that involves cardiac pre-excitation syndrome [50,61]. The m.G14459A mutation has been suggested to alter the coenzyme Q binding site of complex I by reducing the protein activity [65]. Mutations in the genes coding for rRNAs and tRNAs are also associated with disease, for example the syndrome of mitochondrial encephalo-myopathy with lactic acidosis and cerebrovascular accident episodes (MELAS) is associated with mutations m.A3243G, m.C3256T, m.T3271C and m.T3291C in the tRNA

leucine 1 [50,66,67,68,69]. The frequencies of these mutations in different populations are poorly characterised.

2.5.1. Mitochondrial diseases and the risk of neurodegeneration with infections

Mitochondrial disorders also underlies sensorineural hearing loss/aminoglycoside induced deafness (SNHL/DEAF) [70]. Upper respiratory tract infections were observed to trigger the hearing loss and neurodegenerative episodes in patients with mitochondrial disease [71]. A mechanism by which these infections may cause hearing loss in patients with mitochondrial disease is not well established. In addition, cells with mitochondrial mutations are susceptible to Legionella pneumophila, an intracellular pathogen that cause Legionnaire's disease, a severe form of pneumonia [72].

2.5.2. Mitochondrial polymorphisms and risk of metabolic disorders

Mitochondrial DNA polymorphisms have been implicated in the pathophysiology of several metabolic diseases including diabetes and obesity [73]. It has been observed that mitochondrial dysfunction in pancreatic beta cells caused by the tRNA (Leu(uur)) gene mutation A3243G on the tRNALeu gene usually occurs in the heteroplasmic form. This variant impairs insulin secretion in response to an increase of glucose levels, which may lead to development of hyperglycemia [55,74]. An increase in insulin secretion is needed to enhance glucose or lipids entry into the cells in order to increase the ATP production [74].

On the other hand metabolic disorder (MELAS) may occur in individual carrying mutation A3243G in the tRNALyS gene when its heteroplasmy level is more than 85% [47,67]. This disease is clinically characterised by generalized convulsions, headaches, deafness, muscle weakness, vomiting, loss of appetite, dementia and, at times, red-ripped fibers [67]. Biochemical studies have shown that substitution of adenine to guanine has transition effects on the tertiary structure of transfer RNA lysine (tRNALys) gene, as well as on methylation, acetylation and taurine modification of the anticodon, thus distorting translation [75]. Further investigations have revealed that m.T4291C mutations in the mitochondrial tRNA gene are associated with metabolic defects including hypercholesterolemia and hypomagnesaemia [76]. Dyslipidemia is a

risk factor for cardiovascular diseases, including myocardial infarction, stroke, and congestive heart failure [76]. Cytidine substitution causes a change in the amino acid and cannot form hydrogen bonds with the other base, therefore the substitution of cytidine for uridine markedly impairs ribosome binding which leads to phenotypic consequences [76,77]. Studies have demonstrated that stavudine is associated with dyslipidemia in HIV/AIDs patients, but its relation to mitochondrial mutations is not well established.

2.5.3. Mitochondrial polymorphisms and drug induced toxicities

Many mitochondrial clinical symptoms manifest when the bearer is exposed to environmental toxins; examples are presented in Table 2.2. Many drugs have been withdrawn from use due to mitochondrial associated toxicities (Table 2.2) and some have received black box warning (Table 2.3). The mechanism by which mitochondria are damaged by medications can be described as either direct or indirect. Thus, drugs can directly inhibit the mtDNA transcription or affect the activity of enzymes required for any of the steps of glycolysis and oxidative phosphorylation [78]. For instance, barbiturates directly inhibit NADH dehydrogenase which is part of complex I of the electron transport chain while aspirin affect coenzyme A (CoA) [78]. Other examples are aminoglycosides, which includes gentamicin and streptomycin (used for treatment of tuberculosis), neomycin and amikacin among others. Pharmacologically they inhibit the ribosomal translation hence blocking protein synthesis resulting into inhibition of bacterial growth and ultimately killing of bacteria. There are similarities in the structure of the bacterial ribosome and the mitochondrial ribosome residing in all eukaryote cells [79]. Studies in humans have shown that patients harboring mutations at m.1555A>G in the m.12SrRNA gene develop aminoglycoside-induced hearing loss when exposed to aminoglycoside [80].

Most of the idiosyncratic adverse drug responses due to nucleoside reverse transcriptase inhibitors (NRTIs) resemble inherited mitochondrial diseases (i.e. hepatic steatosis, lactic acidosis, myopathy, nephrotoxicity, peripheral neuropathy and pancreatitis). Histologic evidence demonstrates abnormal mitochondria and/or mtDNA depletion in affected tissues [81,82]. Studies have demonstrated that stavudine is associated with dyslipidemia in HIV/AIDs patients [83], but its relation to mitochondrial mutations is not well established. Although NRTI toxicities are a major problem for patients infected with HIV [84,85,86,87,88], most studies related to

stavudine associated toxicities have been carried in Caucasians where it is no longer used, while it is still used in resource poor countries where pharmacogenetic research in this area among African populations is limited.

Table 2.2: Drugs withdrawn from the market due mitochondrial toxicity since 1960s

Drug name	Indications	Reason for withdraw	Withdrawn
Thalidomide	Erythema Nodosum Leprosum (ENL)	Teratogenicity	1960s
Lysergic Acid Diethylamide	Psychotherapy	Abused	1960s
Diesthystilbestrol	Palliative therapy of inoperable prostate carcinoma	Teratogenicity	1970s
Phenformin and Buformin	Antihyperglycemic	Lactic acidosis	1978
Ticrynafen	Diuretic	Hepatitis	1982
Zimelidine	Antidepressant	Guillain- Barre	1983
Methaqualone	Depressant	Syndrome	1984
Triazolam	Insomnia	Addiction and overdose	1991
Fenfluramine	Antiobesity	UK-Psychiatric	1997
Dexfenfluramine	Antiobesity	Hepatotoxicity	1997
Terfenadine	Antihistamine	Hepatotoxicity	1998
Mibefradil	Angina and High Blood Pressure	Arrhythmias	1998
Troglitazone	Antihyperglycemic	Interactions	2000
Alosetron	Irritable bowel syndrome (IBS)	Hepatotoxicity	2000
Cisapride	Gastrointestinal Dysmotility	Constipation	2000s
Cerivastatin	Primary hypercholesterolemia, mixed dyslipidemia	Arrhythmiasis Rhabdomyolysis	2001
Rapacuronium	General Anesthesia		2001
Rofecoxib	Rheumatoid Arthritis	Bronchospasm	2004
Adderall (XR)	Attention Deficit Hyperactivity Disorder (ADHD) and Narcolepsy	Myocardial Infarction Canada - stroke	2005
Pemoline	Attention Deficit Hyperactivity Disorder (ADHD)	Hepatotoxicity	2005
Hydromorphone	Analgesic		2005
Natalizumab	Multiple Sclerosis (MS)	Overdose with alcohol CNS viral inflammation	2005 - 2006

Modified from Neustadt J and Pieczenik SR [78]; Dyken [89]; Labbea et al [90]

Table 2.3: Drugs receiving black box warnings due to mitochondrial impairment that are in use currently

Hepatoxicity		**Cardiovascular**		
Antivirals	**Antibiotics**	**Anthracyclines**	**Anaesthetic**	**Beta-Blockers 1**
Abacavir	Isoniazid	Dauncrubicin	Bupivacaine	Atenolol
Didanosine	Ketoconazole	Doxorubicin		
Emtricitabine	(oral)	Epirubicin		**Antiarhythmic**
Lamuvidine	Streptozocin	Idarubicin		Amiodarone (oral)
Nevirapine	Trovafloxacin		**Anticancer**	Disopyramide
Telbivudine		**NSAIDs**	**Drugs**	Dofetilide
Tenofovir	**CNS**	Celecoxib	Arsenic Trioxide	Ibutilide
Tipranavir	Dantrolene	Diclofenac	Cetuximab	
Stavudine	Divalproex Sodium	Diflunisal	Denileukin	**CNS**
Zalcitabine	Felbamate	Edodolac	diftitox	Amphetamines
Zidovudine	Naltrexone	Feneprofen	Mitoxantrone	Atomoxutin
	Nefazodone	Ibruprofen	Tamoxifen	Droperidol
Anticancer		Indomethacin		Methamphetamine
Flutamide	**Hypertesion**	Ketoprofen	**Diabetes**	Pergolide
Decarbazine	Bosentan	Mefenamic	Pioglitazone	
Gemtuzumab		Meloxicam	Roiglitazone	
Methotrexate		Naproxen		
Pentostatin		Nabumetone		
Tamoxifen		Oxaprozin		
		Piroxicam		
		Salsalate		
		Sulindae		
		Thioridazine		
		Tolmetin		

Lactic acidosis is a reflection of mitochondrial impairment. Modified from Neustadt J and Pieczenik SR [78]; Dyken [89]; Labbea et al [90]; Kakuda TN [82]

2.6. Stavudine disposition and pharmacokinetics

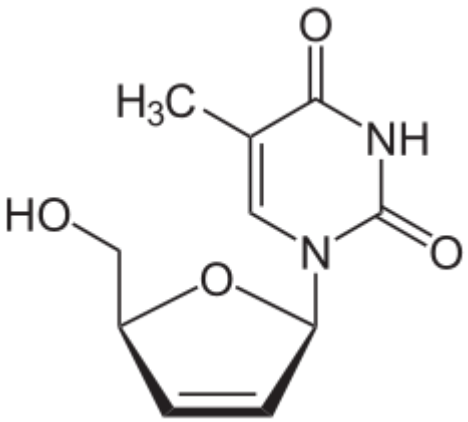
Stavudine is an analogue of thymidine, one of the building blocks of deoxyribonucleic acid and is a potent, selective, and orally active antiretroviral agent. The difference between stavudine and thymidine is the lack of a 3'-hydroxyl group on the stavudine deoxyribose moiety, thus, incorporation of stavudine in a new DNA strand leads to termination of the elongation of the strand because the next incoming deoxynucleotide cannot form the next 5'-3' phosphodiester bond needed to extend the DNA chain (Figure 2.5). Stavudine is lipophilic and is rapidly absorbed with peak plasma concentrations occurring within 1 hour after dosing. The absolute bioavailability is between 82 to 99% [91,92]. The prodrug enters the cells by non-facilitated diffusion where it is transformed intracellularly by nucleoside kinases into the pharmacologically

active d4Ttriphosphates moiety (d4T-TP) [93,94] as shown in Figure 2.5. The phosphorylated NRTIs in the cytoplasm are also transported into the mitochondrial matrix by mitochondrial protein carriers which act as deoxynucleotide carriers (DNC) [95]. Phosphorylated molecules in the mitochondrial matrix inhibit the activity of mitochondrial polymerase gamma by competing with dNTPs for the binding site and then as they get incorporated into the growing DNA strands by causing inhibition in the synthesis of mtDNA, thus prematurely terminating chain elongation [82,96].

Phosphorylated stavudine inhibits the activity of HIV-1 reverse transcriptase by competing with natural substrates of DNA polymerization, thymidine triphosphates. Thymidine kinase is the only rate limiting enzyme, hence the intracellular concentrations of stavudine monophosphates (d4T-MP), stavudine diphosphates (d4T-DP) and stavudine triphosphates (d4T-TP) have been observed to be similar in vitro. Differences in this rate limiting enzyme may cause variations in intracellular stavudine-TP levels [97,98]. The absorption of the parent compound is rapid, and it has a terminal elimination half-life of 1.3-1.4 hours but the phosphorylated drug (d4T-TP) has a longer half life of around 7 hours [92,99]. The main elimination route is renal, studies have shown that about 40% of the parent compound is eliminated unchanged through the kidney [91]

Higher NRTI drug levels have proven to be associated with toxicities [91,94]. In 2006 the World Health Organization (WHO) recommended a reduction in dosage of stavudine from 40mg to 30mg in patients weighing >60 kg (the recommended dose for patients <60 kg was already 30mg), following reports of reduced toxicities at the lower dose with retention of efficacy [100]. Despite this recommendation, stavudine associated side effects continue to occur in HIV patient on the regimen containing stavudine. Nevertheless, data on a minimum recommended dose of 30 mg/kg/day in relation to intracellular tri-phosphorylated drug levels that retain robust antiviral activity while reducing drug related toxicities is limited.

A



B

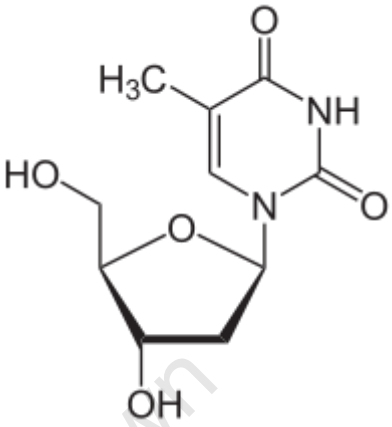


Figure 2.5: A comparison of the molecular structures of stavudine (d4T) and thymidine. A: **stavudine;** B, **thymidine**

2.6.1. Stavudine induced/associated mitochondrial toxicities

Human organs such as bone marrow, liver, peripheral nerves, muscle, and pancreas, depend heavily on oxidative phosphorylation in mitochondria for supplying their energy. These organs are at particular risk of toxicity when exposed to NRTIs. There are various suggested mechanisms by which stavudine causes mitochondrial toxicities as shown in Figure 2.6 [81]. Stavudine is one of the nucleoside reverse transcriptase inhibitor (NRTIs) that lacks a 3'-hydroxyl group on the deoxyribose moiety

Because of its chemical properties phosphorylated stavudine is transported into the mitochondrial matrix and competes with dNTP. Stavudine has been implicated in the development of mitochondrial toxicity related adverse effects such as peripheral neuropathy, lactic acidosis, pancreatitis, lipoatrophy and hepatic steatosis [101,102,103]. These toxicities are believed to be related to defective mitochondrial DNA replication due to the inhibition of the mitochondrial DNA polymerase gamma [102,104]. In addition to mitochondrial DNA

polymerase gamma inhibition, mitochondria dysfunction could be the a result of mtDNA mutations as well as oxidative stress (an imbalance between reactive oxygen species and antioxidants) [104]. Stavudine's efficacy has been compromised by associated adverse effects.

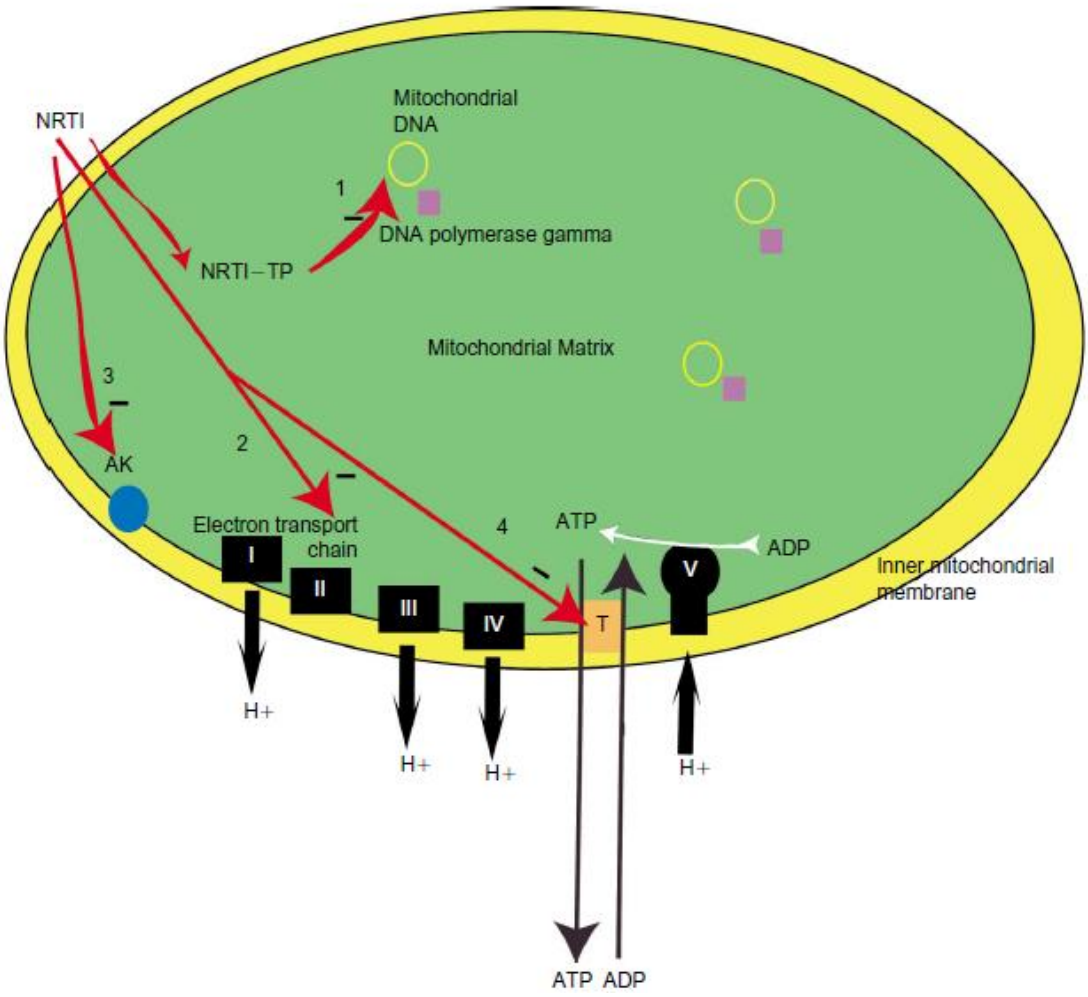


Figure 2.6: Indicating proposed mechanisms of NRTI mitochondrial toxicity.
These includes (1) inhibition of polymerase gamma activities which result in mitochondrial DNA depletion; (2) getting inserted in the electron transport chain genes which may result in termination of replication of ETC as well as cause leakage of the electrons from the system which react with oxygen producing more reactive oxygen species (ROS) which in turn damages the mtDNA (electron transport chain I –V) a consequence which results into oxidative phosphorylation failure and lead to ATP depletion; (3,4) interferes with adenylate kinase (AK) and ADP/ATP translocator activities which results into less ATP being produced. Adapted from Keswani et al. [81].

2.6.2. Peripheral neuropathy

Peripheral neuropathy refers to the damage of the peripheral nervous system. It is the most common neurological complications associated with HIV infection, occurring in up to 35% of the patients [83,105]. Patients may experience numbness, tingling, and pricking sensations (paresthesia), sensitivity to touch, or muscle weakness. Others may suffer more extreme symptoms, such as burning pain, muscle wasting, paralysis or organ or gland dysfunction (autonomic sensory neuropathy) [106]. These complications may results from HIV infection itself or from neurotoxic antiretroviral therapy such as NRTIs [105,107]. It has been clinically difficult to distinguish between drug induced and HIV associated neuropathy, although the timing of symptom onset may help to differentiate the aetiology [108,109].

2.6.3. HIV associated peripheral neuropathy

HIV associated peripheral nerve syndrome is an axonal neuropathy that is distal, symmetric, painful, and predominantly sensory. The symptoms may differ in different stages of HIV infection; and increase with progression of disease [110]. It is also more common in HIV/AIDS patients with CD4 count less than 200 X 10^6/L and HIV RNA >10,000 copies/mL [82,110]. HIV-1 viruses found in the brains of AIDS patients are chemokine receptor type 5 (CCR5) dependent and macrophage-tropic. The binding of virus on the CCR5 receptor on macrophages induces the proliferation of pro-inflammatory cytokine interleukin-1 beta 1 (IL-1β1) in response to HIV-1 infection [111]. Studies of the central nervous system have led to the suggestion that HIV-1 peripheral neuropathy is mediated by the pro-inflammatory cytokine interleukin-1 beta (IL-1β) that is both produced by neurons and macrophages and acts by promoting neurite degeneration [111]. Damage to peripheral nerves can interfere with the communication between the area it serves and brain, therefore affecting the ability to move certain muscles or feel normal sensations. Neurons are heavily energy dependent suggesting that they have the greatest concentration of mitochondria [112]. Reports show that mitochondria are found close to the terminal neurite membrane, ideally located for supplying energy for membrane mechanisms [112], therefore mitochondrial depletion may result into neurite degeneration.

2.6.4. Stavudine associated neuropathy

Although it is well established that HIV virus infection causes neuropathy, an increase in incident cases has been observed since the introduction of antiretroviral therapy, particularly the NRTIs [113]. Cui et al [114] showed that stavudine inhibits neurite regeneration, however the mechanism by which it induces neurotoxicity is not well established [114]. In support Lake-Bakaar et al (2001) observed that the NRTIs didanosine (ddI), zidovudine (AZT), zalcitabine (ddC) stavudine (d4T), damage the mitochondrial DNA by inhibiting the expression of cytochrome *c* oxidase II and ribosomal RNA suggesting that this could be due to chain termination, a possible mechanism of inhibition of mtDNA replication [115]. Recently observed polymorphisms in mitochondrial genes have been suggested as possible explanation for variations in the response to therapy between individuals [102]. Mitochondrial mutations at positions m.4216T>C, m.4917A>G, m.7028C>T, m.10398G>A, and m.13368G>A in mitochondrial haplogroup T have been shown to be associated with increased risk of developing peripheral neuropathy in patients on stavudine containing anti-retroviral regimens [102,116]. Mitochondrial DNA variant m.4216T>C, m.4917A>G, and m.10398G>A variants are non-synonymous resulting in amino acid change in ND1:p.Tyr304His, ND2:p.Asp150Asn and ND3:p.Thr114Ala respectively. Variant m.4216C has been suggested to affect the structural integrity of complex 1 associated with a decrease in the activity of the enzyme [117]. On the contrary, point mutations m.7028C>T and m.13368A>G in the cytochrome C oxidase and ND5 do not result in amino acid changes. However these polymorphisms in the mitochondrial DNA may affect efficiency of oxidative phosphorylation and impair energy production, that may trigger the clinical onset of peripheral neuropathy after the exposure to some NRTIs [118]. Molecular mechanisms underlying functional differences between mtDNA haplogroups are not fully understood. Most studies that have reported polymorphisms related to mtDNA haplogroups are not relevant to African populations, as they report non mtDNA L lineage haplogroups [102,116,119] except for a study conducted by Canter et al [120].

Although African mtDNA genetics has been studied with the aim of unraveling the demographic phenomena that has contributed to the settlement of populations and ethnic groups on the continent, little is known about population mtDNA characteristic in relation to drug toxicities in Southeastern Africa, especially Malawi, Zambia and Zimbabwe [37]. In addition, population

dispersal, migration, consanguinity and other population characteristics of these countries vary widely. Therefore, more data is required from African populations to describe mtDNA haplogroups and their role in the observed differential response to drug treatment as well as emergence of adverse effects.

2.6.5. Mitochondrial polymorphisms and stavudine induced lactic acidosis

Lactic acidosis is a life threatening metabolic complication that results from overproduction of lactic acid [121,122], with increased lactate levels in the blood. A differentiation between severe and symptomatic hyperlactatemia has been suggested, with severe hyperlactatemia being defined as lactate levels greater than 5 mmol/L without symptoms or metabolic acidosis; symptomatic hyperlactatemia as lactate levels greater than 5.0 mmol/L with symptoms but no metabolic acidosis; whereas lactic acidosis is characterised by a lactate level greater than 5.0 mmol/L, with symptoms and an arterial blood pH <7.35, bicarbonate (HCO3) level of <20 mmol/L and anion gap >20 mEq/L [122]. During cellular respiration glycolysis produces lactate as the end product of oxygen-independent cellular energy production. In the mitochondrial matrix the pyruvate dehydrogenase complex (PDC) controls the accumulation of lactate and decarboxylates it into acetyl CoA which is a substrate of the Krebs cycle. The acetyl CoA is further metabolized into carbon dioxide and water by the Krebs cycle and oxidative phosphorylation [5,7]**.** Post-translational regulation of pyruvate dehydrogenase complex expression is controlled by substrate activation, end point inhibition and reversible phosphorylation in which the enzyme is inactivated [123]. In normal physiological conditions blood lactate concentration is maintained below 2 mmol/l. However lactate production increases when the rate of pyruvate formation in the cytosol exceeds rate of its use by the mitochondria. The liver and kidneys play an important role in the metabolism and removal of lactate [124]. Hyperlactatemia in normal aerobic conditions is classified as Type B and may indicate excessive tissue lactate production and impaired hepatic metabolism attributed to mitochondrial dysfunction [123]. High incidences of hyperlactatemia and lactic acidosis have been observed in association with drugs such as stavudine [122]. If mitochondrial function is affected following treatment with NRTIs, NADH formation from glycolysis, by way of oxidation of pyruvate via the citric acid cycle may be impaired. This results in a shift towards anaerobic oxidation of pyruvate by pyruvate dehydrogenase and the formation of lactate. Accordingly, elevated lactate

levels are a sign of mitochondrial dysfunction. Abnormal functioning of the mitochondria will result into a shift towards anaerobic oxidation of pyruvate by lactate dehydrogenase and the formation of lactate which does not contribute to ATP production. Increase in glycolysis induces overproduction of lactate, resulting in imbalances in the lactate:pyruvate balance [125]. Mitochondrial DNA chain elongation termination results also into impaired β-oxidation of fatty acids that lead to accumulation of fat droplets within cells as shown in Figure 2.7 [125,126,127]. Despite the wide-spread use of stavudine in first line treatment regimens in many low income countries there is limited data about the effect of this drug in African populations. In particular, there is no established information on the relationship between mitochondrial polymorphisms and the use of nucleoside reverse transcriptase inhibitors lactic acidosis and other toxicities.

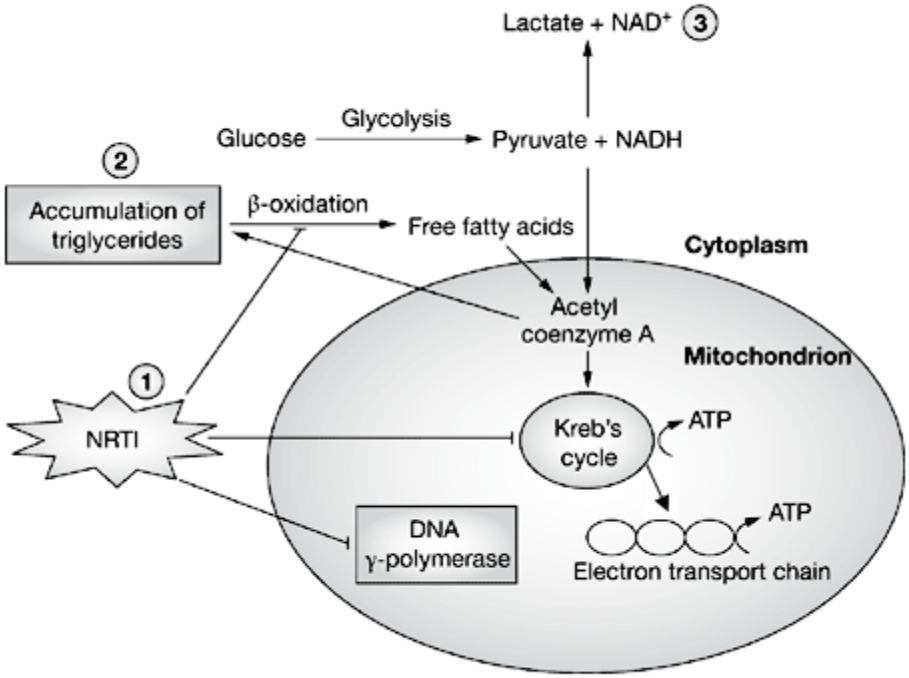


Figure 2.7: Mechanisms of nucleoside reverse transcriptase inhibitor (NRTI)-induced lactic acidosis.

NRTIs disrupt β-oxidation, the Kreb's cycle and transcription of essential enzymes needed for ATP production by inhibiting DNA polymerase-γ; Accumulated triglycerides cause hepatic steatosis; Lactic acid increases more rapidly than it can be excreted by the liver and kidney. Adapted from Claessens et al [125].

2.6.6. Stavudine induced pancreatitis

The pancreas is a very important organ in metabolism that releases the hormones, insulin and glucagon, as well as digestive enzymes that help in food digestion and absorption. Enzymes produced are in an inactive form and only become active in the small intestine. Pancreatitis is inflammation of the pancreas where pancreatic enzymes autodigest the gland, a situation which occurs when pancreatic enzymes such as trypsin are activated within the pancreas instead of in the small intestine. The condition is classified as either acute or chronic pancreatitis. Acute pancreatitis is mostly caused by gallstones, alcohol ingestion, infections, for example, HIV, trauma and medications, while chronic pancreatitis is commonly associated with increased alcohol abuse, hyperparathyroidism and also can be hereditary [128,129,130,131]. Genetic defects and depletion of mitochondria are also associated with pancreatic abnormalities [132]. Medications such as cortosteroids (e.g. prednisolone), diuretics, anticonvulsant (e.g. valproic acid), antihyperglycemic agent (e.g. sitagliptin) and antiretroviral drugs (e.g. nucleoside reverse transcriptase inhibitors) can cause pancreatitis [133,134]. Acute pancreatitis is a potentially life-threatening condition which occurs more frequently in HIV infected patients [131] and may also be induced by antiretroviral drugs, particularly stavudine, didanosine and lamivudine [126,135]. Suggestive clinical manifestations of pancreatitis include abdominal pain, nausea, vomiting, hyperamylasemia with dysglycemia, rising triglycerides, decreasing serum calcium and fever. Antiretroviral therapy should be discontinued if clinical pancreatitis develops [135,136]. It has been postulated that pancreatitis in HIV patients on treatment could be a result of mitochondrial toxicity from the NRTIs [126,136]. The mechanism by which NRTIs cause pancreatitis in relation to mitochondrial toxicity is not known. It has been suggested that depletion of mitochondrial DNA which result in a drop of ATP production below a certain required threshold can lead to tissue and organ malfunction and apparent clinical manifestation [137]. CD4 cell count of less than 200 $X10^6$ cells/microlitres in AIDS patients on NRTI containing therapy has been regarded as a potential confounding factor for pancreatitis [136]. Furthermore, pancreatitis may also be an indicator of the lactic acidosis syndrome [126].

2.6.7. Mitochondria dysfunction and stavudine mediated lipodystrophy

HIV-associated lipodystrophy is a condition characterised by peripheral fat loss especially of the limbs, hollowing of the cheeks, and/or flattening of the buttocks (lipoatrophy), or relative accumulation of central fat in the abdomen, neck and/or breasts (lipohypertrophy). In HIV-infected subjects it can be characterised as metabolic abnormalities with peripheral fat loss and abdominal fat accumulation; metabolic abnormalities with fat accumulation but without fat loss; metabolic abnormalities with fat loss but without fat accumulation; and body shape changes [138]. Even though lipoatrophy and lipohypertrophy are influenced by different risk factors and occur independently from each other, they coexist [139]. Lipoatrophy syndrome has been associated with nucleoside analogue therapy mainly stavudine [140]. Mitochondrial dysfunction in the liver results in the inhibition of fatty acid oxidation, a mechanism which results in accumulation of triglycerides and fatty acids in vesicles [125].

Skeletal muscles are one of the tissues with a high energy demand and are affected by mitochondria dysfunction. It has been suggested that the mechanism of fat loss depends on the activity of adiponectin on adipose tissues [141]. Adiponectin a protein that is secreted exclusively by body fat cells, up-regulates insulin activities and energy homeostasis, by increasing the insulin level to suppress gluconeogenesis, accelerating the oxidation of nonesterified fatty acids by muscles, lowering free fatty acids and plasma triglycerides [125,141]. Studies have shown that in patients treated with stavudine, levels of the enzyme adiponectin decrease [141]. This decrease accelerates the oxidation of nonesterified fatty acids by muscle, lowers circulating fatty acid, reduces the plasma levels of triglycerides and prevents lipid accumulation in skeletal muscle and liver which result into fat wasting syndrome called lipodystrophy [141]. The pathogenesis of medication induced lipodystrophy is possibly associated with adipocyte mtDNA depletion and mitochondrial cell proliferation inhibition [142]. That is the NRTIs inhibit the activities of polymerase gamma an enzyme responsible for replication of the mtDNA. The interference with the replication mechanism result in depletion of mtDNA encoded enzymes and finally leads to mitochondrial dysfunction [137,143,144].

However, phenotype features of lipodystrophy in HIV infected patients receiving antiretroviral therapy resemble Madelung's disease (multiple symmetric lipomatosis), a disease associated with

mtDNA mutations at position mt.8344 A>G [145,146]. These mutations have been found to affect the structure and functional activity of the mtDNA ATPase gene [146]. Similarly, NRTIs have been associated with mitochondrial disorder a consequence which results in lipodystrophy [147]. The disorder has been common in specific subpopulation who carry the specific mutation mt.9055 G>A in the ATP6 gene of mtDNA, for example, subpopulations characterised with mitochondrial haplogroup K [148]. In two separate studies carried out among European patients, enrolled in a Multicenter AIDS cohort study and at the Clinic of Infectious Diseases of the Catholic University in Rome, analysis showed that individuals carrying the haplogroup H had increased risks of atrophy while those harboring the haplogroup T exhibited decreased risk, further confirming that specific subpopulations have different risk profiles in developing mitochondrial associated disorder [119,148]. However, the question of whether mutations in the mtDNA are associated with stavudine induced lipodystrophy has not been fully addressed in African populations where a different pattern of mtDNA haplogroups exist which characteristically define African populations.

2.7. Polymerase Gamma (POL-γ) gene

The nuclear-encoded DNA polymerase gamma (POL-γ) is the enzyme solely responsible for replication and repair of mitochondrial DNA [74]. Mitochondrial DNA replication requires the combined action of many proteins including mitochondrial single stranded DNA-binding protein (mtSSB) which stimulates POL-γ activity and the accessory subunits of DNA polymerase gamma that confer processivity [149]. The human POL-γ is a heterotrimer composed of trimeric protein complex of a catalytic subunit of 140 kDA referred to as POL-γ and coded from chromosome 15q25 and a dimeric accessory subunit of 55 kDa referred to as POL-β whose gene is located on chromosome 17q [150]. The catalytic subunit is divided into four active domains;

- the N-terminal domain (26 -170) which is responsible for stimulating the unwinding activity of the DNA.
- the exonuclease domain (171 - 440) which proof reads and repairs the nascent strand
- the spacer (476 – 785) where the accessory subunits bind
- polymerase domain (441-475 and 786 – 1239) which replicates mtDNA [151].

POL-γ increases the affinity of nucleotides to the enzyme DNA complex and also enhances processivity by simultaneously accelerating the polymerization rate and suppressing exonuclease activity which may help maintain the integrity of the replisome at the mitochondrial replication forks [74].

2.7.1. POL-γ structure and function

The POL-γ active site domain conforms to canonical polymerase "right hand" configuration that comprises of finger, palm and thumb subdomains important in catalyzing a nucleotidyl transfer reaction [74,152]. The palm, which lies between 816-910 and 1096 -1239, is the most conserved subdomain [151,153]; It is positively charged, plays an important role in the attraction of the domain to the negatively charged DNA backbone. In addition, the magnesium ions within the aspartate residue in the domain increases the affinity by forming phosphodiester bonds between the 3´- OH end of the growing nascent strand and phosphate groups on the incoming nucleotide. The finger binds to the DNA template and incoming dNTPs. In order to release the incoming dNTP into the catalytic groove of the palm and bring it into contact with the magnesium ions that will enhance the formation of the new phosphodiester bonds. The finger domain changes its conformation immediately after the base of the dNTP has been proved to correctly match with the corresponding base at the 3´-end of the template [4,152]. Further conformational changes in the DNA are due to the significant movement of the thumb sub domain, a residue at 441-445 polymerase domains. The thumb interacts with the growing DNA strand and helps to keep the polymerase on its template DNA, thereby increasing processivity [4,151,152] .

The POL-γ also contains a domain for exonuclease (*exo*) which is separated by a linker (spacer) and is important in repairing replication errors by excision [153,154]. Exonuclease proofreading activity exhibits a preference for mismatched rather than matched bases [155]. The kinetic partitioning of polymerase and exonuclease activities determines the selectivity between forward polymerization and excision repair [154]. The rate of polymerization for suitable nucleotide over the mismatch is nucleotide concentration-dependent such that forward polymerization is fast (300 bases/second) compared to the rate of transfer of correctly base-paired DNA from the polymerase to the exonuclease active site which is 0.2 bases/second, resulting in low proofreading rate [154]. To achieve fidelity during mtDNA synthesis when a new nucleotide is

added to the polymerization at the 3'-end, two things may happen; POL- γ may either proceed adding the next nucleotide, or may allow the exonuclease activity to remove the newly incorporated nucleotide [4,155]. However when a mismatched nucleotide has been inserted there is a reduction in forward polymerization activity, while the rate of dNTP transfer to the exonuclease active site increases strongly, favouring exonuclease removal [154,156]. Though some studies show that the exonuclease activity remains highly active for excision of A> C and T>C mispairs even under extreme reaction conditions [155], the exact mechanisms by which base mismatches are recognized by the human mitochondrial DNA polymerase remains unknown. Johnson et al [167] reported that incorrect nucleotides bind with a tighter affinity than the correct indicating that lack of hydrogen bonding may also play an important role in low rate of excision [154]. This was supported by Bailey et al [157] who found that polymerase gamma's binding affinity of a phosphorylated stavudine (d4T-TP) was twofold tighter than for the natural substrate 2'-deoxythymidine 5'-triphosphate (dTTP).

The polymerase and exonuclease domains are separated by a spacer or linker region that interacts with POL- β. The subdomain comprises of an intrinsic processivity subdomain between amino acid residues 475-510 and 571-785 and an accessory interacting determinant subdomain between amino acid residues 511-570 which binds to the accessory subunit [158]. The POL-β activity through interaction with the spacer subdomain increases holoenzyme processivity and reduces fidelity by enhancing the DNA affinity and polymerization rate and at the same time suppressing exonuclease activity [159].

It has been postulated that the mechanisms by which the accessory subunit increases processivity of the holoenzyme depends on the intrinsic processivity subdomain that contains an area rich in positively charged amino acids termed the K tract. When the accessory subunit binds the spacer interacting determinant subdomain, it brings up a conformational change which exposes positively charged amino acids, that then interact with the template DNA, increasing its contact length with the holoenzyme [158].

2.7.2. Polymerase gamma defects and human disease

Over 160 mutations have been identified in all domains of mitochondrial polymerase gamma (MIM 174763; http://www.tools.niehs.nih.gov/polg/) and these mutations have been associated with many human diseases [151]. Diseases due to mitochondrial dysfunction are related to mitochondrial DNA depletion or deletion disorders both leading inhibition of POL-γ activities [74,151,153]. The clinical manifestations of diseases associated with polymerase gamma mutations include: sensory ataxia neuropathy with (SANDO) or without ophthalmoplegia; autosomal dominant (ad) (adPEO; MIM 157640) and recessive (arPEO; MIM 258450) forms of chronic progressive external ophthalmoplegia (CPEO); a mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)-like syndrome; and MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) [151]. A few of the syndromes or disorders with mitochondrial DNA mutations as basis are described below.

2.7.2.1. Alpers' Syndrome

Alpers' Huttenlocher syndrome is an autosomal recessive inherited disease within the group of encephalopathy's related to progressive degenerative disease of the central nervous system that occurs mostly in infants and children due to mutations in the catalytic domains of the POL-γ [160]. It is an autosomal recessive disorder that is sometimes seen in siblings and is characterised by epilepsy, psychomotor retardation and liver failure that leads to early death [160,161]. The most common POL-γ mutations necessary for the disease to manifest are 1399G >A (A467T) and 2243G > C (W748S) [161,162]. Disorders associated with polymerase gamma would result in damage of mitochondrial DNA which leads to low ATP production [161]. Likewise drugs such as didanosine (ddI; Videx) and stavudine (d4T; Zerit) inhibits mtDNA polymerase gamma activities which in turn depletes/damages mtDNA a mechanism which results in liver steatosis and enlargement [125]. Whether patients with these mutations are at increased risk when exposed to stavudine containing regimen is not known.

2.7.2.2. POL γ myopathies - Progressive external ophthalmoplegia (PEO)

Progressive external ophthalmoplegia (PEO) is an autosomal dominant (adPEO) mitochondrial disorder caused by depletion of mitochondrial DNA and/or accumulation of mutations within mtDNA [163]. Although reports indicate that mean age of onset of the disease is 29 ± 11 years,

patients may present initial symptoms at almost any age from early childhood to over 70 years. PEO is a progressive weakness of the external eye muscles accompanied by proximal limb and neck muscle weakness [163]. Interestingly, most of the mutations associated with PEO occur in the polymerase domain. This domain is associated with binding of the incoming dNTP and increasing polymerization of the mtDNA [4,151,152,163]. Mutations at position 2828G>A and 2864A>G (R943H and Y955C) in the active site of POLG have been associated with a decrease in processivity, increases in nucleotide misinsertion and replication errors increase by 10–100 fold in the absence of exonucleolytic proofreading [163]. The 3428A>G (E1143G) mutation has been found in the region has been associated with PEO features [163]. Several studies have indicated that POL-γ mutations were associated with sporadic mitochondrial disorders, however very little information is available on the role POL-γ mutations play in stavudine associated toxicities experienced by patients on HIV antiretrovirals treatment.

2.7.3. POL-γ Mutations and NRTI associated toxicities

Studies have shown that mutations in the active domain of the POL-γ at position 18939C>T (2890C>T, R964C) of the gene are associated with hyperlatactemia [164]. The mechanistic studies show that these mutations decrease POL-γ catalytic domain activities by 10% [96,164]. The mutation 2890C>T decreases the incorporation of the natural substrate by 33% and a threefold lower d4TTP discrimination when compared with wild type polymerase, indicating that mutations at this position result in loss of selectivity for the natural dTTP substrate [96].

Furthermore, Bailey et al (2009) reported that mutated polymerase gamma's binding affinity to d4T-TP was stronger for the natural substrate dTTP. The affinity of the incorrect nucleotide is stronger compared to wild type resulting in a decrease in rate of excision [154]. Lack of hydrogen bonding and inability to remove the incorrect nucleotide will result in mtDNA chain elongation termination [154], a mechanism which has been proposed to explain mtDNA depletion. In another study it was found that individuals with mutation 3428A>G on amino acid E1143G are at high risk of developing lipodystrophy when exposed to stavudine [154,165]. Although several studies have looked at mutations in POLG and predicted their role in diseases, little is known about the role of these mutations in relation to stavudine toxicities such as peripheral neuropathy, lactic acidosis, lipodystrophy, and steatosis among African populations.

2.8. AIM

The aim of this study was therefore, to investigate the extent of genetic variation in mtDNA and polymerase gamma among Malawians and to evaluate the effects of the observed variation on how HIV/AIDS patients responded to stavudine- containing antiretroviral therapy.

2.9. Specific Objectives

- To characterise the Malawi population with respect to mtDNA variation and how this can be used to genetically differentiate the different ethnic groups .
- To evaluate specific mtDNA haplotypes on their effects on treatment outcome among HIV/AIDS patients using HAART containing stavudine (e.g. effects on immunological and virological failure)
- To correlate the specific mtDNA subhaplogroups are associated with stavudine induced metabolic syndromes, such as lipodystrophy and hyperlactatemia and the neurological outcome, peripheral neuropathy.
- To characterise the extent of POL-γ variation among Malawian ethnic groups and evaluate if any of the POL-γ variants are associated with stavudine induced toxicities.
- To measure mitochondrial level and evaluate its relationship with stavudine induced toxicities

2.10. References

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3. CHAPTER 3: EFFECTS OF STAVUDINE USE AND ITS TREATMENT OUTCOMES

3.1. Materials and Methods

3.1.1. Participants recruitment

Unrelated adults were recruited from an HIV/AIDS treatment cohort at Queen Elizabeth Central Hospital in Blantyre, Malawi as part of a study to determine factors associated with antiretroviral drug toxicities. Eligibility criteria included the following: 1) participants had to be on stavudine containing antiretroviral therapy for at least 6 months and 2) participants had to have 100% adherence to therapy in the past three days before entry into the study. Patients were excluded if they were on antituberculosis treatment, pregnant or unable to provide informed consent. All completed a structured questionnaire that collected demographic information, medical records as well as individuals' ancestry up to their grandparent's level. The protocol was approved by the College of Medicine Research Ethics Committee (COMREC) protocol NO. P.02/10/861, University of Malawi and Human Research Ethics Committee University of Cape Town (UCT) protocol NO. HREC REF 231/2010. The participants gave written informed consent and the study conformed to the declarations of Helsinki 2008.

3.1.2. Clinical measurements

For all patients, data were collected on the following parameters: patients' clinical status at visit, and past medical history including any previous ART regimen, potential stavudine related clinical complications (including peripheral neuropathy, lipodystrophy, lactic acidosis and hypertension). Data were collected using personal interviews, physical examination of the participants as well as through an extensive systemic review of medical records. Participants with history of peripheral neuropathy that predated the start of ART and those on TB treatment were excluded from the study. Peripheral neuropathy due to HAART was based on patients' presentation with symptoms of numbness or dysesthesia (tingling, and pricking sensations (paresthesia), sensitivity to touch, or muscle weakness) after 6 months of initiation of treatment as defined according by van Oosterhout [1]. Lipodystrophy was diagnosed based on a predetermined case definition. The study questionnaire captured information that combined patient self-assessment and physical examination, by a trained clinician, of seven body areas

(face, neck, chest, abdomen, arms, legs and buttocks) [2]. The procedure is based on objective approach of quantifying the impacts of HAART associated lipodystrophy syndrome (HALS) and other lipodystrophy syndromes in Africa settings.

3.1.3. Laboratory measurements

3.1.3.1. Blood Collection

Venous blood was collected in three 5 ml ethylenediaminetetraacetic acid (EDTA) coated tubes and a plain vacutainer tube labeled for each participants´ identification. The first sample of blood collected in the EDTA coated tube was used for CD4 cell count measurement, the second blood sample was stored at -20^{0}C for genetic analysis (DNA extraction) while the remaining samples in EDTA coated tube and plain vacutainer tubes were used for the collection of plasma and serum, respectively, aliquoted into 250 µl vials each. Plasma was used for viral load determination while serum aliquots were used for blood chemistries (creatinine, triglycerides and cholesterol).

3.1.3.2. CD4 T-cell absolute count

CD4 receptors are molecules on T-lymphocytes cells that send signals to start the immune response that protects the body from infectious invaders such as bacteria and viruses. The normal CD4 cell count range for adults is between 500 and 1,500 cells per microliter. HIV/AIDS patients CD4 cell count is one of the important parameters to predict how disease is progressing, and individuals with CD4 cell count below 200 cells per microliter are at high risk of developing opportunistic infections. The absolute CD4 cell count (a measurement of how many functional CD4 T-cells is circulating in the blood) was carried on BD FACScount flow cytometer (BD Biosciences, CA, USA) within 6 hours of sample collection. Fifty (50 µl) microlitres of patients fresh whole blood from one of the 5 ml EDTA coated tube was mixed and pipetted into each of FACSCount fluorochrome-labeled antibodies reagent tubes. The tubes were capped and the mixture was vortexed and then incubated in the dark for 60-120 minutes at room temperature (20-25° C). Fifty microlitres (50µl) of fixative solution was added into reaction mixture, vortexed and further incubated for at least 30 minutes at room temperature (20-25 °C) in the dark. The product contents were run on the FACSCount instrument within 2 hours of fixation. The analyzer performed all calculations internally to produce the final CD4 reported result in

absolute values. The CD4 T-cell count was computed by the Multiset software (BD Biosciences, CA, USA) using the formula: CD4 T cell count = (sample CD4 events/bead events) × (beads per test/volume) room temperature (20-25 °C) in the dark.

3.1.3.3. Clinical chemistry parameters (Creatinine, cholesterol, triglycerides and lactate)

Clinical chemistry parameters are important in elucidating the aetiology of chemical and biochemical mechanisms of the body in relation to disease. Stavudine has been associated with changes in the biochemical levels (such as lactate, triglycerides cholesterol) of the body. Although studies have shown that stavudine has not been associated with renal failure, creatinine levels were measured in the study participants. Creatinine, triglycerides and cholesterol measurements were run on Beckman SYNCHRON CX5 analyzer Pro (Beckman Coulter Inc., Massachuessate, USA) following the CX5 protocol manual and reagent insert according to College of Medicine - Johns Hopkins laboratories (JHP) laboratories standard operating procedures.

Creatinine is generated from muscle metabolism and excreted through the kidney. Creatinine levels with normal kidney function results are 0.7 to 1.3 mg/dL for men and 0.6 to 1.1 mg/dL for women. Elevated creatinine level signifies impaired kidney function or kidney disease. It has been shown that HIV infection and antiretroviral drugs are associated with the abnormal renal function [3,4]. Creatinine levels were determined by an end point method where creatinine reagents were used to measure the creatinine concentration by the modified rate Jaffé method [5]. Five hundred microlitres (0.5 ml) of each freshly drawn serum specimen was pipetted into a cuvette. In the reaction, creatinine in the sample combined with picrate in an alkaline solution to form a creatinine-picrate complex. The system monitored changes in absorbance at 520nm wavelength. Change in absorbance was directly proportional to the concentration of creatinine, in the sample and results were computed to calculate and express creatinine concentration.

Cholesterol is an important in membrane structure as well as being a precursor for the steroid hormones and bile acid and is synthesized in the liver from acetyl-CoA. It is categorized as low density lipoprotein (LDL) cholesterol which is transported from liver to the cell and high density lipoprotein (HDL) cholesterol which is transported from cell to liver. Low density lipoprotein cholesterol is also referred to as bad cholesterol because it turns to accumulate in the artery when in excess causing atherosclerosis. According to US National Cholesterol Education Program (NCEP) the normal values of cholesterol are as follows; total cholesterol = <5.1 mmol/L, LDL = <2.6 mmol/L and HDL = <1.0 mmol/L. Cholesterol levels were quantified from serum by use of cholesterol reagent, in conjunction with SYNCHRON CX® System(s) and SYNCHRON CX MULTI™ Calibrator. Five hundred microlitres (0.5 ml) of each freshly drawn serum specimen was pipetted into a cuvette and the SYNCHRON CX® System(s) automatically proportioned the appropriate sample and reagent volumes in the ratio of one part sample to 100 parts reagent. In the reaction, cholesterol esterase (CE) hydrolyzes cholesterol esters to free cholesterol and fatty acids which were further oxidized to cholestene-3-one and hydrogen peroxide by cholesterol oxidase (CO). Furthermore the enzyme peroxidase catalysed hydrogen peroxide reaction with 4-aminoantipyrine (4-AAP) and phenol to produce a colored quinoneimine product. The change in absorbance at 520 nanometres was directly proportional to the concentration of cholesterol in the sample and values were used by the system to calculate and express cholesterol concentration.

Triglycerides and another form of lipids produced as end product of fat digesting and break down. An increase in triglyceride levels in the body is mostly associated with being overweight and may contribute to atherosclerosis. The normal levels for triglycerides according to American Heart Association are 1.7 mmol/L. Drugs such as NRTIs have been associated with lipid abnormalities [6]. Total serum triglyceride levels were quantified by use of triglyceride reagent, in conjunction with SYNCHRON CX® System(s) and SYNCHRON CX MULTI™ Calibrator. Triglyceride measurements are used in the diagnosis of stavudine associated lipid metabolic disorder. Lipases in reagent hydrolyzed the sample into glycerol and free fatty acids. Five hundred microlitres (0.5ml) of each freshly drawn serum specimen was pipetted into a cuvette and the SYNCHRON CX® System(s) automatically proportioned the appropriate sample and reagent volumes in the ratio of one part sample to 100 parts

reagent. In a sequence of three coupled enzymatic steps using glycerol kinase (GK), glycerophosphate oxidase (GPO), and horseradish peroxidase (HPO) catalyzed by peroxidase caused the oxidative coupling of 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) with 4-aminoantipyrine to form a red quinoneimine dye. The intensity of the color produced was directly proportional to the concentration of triglycerides in the sample when measured at 520 nanometre.

Glycolysis is a process that involves glucose breakdown to ATP, NADH and pyruvate production. Pyruvate is converted to lactic acid under anaerobic conditions, which dissociates into lactate molecules and hydrogen H^+ [7]. An increase in the lactate levels is directly proportional to H^+ (measure of acidosis); therefore, lactate measurement is an indirect indicator of acidosis. The normal values of lactate are 0.3 to 1.3 mmol/L [7]. Presence of lactate in the blood was measured with the hand-held Lactate ProH (Arkray Europe B.V., Amstelveen, Netherlands) at the point-of-care. Patients were rested and checked for dehydration before sampling. Lactate levels > 2.5 mmol/L were considered high.

3.1.3.4. Viral Load

The aim of ART treatment is to reduce HIV viral load and thus stopping progression to AIDS. However, there are interindividual variations in the way patients respond to ART. In order to determine whether there is treatment success/and or failure in individuals, viral load (number of virus per microlitre) is monitored. Treatment success is regarded as decrease in copy number of virus to undetectable levels after treatment initiation and failure is defined as continuous increase in copy number of virus despite medication a consequence that may lead to disease progression. Viral load monitoring was done using the Amplicor HIV Monitor kit version 1.5 (Roche Diagnostic Systems, Basel, Switzerland) as adopted by the College of Medicine -Johns Hopkins laboratories**.** The test was an in vitro nucleic acid amplification test for the quantitation of human immunodeficiency virus Type – 1 (HIV-) viremia in the plasma with lower limit of detection of 400 copies/mL.

The Amplicor HIV-1 monitor test, v 1.5, was based on five major processes:

1. Specimen preparation
2. Reverse transcription of target RNA to generate complementary DNA (cDNA)
3. Polymerase chain reaction (PCR) amplification of target cDNA, using HIV-1 specific complementary primers
4. Hybridization of the amplified products to oligonucleotide probes specific to target(s)
5. Detection of probe bound amplified products by calorimetric determination.

The test allowed reverse transcription, amplification of HIV-1 and Quantitation Standard (QS) RNA reactions to occur simultaneously. The Master Mix reagent contained a biotinylated primer pair specific for both HIV-1 and QS target nucleic acid. The quantitation of the virus RNA was performed using a Quantitation Standard (QS). The Quantitation Standard was added to each specimen at a known copy number and carried throughout the specimen preparation, reverse transcription, PCR amplification, hybridization, and detection steps along with the HIV-1 target. HIV-1 RNA levels were determined by comparing the absorbance of the specimen to the absorbance obtained for the QS.

A. Specimen preparation

Two hundred microlitres (200 µL) each of patient plasma prepared from blood that had been collected into EDTA-containing tubes was added to appropriately labeled tubes containing 600 µL standard working lysis reagent mixed with quantitation standard (QS). The tubes were capped and vortexed for 3-5 seconds. Specimen and control tubes were incubated for 10 minutes at room temperature. 800 µL of 100% isopropyl alcohol (at room temperature) were added to each specimen and control reactions and vortexed for 3-5 seconds. Each tube was marked with an orientation mark and micro centrifuged at maximum speed of 12,500 x g for 15 minutes at room temperature with the orientation marks facing outward, so that the pellets aligned with the orientation marks. The supernatant from each tube was discarded and 1.0 mL of room temperature 70% ethanol t) was added and tubes vortexed for 3-5 seconds. Tubes were micro centrifuged still with the orientation marks facing outward for 5 minutes at maximum speed of 12,500 x g at room temperature. The supernatant was discarded, and 400 µL HIV-1 diluents were added to each reaction and vortexed for 10 seconds.

B. Reverse transcription of target RNA to generate complementary DNA (cDNA)

Reverse transcription followed within 2 hours of the specimen preparation. It started with master mix preparation of 100 µl of AMPLICOR HIV-1 MONITOR manganese solution added to AMPLICOR HIV-1 MONITOR Master Mix and the mixture was stored at 2 - 8°C prior and use. Using a pipette with aerosol barrier or positive displacement tip, 50 µL of each processed specimen and control were added to the reaction tubes containing 50 µL working master mix. The tubes were capped and sealed using the MicroAmp cap installing tool.

C. Reverse transcription and amplification – post- amplification/detection

The samples were amplified by GeneAmp 9600 thermal cycler (Applied Biosystem, California, USA) under the following conditions: The initial denaturation step was set at 50°C for 2 minutes, followed by 60°C incubation for 30 minutes, 8 cycles of incubation at 95°C for 10 seconds, 52°C for 10 seconds and 72°C at for 10 seconds, further 23 cycles of incubation at 90°C for 10 seconds, 55°C for 10 seconds and 72°C for 10 seconds, annealing and extension temperatures respectively, with a final extension at 72°C for 5 minutes

D. Hybridization reaction

In order for the amplicon to form single stranded DNA, 100 µL of monitor denaturation solution 1 was added to each amplicon tube. Following the denaturation, aliquots were added to separate wells of a microwell plate coated with HIV-1 specific (SK102) and QS-specific (CP35) oligonucleotide probes. HIV-1 and QS amplicons were bound to microwell plate probes by hybridization. The amplicon was left at room temperature for detection within 2hrs.

E. Detection of HIV post amplification

The detection stage started with preparation of working wash solution. The 10X wash buffer was diluted with distilled water at the ratio of 1:10. One hundred microlitres (100 µL) of monitoring hybridization buffer was pipetted into each micro well plate with rows labeled A to H. The 25 µL of denatured amplicons to the HIV-1 were added to row A of the detection plate which was followed with mixing, and 5-fold serial dilutions were made in the HIV-1 wells in rows B through F by transferring 25 µL from row A to row B and mix, continue through row F

with final 25 µL discard. Twenty-five microlitres (25 µL) of denatured amplicon was added and mixed to the quantitative standard wells in row G and 25µl was transfered from row G to row H mixed, then same amount was removed from row H and discarded. The plates were washed with working wash solution, using an automated ELx50 Microplate Strip Washer, (BioTek; Winooski, U.S). The plates were dried and then 100 µL of avidin horseradish peroxidase (AV-HRP) was added, the reactions were incubated for 15 minutes at 37°C. After a second wash, 100µl of working substrate solution was added to the amplicons. The working substrate solution was prepared by mixing 12 ml substrate A and 3 ml substrate B. Stop solution (100µl) was added to the amplicon reaction and measured at optical density (OD) of 450 nm and recorded by Stat Fax® 3200 Microplate Reader, **(**Diagnostic Automation/Cortez Diagnostics, Inc**.** California**,** USA**).**

3.2. Results

This section will start with a description of the studied population, followed by demographic characteristics. The next section will describe the medical characteristics of the participants and their clinical outcomes. Finally the clinical outcome between groups will be compared.

3.2.1. Population Description and demographics

Two hundred and fifteen (n = 215) adult participants were enrolled in this study and all were on a stavudine containing antiretroviral therapy (ART) regimen. Participants were Malawian Bantu speakers from seven ethnic groups (Chewa, Lhomwe, Ngoni, Sena, Tonga, Tumbuka and Yao) maternally unrelated (according to family-history information) as shown in Figure 3.1. One hundred and thirty two (61%) were women with mean age of 38 years. Participants were grouped into young adults (range = 22 - 40 years) and older adults (range = 41- 69 years) with median age of 40 (IQR = 32 – 46). Young adults were the most frequent (n = 123; 57%) of participants than older adult. Significant differences were observed between ages of women median age of 36 (IQR = 30 – 46) years and men median age of 39 (IQR = 34 – 48) years P = 0.027. Women were shorter (median = 158 cm; IQR = 152 – 158 cm) than men (median = 163 cm; IQR = 159 – 163 cm) P = 0.001. There was no difference in weight between women (median = 57.2kg; IQR = 51.55 – 57.2kg) and men (median = 58.9; IQR = 54.4 -58.9) with P = 0.152. Median body mass

index (BMI) of participants was 23 (IQR = 21 – 25) kg/m^2 with 20% of them having body mass index above the normal range of 25 kg/m^2. Median BMI for women, (23.2; IQR = 21- 25) kg/m^2 was significantly higher (P = 0.006) when compared to that of men (22.2; IQR = 20.57 – 23.75) kg/m^2. There was no difference in BMI between age groups (P = 0.974). Two (1%) patients had severely elevated serum creatinine levels of 5.4mg/dl and 6.0mg/dl with an estimated GFR of 15.4 ml/min/1.73m^2 and 10.3 ml/min/1.73m^2, respectively. Figure 3.1 shows the ethnic distribution of the participants and detailed analysis of the ethnic groups are presented in chapter 4.

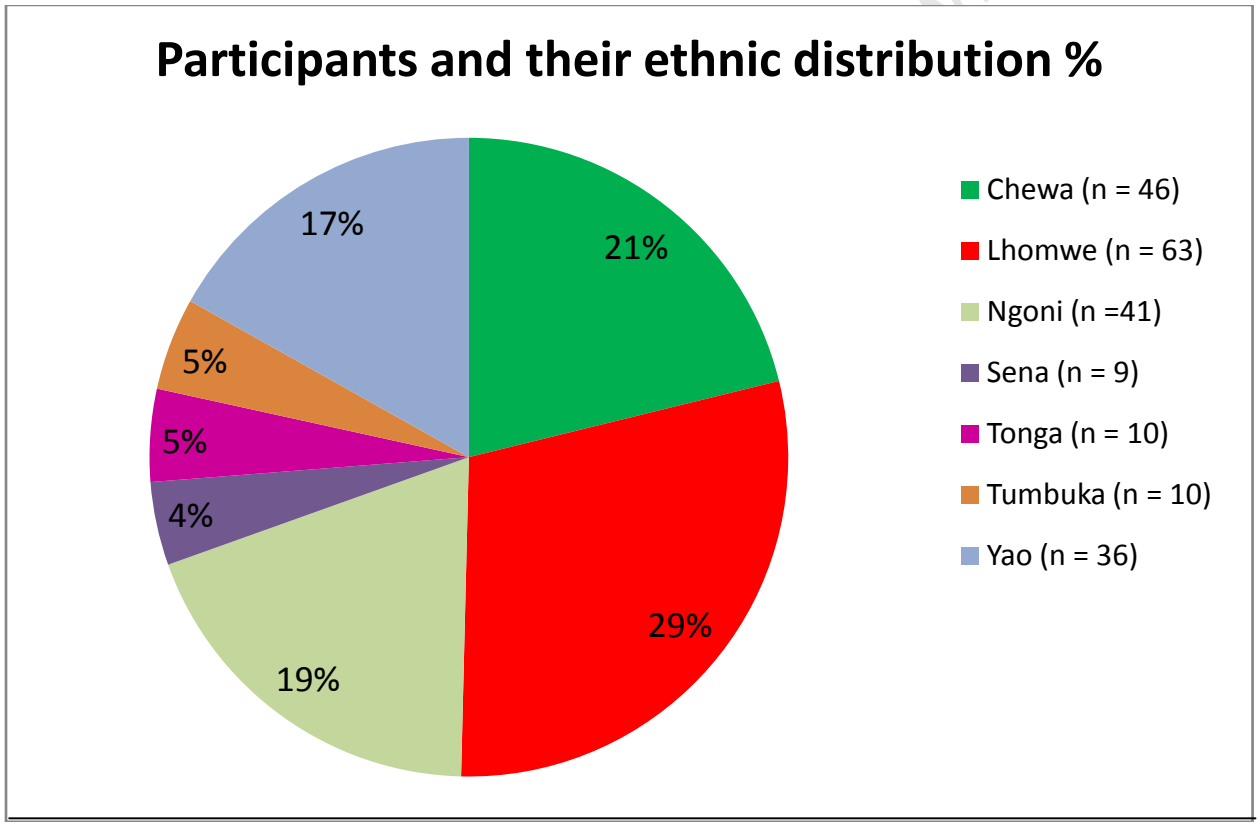

Figure 3.1: Ethnic distribution of the study participants

3.2.2. Participants medical characteristics

All participants were on stavudine containing antiretroviral therapy regimen for median duration of 25 months (range 23 -29). Two (0.01%) and 3% (n = 6) participants were smokers and

alcoholics respectively. Participants were on cotrimoxazole prophylaxis; 10%, 3%, 2% and 1% were also on antihypertensive drugs (nifedipine, hydrochlorothiazide, ACE inhibitors), amitriptyline, pyridoxine and fluconazole, respectively. Triglycerides and lactate did not show any difference between different women and men. In contrast cholesterol levels were significantly higher in women than men (P = 0.013). However, in a separate analysis total cholesterol levels (>5mmol/L) were not associated with low BMI (BMI <18.5kg/m^2 = OR, 1.62, 95% CI, 0.18 – 19.11; P = 0.699) or high BMI (BMI >25kg/m^2 = OR, 2.53; 95% CI, 0.81 – 7.87; P = 0.109). Accordingly no differences were observed between body mass index with triglycerides levels (OR, 4.33; 95% CI, 0.83 – 13.86; P = 0.089). Table 3.1 provides detailed information of participants' demographics and clinical information.

Table 3.1: Patients demographic and clinical characteristics.

Phenotypes	Male (%)	Female (%)	Total	P-Value
Age				
<40	44 (0.53)	79 (0.60)	123 (0.57)	0.324
≥40	39 (0.47)	53 (0.40)	92 (0.43)	
Viral Load (copies/ml)				
<400	68 (0.82)	112 (0.85)	180 (0.84)	0.833
>400	10 (0.12)	14 (0.11)	24 (0.11)	
Missed	5 (0.06)	6 (0.04)	11 (0.05)	
CD4 (cells/µL)	**292 (157 – 436)**	**392 (297 – 534)**	**344 (227 – 489)**	**0.001**
Missing	4 (0.05)	6 (0.05)	10 (0.05)	
BMI categories				
Underweight (<18.5 kg/m^2)	**5 (0.06)**	**1 (0.01)**	**6 (0.03)**	**0.001**
Normal (18-25 kg/m^2)	66 (0.79)	100 (0.76)	166 (0.77)	
Overweight (>25 kg/m^2)	12 (0.14)	30 (0.23)	42 (0.20)	0.089
eGFR categories (ml/min)				
≥90	77 (.93)	112 (0.85)	189 (0.88)	0.133
60- 89	1 (0.01)	12 (0.09)	13 (0.06)	
30 - 59	0 (0)	1 (0.01)	1 (0.01)	
15- 29	1 (0.01)	0 (0)	1 (0.01)	
<15	0 (0)	1 (0.01)	1 (0.01)	
Missing	4 (0.05)	6 (0.05)	10 (0.055)	
Lactate (U/L)				
≤2.5	26 (0.70)	48 (0.75)	74 (0.73)	0.534
>2.5	8 (0.22)	13 (0.20)	21 (0.21)	
Missing	3 (0.08)	3 (0.05)	6 (0.06)	
Cholesterol (IQR) mmol/L	**3.55 (3.03 – 4.87)**	**4.53 (3.52 – 5.06)**	**4.19 (3.3-5.03)**	**0.031**
Triglycerides (IQR) mmol/L	0.96 (0.64 – 1.29)	1.05 (0.67 – 1..36)	0.97 (0.65 – 1.35)	0.296
Current smokers				
Yes	2 (0.02)	0 (0.0)	2 (0.09)	0.788
No	81 (0.98)	132 (100)	213 (0.99)	
Current alcoholic				
Yes	2 (0.02)	4 (0.03)	6 (0.03)	0.073
No	81 (0.98)	128 (0.97)	209 (0.97)	
Toxicities				
Peripheral Neuropathy				
Yes	24 (0.29)	29 (0.22)	53 (0.25)	0.250
No	59 (0.71)	103 (0.78)	162 (0.75)	
Lipodystrophy				
Yes	6 (0.14)	13 (0.11)	19 (0.16)	0.554
No	38 (0.86)	60 (0.89)	98 (0.91)	
Lactic Acidosis				
Yes	0 (0)	1 (0.76)	1 (0.47)	0.427
No	83 (100)	131 (99.24)	214 (99.53)	
Pancreatitis	0	0	0	

Notes: *P* values obtained from Chi-squared, Fisher's Exact. IQR = interquartile range.

3.2.3. Participants´ Clinical measurements

3.2.3.1. Patients' clinical-immunological outcomes

The median CD4 cell count for 205 individuals was 344 (IQR = 227 – 489). Men were more likely to have lower CD4 cell count compared to women (P = 001).The median CD4 cell count for women was 392 (IQR = 297 – 534) cells/µL while median CD4 cell count of men was 292 (IQR = 157 – 436) cells/µL. Forty seven percent (n = 101) of the participants had CD4 cell count above 350 cells/µL; 49% (n = 105) had CD4 cell count of less than 350 cells/µL and 4% (n = 10) had missing data.

3.2.3.2. Patients' clinical-virological outcomes

The median viral load was 202 copies/ ml (IQR 146 – 268) and virological responses between women and men were not different (P = 0.766). The majority of participants had undetectable viral load (n = 181; 84%) at a cutoff point of 400 copies/ml while 24 (11%) detectable viral load (above 400 copies/ml) at the time of the study. Viral load above 10 000 copies/ml was observed in seven (3.26%) of patients. Viral load data was missing on 5% (11/215) of the recruited participants. Table 3.2 provides detailed viral distribution of levels among participants.

Table 3.2 Distribution of viral load levels (copies/ml) among patients in our study

Viral Load (copies/ml)	N (%)	Frequency (%)	Cumulative (%)	95% CI
2 – 400	181	84.19	84.19	0.050 - 0 .129
>400 – 1000	5	2.33	86.51	0.008 – 0.053
>1000 – 5000	7	3.26	89.77	0.013 – 0.066
>5000 – 10000	4	1.86	91.63	0.005 – 0.047
>10000	7	3.26	94.88	0.013 – 0.066
Missed	11	5.12	100	
Total	215	100		

3.2.3.3. Participants drug associated toxicities

Stavudine induced adverse reactions, such as peripheral neuropathy and lipodystrophy, were reported in 30% (n = 65) of the patients. The commonest toxicity was peripheral neuropathy elicited in 25% (53/215) of the study population, followed by lipodystrophy 16% (19/117). Only 113 patients had their lactate levels investigated and of these 17% (n = 19) had high lactate levels (>2.5 mmol/L).

3.2.3.3.1. Peripheral neuropathy

Twenty five percent (53/215) of participants presented with peripheral neuropathy and 29 (13%) of them were women. There was no significant difference (P = 0.250) observed between men and women with regard to peripheral neuropathy. No differences in CD4 cell count between those presenting with and without peripheral neuropathy (n = 205; P = 0.880). Likewise virologic response (above or below 400 copies/ml) between participants presenting with and without peripheral neuropathy was not different. The duration on treatment was not different in participants presenting and not presenting peripheral neuropathy. Although being over > 40 years of age was associated with increased risk of developing peripheral neuropathy (OR, 1.90; 95%, CI 1.01 – 3.55; P = 0.045) in a univariate analysis, this observation was not reflected in the multivariate analysis (OR, 1.90, 95%, CI 0.96 – 3.73); P = 0.064). Height (median 159cm) showed a trend towards reduced risk of developing peripheral neuropathy (OR, 0.96; 95% CI, 0.93 -1.00; P = 0.058) in the study. Further results are presented in chapter 5.

3.2.3.3.2. Lipodystrophy

Only 117 of the 215 participants had their lipodystrophy measured or evaluated, of which 62% (n=73) were women. Sixteen percent of the 117 participants (n=19) presented with lipodystrophy. There was no difference in the duration time on treatment between participants presenting lipodystrophy and those without lipodystrophy (P = 0.229). However older (>40 years) participants were more likely to present with lipodystrophy compared to those less than 40 years with significance of (P = 0.017). Total median body mass index (BMI) of participants was 24 (IQR = 24- 25) kg/m^2 and no significant differences were observed in BMI between presenting lipodystrophy compared to participants without lipodystrophy (P = 0.527). After

further classification of body mass index according WHO categories of underweight, normal and above normal, it was observed that 3 (12%) participants with lipodystrophy had BMI above the normal range of 25 kg/m^2. Triglycerides and cholesterol were not a risk factor to lipodystrophy. Markers of disease progression CD4 cell count and viral load were not associated with lipodystrophy. In a multivariate analysis, older age was associated with increased risk of developing lipodystrophy (OR, 3.73; 95% CI, 1.22 – 11.40; P = 0.021) (Table 3.3). Chapter 6 provides further results.

Table 3.3: Risk factors for lipodystrophy

Covariate	Unadjusted OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
Gender (Female)	1.37 (0.48 – 3.92)	0.484	1.51 (0.46 – 4.96)	0.499
Age (yrs) ≥40	**3.57 (1.25 – 10.21)**	**0.017****	**3.73 (1.22 – 11.40)**	**0.021****
BMI (kg/m^2) >25	0.65 (0.17 – 2.45)	0.527	0.60 (0.15 – 2.46)	0.488
Duration (months)	1.28 (0.86 – 1.91)	0.229	1.17 (0.75 – 1.83)	0.481
Viral Load (CPs/ml)	0.41 (0.0.05 – 3.34)	0.403	0.28 (0.03 – 2.45)	0.253
CD4 count (cells/µL)	0.99 (0.99 – 1.00)	0.724	1.00 (0.99 – 1.00)	0.501

Cholesterol and triglycerides excluded because 26% (n = 30) of the participants did not have the results

3.2.3.3.3. Lactate

One hundred and thirteen (n = 113, 52%) participants had their lactate levels determined. Using Mann Whitney, lactate levels were not different between gender groups. Age was not associated with hyperlactatemia (P = 0.480). Metabolic parameters (cholesterol and triglycerides) did not show any degree of association with hyperlactatemia with P =0.239 and 0.429 respectively. There was no difference in levels of lactate between participants experiencing or not experiencing adverse effects such as peripheral neuropathy (P = 0.989), lipodystrophy (P = 0.627) and hypertension (P = 0.723). Body mass index (BMI) not associated with

hyperlactatemia. In addition measure of immunological (P = 0.911) and treatment failure (P = 0.269) were not associated with increase in lactate levels

3.3. Discussion

This part of the study was carried out to evaluate the response of HIV/AIDS participants to stavudine containing antiretroviral therapy using immunological markers (CD4 cell count) as well as virologic response. We also determined some important, biochemical parameters such as cholesterol, triglycerides, lactate levels as well as stavudine associated toxicities including peripheral neuropathy and lipodystrophy.

It has been demonstrated in this cross sectional study that stavudine containing antiretroviral therapy regimen results in favorable virological and immunological response in a large proportion of patients (84% in this case); these outcomes are consistent with findings of other studies including our earlier study [1,8,9] . While virological outcomes were similar between women and men, it was observed that CD4 cell count was higher in women when compared to men with a significant difference of P = 0.001, these results are similar to Kumarasamy et al [10]. Women tended to have lower BMIs (< 18.5kg/m^2) P = 0.001 compared to men.

Stavudine has been associated with metabolic disorder which includes dyslipidemia (hypercholesterolemia and/or hypertriglyceridemia), insulin resistance with hyperinsulinaemia and hyperlactatemia, kidney failure and liver toxicities [11]. In this cross sectional study we found high prevalence of mild hypercholesterolemia especially in women confirming what has been shown by others [11], although the increase in cholesterol levels in this study was mild.

The prevalence of peripheral neuropathy and lipodystrophy in the study, confirms what has been established by other studies. Peripheral neuropathy was found in 25% of the study participants and was the most common adverse drug effect. This is in line with findings from other studies [12,13]. The prevalence of peripheral neuropathy (25%) in the studied population was higher than that reported in South African and Rwandan population groups but lower than in Ugandans [13,14,15].

Older age has previously been independently associated with peripheral neuropathy [12,16]. Although older age appeared significant during univariate analysis, it was not significant in a multivariate model after controlling for other clinical parameters. Possibly other confounding factors for example height (median height of 159cm) which was observed having a trend towards reduced risk of developing peripheral neuropathy could have played a role. Other studies have shown that patients taller than 176cm are at an increased risk of peripheral neuropathy [12,16]. In some studies the risk of peripheral neuropathy was associated with malnutrition [17,18], but body mass index (BMI) was not associated with risk of developing peripheral neuropathy in this study. Good outcome in both CD4 cell counts and viral load suggest that HIV infection itself may not be the cause of peripheral neuropathy and strongly point to stavudine-induced toxicity as the cause. CD4 counts less than 200 X 10^6/L and HIV RNA >10,000 copies/mL were found to be risk factors for peripheral neuropathy [19,20,21].

The analysis of risk factors for lipodystrophy by gender showed no difference between female and male in contrast to a study conducted in Rwanda which showed that the female gender was associated with increased risk of stavudine-induced lipodystrophy [8]. Body mass index, CD4 cell count and viral load parameters were not associated with lipodystrophy. The result could be due to the fact that most of the participants had improved clinical response to treatment which was indicated by increased CD4 count and low viral load. Metabolic abnormalities such as dyslipidemia (increase in triglycerides and cholesterol levels) has been associated with lipodystrophy in HIV patients on stavudine containing therapy including our earlier study [1,6,11,22], an outcome which was not the same with this study.

Although there was no association between gender and hyperlactatemia, high prevalence of hyperlactatemia in women (n = 13; 61%) in this study confirms the previous findings that women are more likely to experience hyperlactatemia and lactic acidosis [23]. Unlike our earlier study [24] where age, lipodystrophy, higher BMI and peripheral neuropathy, were associated with hyperlactatemia in univariate analysis we did not have similar observation in this study. Possibly small sample size might have affected the findings as the analysis was based on only 46% of the investigated participants.

The findings of this part of the study are limited by its design as well as limited laboratory parameters (triglycerides, cholesterol and lactate) as not all participants may have been identified in order to establish relationship between clinical parameters and stavudine associated toxicities. However, stavudine associated adverse reactions including, peripheral neuropathy, lipodystrophy, and lactic acidosis have been associated with mitochondrial dysfunction [25,26]. These toxicities are prevalent in specific populations which have specific mutations in the mitochondrial DNA. On the other hand, there remains controversy regarding the NRTI mitochondrial toxicities in Africans and relatively little is known in regards to polymorphic variation of these genes in African population [27]. Detailed information on the stavudine associated toxicities and mitochondrial dysfunction have been discussed in chapters 5 – 7.

3.4. References

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4. CHAPTERS 4: DIVERSITY OF MTDNA IN THE MALAWIAN POPULATION

Diverse mtDNA subhaplogroups in seven Malawian ethnic groups *(submitted to Plos One and now under revision)*

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Running title: Mitochondrial DNA variation in Malawians.

Abstract

Introduction: Human population diversity has been characterised mainly on the basis of Y-chromosome and mitochondrial DNA (mtDNA) variation. With regards to mtDNA variation, Sub-Sahara Africa is defined by the mitochondrial lineage L which is further divided into haplogroups L0 and L1-6. The distribution of these subhaplogroups is geographically and ethnically structured. We aimed to describe the mtDNA variation in seven ethnic groups from Malawi, a previously understudied population and draw comparisons with other populations in Africa, especially Southern Africa where L0 mtDNA subhaplogroup are deeply-rooted.

Method: Two hundred and fifteen (n=215) Malawian subjects were recruited as part of a study that investigated genetic correlates of differential susceptibility and response to highly-reactive antiretroviral therapy (HAART) among HIV/AIDS patients. Whole mtDNA coding region was sequenced in patients, comprising of Chewa/Nyanja, Lhomwe, Ngoni, Sena, Tonga, Tumbuka and Yao and the sequences were aligned with the revised Cambridge reference sequence. A phylotree for the seven Malawi ethnic groups was constructed. Molecular indices were measured using Arlequin software and the genetic structure of the Malawian ethnic groups was investigated using principal component analysis and also compared to other African ethnic groups.

Results: The Malawian population presented with 184 haplotypes, resolving into 9 subhaplogroups with the following frequencies L0a1 (0.06), L0a2 (0.28), L0d (0.07), L0f (0.03), L0k (0.04), L1c (0.13), L2a (0.25), L3d (0.02) and L3e (0.12). We report the presence of subhaplogroups L0d and L0k that are concentrated in Southern Africa. The Malawian ethnic groups correlate well with findings in other Southern African populations with a 48% prevalence of haplogroup L0 followed by the L2a (25%), while L3d had the lowest frequency (2%).

Conclusion: This study further confirms the genetic-relatedness of the Bantu linguistic group and place Malawian populations more closely with southeast and southern African Bantu population groups.

Keywords: Malawi, Bantu, haplogroups, ethnicity, mitochondrial DNA

Introduction

There is no doubt that Sub-Sahara Africa region is home to the earliest lineages of mtDNA. Bantu-speaking people migrated from northwestern Cameroon/Southern Nigeria and spread throughout Sub-Saharan Africa [1]. Before the Bantu expansion, Sub-Sahara Africa was populated by hunting and foraging peoples, such as the Pygmies in central Africa, proto-Khoisan around the Kalahari Desert, Hadza and Sandawe in East Africa and Khoe and San in South Africa and Namibia [2,3]. The Bantu migration followed two major routes, one group took a western route, travelling south through what is now the Democratic Republic of Congo (DRC) and following the Atlantic coastline [1]. A second group moved eastwards and settled near the Great Lakes of East Africa [4,5]. The eastern Bantu went further southwards into two parallel directions, one along the Ruvuma river towards the coast, reaching present-day Natal by the end of the third century A.D., and another along the shores of Lake Malawi, through what is now eastern Zimbabwe, reaching northern Transvaal (current Mpumalanga) around A.D. 500. [5]. The Malawian population is believed to have emerged from this last migration.

The Republic of Malawi is located in the southeast of Africa and its 16 million populations is made up of eight major ethnic groups comprising the following: Chewa/Nyanja (38%, ~ 6 million); Lhomwe (18%, ~ 3 million) Yao (13%, ~ 2 million); Ngoni (12%, ~ 2 million); Tumbuka (9%, ~ 1.5 million); Sena (3.5%, ~ 700,000); Tonga (2%, ~ 300,000); and Ngonde (1%, ~ 160,000) and other small ethnic groups as well as a few Asians and Europeans (3.5%, ~ 700,000) [6,7]. Apart from the Asians and Europeans all the ethnic groups belong to the Bantu speakers [8]. According to history, the Chewa tribe also known as Nyanja in other countries, migrated from the Luba area known as Malambo in what is now called the Democratic Republic of Congo and is one of the tribes which is highly concentrated in the central region [9]. Earlier settlement shows that the Chewa were the first Bantu speakers to settle in Malawi where they are concentrated in the central region but also show a presence Chikwawa and in Nkhata-Bay districts in southern and northern Malawi, respectively, which are dominated by other ethnic groups. Generally people identify themselves by languages of major tribes in their area of geographical residence [9]. They follow matrilineality system and their language (called Chichewa or Chinyanja) is the official language for Malawi. The Chewa are also found in Botswana, Mozambique, Namibia, Tanzania, Zambia and Zimbabwe [10].

The Lhomwe people migrated into Malawi from Mozambique in the 1930s with the majority settling along the Zambezi province [8]. They are thought to be descendants of the Bantu speakers who migrated East from Central Africa [1]. Lhomwe are small scale farmers and similar to the Chewa, practice matrilineal kinship. Although the Lhomwe ethnic group constitutes the second largest ethnic group in Malawi (18% of the country), their language is spoken by a mere 2.4% of the population.

Ngoni is one of the tribes whose distribution is highly dispersed throughout Malawi as shown in Figure 1. According to history, the Ngoni ethnic group (also referred to as Nguni) of Malawi, are offshoots of the Zulu-Swazi who emigrated to Natal and Swaziland in Southern Africa [11,12]. The name Ngoni was changed from Nguni after crossing Zambezi river and defeating the Shona, Thonga and Chewa. They migrated in two groups; one group moving towards the north and settling in Mzimba (northern Malawi), while the other group moved southwest and settled in Ntcheu (central Malawi) [12]. Later a number of offshoots arose from these groups in the nineteenth and early twentieth century and settled in various parts of the country (including Dedza, Mchinji, Mwanza, Neno and Thyolo) where their culture and language were assimilated by the tribes they found in these areas, for example, the Tumbuka and Chewa [13].

Another ethnic group found in southern Malawi is the Sena whose name derives from a Mozambican town of Sena where they are thought to have migrated from. They speak a Bantu language referred to as Chisena. The majority of the Sena are found in Nsanje and Chikwawa districts and to a lesser extent in parts of Mwanza and Thyolo highlands which are dominated by the current the Chewa, Ngoni and Lhomwe [14].

The Tumbuka are part of Bantu people of Malawi and are mainly found in the northern part of Malawi [15] and also in neighboring countries such as Zambia and Tanzania [10]. Their primary language is called Chitumbuka, and in addition Senga, Fungwe and Yombe of Zambia speak the same language as Tumbuka [16]. The Tonga is another tribe which is found in the Northern part of Malawi and their language is known as Chitonga. While traditionally the Tumbuka are agriculturists, the Tonga are fishermen. Like the northern Ngoni and Sena, Tumbuka and Tonga believe in patrilineal descent.

Yao people are widely distributed in the southern part of Malawi, originating from northern Mozambique and parts of Tanzania [10]. They established groups of settlements based on fishing due to their proximity to the lake and were the first people to be agents of Arabs during slave trade. Yao people believe in the matrilineal lineage. Despite political boundaries, it has proven difficult to distinguish the Malawi population by language.

Current efforts to understand population history relies heavily on archaeology and genetic characterization of both Y-chromosome and mitochondrial DNA. However, there has been no published data and parameter estimation to characterise genetic differences in the mtDNA of the Malawian ethnic groups in comparison to other African ethnic groups. Analysis of mtDNA is one of the tools available to describe the relatedness, movements and settlements of populations. However, populations within Southeast African countries such as Zimbabwe, Malawi and parts of Zambia including South Africa and Angola are yet to be fully characterised genetically [10,17]. Studies on mitochondrial genetic diversity have until recently been mostly confined to East African populations of Uganda, Kenya and Tanzania while from southeast Africa we have data only from few studies on Zimbabwean, Mozambican and Zambian ethnic groups [1,17,18,19].

The human mtDNA is characterised by distinct lineages or haplogroups. These haplogroups are characterised by specific sets of single nucleotide polymorphisms (SNPs) [20]. Many of the described haplogroups are characteristic of different populations and/ or ethnic groups [21]. The mtDNA L lineage are characteristic of Sub-Sahara African populations [20]. As a result of the African population genetic diversity of mtDNA lineage L, it is divided into two branches, L0 and L1-6 [22]. These haplogroups are further differentiated into subhaplogroups and the distribution and frequency of these subhaplogoups appear to have distinctive patterns in different geographic regions or ethnic groups [18,21,23,24]. Furthermore, the L0 haplogroup is divided into L0a, L0d, L0f and L0k subhaplogroups with varying frequencies. For example, over 90% of the Khoisan group in South Africa carry the haplogroup L0 with L0k and L0d being dominant while the L0 haplogroup is found in only 20% of East Africans [18]. The distribution of L0 among Southern and East African populations suggest related origins and reflects a possible shared migration route and population admixture [18].

We aimed to describe the phylogenetic relationship, the pattern of diversity and phylogeography of seven Malawian ethnic groups with respect to mtDNA variation. Malawi represents an important area of the African continent that had not yet been sampled [17]. It is also hoped that a better understanding of this diversity could lead to insight into patterns of genetic diseases and genetic variations relevant for pharmacokinetic profiles [25].

Materials and Methods

Participant recruitment and sample collection

Two hundred and fifteen (n=215) participants comprising of 46 (22%) Chewa (CH), 63(31%) Lhomwe (LO), 41(19%) Ngoni (NG), 9(4%) Sena (SE), 10(5%) Tonga (TO), 10(10%) Tumbuka (TU), and 36 (16%) Yao (YA) with their origin as indicated on map in Figure 1. All participants were unrelated adults recruited from an HIV/AIDS treatment cohort at Queen Elizabeth Central Hospital in Blantyre, Malawi, as part of a study to determine factors associated with antiretroviral drug toxicities. Participants completed a structured questionnaire that collected demographic information, medical history as well as individuals' ancestry up to their grandparent's level. A 5 ml blood sample was collected from each participant for genetic analysis. The protocol was approved by the College of Medicine Research Ethics Committee (COMREC), University of Malawi and the Human Research Ethics Committee of the University of Cape Town (UCT). The participants gave written informed consent.

Whole mitochondrial DNA amplification and sequencing

Blood samples were collected in EDTA coated tubes and were kept at -20^{o}C until DNA extraction. DNA was isolated by use of a GenElute Blood Genomic DNA Kit (Sigma-Aldrich, ST Louis, US) according to the manufacturer's protocol. Whole mitochondrial genome of each participant was amplified through a series of 9 partially overlapping fragments using the primers according to the method of Ramos et al. [26]. Each time mtDNA templates were amplified in a total volume of 25 µl in a reaction mixture that consisted of 1X green GoTaq reaction buffer, 200 µM of dNTP, 1.0 mM of $MgCl_2$, 0.4 µmol of each primer, 0.5 U of Taq DNA polymerase and 20 ng of DNA. The PCR programs each consisted of an initial denaturation step at 94 ^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 1 min, annealing step at 61 ^{o}C for 40s and

extension at 72 ^{o}C, for 2.5 min respectively, with a final extension step of 5 min at 72 ^{o}C [26]. The quality of the amplified fragments was visualized on a 1.5% agarose gel by electrophoresis. PCR products were purified using the Exonuclease and Shrimp Alkaline Phosphatase (ExoSap) before sequencing. The 9 overlapping mtDNA PCR fragmented from each participants, were sequenced from position 478 to 15953 (which includes the mtDNA hypervariable segment 3 and whole mtDNA coding region) using a series of forward and reverse primers and additional internal primers. The control region of mtDNA was not sequenced.

Mitochondrial DNA variation analysis

Sequences were aligned with the rCRS for the human mitochondrion NC_012920.1 [27] and assembled using Lasergene 10 Core Suite software, DNASTAR package (Madison, Wisconsin USA) for mutation scoring [27]. Haplogroups and subhaplogroups were classified according to software and data bases developed by of van Oven and Kayser and Accetturo et al [22,28]. Network 4.6.1.1[29] was used to construct a phylogenetic network and confirm the grouping of haplotypes [30]. Post analysis of median joining network was carried out using maximum parsimony algorithm and the network was redrawn [31] within the software with network calculation (epsilon parameter) set at default 1:1 and transition/transversion ratio of 3:1. The parsimony network of haplotypes was reconstructed by hand. Stata® for windows software, version SE/11 (Stata corp; Texas, USA) was used to analyze haplogroup and subhaplogroup frequencies.

Arlequin 3.5 [32] was used to determine the molecular indices which included gene-diversity, haplotype diversity, nucleotide diversity, Tajima's D and FU's Fs tests within and between ethnic groups. Genetic structure between ethnic groups' was calculated based on F_{ST} values [33] which estimates evolutionary distances between sequences by incorporating information on both haplotype frequencies and genetic distances between haplotypes, as the number of nucleotide position differences between each pair of sequences and gamma correction of 0.5 using the program Arlequin version 3.5 [32]. The distance values were used to construct a Neighbor – joining tree using software program in MEGA version 5.2.2 under the Tamura and Nei nucleotide substitution model [34].

Principal Component Analysis (PCA) was performed with software R foundation for statistical package ade4 [35,36] in order to visualize the diversity and relative weight of genetic variables among the Malawian ethnic groups and also in comparison to 52 ethnic groups (Table S1) from the Sub-Saharan African region using subhaplogroup frequencies (Table S2) . These samples were from west, central, southern, southeast and east Africa.

Results

Mitochondrial subhaplogroup distribution among the 7 Malawi ethnic groups

All the 215 DNA samples from the participants were successfully sequenced and 184 positions showed nucleotide differences when compared with bases on the rCRS [27]. Of these 184 mutations 175 (95%) have been reported in specific L subhaplogroups before; 7 (4%), m.3579 A>G (m.3606 A>G, m.5090 T>C , m.647 A>G, m.10463 T>C, m.12192 G>A and m.13104 A>G with frequencies of 2%, 2%, 6%, 2%, 1%, 3% and 7%, respectively, are being reported for the first time in an African populations although they have been reported in other populations before [22,37,38,39], while two, m. 12769 G>A and m.14612 G>A with frequencies of 2% and 4%, respectively, are novel and have not yet been included on the mtDNA databases (MITOMAP or Phylotree). Mutation m.12679 G>A is non-synonymous; while and m.14612 G>A is synonymous resulting ND5:p.Glu145Lys and ND6:p.Arg154Lys changes, respectively. It is well known that the high mutation rate of mtDNA leads to numerous instances of both parallel mutations at the same site and of reversion (i.e., homoplasmy) in populations [21]. Of Figure 2 shows a maximum parsimony tree of the subhaplogroups in our dataset.

Haplogroups L0, L1, L2 and L3 were observed in the seven Malawian ethnic groups with frequencies of 48%, 13%, 25% and14%, respectively. These haplogroups were further grouped into 9 subhaplogroups (L0d, L0k, L0f, L0a1, L0a2, L1c, L2a, L3d and L3e) as shown in Figure 2. There were both quantitative and qualitative differences in the distribution of the subhaplogroups in the Malawian ethnic groups, with the Lhomwe, Ngoni and Yao each having 8 subhaplogroups whilst the least diverse was the Sena with only four subhaplogroups. The L1c (P=0.001) and L2a (P=0.015), were distributed significantly different in the Sena and the Chewa, respectively, when compared to other ethnic groups. Figure 3 shows the distribution of the mtDNA subhaplogroup in each of the seven Malawian ethnic groups. Subhaplogroups L1c and

L3e were present in all ethnic groups while L0a1 and L0k were observed in only 3 ethnic groups each. Among these seven Malawian ethnic groups, L4, L5 and L6 haplogroups were not observed.

Evaluating genetic diversity among the Malawi ethnic groups

Genetic diversity indices for the seven ethnic groups are presented in Table 1. There were similarities in genetic diversity with value of 1; haplotype diversity within the population with all ethnic groups indicating an excess of rare haplotypes variants compared to the expectation under a neutral model of evolution by negative Tajima's D and of Fu's F_S values. Although overall diversity was similar among ethnic groups, mean pairwise distances and nucleotide diversity were both lowest within the Yao ethnic group as reflected by significant Tajima's D and of Fu's F_S.

Populations that are closely related are more likely to present with similar frequencies of mtDNA haplogroups. Pairwise F_{ST} values between ethnic groups ranged from 0.001 to 0.141 (Table 2). Comparison between the Sena and other ethnic groups showed highly significant differentiation pairwise distances. In contrast the least differentiation was observed between the Lhomwe and the Tonga (F_{ST} = 0.001) ethnic groups. In addition, to understanding genetic relationship between populations a neighbor-joining tree based on genetic distance was constructed (Figure 4) to determine the nucleotide diversity between the different ethnic groups using data from Table 2.The ethnic groups clustered into two distinct groups with the Sena ethnic group completely isolated from the other groups and also reflecting the first to diverge. The inner shorter branches separating the Lhomwe and Tonga, Ngoni and Yao, respectively, indicate lack of genetic differentiation among these ethnic groups.

Comparison of mtDNA variation in the Malawian population to other African populations

Characteristics of Southeastern African populations with respect to mtDNA subhaplogroup variation are presented in Figure 5. Data on geographical origin, mtDNA subhaplogroup distribution and language classification for the Malawi ethnic groups were compared to other

African ethnic groups (Table S1). Using database of mtDNA subhaplogroup frequencies (Table S2), principal component analysis was performed to check for population stratification in the Malawi and other African ethnic groups. PCA axes were plotted basing on the 27% variance observed and clearly separated the African populations based on the geographical placement on the continent. Ethnic groups from West African were clustered together to the bottom left, Central, Southeast and Southern African populations clustered at the centre and East African ethnic groups clustered together to the bottom right of the PCA (Figure 6). The Malawi ethnic groups were clustered between ethnic groups from Southeast and Southern Africa. It was also observed that clustering of ethnic groups followed a pattern dependent on language, with Bantu speakers (including the Malawian ethnic groups) clustered away from Afro-asiatic, Nilotic and Click speakers (Figure 6). The Bantu speakers were clustered to the left while Afro-asiatic, Nilotic and Click speakers to the right of the PCA. The Malawi population was clustered with the Bantu speakers to the left.

Discussion

Very little genomics research has been carried out among populations of Malawi [10] including mtDNA variation. We characterised mtDNA variation in seven Malawian ethnic groups and compared the observed haplogroups and subhaplogroups to those reported from other ethnic groups in Africa. The Malawi population is characterised with the L haplogroups, L0, L1, L2, and L3, which define African populations and further support the observation that sub-Saharan mtDNA gene pool is mixture of L subhaplogroups occurring at varying frequencies throughout the continent [40]. L4, L5 and L6 were not observed, confirming earlier reports of their confinement to West and East African populations and their absence in Southeast African populations [1,19]. High prevalent of L0a (34%) subhaplogroup among the Malawi population is consistent with earlier studies, which have reported the subhaplogroup as the most common in Southeast African populations [1,19]. Presence of L0d and L0k subhaplogroups in Southeast African population which was previously reported [17] is further confirmed here, pointing to possible population admixture with the Khoisan. Subhaplogroup distribution within the ethnic groups showed variations with Chewa and Sena being associated more with L2a ($n = 22$, $P = 0.029$) and L1c ($n = 6$, $P = 0.001$), respectively, suggesting that haplotypes are not randomly distributed among ethnic groups. The genetic distinctiveness of the Sena ethnic group compared

to the other six Malawian ethnic groups could be attributed to the unique culture of patrilineal kinship which prevents them from marrying among surrounding ethnic groups with different cultures. In contrast Lhomwe, Ngoni, Tonga, Tumbuka and Yao, showed a lot of genetic admixture such that they could not be assigned with specific subhaplogroups, possibly due to their migration history as well as shared common languages [41] which makes it difficult to establish their real ethnicity. For example, intermarriages are very common between Tumbuka and Ngoni from the Northern part of Malawi such that most Ngoni are masked under the Tumbuka and these groups have patriarchal culture where ethnicity is defined by the male lineage. In addition, industrialization in the southern region of Malawi an area which is mainly dominated by Yao and Lhomwe might have also contributed to the failure to assign the tribes into specific haplogroups because more people of different ethnic groups moved and settled in the Lhomwe and Yao settlements.

Comparing the subhaplogroup frequencies among Malawi, Zimbabwean and Mozambican populations, all from Southeast Africa shows, show differences in the distribution of most of the subhaplogroups. A comparison of the distribution of L0a, L2a and L3e subhaplogroups among Malawian and Zimbabwean populations reveals a possible genetic relatedness that needs further investigations. The clustering of African ethnic groups on PCA was based on geographical origin similar to what was also observed in Coelho's study [42] suggesting variability in the genetic structure within ethnic groups in the Sub Sahara African region. Furthermore, the analysis also shows that the Malawi Yao and Kaskazi Yao of Mozambique are very similar, supporting their common maternal ethnic origin or influence [43]. Alves et al [44] described that the Mozambican Yao are linguistically closer to Bantu people from Tanzania and Kenya suggesting relationships to ethnic groups in an extended geographical area. There is need for a comprehensive molecular evolutionary analysis of the ethnic groups in Southeast Africa focusing on more markers including Y-chromosome DNA. The Ngoni, Tonga and Tumbuka and from Malawi are scattered between the Southern African Bantus speakers (i.e. Zulu/Xhosa from South Africa, Swazi from Swaziland, Sotho from South Africa and Makonde of Mozambique). Taking into account gene variation, these ethnic groups share similarities in the distribution of the L0 haplogroups which is more frequent in Southern African ethnic groups [1,18,45].

Interestingly, there seems to be less genetic relatedness between Lhomwe ethnic group of Malawi and the Lhomwe from Mozambique possibly suggesting population admixture in support of the ethnologue information [43]. This observation defeats the use of language to solely characterise populations. Using PCA, clear separation is observed between Malawi Sena and Nyasa speakers of Mozambique (Sena and Nyungu). However the Sena is an umbrella term for sub-ethnic groups of the major Kusi-speaking groups of Makua, Shona, Rabu and Phodzo and other Southeast Bantu [14,44]. Therefore the separation from the cluster of the Kusi-speakers as observed on PCA may reflect genetic variation still to be explained. Nonetheless, further work to be carried in order to investigate the relationship of the Malawi Sena ethnic group with the Kusi-speaking ethnic groups. One must however take precaution when predicting genetic similarity from language because during population dispersal there were tribal wars and conquering victors often imposed their language on the losers.

MtDNA variation has been used in human phylogeography and also in studies trying to define the risk mtDNA variants in common disease [21,46]. The presence of mtDNA variants which have not been reported in L subhaplogroups could suggest population admixture possibly due to colonisation, slavetrade and exploration [41,47,48,49] which could have increased admixture. Data on the mtDNA landscape of Malawi has not been available up to this point and this is a contribution towards unraveling the deep-rootedness of mtDNA lineages in Southern African populations. However, one of the limitations of this study was small sample sizes for ethnic groups because participants were primarily recruited to investigate the pharmacogenetics correlates of differential response to stavudine-containing antiretroviral therapy and evaluating population history was a secondary. However, still our sample sizes for the ethnic groups are comparable to other current studies [50]. In addition, mtDNA variation, may not completely explain the population characteristics of Malawian ethnic groups because of the focus on matrilineality, thus, need to be complemented with studies looking at Y-chromosome variation. For future studies, we recommend focused sampling for an in depth population history study of the Malawian ethnic groups in their respective geographical areas than the current study which relied on participants presenting themselves for HIV/AIDS treatment at Queen Elizabeth Hospital in Blantyre, although this hospital draws its patients from across the whole country.

However, our study gives a glimpse into the population history of the Malawian people.

Conclusion

The results presented here reveal the genetic characteristics of the of Malawi population with respect to mtDNA variation. The majority of subhaplogroup distribution patterns in the 7 Malawian ethnic groups bear resemblance to that of Southern and Southeastern African populations. The observation of L0d and L0k in the Malawi population further suggests that there could maternal relationship of the Malawi Bantu speakers to L0d and L0K lineages of Khoisan. It is now well recognized that mutations of mtDNA are an important cause of inherited neuromuscular disorders in man [51]. A great deal of work has been done to identify and classify these mutations in Caucasian European populations [52,53] and efforts in Asian populations have been growing for a number of years [54,55]. However, progress in populations of African origin has been limited, what has been deduced is that the spectrum of mutations responsible for clinically-manifesting mtDNA are likely to have some important differences in African populations when compared to others [56]. This suggests that haplogroup context is playing an important role in the penetrance of mutations, and this is something that we have seen illustrated before by the variable penetrance of Leber's hereditary optic neuropathy (LHON) in different haplogroups in both European and Asian populations [57,58]. Furthermore, clinically manifesting mutations such as those that can result in the LHON are thought by many to be only one facet of the role played by mtDNA variants in human disease, with many studies suggesting a role for common mtDNA variants in common complex disease either affecting likelihood of disease [59] or course of disease [60]. Therefore observation from this data makes it imperative for studies to investigate the clinical significances of the common mtDNA haplogroups in Southern and southeast African populations that bears such a huge burden of common diseases.

The effective identification of clinically manifesting mutations and construction of association studies alike requires a detailed and accurate understanding of phylogeny [61], and for many African populations this has been lacking. While there has been much needed growth in the numbers of such sequences and many geographical areas now being well characterised [1]; the

variation in Malawi is as yet unexplored, as such we offer here a tentative first exploration of the mtDNA diversity of the ethnic groups of this nation.

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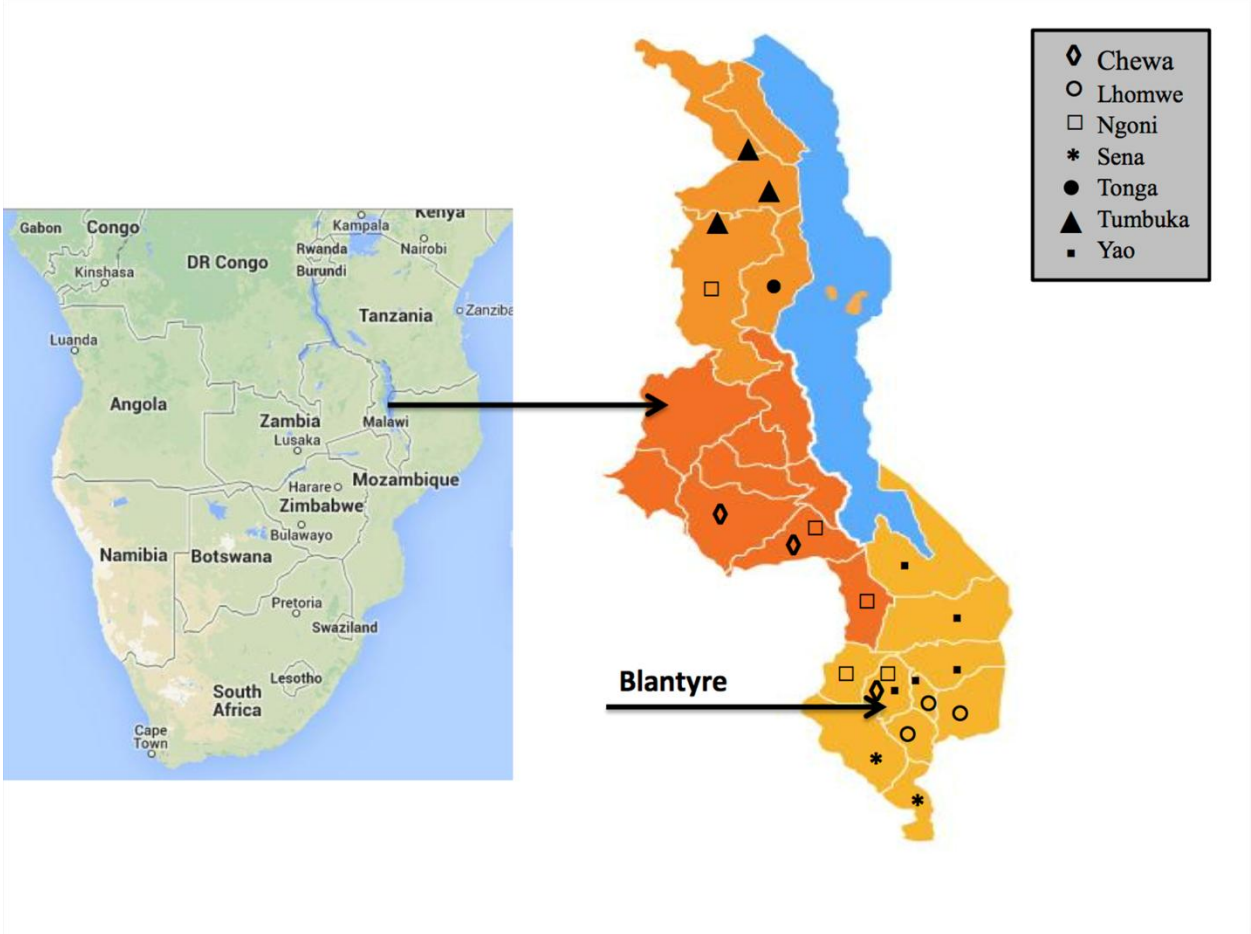

Figure 1: Map of Malawi showing ethnic groups in the study and their settlement pattern.
Malawi is bordered by Tanzania, Mozambique and Zambia. CH = Chewa/Nyanja; LO = Lomwe; NG = Ngoni; SE = Sena; TO = Tonga; TU = Tumbuka; YA = Yao. The participants were recruited from Queen Elizabeth central Hospital in Blantyre as part of a pharmacogenetics study on mitochondrial stavudine induced toxicities.

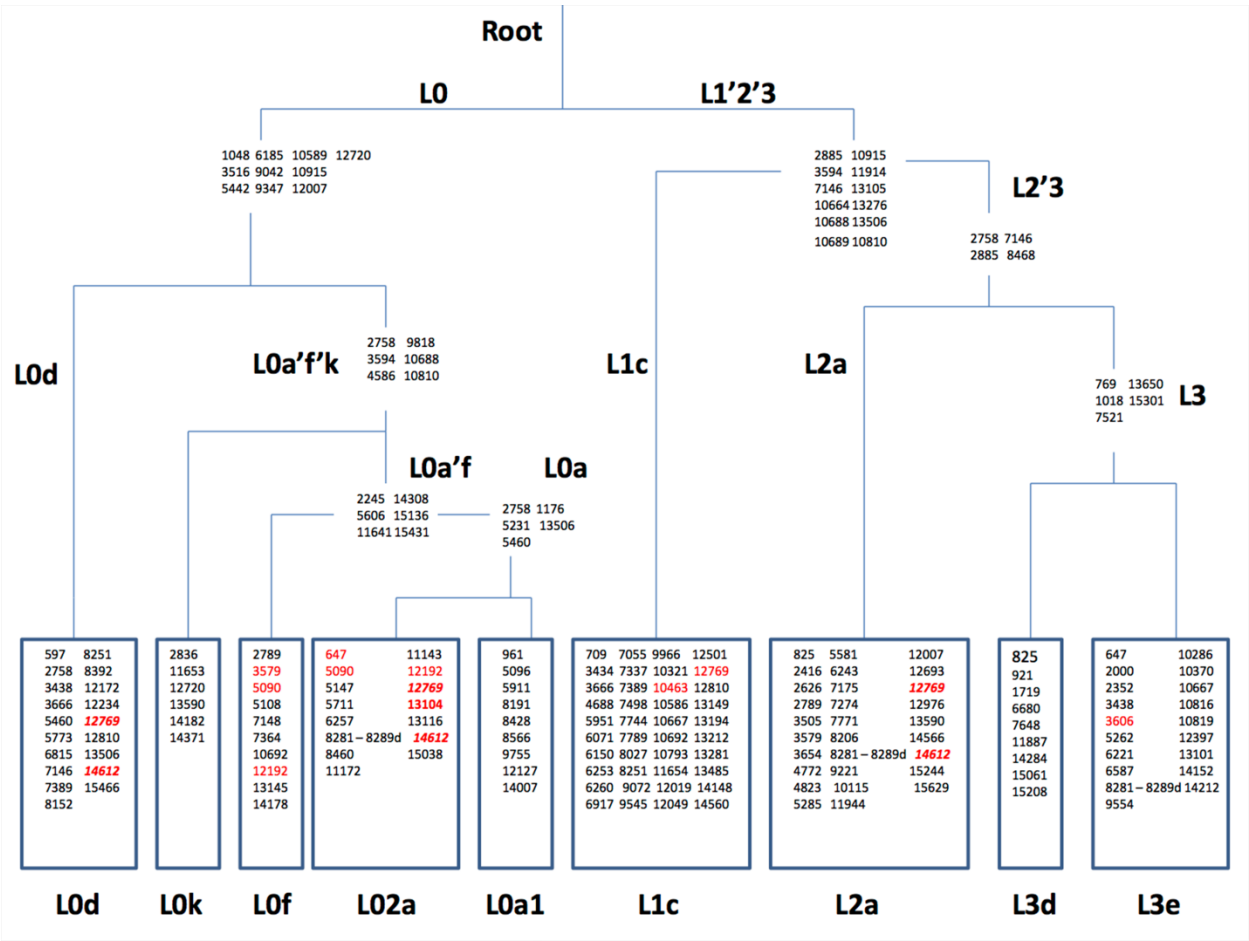

Figure 2: Maximum parsimony network of haplotypes for mtDNA of Malawian samples.
The network was reconstructed from maximum parsimony phylogenetic tree. It is showing subhaplogroup diversity in Malawian population. Numbers refer to nucleotide (haplotype) position with respect to revised Cambridge Reference Sequence. Positions of nucleotides in red board are reported for the first time in the mtDNA L lineage. Nucleotides in red board and italised are novel. 8281-8298d was a nine base pair deletion (CCCCCTCTA).

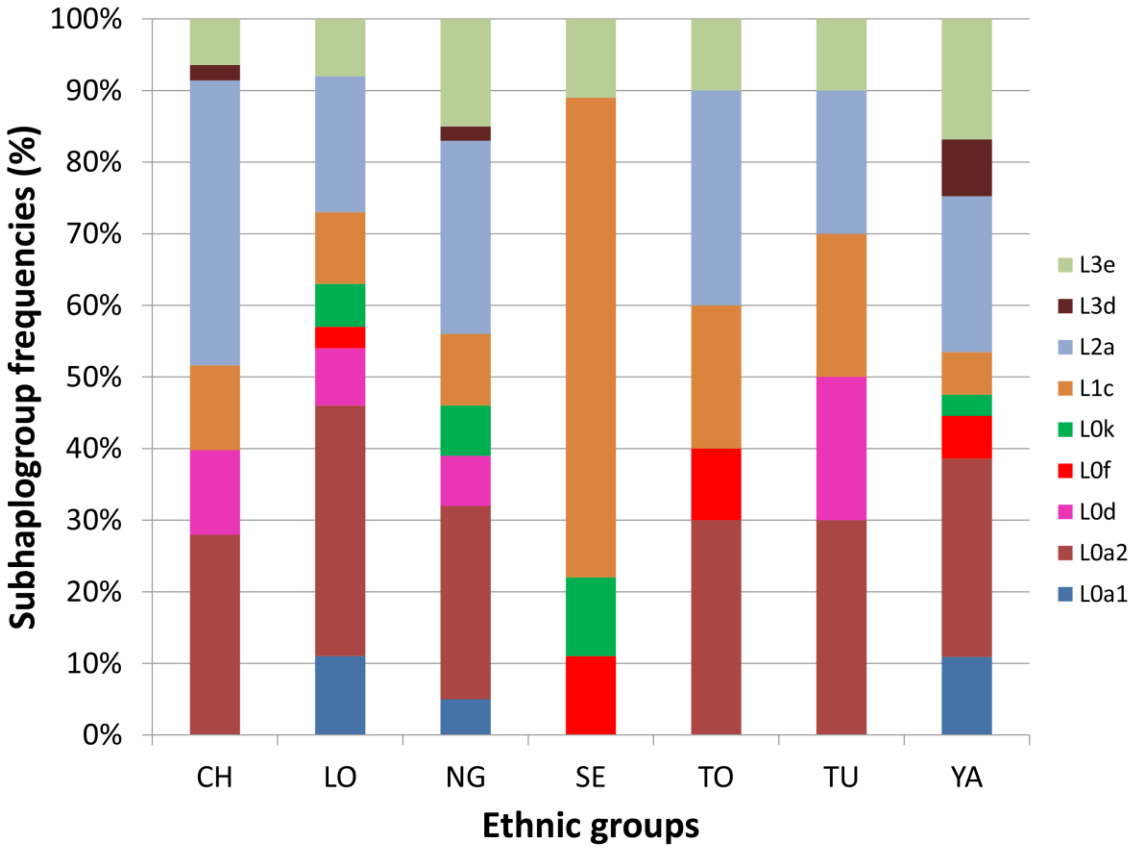

Figure 3: Subhaplogroup distributions among Malawian ethnic groups.
CH = Chewa; LO = Lomwe; NG = Ngoni; SE = Sena; TO = Tonga; TU = Tumbuka; YA = Yao. The most diverse ethnic groups in terms of mtDNA subhaplogroups were Lhomwe, Ngoni and Yao, while the least was Sena with only 4 subhaplogroups

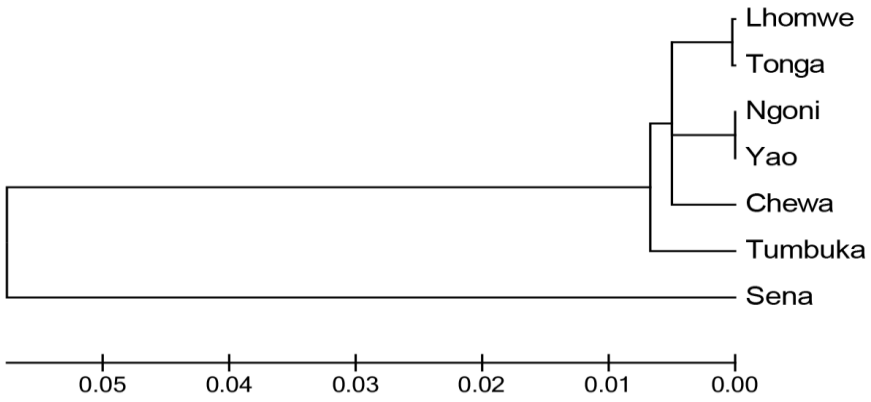

Figure 4: Neighbor joining tree based on genetic distances between seven Malawi ethnic groups.

The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances that were used to infer the phylogeny tree (scale bar, 0.01 nucleotide substitutions per site). CH = Chewa; LO = Lomwe; NG = Ngoni; SE = Sena; TO = Tonga; TU = Tumbuka; YA = Yao

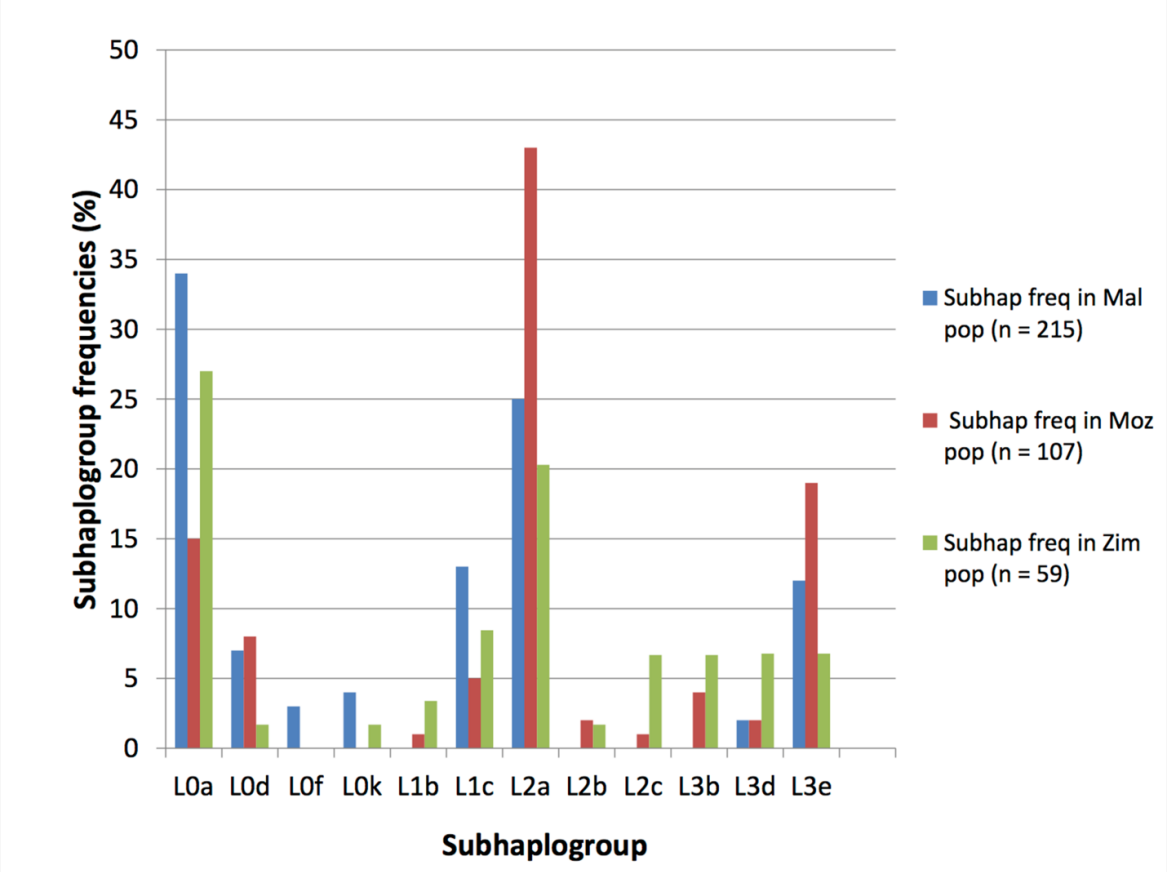

Figure 5: Comparison of mtDNA subhaplogroup distribution within Southeast African (Malawi, Mozambique and Zimbabwe) populations.

Presence of L0f, L0k, L1b, L2b, L2c, L3b and L4g is population dependent. L0a, L0d, L1c, L2a, L3d and L3e are present in all the populations. Mal = Malawi; Moz = Mozambique; Zim = Zimbabwe; pop = population. When haplogroups frequencies Malawi population were compared to Mozambican population significant differences P – value of 0.022 and 0.011 were observed in haplogroup L0a and L2a respectively

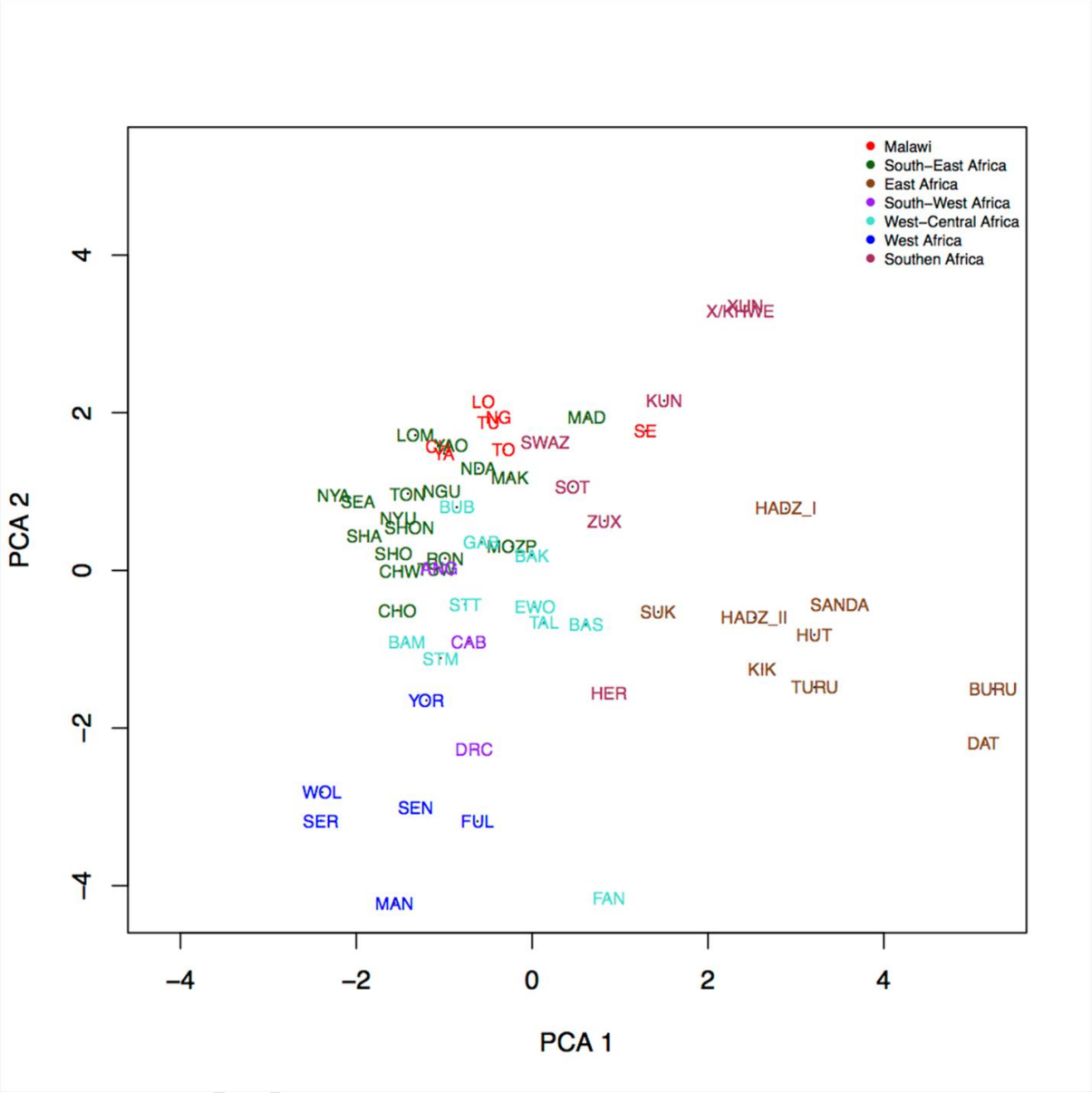

Figure 6: Principal component analysis showing genetic affinities between 60 ethnic groups in Sub-Sahara Africa.

The ethnic groups are classified as of Niger-Congo, Nilo-Saharan and Khoisan speakers from West, Central, Southwest, Southeast, Southern Africa and East Africa. The codes in red are representing the Malawi population and the codes in blue, bright turquoise, purple, dark green, brown, bright maroon are representing the other African population in the west, west central, central, southeast, east and southern Africa respectively. Codes bottom left of the PCA are for Bantu population from West Africa and bottom right is for population from East Africa. The population from central and southeast Africa is clustered to the upper left centre of the PCA. The Southern African Click speakers are to upper right of the diagram. Populations are abbreviated as follows: CH = Chewa; LO = Lomwe; NG = Ngoni; SE = Sena; TO = Tonga; TU = Tumbuka; YA = Yao; CHO = 5 Chopi; CHW = Chwabo; LOM = Lomwe; MAK = Makhuwa; MAD = Makonde; MOZP = Mozambicans; NDA = Ndau; NGU = Ngoni; NYA = Nyanja; NYU = Nyungwe; RON = Ronga; SEA= Sena; SHA = Shangaan; SHO = Shona; TON = Tonga; TSW = Tswa; YAO = Yao; SHON = Shona; KIK = Kikuyu; HUT = Hutu; SUK = Sukuma; ANG = Mbundu; CAB = Cabinda; HER = Herero; BAM = Bamileke; BUB = Bubi; EWO = Ewondo; FAN = Fang; BAK = Bakaka; BAS = Bassa; STM = Sa˜o TomeM; STT = Sa˜o TomeT; TAL = Tali; FUL = Fulbe; MAN = Mandenka; SEN = Senegal; SER = Serer; WOL = Woloff; YOR = Yoruba; Hadz II = Hadza II; Sanda = Sandawe; Hadz I = Hadza I; X/Khwe = Xun/Khwe; Xun = Xun; Kun = Kung; Manyanga = DRC; Zulu/Xhosa = ZUX; Swazi = Swaz; Sotho = SOT.

Table 1: Intrapopulation diversity indices in the Malawi population

Population	N	NH	H (SD)		MNPD (SD)	π (SD)	Tajima's D (P)	Fu's Fs (P)
Chewa	46	135	1.00 (0.022)	±	22.15 (9.94)	0.127 (0.063)	-1.14 (0.119)	-24.16 (0.001)
Lhomwe	63	134	1.00± (0.016)		20.20 (9.04)	0.116 (0.057)	-1.24 (0.075)	-24.11 (0.001)
Ngoni	41	124	1.00 (0.024)	±	17.68 (8.02)	0.102 (0.051)	-1.39 (0.062)	-24.22 (0.001)
Sena	9	54	1.00 (0.111)	±	15.38 (7.60)	0.088 (0.049)	-1.15 (0.134)	-1.85 (0.099)
Tonga	10	71	1.00 (0.100)	±	16.49 (8.03)	0.095 (0.052)	-1.48 (0.058)	-2.21 (0.072)
Tumbuka	10	82	1.00 (0.100)	±	19.87 (9.61)	0.115 0.0620	-1.52 (0.059)	-1.88 (0.104)
Yao	36	96	1.00 (0.028)	±	13.67 (6.28)	0.078 (0.041)	-1.59 (0.022)*	-24.35 (0.001)*

N, number of sequences; NH, number of different haplotypes; H, haplotype diversity (defined as $H = (1-\sum x^2_i) N/(N-1)$, where x is the frequency of haplotype; π, nucleotide diversity (average over loci); MNPD, mean pairwise distance. *Significant difference

Table 2: Pairwise differences (F_{ST}) between the 7 ethnic groups

	Chewa	Lhomwe	Ngoni	Sena	Tonga	Tumbuka	Yao
Chewa	-	-	-	-	-	-	-
Lhomwe	0.0288*		-	-	-	-	-
Ngoni	0.016	0.018		-	-	-	-
Sena	0.081*	0.152*	0.107*		-	-	-
Tonga	0.0436	0.001	0.029	0.132*		-	-
Tumbuka	0.007	0.012	0.013	0.072*	0.004		
Yao	0.011	0.037*	0.004	0.141*	0.005	0.040	-

Table 3: P-values for Pairwise differences (F_{ST}) between the 7 ethnic groups

	Chewa	Lhomwe	Ngoni	Sena	Tonga	Tumbuka	Yao
Chewa	-	*0.018*	*0.968*	*0.022*	*0.994*	*0.377*	*0.142*
Lhomwe	-		*0.055*	*0.004*	*0.380*	*0.276*	*0.016*
Ngoni	-	-		*0.006*	*0.866*	*0.241*	*0.430*
Sena	-	-	-		*0.002*	*0.041*	*0.001*
Tonga	-	-	-	-		*0.540*	*0.069*
Tumbuka	-	-	-	-	-		
Yao	-	-	-	-	-	-	-

Table S1: Population groups, geographical origin, language classification sample size, and references for the mtDNA subhaplogroup data

Bantu	CODE	Geographical Location West Africa	Language	No. of Population	Study
Fulbe	FUL	Niger	Niger- Congo, Atlantic, North Senegambian	57	{Watson E, 1996 #234}
Mandenka	MAN	Senegal	Niger- Congo, Mande, Central	117	{Graven L, 1995 #226}
Senegal	SEN	Senegal	Niger- Congo, Atlantic, mixed	50	{Rando JC, 1998 #231}
Serer	SER	Senegal	Niger- Congo, Atlantic, North Senegambian	23	{Rando JC, 1998 #231}
Woloff	WOL	Senegal	Niger- Congo, Atlantic, North Senegambian	48	{Rando JC, 1998 #231}
Yoruba	YOR	Nigeria	Niger- Congo, Volta-Congo, Defoid	34	{Watson E, 1996 #234}
Gabon	GAB	Gabon	Niger- Congo, Mixed	489	{Quintana-Murci L, 2008 #216}
Bantu		**West- Central Africa**			
Bamileke	BAM	Cameroon	Niger- Congo, Bantoid, Narrow Grassfields	48	{Destro-Bisol G, 2004 #225}
Bubi	BUB	Bioko	Niger- Congo, Narrow Bantu, Northwest, B	45	{Mateu E, 1997 #228}
Ewondo	EWO	Cameroon	Niger- Congo, Narrow Bantu, Northwest, A	53	Destro-Bisol G and others 2004)
Fang	FAN	Guinea	Niger- Congo, Narrow Bantu, Northwest, A	11	{Pinto F, 1996 #229}
Bakaka	BAK	Cameroon	Niger- Congo, Narrow Bantu, Northwest, A	50	{Coia V, 2005 #224}
Bassa	BAS	Cameroon	Niger- Congo, Narrow Bantu, Northwest, A	47	{Coia V, 2005 #224}
São TomémM	STM	São Tomé	Niger- Congo, Narrow Bantu, mixed	50	{Mateu E, 1997 #228}
São TomémT	STT	São Tomé	Niger- Congo, Narrow Bantu, mixed	103	{Trovoada MJ, 2004 #233}
Tali	TAL	Cameroon	Niger- Congo, Volta Congo, Adamawa	20	{Coia V, 2005 #224}
Bantu		**South West Africa**			
Mbundu	ANG	Angola	Niger- Congo, Narrow Bantu,	44	{Plaza S, 2004 #199}

			Central, H		
Cabinda	CAB	Angola	Niger- Congo, Narrow Bantu, Central, H	110	{Beleza S, 2005 #200}
Herero	HER	Namibia	Niger- Congo, Narrow Bantu, Central, R	27	{Soodyall H, 1996 #232}
Bantu		**South East Africa**			
Chewa	CH	Malawi	Niger- Congo, Narrow Bantu, Central N	46	present study
Lomwe	LO	Malawi	Niger - Congo, Mixed (Makhua-Nyanja)	63	present study
Ngoni	NG	Malawi	Niger - Congo, Narrow Bantu, Central S	41	present study
Sena	SE	Malawi	Niger - Congo, Narrow Bantu, Central N	9	present study
Tonga	TO	Malawi	Niger - Congo, Narrow Bantu, Central N	10	present study
Tumbuka	TU	Malawi	Niger - Congo, Narrow Bantu, Central N	10	present study
Yao	YA	Malawi	Niger - Congo, Narrow Bantu, Central P	36	present study
Chopi	CHO	Mozambique	Niger- Congo, Narrow Bantu, Central, S	27	{Salas Antonio, 2002 #184}
Chwabo	CHW	Mozambique	Niger- Congo, Narrow Bantu, Central, P	20	{Salas Antonio, 2002 #184}
Lomwe	LOM	Mozambique	Niger- Congo, Narrow Bantu, Central, P	20	{Salas Antonio, 2002 #184}
Makhuwa	MAK	Mozambique	Niger- Congo, Narrow Bantu, Central, P	20	{Salas Antonio, 2002 #184}
Makonde	MAD	Mozambique	Niger- Congo, Narrow Bantu, Central, P	19	{Salas Antonio, 2002 #184}
Mozambicans	MOZP	Mozambique	Niger- Congo, Narrow Bantu, Central, mixed	107	{Pereira, 2001 #2}
Ndau	NDA	Mozambique	Niger- Congo, Narrow Bantu, Central, S	19	{Salas Antonio, 2002 #184}
Nguni	NGU	Mozambique	Niger- Congo, Narrow Bantu, Central, N	11	{Salas Antonio, 2002 #184}
Nyanja	NYA	Mozambique	Niger- Congo, Narrow Bantu, Central, N	20	{Salas Antonio, 2002 #184}
Nyungwe	NYU	Mozambique	Niger- Congo, Narrow Bantu, Central, N	20	{Salas Antonio, 2002 #184}
Ronga	RON	Mozambique	Niger- Congo, Narrow Bantu, Central, S	22	{Salas Antonio, 2002 #184}
Sena	SEA	Mozambique	Niger- Congo, Narrow Bantu, Central, N	21	{Salas Antonio, 2002 #184}
Shangaan	SHA	Mozambique	Niger- Congo, Narrow Bantu, Central, S	22	{Salas Antonio, 2002 #184}
Shona Moz	SHO	Mozambique	Niger- Congo, Narrow Bantu, Central, S	17	{Salas Antonio, 2002 #184}
Tonga	TON	Mozambique	Niger- Congo, Narrow Bantu, Central, S	20	{Salas Antonio, 2002 #184}
Tswa	TSW	Mozambique	Niger- Congo, Narrow Bantu, Central, S	19	{Salas Antonio, 2002 #184}
Yao	YAO	Mozambique	Niger- Congo, Narrow Bantu, Central, P	10	{Salas Antonio, 2002 #184}

Shona Zim	SHON	Zimbabwe	Niger- Congo, Narrow Bantu, Central, S	59	{Castrì, 2009 #221}
Khoisan		**Southern Africa / East Africa**			
Hadza II	Hadz II	Tanzania	Khoisan, Hatsa	79	{Gonder, 2007 #147}
sandawe	Sanda	Tanzania	Khoisan, Sandawe	82	{Gonder, 2007 #147}
Hadza I	Hadz I	Tanzania	Khoisan, Hatsa	12	{Knight A, 2003 #227}
Xun/Khwe	X/Khwe	South Africa	Khoisan, Southern Africa, Central	18	{Gonder, 2007 #147}
Xun	Xun	Namibia	Khoisan, Southern Africa, Southern	43	{Vigilant L, 1991 #235}
Kung	Kun	Botswana	Khoisan, Southern Africa, Northern	26	{Vigilant L, 1991 #235}
Bantu		**Southern Africa**			
Zulu/Xhosa	ZUX	South Africa	Niger-Congo, Atlantic-Congo, Volta-Congo, Southern, Narrow Bantu, Central, S	36	{Schlebusch, 2009 #194}
Swazi	SWAZ	South Africa	Niger-Congo, Atlantic-Congo, Volta-Congo, Narrow Bantu, Central, S,	5	{Schlebusch, 2009 #194}
Sotho	SOT	South Africa	Niger-Congo, Atlantic-Congo, Narrow Bantu, Central, S	22	{Schlebusch, 2009 #194}
Bantu		**East Africa**			
Kikuyu	KIK	Kenya	Niger- Congo, Narrow Bantu, Central, E	25	{Watson E, 1996 #234}
Hutu	HUT	Rwanda	Niger- Congo, Narrow Bantu, Central, J	42	{Castrì, 2009 #221}
Sukuma	SUK	Tanzania	Niger- Congo, Narrow Bantu, Central, F	21	{Knight A, 2003 #227}
Turu	TURU	Tanzania	Niger- Congo, Narrow Bantu, Central, F	29	{Tishkoff, 2007 #183}
Nilo - Saharan		**East Africa**			
Datoga	DAT	Tanzania	Nilo - Sahara, Eastern Sudanic, Nilotic, Southern	39	{Tishkoff, 2007 #183}
Afro - Asiatic		**East Africa**			
Burunge	BURU	Tanzania	Afro - Asiatic, Cushitic, South	38	{Tishkoff, 2007 #183}

Table S2: Mitochondria Haplogroup frequencies in different population in Sub-Sahara Africa

	L0a	L0a1	L0a2	L0d	L0f	L0k	L1b	L1c	L2	L2a	L2b	L2c	L2d	L3	L3b	L3d	L3e	L3f	L3x1	L4g	L5	Other
CH	0	0	26	11	0	0	0	11	0	37	0	0	0	0	0	2	13	0	0	0	0	0
LO	0	11	35	8	3	6	0	10	0	19	0	0	0	0	0	0	8	0	0	0	0	0
NG	0	5	27	7	0	7	0	10	0	27	0	0	0	0	0	2	15	0	0	0	0	0
SE	0	0	0	0	11	11	0	67	0	0	0	0	0	0	0	0	11	0	0	0	0	0
TO	0	0	30	0	10	0	0	20	0	30	0	0	0	0	0	0	10	0	0	0	0	0
TU	0	0	30	20	0	0	0	20	0	20	0	0	0	0	0	0	10	0	0	0	0	0
YA	0	11	27	0	0	3	0	6	0	22	0	0	0	0	0	8	17	0	0	0	0	0
CHO	0	4	11	0	0	0	4	7	0	30	0	0	0	7	0	15	11	7	0	0	0	0
CHW	0	10	5	0	0	0	5	5	0	40	0	0	5	0	10	10	5	0	0	0	0	0
LOM	0	20	35	0	0	0	0	10	0	25	4	0	5	0	0	0	5	0	0	0	0	0
MAK	15	0	30	0	0	0	0	10	0	20	0	0	0	0	0	5	20	0	0	0	0	0
MAD	0	0	16	5	0	0	0	11	21	0	0	0	0	0	11	0	37	0	0	0	0	0
MOZP	15	0	0	7	0	0	1	6	0	43	0	1	0	2	4	2	17	0	0	0	0	0
NDA	0	6	33	17	0	0	0	6	0	28	0	0	0	8	6	6	0	0	0	0	0	0
NGU	8.33	25	17	0	0	0	0	0	0	17	2	0	0	0	0	8	17	0	0	0	0	0
NYA	0	5	25	0	0	0	0	0	0	45	0	0	0	0	0	15	10	0	0	0	0	0
NYU	0	30	0	0	0	0	0	10	0	20	0	0	0	0	0	10	15	0	0	0	0	0
RON	0	10	10	19	0	0	0	0	0	24	0	5	0	0	0	0	19	10	0	0	0	0
SEA	0	5	32	0	0	0	0	5	0	36	0	0	0	0	0	14	5	5	0	0	0	0
SHA	0	14	14	5	0	0	5	9	0	27	5	0	0	0	0	9	14	0	0	0	0	0
SHO	0	13	13	0	0	0	6	0	0	44	0	6	0	0	0	0	13	6	0	0	0	0
TON	0	5	10	5	0	0	0	10	0	35	5	0	0	0	0	0	30	0	0	0	0	0
TSW	0	11	0	16	0	0	0	0	0	37	0	0	5	0	11	11	11	0	0	0	0	0
YAO	0	10	30	0	0	0	0	0	0	10	0	0	0	10	0	40	0	0	0	0	0	0
SHON	0	7	20	2	0	2	3	8	0	20	2	0	0	7	7	1	0	0	0	2	0	0
KIK	0	8	4	0	0	0	0	0	0	12	0	0	20	0	0	19	0	0	0	4	8	4
HUT	0	12	5	0	17	0	0	2	0	7	2	2	10	17	2	20	0	2	0	7	5	2
SUK	0	14	10	0	0	0	0	14	0	5	0	0	0	5	5	7	0	0	0	24	5	14
ANG	0	7	7	0	0	0	5	16	0	26	5	0	0	2	5	5	2	0	0	0	2	0
CAB	0	10	3	0	0	0	3	25	0	5	6	0	0	4	5	21	13	0	0	1	0	3
HER	0	0	0	0	0	0	0	0	0	0	0	0	0	81	0	7	0	0	0	0	0	7
BAM	6	4	4	0	0	0	2	6	0	29	4	4	0	4	13	10	10	0	0	0	0	0
BUB	0	0	9	0	0	0	0	22	0	18	2	16	0	0	0	33	0	0	0	0	0	0
EWO	0	8	2	0	0	0	6	20	0	18	0	8	0	6	0	8	4	0	0	8	0	4
FAN	0	8	0	0	0	0	8	17	0	0	0	0	0	0	0	0	25	0	0	0	0	25
BAK	0	10	10	0	0	0	6	14	2	10	2	0	12	2	0	26	4	0	0	2	0	0
BAS	4	0	0	0	0	0	7	24	2	15	0	4	13	2	2	13	11	0	0	2	0	0
STM	0	6	2	0	0	0	18	8	0	20	0	0	0	4	0	28	6	0	0	0	0	4
STT	0	6	1	0	0	0	14	3	16	14	3	1	0	4	6	27	4	0	0	0	0	2
TAL	0	0	0	0	0	0	5	5	5	26	0	0	11	11	0	21	16	0	0	0	0	0
FUL	0	0	0	0	0	0	16	0	0	16	0	0	0	14	11	9	9	0	0	2	0	14
MAN	0	2	0	0	0	0	20	0	0	12	3	1	0	5	9	7	1	0	0	0	0	5
SEN	0	0	0	0	0	0	8	4	0	20	8	0	0	14	10	6	8	0	0	0	0	8
SER	0	0	0	0	0	0	22	0	0	26	17	0	0	17	4	0	0	0	0	0	0	0
WOL	0	0	0	0	0	0	23	0	0	25	15	6	0	2	4	8	0	0	0	0	0	0
YOR	0	3	0	0	0	0	18	6	0	21	3	0	0	9	6	21	3	0	0	3	0	6
GAB	0	7	3	0	0	0	0	36	0	14	2	2	1	2	3	4	15	6	0	1	0	0
TURU	10	0	0	0	14	0	0	3	0	7	0	0	8	10	7	3	3	3	0	28	0	12
DAT	28	0	0	0	5	0	0	0	0	0	0	0	0	27	0	0	0	0	0	13	0	19
BURU	25	0	0	3	30	0	0	0	3	0	0	0	0	18	0	3	0	8	0	8	0	6
Hadz_II	5	0	0	3	0	0	0	0	1	10	0	0	0	8	4	1	0	5	0	60	1	3
Sanda	17	0	0	5	4	0	0	0	0	1	0	0	0	0	0	0	13	2	0	48	5	4
0Hadz_I	0	0	0	0	0	0	0	0	17	0	0	0	0	0	0	0	0	0	0	83	0	0
X/Khwe	0	0	0	61	0	22	0	0	17	0	0	0	0	0	0	0	0	0	0	0	0	0
Xun	**2**	**0**	**0**	**51**	**0**	**26**	**0**	**0**	**16**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**5**	**0**	**0**	0	0	0
Kun	**0**	**0**	**0**	**96**	**0**	**4**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	0	0	0
DRC	**7**	**0**	**0**	**0**	**0**	**0**	**14**	**0**	**0**	**7**	**0**	**7**	**0**	**0**	**7**	**7**	**29**	**21**	**0**	0	0	0
ZUX	**8**	**0**	**0**	**44**	**0**	**3**	**0**	**6**	**0**	**14**	**0**	**3**	**0**	**0**	**0**	**8**	**8**	**3**	**0**	0	2.8	0
Swaz	**0**	**0**	**0**	**40**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**60**	**0**	**0**	0	0	0
SOT	**14**	**0**	**0**	**23**	**0**	**0**	**0**	**5**	**0**	**27**	**0**	**0**	**0**	**0**	**0**	**5**	**10**	**0**	**0**	0	0	0

5. Chapter 5: MITOCHONDRIAL DNA VARIATION AND SUSCEPTIBILITY TO PERIPHERAL NEUROPATHY

Mitochondrial DNA subhaplogroups L0a2 and L2a modify susceptibility to peripheral neuropathy in Malawian adults on stavudine containing highly active antiretroviral therapy (*published in JAIDS 2013, vol 63(5)647 -652*)

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Abstract

Introduction: Peripheral neuropathy is one of the main toxicities associated with stavudine. Genetic variants in mitochondrial DNA haplogroups have been associated with increased risk of developing peripheral neuropathy 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 antiretroviral therapy.

Method: Two hundred and fifteen (n = 215) 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 mitochondrial DNA of individuals in relation to response to treatment. Patients were categorized according to whether or not they had developed peripheral neuropathy after a minimum of six months on stavudine containing ART. Whole mitochondrial DNA coding regions of each patient were sequenced, CD4 count, viral load and creatinine were determined. Mitochondrial DNA variation was correlated with clinical characteristics.

Results: Fifty three (25%) of the participants developed peripheral neuropathy after starting stavudine containing antiretroviral therapy. Mitochondrial DNA subhaplogroup L0a2 was independetly associated with increased risk of peripheral neuropathy in a multivariate model (OR 2.23; 95% CI, 1.14 – 4.39; $p = 0.019$) and subhaplogroup L2a was independently associated with reduced risk of peripheral neuropathy (OR 0.39; 95% CI, 0.16 – 0.94; $p = 0.036$).

Conclusion: Genetic variation in mtDNA confers differential risk of developing peripheral neuropathy in patients on stavudine containing ART among Malawians.

Key words: Stavudine, mtDNA, subhaplogroup, peripheral neuropathy, toxicities

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]. Peripheral neuropathy 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 peripheral neuropathy. 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 with deoxyribonucleotide triphosphates (dNTPs) for 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 mitochondrial DNA polymerase gamma inhibition, mitochondria dysfunction could be the a result of increased mtDNA polymorphisms as well as oxidative stress caused by NRTI which 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 ART between individuals [8].

The evolution of the human mtDNA is characterised by the emergence of distinct lineages or haplogroups. These haplogroups are characterised 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 defines African populations and is divided into two branches, L0 and L1-6 [12] see Figure 1. The distribution and frequency of these mtDNA L subhaplogoups within Africa appear to have distinctive patterns in different geographic regions or ethnic groups [11, 13-15]. For example, over 90% of Khoisan group of South Africa carry the haplogroup L0 while this haplogroup is found in only 20% of East Africans [14].

Analysis of mitochondrial subhaplogroups among European patients enrolled in AIDS clinical Trials Group (ACTG) studies showed that individuals in haplogroup T had increased risk of developing peripheral neuropathy 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, while 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 peripheral neuropathy 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 as well as ancestry of each participant up to their grandparent's level. Pregnant women, patients on tuberculosis treatment, persons who experienced peripheral neuropathy prior to 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 (UCT). 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 enrolment. Stavudine associated peripheral neuropathy 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, New Jersey, USA) 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 EDTA coated tubes and were kept at -20^{o}C until DNA extraction. DNA was isolated by use of a GenElute Blood Genomic DNA Kit (Sigma-Aldrich, ST Louis, US) according to the manufacturer's protocol. Mitochondrial DNA 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 25µl in a reaction that consisted of 1X green GoTaq reaction buffer, 200µM of dNTP, 1.0mM of $MgCl_2$, 0.4µmol of each primer, 0.5U of Taq DNA polymerase and 20ng of DNA were performed in GeneAmp® PCR System 9700 by Life technologies (New York, USA). The PCR programmes each consisted of an initial denaturation step at 94^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 1 min and annealing and extension step at 57 - 64.4 ^{o}C , 72^{o}C for 40s and 2.5 min respectively, with a final extension step of 5 min at 72^{o}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 in order 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 the revised Cambridge reference sequence Locus NC_012920.1. Capillary electrophoresis for sequencing reactions was run on an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, USA). Sequences were aligned to the revised Cambridge Reference Sequence for the human mitochondrion Locus NC_012920.1 and assembled using Lasergene 10 Core Suite software supplied by DNASTAR package (Madison, Wisconsin USA). After assembly of the sequences, mutations (polymorphisms) were determined as nucleotide differences when compared to the Cambridge reference sequence.

Subhaplogroup Analysis and Statistical Analysis

We classified mtDNA mutations into haplogroups according to the data bases of van Oven and Kayser [12] *www.phylotree.org/* and Accetturo et al [20]. Stata for windows software (version SE/11; 4905; Stata Corp; Texas, USA) was used for statistical analysis. Subhaplogroup frequencies were

compared between participants presenting with and without peripheral neuropathy using Fisher's exact tests.

In order to assess the relationship between independent variables (sex, BMI, age, duration on ART, CD4 count, viral load, eGFR and subhaplogroups) and the presence of peripheral neuropathy, univariate logistic regression model was performed. Variables which showed a degree of association with peripheral neuropathy ($P<0.1$) were included in multivariate logistic regression models, where one 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 antiretroviral therapy for at least 23 months, median duration of 25 months (range 23 -29). Two (1%) patients had severely elevated serum creatinine levels of 5.4mg/dl and 6.0mg/dl with an estimated GFR of 15.4 ml/min/1.73m^2 and 10.3 ml/min/1.73m^2, respectively. One percent (1%) and 3% of patients were current smokers and alcoholics respectively. Fifty-three (25%) patients had peripheral neuropathy. 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 revised Cambridge Reference Sequence (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 two (1%); m. 12769 G>A with a frequency of 2% and m.14612 G>A at a frequency of 4% were novel and are not on either MITOMAP or Phylotree. The 134 single nucleotide polymorphisms 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 characterised into 9 subhaplogroups, namely; L0a1, L0a2, L0d, L0f, L0k, L1c, L2a, L3d and L3e (Table 2). Subhaplogroup L0a2 had the highest frequency (28%) while L3d (2%) was the least common. We did not observe haplogroups L4, L5 and L6 as well as subhaplogroup L1b which have been reported in other African populations [14, 24]. Two mitochondrial subhaplogroups were associated with the presence of peripheral neuropathy (see Table 2). In a multivariate logistic regression model, the L0a2 subhaplogroup was an independent risk factor for peripheral neuropathy (OR, 2.23; 95% CI, 1.14 – 4.39; p = 0.019) (Table 3). On the other hand, the presence of the L2a subhaplogroup was associated with reduced risk for peripheral neuropathy (OR, 0.39; 95% CI, 0.16 – 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 peripheral neuropathy in HIV/AIDS patients from Malawi. We observed that there was no relationship between gender and the risk of peripheral neuropathy. The role of age on peripheral neuropathy is in contrast to our earlier findings that reported there was association between age and risk of peripheral neuropathy [2]. Unlike in other studies where height has been associated with increased risk for peripheral neuropathy, this was not the case in the Malawi cohort [25]. In antiretroviral naïve patients, peripheral neuropathy has been reported to be more common with CD4 counts <200 cells/µL and HIV-1 RNA >10,000 copies/mL [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 peripheral neuropathy.

In some studies the risk of PN was associated with malnutrition [28, 29], therefore our finding that high body mass index (BMI) (>25Kg/m^2) with borderline significance (P-value = 0.055) in patients experiencing peripheral neuropathy 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].

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]. Prevous studies have demonstrated that European polulations with haplogroup T are more susciptible to developing stavudine associated peripheral neuropathy compared to 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 peripheral neuropathy [34] , while in this Malawi population, two 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 which include a small sample size which makes it difficult to determine the effects of subhaplogroups with low frequencies (e.g. L3d with 2%), a weakness in the objective assessment of peripheral neuropathy (for example, clinical findings including nerve conduction velocity and/or intraepidermal nerve fiber density) , possibility of undiagnosed pre-ART neuropathy and lack of information on the role of other factors associated with stavudine-induced peripheral neuropathy such as polymorphisms in host cytokine genes [35] .

Conclusion

We report a significant association between L02a with increased risk of peripheral neuropathy 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 toxico-pathology 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.

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Table 1: Patient characteristics and peripheral neuropathy diagnosis

Characteristic	No PN	PN	Total (n = 215)	P-Values
Gender (Female)	103 (63)	29 (56)	132 (61)	0.251
Median age (IQR), (years)	37 (31– 46)	41 (36 – 48)	38 (32 – 46)	0.063
Age Categories				
Age < 40 years	99 (61)	24 (45)	123 (57)	Ref
Age ≥ 40 years	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 (18-25 kg/m^2)	130 (80)	37 (70)	167 (78)	Ref
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)	Ref
Viral Load (CPs/ml)				
<400	135 (87)	45 (92)	180 (88)	
≥400	20 (13)	4 (8)	24 (12)	0.374
Duration on ART(IQR) (months)	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 is expressed as N (%) except for age, BMI, CD4 count, viral load, duration on ART and eGFR (median, IQR); †† IQR, inter-quartile range; BMI, body mass index; ART, antiretroviral therapy; eGFR, estimated glomerular filtrate Rate; PN, peripheral neuropathy; ‡ BMI (calculated as weight in kilograms divided by meters squared); ‡‡ eGFR (calculated using US K-DOQI group method); * 11 samples (7 without PN and 4 with PN did not have CD4 and viral load data); ** 9 samples (6 without PN and 3 with PN did not have eGFR data)

Table 2: The association of mtDNA subhaplogroup with peripheral neuropathy among Malawian ART patients

Subhaplogroups	Total (%)	no PN (%)	PN (%)	P-Value
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)	

*PN, peripheral neuropathy; ** Significant differences are shown in bold

Table 3: Multivariate logistic regression analyses of factors associated with peripheral neuropathy

	Model 1		Model 2	
Covariate	**Adjusted OR (95% CI) L2a group**	**P-Value**	**Adjusted OR (95% CI) L0a2 group**	**P-Value**
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	0.93 (0.10 – 8.43)	0.948	0.80 (0.08 – 7.33)	0.846
18.5 – 25				
>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**

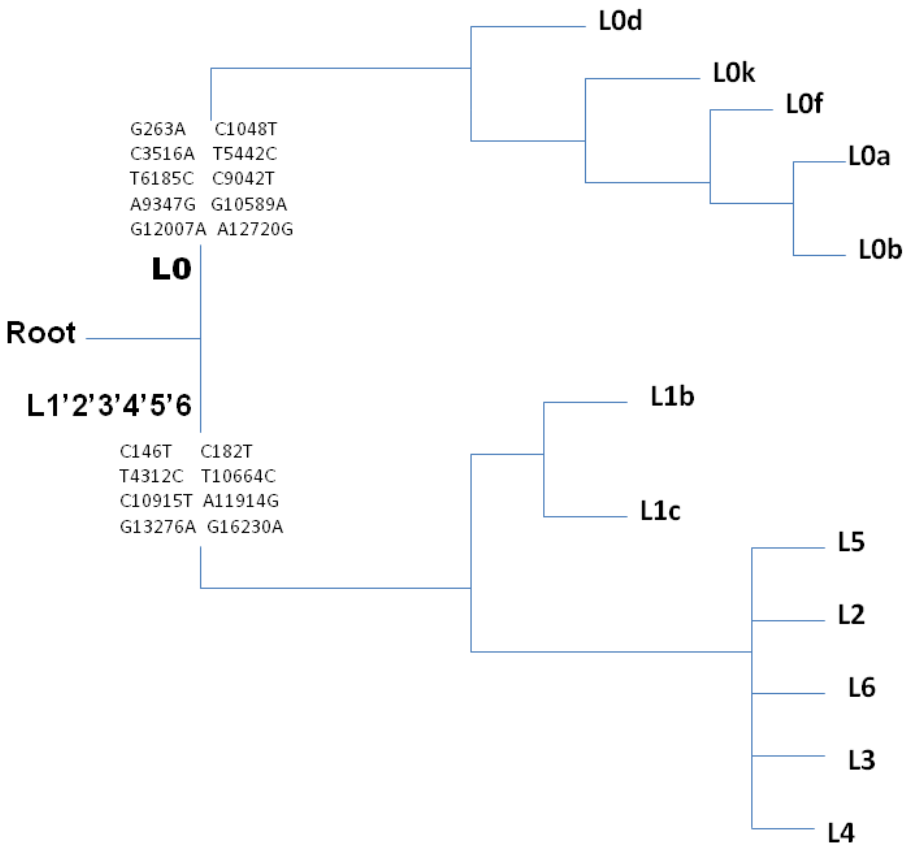

Figure 1. Phylotree describing the mtDNA haplogroup L and its subhaplogroup.

The haplogroups are differentiated by mutations at specific positions. Numbers refer to nucleotide (mutation) position with respect to revised Cambridge Reference Sequence. The letter to the left side of the number refers to ancestor allele and the letter to the right side refers to derived allele. Haplogroups are found at varying frequencies throughout the Sub-Sahara Africa [36].

6. CHAPTER 6: MITOCHONDRIAL DNA VARIATION AND SUSCEPTIBILITY TO LIPODYSTROPHY

Mitochondrial subhaplogroups and differential risk of stavudine induced lipodystrophy in Malawian HIV/AIDS patients *(Accepted for publication in Pharmacogenomics journal, December 2013 issue)*

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Running title: Mitochondrial subhaplogroup susceptibility to lipodystrophy

Abstract

Introduction: Lipodystrophy remains a significant problem in HIV/AIDS patients especially those on regimens containing either protease inhibitors or thymidine analogues (stavudine or zidovudine). Many of the manifestations of lipodystrophy have been linked to mitochondrial dysfunction. We set out to investigate if mitochondrial DNA variation is associated with development of stavudine induced lipodystrophy among adult Malawian HIV/AIDS patients on antiretroviral therapy that included stavudine.

Method: One hundred and seventeen (n = 117) adult HIV/AIDS patients on stavudine containing ART were recruited from the antiretroviral therapy (ART) clinic at Queen Elizabeth Central Hospital, Blantyre. The patients were categorized according to whether or not they had developed lipodystrophy after being on stavudine containing ART regimen for at least 6 months. Whole mitochondrial DNA coding region of each patient was sequenced and correlated to clinical characteristics.

Results: Lipodystrophy was apparent in 16% (n=19) of the participants. In multivariate analysis, Age >40 years (OR, 4.43; 95% CI, 1.36 – 14.47; P = 0.014) was significantly associated with presence of lipodystrophy. The mtDNA subhaplogroup L3e appeared to be protective against lipodystrophy as none of 11 subjects with this subhaplogroup presented with lipodystrophy.

Conclusion: mtDNA subhaplogroups seem to differentially affect susceptibility to lipodystrophy. More research is required to identify patients who are more or less likely to benefit from stavudine containing ART.

Key words: lipodystrophy, mitochondrial DNA, subhaplogroups, toxicities, dyslipidemia

Introduction

Lipodystrophy is one of the metabolic disorders associated with HIV infection and antiretroviral therapy (ART). Lipodystrophy is characterised by peripheral fat loss of the limbs, cheeks, and/or buttocks (lipoatrophy), and by accumulation of fat in the abdomen, neck and/or breasts (fat accumulation) as reviewed by Cossarizza et al [1]. In many cases, lipodystrophy is accompanied by metabolic features such as hypertriglyceridemia, hypercholesterolemia, low high-density lipoprotein cholesterol levels, insulin resistance, type 2 diabetes mellitus, and steatosis of the liver [2, 3]. These conditions may elevate the risk of cardiovascular disease in ART patients [4]. Although lipoatrophy and lipohypertrophy may have different pathogenic components and can occur independently from each other, they often coexist [5]. Risk factors for lipodystrophy that have been identified in ART patients include the use of stavudine and didanosine, protease inhibitors (PIs), longer duration of ART, age over 40 years, female gender, lower baseline CD4 cell count and BMI >25 kg/m^2 [6-9]

Lipodystrophy occurs in 12% - 34% of HIV/AIDS patients in sub-Saharan Africa, and the risk is especially high in patients on stavudine containing ART [2, 10]. Despite having an extensive pattern of toxicities, stavudine is still widely used in low and medium income countries (LMIC), mainly because of its efficacy and low price. Rather than a complete ban of stavudine, it can be argued that if biomarkers of poor response to and toxicity of stavudine could be identified, only certain patients may be excluded from its use. Mitochondrial dysfunction together with effects from variation in mitochondrial polymerase-γ have been suggested as effectors of stavudine induced lipodystrophy as reviewed in Lewis et al [11]. In addition, genetic variation in HLA genes has been shown to play a role in the pathogenesis of lipodystrophy. For example, HLA A01, B08 or DQ2 supertype alleles were protective against development of lipoatrophy among Australians, while in the Thai population, the *HLA-B*4001* allele was associated with increased risk of stavudine associated lipodystrophy [12, 13]. It was also observed that lipodystrophy is overrepresented in patients carrying the m.9055 G>A single nucleotide polymorphism (SNP) in mitochondrial DNA (mtDNA) which was associated with disruption of the normal functions of the mitochondrial ATP6 enzyme [14]. In two European studies, patients carrying the mtDNA haplogroup K presented with increased risk of lipoatrophy while those carrying haplogroup T appeared protected [14, 15]. Different mitochondrial subhaplogroups have been mapped in

various populations worldwide, and it is therefore important to investigate if there is a role for mtDNA subhaplogroups specific for Africans, premised under the L haplogroup, in the susceptibility to stavudine toxicity. Particularly few data still exist about the effects of L subhaplogroups on the development of lipodystrophy [16], and the aim of this project therefore was to investigate the association of mtDNA subhaplogroups with lipodystrophy in adult Malawian HIV/AIDS patients on stavudine containing ART.

Materials and methods

Participants

The participants in this study were recruited from the ART clinic of Queen Elizabeth Central Hospital, in Blantyre, Malawi and were part of an earlier longitudinal study that reported on stavudine toxicities [2]. To be eligible for the study, participants had to be on the standard first line ART regimen (stavudine, lamivudine and nevirapine), be maternally unrelated and willing to provide blood samples for genetic analysis. The exclusion criteria were pregnancy, being on tuberculosis treatment, being on ART treatment for less than 6 months and having missed medication for the past 3 days. The protocol conformed to the declarations of Helsinki 2008 and was approved by the College of Medicine Research Ethics Committee of the University of Malawi and the University of Cape Town, Faculty of Health Sciences, Research Ethics Committee. All participants gave written informed consent.

Clinical and laboratory measurements

Lipodystrophy was diagnosed with a validated questionnaire that uses a combination of patient self-assessment and inspection of seven body areas (face, neck, chest, abdomen, arms, legs and buttocks) by clinician as outlined by Carr et al [17]. An experienced ART clinician was trained in the use of the tool before the study by one of the investigators. CD4 cell counts were done with a FACSCount flow cytometer (Beckton Dickinson, New Jersey, USA), HIV-1 RNA with the Amplicor HIV Monitor version 1.5 (Roche Diagnostic Systems, Basel, Switzerland). Creatinine levels were measured using the end point Jaffe method [18] and the glomerular filtrate rate was estimated from creatinine with the US K-DOQI group method *[19]*.

Total DNA was isolated from peripheral blood samples collected in EDTA coated tubes and used for mtDNA genotyping. MtDNA from each patient was amplified in 9 partially overlapping fragments to cover the whole mtDNA sequence using the primers reported by Ramos et al. [20, 21]. Total volumes of 25 µl in a reaction mixture that consisted of 1X green GoTaq reaction buffer, 200 µM of dNTP, 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 amplified in the GeneAmp® PCR System 9700 by Life technologies (New York, USA). The PCR programs each consisted of an initial denaturation step at 94 ^{o}C for 5 min, followed by 35 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 57 - 64.4 ^{o}C for 40s and extension at 72^{o}C 2.5 min, with a final extension step of 5 min at 72^{o}C [20]. PCR products were purified to remove excess primers and dNTP using exonuclease and shrimp alkaline phosphatase (ExoSap).

Following polymerase chain reaction of 9 overlapping fragments sequencing reactions were performed using forward and reverse primers and additional internal primers. Mitochondrial DNA coding region was sequenced from nucleotide position 577 – 15953 according the revised mtDNA Cambridge reference sequence Locus NC_012920.1. ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, California, USA) was used for capillary electrophoresis and Lasergene 10 Core Suite software (DNASTAR, Wisconsin, USA) to align and assemble sequences. Mutations (polymorphisms) were determined as nucleotide differences when compared to the Cambridge reference sequence.

Subhaplogroup Analysis and Statistical Analysis

Stata for windows software version SE/11 (Stata Corp; Texas, USA) was used for statistical analysis. We assessed the association of independent variables (sex, BMI, age, duration on ART, CD4 count, viral load and eGFR) with lipodystrophy, as well as the subhaplogroup distribution between patients presenting with and those without lipodystrophy with univariate logistic regression modelling. Multivariate logistic regression analysis was performed with those patient characteristics and subhaplogroups that had shown some degree of association ($P < 0.12$) with lipodystrophy in univariate analysis. Odds ratios are reported with 95% confidence intervals and a p-value of <0.05 was considered as statistically significant.

Results

A total of 117 participants on the standard first-line stavudine containing regimen were recruited and sixty-two per cent of the participants (n = 73) were female. The median age was 37 (IQR = 31 - 46) years. The median duration of ART at enrolment was 24 months (range 24 -25). The median body mass index (BMI) was 23 kg/m^2 and 21% had a BMI above the normal range (>25 kg/m^2). Lipodystrophy was diagnosed in 16% (n = 19). Two (1%) patients presented with severely elevated serum creatinine levels of 5.4 mg/dl and 6.0 mg/dl (normal range = 0.5 – 1.5 mg/dL). One percent (n = 1) and 2% (n = 2) were current smokers and consumed alcohol, respectively. Table 1 gives a summary of the demographic and clinical characteristics of the study participants' time point at first lipodystrophy diagnosis. A significantly larger proportion of patients with lipodystrophy were above 40 years of age compared to those without lipodystrophy.

Mitochondrial variation and haplogroup analysis

After sequencing and alignment, 184 positions exhibited polymorphisms that were used to groups the participants into four mitochondrial haplogroups L0, L1, L2 and L3 were identified and further classified into subhaplogroup L0a1, L0a2, L0d, L0f, L0k, L1c, L2a, L3d and L3e with L0a2 being the most frequent (27%) and L3d (3%) the least. None of the individuals carrying the mtDNA L3e subhaplogroup had lipodystrophy (table 2). No subhaplogroups were significantly associated with presence of lipodystrophy in univariate analysis.

In multivariate logistic regression models, age >40 years was independently associated with lipodystrophy (Table 3). Subhaplogroup L2a which had shown a degree of association with lipodystrophy in the univariate analysis however this was no longer significant after multivariate analysis. Heteroplasmy was observed in 19 mtDNA positions with the 5 positions, 6917, 7055, 7274, 10589 and 10792, each being reported in 2 different samples, while the rest of the heteroplasmic positions were only observed once. Only one patient with lipodystrophy exhibited heteroplasmy (mtDNA position 10792).

Discussion

The study was undertaken to explore the role of mtDNA subhaplogroups in susceptibility to develop lipodystrophy in HIV/AIDS patients on stavudine from Malawi. Subhaplogroup L3e appeared to protect patients against lipodystrophy. Variation in susceptibility to lipodystrophy observed with respect to mtDNA subhaplogroups could point to effects of subhaplogroup specific mutations on the structure of mitochondria and associated proteins. Studies conducted in HIV/AIDS patients of Caucasian origin by Hendrickson et al [15] showed that the mitochondrial haplogroup K was a risk factor for lipodystrophy. The absence of lipodystrophy in our patients with the mtDNA subhaplogroup L3e confirms earlier observations in South African HIV/AIDS patients [16]. Our findings and those of others show that each population group may have specific subhaplogroups that are associated with differential susceptibility to side effects of drugs. This notion has implications for the study of biomarkers of the response to medication, stressing the need to include all population groups in such studies to determine population and group specific biomarkers.

Of other characteristics that were studied, only age above 40 years was significantly associated with the presence of lipodystrophy. Our observation about age is comparable to other reports [10, 15]. A study in South Africa found that males were at increased risk of lipodystrophy [22], however we observed no gender bias. In our study, lipodystrophy was not associated with markers of disease progression (viral load and CD4 cell count), although others indicated that the risk was greater in patients with lower CD4 cell count and lower BMI during early stages of ART treatment [23]. This is mainly determined by the fact that our measurements were done at a time that patients had been on ART for 2 years.

Our study in the genetically underresearched Malawian population [24] has several limitations. These include the cross sectional study design, which only considered variables at the point of enrolment and strongly limits conclusions about causality. The study was insufficiently powered, due to a small sample size, to detect associations of multiple haplogroups with lipodystrophy. Therefore we recommend larger prospective studies in Malawi to further investigate mtDNA patterns and ART responses. Secondly, lipodystrophy was diagnosed clinically, without using dual-energy X-ray absorptiometry (DEXA), thus our case definition of lipodystrophy was less

objective and we may not have separated lipo-atrophy from fat accumulation accurately. In addition, other genetic factors that may be associated with stavudine-induced lipodystrophy, such as levels of cytokines including TNF-α, IL-6; proteins such as leptin and adiponectin and polymorphisms in major histocompatibility complex genes [12, 25] were not considered.

Conclusion

Our study provides indications that susceptibility to lipodystrophy in HIV/AIDS patients on stavudine containing ART is mtDNA subhaplogroup and population specific.

Future perspective

Knowledge about the genetic variation in mtDNA should be integrated with information about variation in other genes especially those coding for drug metabolising enzymes, receptors and transporters, to get a clearer picture of the response to stavudine containing ART.

Executive Summary

- Genomics research especially with respect to pharmacogenetics, promises to reveal genetic biomarkers associated with differential susceptibility and response to treatment.
- Recently, variation in mtDNA has been associated with risk of developing antiretroviral induced metabolic disorders in a subhaplogroup specific manner.
- In addition to the traditional studies evaluating variation in drug metabolising enzyme genes, receptors and transporters, variation that affects mitochondrial function should be studied as it affects both susceptibility to development of metabolic disorders and response to treatment
- More African populations need to be studied in order to capture the breadth of the genetic diversity among its people.
- In this study, we report the association of L3e with decreased risk of developing stavudine associated lipodystrophy in a Malawian HIV/AIDS cohort.

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Table 1: Clinical characteristic of the participants in relation to lipodystrophy

Characteristic	LD (Freq)	No LD (Freq)	Total (N = 215)	Odds Ratio	P-Value
Female gender (%)	15 (0.71)	117 (0.60)	132 (0.61)	1.64 (0.57 – 5.39)	0.320
Age Categories					
Age < 40 years	7 (0.33)	116 (0.60)	123 (0.57)	1.00	
Age ≥ 40 years	**14 (0.67)**	**78 (0.40)**	**92 (0.43)**	**2.97 (1.06 – 9.07)**	**0.02**
BMI categories					
Normal (18-25 kg/m^2)	17 (0.81)	150 (0.77)	167 (0.78)	1.00	1.00
Underweight (<18.5 kg/m^2)	0 (0.0)	6 (0. 3)	6 (0.3)	-	-
Overweight (>25 kg/m^2)	4 (0.19)	38 (0.20)	42 (0.19)	0.93 (0.29 – 2.92)	0.899
*CD4 count (cells/µL)	340 (228 - 451)	344 (227 – 494)	344 (227 – 489)	1.00 (0.99 – 1.01)	0.543
**Viral Load (CPs/ml)					
<400	19 (0.95)	161 (0.87)	180 (0.88)	1.00	1.00
≥400	1 (0.05)	23 (0.13)	24 (0.12)	0.368 (0.01 -2.56)	0.323
Duration on ART(IQR) (months)	25 (24 – 26)	25 (23 – 31)	25 (23 -29)	1.00 (0.95 – 1.04)	0.845
Median eGFR (IQR) (ml/min)	129 (114 – 139)	135 (113 – 154)	136 (113 – 152)	0.99 (0.98 – 1.00)	0.301

Data is expressed as N (%) except for CD4 count and duration on ART
IQR =, inter-quartile range
BMI = body mass index (calculated as weight in kilograms divided by meters squared)
LD = Lipodystrophy
Freq = Frequency
*10 samples (1 with LD and 9 without LD) did not have CD4
**11 samples (1 with LD and 10 without LD) did not have viral load data
eGFR = estimated glomerular filtrate rate
Black bold significant association

Table 2: Association of mtDNA haplogroup with lipodystrophy in Malawian ART patients

Subhaplogroups	Total (Freq)	LD (Freq)	No LD (Freq)	Unadjusted OR (95%CI)	P-Value
L0a1	13 (0.06)	2 (0.10)	11 (0.06)	1.75 (0.17 – 8.94)	0.481
L0a2	61 (0.28)	4 (0.19)	57 (0.29)	0.56 (0.13 – 1.84)	0.318
L0d	15 (0.07)	2 (0.10)	13 (0.07)	1.46 (0.15 – 7.26)	0.630
L0f	6 (0.03)	2 (0.10)	4 (0.02)	5.00 (0.42 – 37.12)	0.049
L0k	**9 (0.04)**	**3 (0.14)**	**6 (0.03)**	**5.22 (0.77 – 26.67)**	**0.015**
L1c	27 (0.13)	4 (0.19)	23 (0.12)	1.75 (0.39 – 6.01)	0.345
L2a	53 (0.25)	2 (0.10)	51 (0.26)	0.29 (0.032 – 1.30)	0.090
L3d	5 (0.02)	2 (0.10)	3 (0.02)	6.70 (0.52 – 61.35)	0.021
L3e	26 (0.12)	0 (0.0)	26 (0.13)	-	-
Total	215 (100)	21 (100)	195 (100)		

Black bold significant association

Table 3: Multivariate logistic regression analyses of factors associated with lipodystrophy

Covariate	Model 1 (L0k versus all other L subhaplogroups) OR (95% CI)	P-value	Model 2 (L2a versus all other L subhaplogroups) OR (95% CI)	P-value
Gender (Female)	1.69 (0.55 – 5.15)	0.356	1.94 (0.64 – 5.79)	0.236
Age (yrs) ≥40	**3.70 (1.30 – 10.58)**	**0.014**	**3.09 (1.14 – 8.38)**	**0.027**
BMI (kg/m^2) >25	0.91 (0.27 – 3.10)	0.270	0.86 (0.26 – 2.85)	0.802
Duration on ART (months)	1.00 (0.95 – 1.05)	0.927	1.00 (0.95 – 1.05)	0.926
Viral Load (CPs/ml) ≥400	0.33 (0.04 – 2.67)	0.299	0.32 (0.04 – 2.63)	0.291
CD4 count (cells/μL)	1.00 (0.99 – 1.00)	0.346	1.00 (0.99 – 1.00)	0.395
L0k(vs all L subhaplogroups)	**7.45 (1.45 – 38.24)**	**0.016**		
L2a(vs all L subhaplogroups)			0.31 (0.07 – 1.04)	0.129

Phenotype data including gender, age, BMI, duration on treatment, viral load, CD4 cell count) were analysed in the multivariable analysis with either L0k (model 1) or L2a (model). Black and bold significant association. L0f and L3d although their P-value were <0.1 but were not considered in the multivariate analysis because of wide variation in the confidence intervals.

7. CHAPTER 7: EFFECTS OF STAVUDINE-CONTAINING ART ON MITOCHONDRIA LEVELS

Decreased mitochondrial DNA content in HIV-infected Malawian patients on stavudine containing antiretroviral therapy *(For submission to the Omics journal).*

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Running Title: mtDNA depletion during stavudine treatment in Malawian adults

Abstract

Mitochondrial toxicity is a major concern related to nucleoside reverse transcriptase inhibitors and manifests through peripheral neuropathy, lipodystrophy, lactic acidosis and hyperlactatemia. Depletion of mitochondria has been associated with mitochondrial dysfunction. We investigated whether mtDNA levels in peripheral blood can be used as a biomarker of mitochondrial depletion due to stavudine associated toxicity. A cross sectional study of 203 HIV- infected patients on stavudine containing therapy and 64 healthy controls conducted. Peripheral neuropathy was present in 21% (43/203), while lipodystrophy measurement were present in 18% (n = 20) of 112 investigated and out of 113 patients high lactate levels were present in 17% (n = 19) and those with no adverse events group (n = 142). Total DNA was extracted from whole blood and relative mtDNA/nuclear DNA ratio was determined.

The healthy controls had higher relative mtDNA/nDNA ratios compared to patients on ART with board line statistical significance (P = 0.05). Compared to healthy controls, the following groups had statistically significantly decreased relative mtDNA/nDNA ratios; patients on ART presenting with peripheral neuropathy (P=0.039) and patients presenting with high lactate levels (P = 0.024). Comparison between groups significant differences in mtDNA/nDNA ratios were observed between patients with high lactate levels to patients with normal lactate levels (P = 0.018). Significant mtDNA/nDNA ratios (P = 0.01) were observed when subhaplogroup L0a2 was compared with L2a. Stavudine is associated with decreased mitochondrial content in HIV-infected patients. Susceptibility to depletion of peripheral mitochondrial levels could be subhaplogroup specific.

Key Words: HIV, treatment, stavudine, toxicity, mitochondrial DNA

Introduction

The amount of mitochondrial DNA (mtDNA) varies among healthy individuals of different ages and in different tissues within the same individual from 100 to 1000 copies mtDNA per cell (Bai et al., 2004; Pezeshkpour et al., 1987). The quantity of mtDNA in an organ can also have pathological relevance as depletion is associated with respiratory chain defects (Bai et al., 2004). Mitochondrial diseases are characterised by molecular defects in mtDNA or in nuclear genes that code for mitochondrial components. These defects can be acquired or inherited (Mazunin et al., 2010; Naviaux RK, 2003). Certain infections, for example human immunodefiency virus (HIV), and adverse effects of drugs such as nucleoside reverse transcriptase inhibitors (NRTIs), may result in mitochondrial dysfunction (Carrozzo and Lucioli, 2005; Mazunin et al., 2010; Naviaux RK, 2003; van der Watt et al., 2011).

Stavudine is a NRTI used in combination with other antiretroviral drugs for the treatment of HIV infected individuals. It is effective in suppressing HIV replication and affordable in low and medium income countries. However, common and severe adverse drug effects have led to calls for its withdrawal. Most of the adverse drug effects of stavudine resemble patterns found in inherited mitochondrial diseases for instance hepatic steatosis, lactic acidosis, myopathy, nephrotoxicity, peripheral neuropathy, and pancreatitis (Keswani et al., 2002; Nicholas et al., 2007). The proposed mechanism by which mitochondria are affected by stavudine can be described as direct, through inhibition of mtDNA transcription, or indirect, through impact on the activity of mitochondrial polymerase gamma, an enzyme required for mtDNA replication and repair. In both scenarios, mitochondrial DNA becomes depleted, and if this reaches a critical threshold, insufficient energy generation ensues (Casula et al., 2005; White, 2001). Low ATP production results into tissue or organ dysfunction. Stavudine induced mitochondrial dysfunction can be the result of effects on adenylate kinase, by inducing mtDNA mutations as well as by exerting oxidative stress; this has been suggested to trigger the onset of peripheral neuropathy (Lewis et al., 2003; Tozzi, 2010). Histologic evidence demonstrates abnormal mtDNA structure and/or depletion in affected tissues of HIV-infected patients on NRTIs based ART (Morse et al., 2012).

There is great inter-individual variation in the presentation of stavudine toxicities and also within the same individual marked differences in organ susceptibility can exist. The molecular mechanisms underlying these differences are not fully understood. One possible explanation is a differential effect of mtDNA variants on treatment response and susceptibility of toxicity in different populations (Canter et al., 2010; Grady et al., 2011; Kampira et al., 2013)

Mitochondrial determinants may predict the risk of developing drug associated toxicities in individuals as they can be determined before initiation on ART. However determination of mtDNA variants as a measure of risk for drug toxicities is expensive in resource poor countries. We aimed at investigating whether mitochondrial levels in peripheral blood can be used as markers of stavudine-associated toxicities in addition to genotyping for mtDNA variants.

Laboratory measurements

Lactate levels were measured with the hand-held Lactate Pro (Arkray Europe B.V., Amstelveen, the Netherlands) at the point-of-care. Lactate levels above 2.5 mmol/L were considered to be elevated. CD4 cell count and HIV-1 RNA were determined by FACSCount flow cytometer (Beckton Dickinson, New Jersey, USA) and Amplicor HIV Monitor version 1.5 (Roche Diagnostic Systems, Basel, Switzerland) respectively.

Whole blood was kept at -20^{o}C until DNA extraction as described earlier (Kampira et al., 2013). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) nuclear control and ATP synthase (ATPase) 8 mitochondrial DNA gene were quantified by real-time quantitative PCR with use of Light Cycler II Roche (Roche Diagnostic Coorperation, Indianapolis, USA). Primers for RT Q-PCR analysis for GAPDH were: forward: 5'-CCCCACACACATGCACTTACC-3´ and reverse: 5'-CCTAGTCCCAGGGCTTTGATT-3´ while those for mtDNA ATPase 8 were: forward: 5'-AATATTAAACACAAACTACCACCTACC-3´ and reverse: 5'-TGGTTCTCAGGGTTTGTT ATA-3´ (Xia et al., 2009). GAPDH and mtDNA ATPase templates were amplified separately in total volume of 25µl in a reaction that consisted of 12.5 µl KAPA SYBR green qPCR master mix, 0.4 µmol of each primer of 200nM concentration, 2 µl DNA and 9.5 µl water. The RT- PCR amplification consisted of a single denaturation–enzyme-activation step of 10 minutes at 95°C, followed by 45 cycles of 10 seconds at 95°C, 10 seconds at 60°C and 15 seconds at 72°C, with a

temperature-transition rate of 20°C per second. The real-time PCR reactions were performed in duplicate for each gene.

The mean threshold cycle number (Ct) values from real time PCR were obtained to compare the level of expression of GAPDH (nDNA) and ATPase 8 (mtDNA) in healthy controls and HIV-infected patients on ART. According to Gibson et al (Gibson et al., 1996) the Ct value is the lowest cycle number at which the fluorescence detected crosses a threshold level deemed to be significantly higher than background. Therefore the fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial DNA concentration, meaning that low Ct values are detected in samples with high initial DNA template (Bustin, 2000). Threshold cycle values of nDNA and mtDNA were compared using the delta Ct (ΔCt) which is the difference of the average of two measurements Ct of nDNA and of mtDNA (ΔCt = CtnDNA- CtmtDNA) in the same sample. Mitochondrial DNA and nDNA standard dilution curves generated from total genomic DNA with the following concentrations: 5000, 500, 50 and 5pg/ µL. Nuclear DNA and mtDNA concentrations were estimated from a standard efficiency curve using the delta Ct (ΔCt) of average Ct of the same sample versus log DNA concentration. Furthermore, the mtDNA content of the cells was expressed relative to concentration of nDNA (mtDNA/nDNA ratio).

Mitochondrial DNA was isolated from peripheral blood samples collected in EDTA coated tubes and used for mtDNA genotyping. Mitochondrial DNA from each of the 203 patients was amplified in 9 partially overlapping fragments to cover the whole mtDNA sequence using the primers reported by Ramos et al. (Ramos et al., 2009; Ramos et al., 2011). A total volume of 25 µl in a reaction mixture that consisted of 1X green GoTaq reaction buffer, 200 µM of dNTP, 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 amplified in GeneAmp® PCR System 9700 by Life technologies (New York, USA). The PCR programs each consisted of an initial denaturation step at 94 ^{o}C for 5 min, followed by 35 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 57 - 64.4 ^{o}C for 40s and extension at 72^{o}C 2.5 min, with a final extension step of 5 min at 72^{o}C (Ramos et al., 2009). PCR products were purified to remove excess primers and dNTP using exonuclease and shrimp alkaline phosphatase (ExoSap).

Following polymerase chain reaction of 9 overlapping fragments sequencing reactions were performed using forward and reverse primers and additional internal primers. Mitochondrial DNA coding region was sequenced from nucleotide position 577 – 15953 according the revised mtDNA Cambridge reference sequence Locus NC_012920.1. ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, California, USA) was used for capillary electrophoresis. Lasergene 10 Core Suite software (DNASTAR, Wisconsin, USA) was used to align and assemble sequences. Mutations (polymorphisms) were determined as nucleotide differences when compared to the Cambridge reference sequence.

Statistical analysis

Both mtDNA and nDNA threshold cycle values and concentration were analyzed as median, interquartile ranges (1QRs) range and differences in levels between health controls and patients on ART were determined. Spearman rank correlation was used to determine the relationship between mtDNA Ct and nDNA CT values of healthy and patients on ART. Using Graph Pad Prism® 5 software (GraphPad Software Inc., San Diego Canada) we compared the mtDNA/nDNA ratio in blood between healthy controls and patients on ART with or without toxicities with the Mann Whitney U- test. We used MANOVA analysis to determine the association of age, gender, and duration on ART, BMI, CD4 and viral load with mtDNA content. Associations were considered significant when $p < 0.05$ was observed. Stata for windows software version SE/11 (Stata Corp, Texas, USA) was used for statistical analysis of age, gender, duration on ART, BMI, CD4 and viral load with mtDNA content. Kruskal Wallis test was used to compare the content of mtDNA in blood between mtDNA subhaplogroups.

Results

Out of the 215 ART patients that were recruited, 203 had their mitochondrial content successfully analyzed, the other 12 (6%) were excluded from the analysis. Six (9%) of 70 healthy controls were excluded for the same reason. Patients were on stavudine containing first line ART for a median duration of 25 months (range 23 -29). There was a female preponderance (n= 132, 61%) and the mean age was 40 years. Cotrimoxazole prophylaxis was used by all participants. Out of 203 patients, 21% (43) presented with peripheral neuropathy while only 112

participants examined for lipodystrophy, of which 18% (20/112) presented with lipodystrophy. Lactate levels were available from investigated 113 patients and of these 17% (n = 19) presented with elevated levels (>2.5 mmol/L). Healthy controls were determined by physical examination and no information on HIV status was available.

Mitochondrial DNA level

The median Ct values of GAPDH (indicative of nuclear DNA) of healthy controls and of HIV/AIDS patients; were not significantly different (23.58 vs. 23.43; P= 0.998). However, the median Ct value of the ATPase gene (indicative of mtDNA) of healthy controls was significantly lower than that of HIV/AIDS patients (17.88 vs.18.75; P = 0.005). Significant differences were also observed between the relative nDNA Ct levels and mtDNA Ct level of healthy controls and HIV/AIDS patients as illustrated in Table 1. There was no significant correlation between nDNA Ct values and mtDNA Ct values in healthy controls while a significant correlation existed (r=0.232; P = 0.0009) ART patients as illustrated in Figure 1. The standard curves generated from total DNA showed good correlation with threshold Ct values, $r^2 = 0.9966$ for GAPDH and $r^2 = 0.9419$ for ATPase (Figure S1) versus log DNA concentration.

Healthy controls had a significantly higher mtDNA/nDNA ratio than patients on ART, (6.64 vs. 5.08; P = 0.05), as indicated in Figure 2. It was observed patients on ART presenting with peripheral neuropathy had significantly lower (6.64 vs 3.4, P = 0.039) mtDNA/nDNA ratio when compared to healthy controls and no significant differences (P = 0,207) were observed between patients experiencing peripheral neuropathy and those not experiencing peripheral neuropathy. mtDNA/nDNA ratios of patients on ART with elevated lactate levels were much lower than in healthy controls (6.64 vs 0.68, P = 0.024) and also lower in patients with elevated lactate (0.68 vs 5.88, P = 0.018) when compared to the group with normal lactate levels. None of the patient characteristics (age, gender, duration on ART, BMI, viral load, CD4 count and viral load) were associated with peripheral blood mtDNA/nDNA ratio (Table 2).

Nine mtDNA L lineage subhaplogroups L0a1, L0a2, L0d, L0f, L0k, L1c, L2a, L3d and L3e were identified with frequencies of 6%, 29%, 8%, 3%, 4%, 12%, 24%, 2% and 12% respectively. Peripheral blood mtDNA/nDNA ratio in patients on ART categorized in subhaplogroup L0a2

had were significantly lower levels (0.62 vs 8.50, P = 0.01) when compared to subhaplogroup L2a (figure 3).

Discussion

Previous studies have associated NRTIs with development of ART associated toxicities such as peripheral neuropathy, lipodystrophy, lactic acidosis and hypertension (Anderson et al., 2004; Domingo et al., 2010; Hulgan et al., 2005; Menezes et al., 2011). However these findings have been tissue specific and there are no established standard laboratory markers to predict the risk of developing/or the onset of mitochondrial toxicity. Therefore, we set out to investigate the effects of stavudine containing ART on mitochondrial levels in peripheral blood. We report no differences in the GAPDH Ct levels between healthy control and patients on ART indicating that the HIV/AIDS condition does not substantially affect GAPDH levels in agreement with earlier observations (Barber et al., 2005).

There was a trend towards low level (P = 0.05) mitochondrial in HIV/AIDS patients on ART when compared to healthy controls an observation that may reflect possible stavudine effects on mitochondrial that may interfere with mtDNA replication processes (White, 2001). In addition, significantly low mitochondrial levels were associated with peripheral neuropathy suggesting that mitochondrial dysfunction may have casual relationship with peripheral neuropathy. These findings are in support of earlier reports which identified decreased mitochondria in patients with peripheral neuropathy on stavudine containing therapy (Casula et al., 2005; Dalakas et al., 2001; Kakuda, 2000). Lack of evidence of decreased mitochondrial content in the group with lipodystrophy could suggest that drug effect on mitochondrial in relation to this adverse event is cell, tissue and organ specific supporting finding of other studies (Feng et al., 2001; Morse et al., 2012). However, other factors such as sample size could be considered since this study was cross sectional.

Decrease in mitochondrial content in HIV/AIDS patients on ART with elevated that lactate levels (>2.5U/L) are in agreement with previous studies which reported elevated lactate levels (asymptomatic hyperlactatemia) association with low mtDNA/nDNA ratio due to NTRI

(stavudine) inhibition effect on mtDNA proliferation (Montaner et al., 2004). Results of this study support the hypothesis that mitochondrial levels in blood are associated with hyperlactatemia and confirm previous observations (Montaner et al., 2004). Accordingly, elevated lactate levels are indirectly a sign of mitochondrial dysfunction. Abnormal functioning of the mitochondria will result a shift towards anaerobic oxidation of pyruvate by pyruvate dehydrogenase and the formation of lactate dehydrogenase and lactate (Claessens et al., 2003; Côté et al., 2002; Miller et al., 2000). Hyperlactatemia has been reported to be associated with peripheral neuropathy and lipodystrophy (Brew B et al., 2001; Simpson D et al., 2004), although hyperlactatemia was common among patients with peripheral neuropathy and lipodystrophy but we could not establish this relationship in this study possibly because of the study design. However in our earlier study it was postulated suggested that elevated lactate levels may reflect a common underlying pathophysiology of peripheral neuropathy and lipodystrophy (Chagoma et al., 2013). Therefore we further suggest that lactate and mitochondrial levels can be used to predict onset of mitochondrial associated toxicities. Differences observed in mtDNA/nDNA ratio between subhaplogroups L02a and L2a could possibly support our earlier findings (Kampira et al., 2013) that susceptibility to stavudine associated toxicities is subhaplogroup specific.

However our study has limitations which include lack of corresponding information on morphology of cells to confirm observed relationships between abnormalities and toxicities. We propose further investigations on the relationship between hypertension and stavudine associated mitochondrial depletion, lipodystrophy and dyslipidemia. Another factor to be considered is that our controls although being Bantu speakers as patients, they were not from the same population. There was no data on mitochondrial content in specific tissues in order to compare with our results in to establish if there is relationship between mitochondrial content in the blood and other tissues. A bigger sample size for each of the toxicity groups is needed to further confirm our findings.

Conclusion

Large numbers of patients in resource limited settings are still receiving stavudine containing regimen. Quantitative evaluation of mitochondrial levels in the peripheral blood of individual on stavudine may be useful in identifying patients that are likely developing stavudine induced

mitochondrial toxicities (peripheral neuropathy and hyperlactatemia which may lead to irreversible lactic acidosis). Further studies need to be conducted to confirm if peripheral blood mitochondrial levels could be used in monitoring events associated to stavudine induced toxicities inorder to prevent lactic acidosis and cardiac arrest which are proven to be fatal.

Declaration

The authors declare that they have conflicting interests

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Table 1: mtDNA content in peripheral blood of healthy controls and patients on stavudine

	Healthy Controls (n = 64)	**On stavudine (n = 203)**	**Difference (ΔΔ Ct)**	**P-value**
ΔCt ($Ct_{nDNA} - Ct_{mtDNA}$)				
Median	5.73	4.62	1.11	**0.003**
Range	3.08 – 7.71	2.77 – 5.12		
nDNA content (pg/µL)				
Median	1225	1352		0.998
Range	229 - 2698	979 - 1892		
mtDNA content (pg/µL)				
Median	15812	6368		**0.005**
Range	482 – 506991	514 – 17458		
Correlation (Ct_{nDNA} vs Ct_{mtDNA})	r^2 = 0.083	r^2 = 0.232		
	P = 0.513	P = 0.0009		

ΔCt difference in Ct value

Table 2: Patients' characteristics in relation to relative mtDNA/nDNA ratio

	mtDNA/nDNA ratio Median (range)	Overall relative mtDNA/nDNA ratio median (range)	P-value
Gender			
Female	5.26 (0.47 – 11.70)	5.08 (0.37 – 12.34)	0.999
Male	4.70 (0.37 – 13.14)		
Age Categories			
Age < 40 years	5.20 (0.37 -13.14)	5.08 (0.37 – 12.34)	0.154
Age ≥ 40 years	4.70 (0.34 – 11.66)		
BMI categories			
Underweight (<18.5 kg/m^2)	6.81 (2.16 – 17.72)	5.08 (0.37 – 12.34)	0.129
Normal (18-25 kg/m^2)	4.99 (0.31 -12.34)		
Overweight (>25 kg/m^2)	5.20 (1.01 – 11.78)		
CD4 Categories (cells/µL)			
0 - 199	5.30 (0.37 – 13.89)	5.14 (0.39 – 13.14)	0.908
200 - 349	5.51 (0.60 -11.62)		
≥350	4.09 (0.29 – 14.27)		
Viral Load (CPs/ml)			
<400	5.15 (0.37 – 10.93)	5.12 (0.39 – 13.14)	0.501
≥400	5.12 (0.39 – 13.15)		
Duration on ART(months)			
< 24	5.12 (0.44 – 13. 14)	5.08 (0.37 – 12.34)	0.730
>24	4.46 (0.31 – 11.69)		

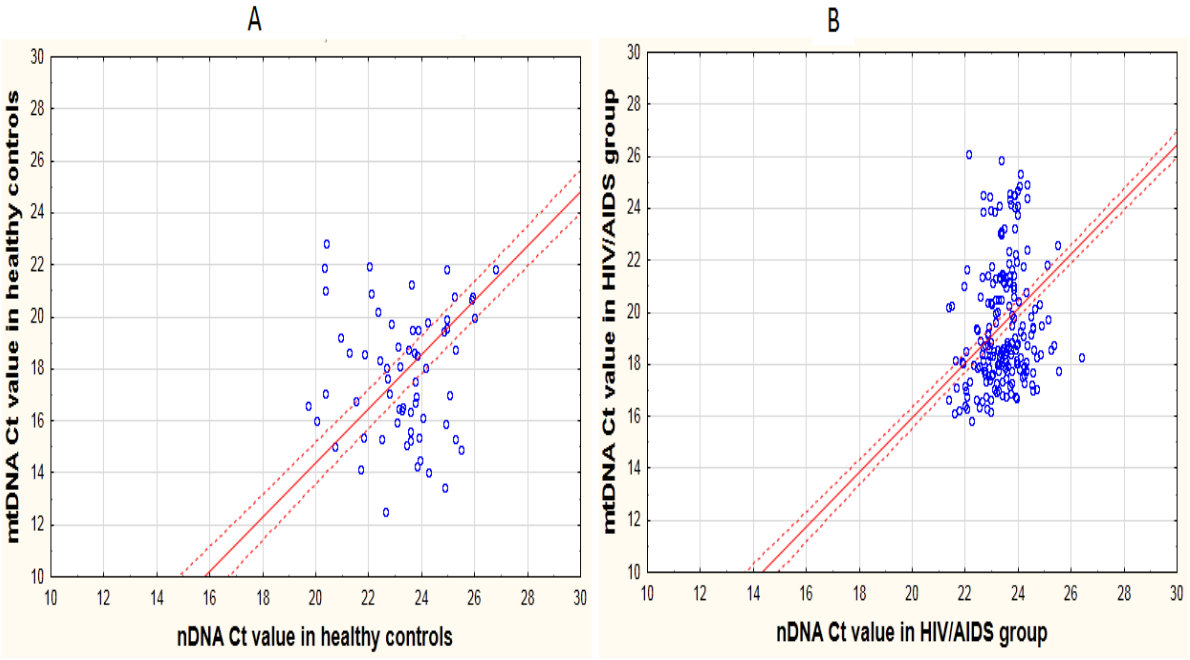

Figure 1: Correlation of nDNA and mtDNA Ct levels in peripheral blood

A: In healthy controls, nDNA and mtDNA Ct values were not significantly correlated (R^2 = 0.043; P = 0.738).B: In patients on stavudine containing ART, nDNA and mtDNA Ct values were significantly correlated R2 = 0.232; P = 0.0009). The red dotted lines are the upper and lower limit of confidence interval at 95%.

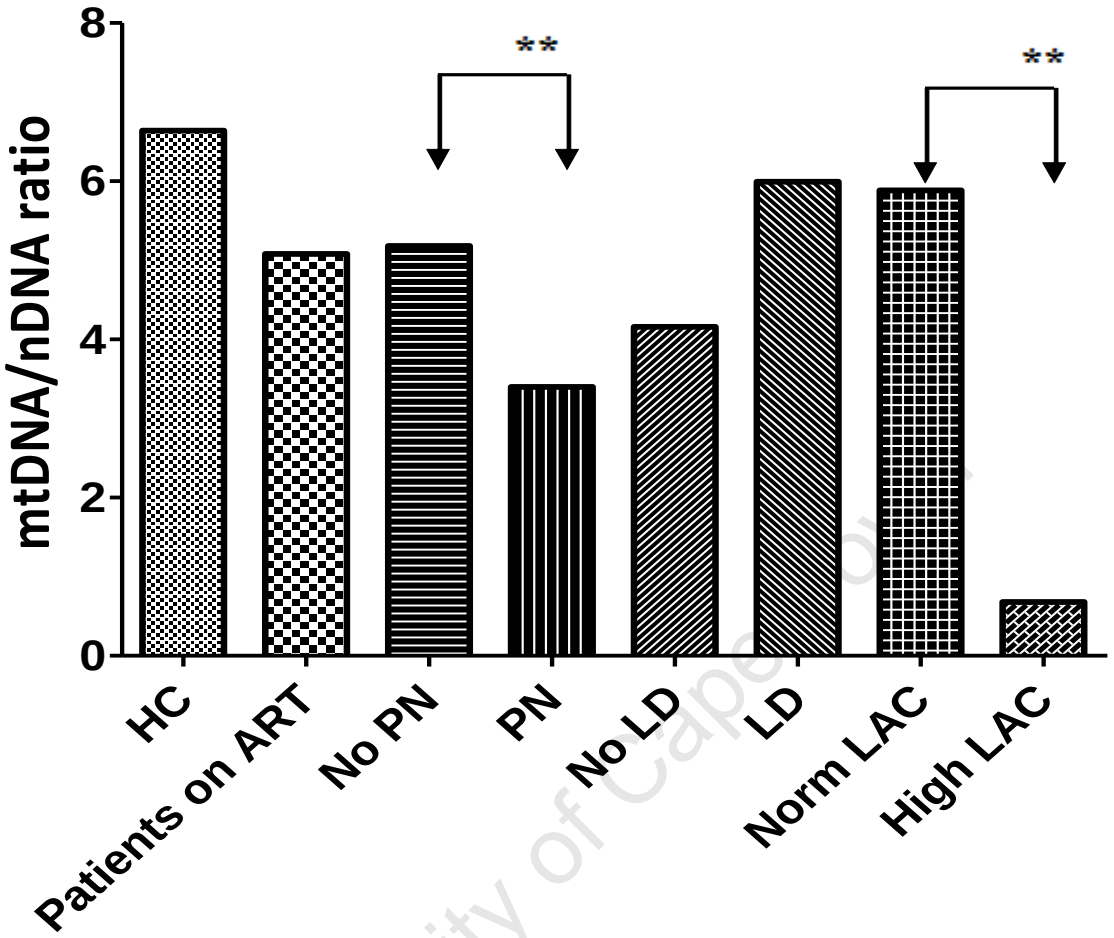

Figure 2: mtDNA/nDNA ratios in healthy controls and ART patients with and without toxicities

1. HC, healthy controls; Patients on ART; 3 PN, peripheral neuropathy; 4 LD, lipodystrophy; 5 LAC, lactate

** Significant when ART group is compared to healthy controls. ↓——↓ Significant when compared between groups

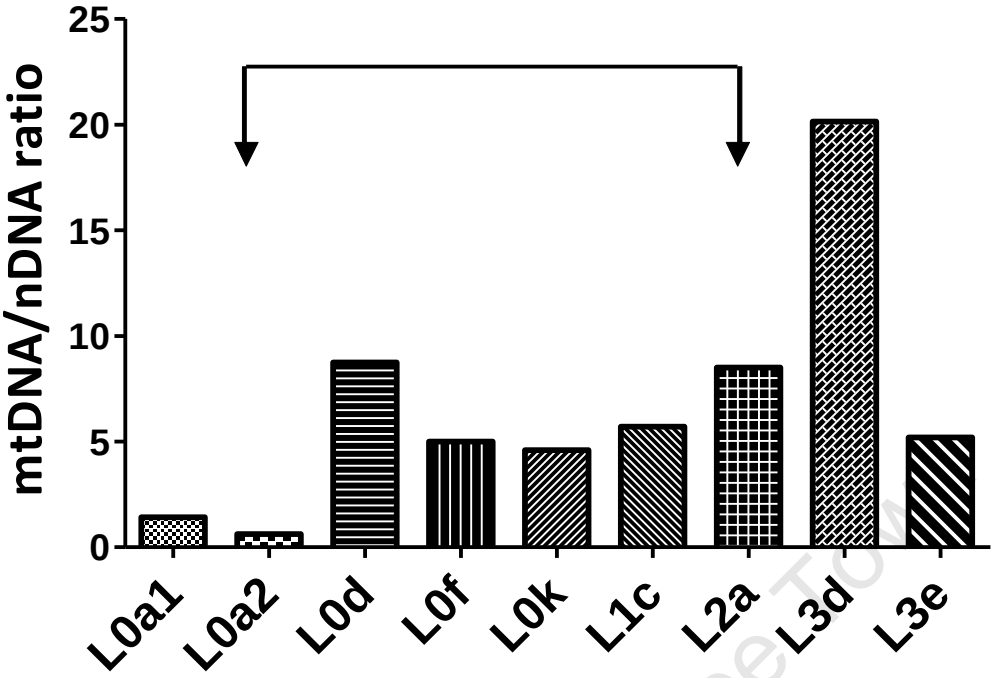

Figure 3: mtDNA/nDNA ratios in ART patients categorized in different mtDNA Subhaplogroups

Significant when compared between groups

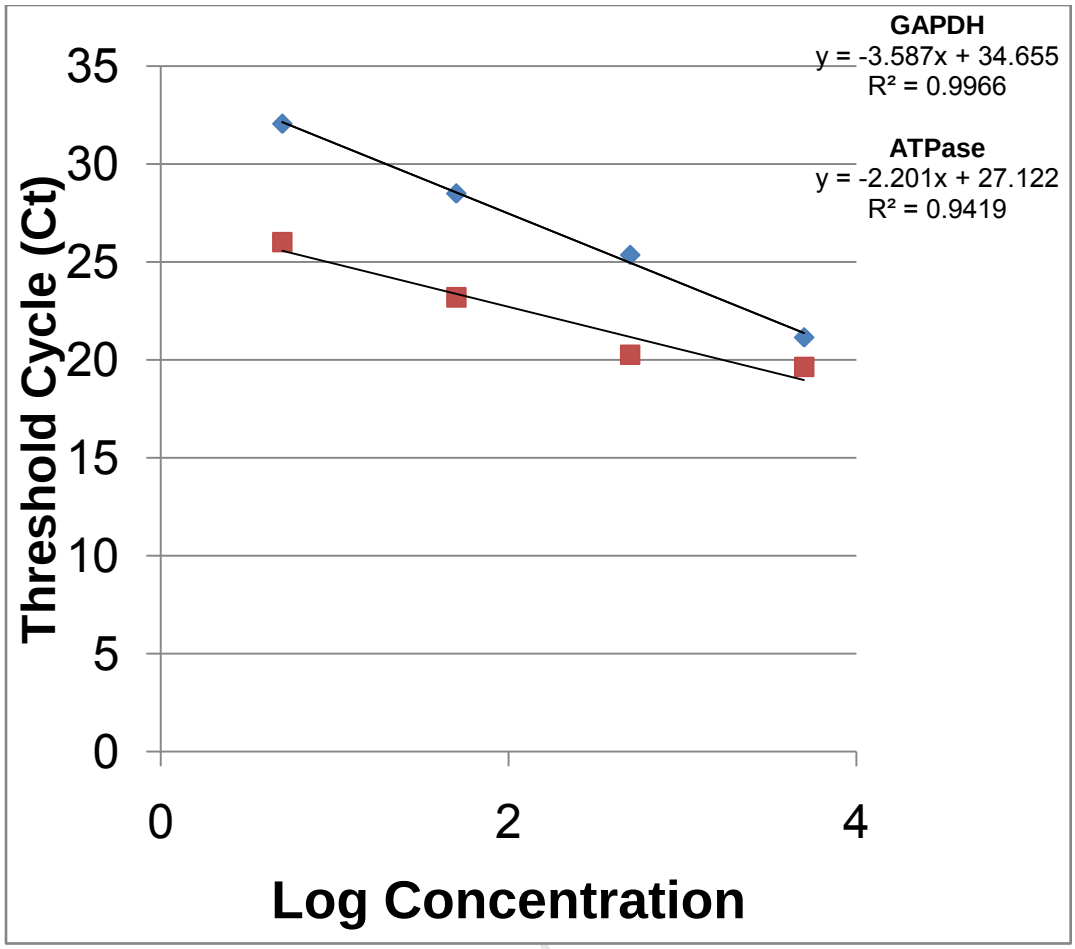

Figure S1: Standard curve plotted from regression analysis of threshold cycle and serial dilution of total genomic amplified DNA.
The top curve is for the GAPD gene and the bottom curve is for ATPase gene.

8. CHAPTER 8: POLYMERASE GAMMA GENE VARIATION AND STAVUDINE ASSOCIATED TOXICITIES

8.1. Methodology

8.1.1. Mitochondrial polymerase amplification and sequencing analysis

Mutations in mitochondrial polymerase gamma have been associated with stavudine induced toxicities but distribution of polymerase gamma variants are population specific [1,2]. In order to investigate the reported variants and possible novel mutations in mitochondrial polymerase gamma, a section of the polymerase gamma domain from exon15 to exon 18, (Figure 8.1) a 1450 base pair region was amplified using the primers according to van Goethem [3] and these were exon 15 forward primer: 5´-ATG GTG GGATGT GGG ATA GATT-3´ and exon 18reverse primer: 5´-GTA ATG GGC AGG AGA TAG AAC AGA-3´. Pol γ DNA template was amplified in total volume of 25 µl in a reaction that consisted of 1X green GoTaq reaction buffer, 200µM of dNTP, 2.0 mM of $MgCl_2$, 0.4 µmol of each primer, 0.5 U of Taq DNA polymerase and 20 ng of DNA in a GeneAmp® PCR System 9700 (Life technologies, New York, USA). The PCR conditions involved an initial denaturation step at 95 ^{o}C for 5 min, followed by 30 cycles of denaturation at 94 ^{o}C for 30 seconds, annealing and extension temperatures of 60 ^{o}C and 72 ^{o}C for 30 seconds and for 1.0 minute, respectively, with a final extension step of 10 minutes at 72 ^{o}C. PCR amplification products were confirmed by electrophoresis on 1.5% agarose gel stained with ethidium bromide. The separated DNA fragments were visualized under UV light using a UV transilluminator (Nucleotech, California, USA) and images were captured using the Nucleovision system and its associated Gel Expert software (Nucleotech, CA,USA).

PCR templates were cleaned-up by using exonuclease 1 in order to remove excess primers and shrimp alkaline phosphatase to degrade the dNTPs in the reaction. Each reaction mixture contained 2 U of exonuclease, 1 U shrimp alkaline phosphatase (Fermentas), 13.9 µl water and 5 µl of PCR product. The samples were then incubated at 37 °C for 15 minutes and at 75 °C for 1hour in GeneAmp® PCR System 9700 by Life technologies (New York, USA).

8.1.2. Mitochondrial polymerase gamma genotyping

Using the cleaned PCR product, sequencing was performed using 0.5 µM of exon 17 forward primer: 5´-CAG GAA TGG GGT AGG AAG AGT C-3´, 1µl of Big Dye Terminator mix Cycle Sequencing Kit (ABI PRISM Big Dye Terminator v3.1 mix (Applied Biosystems, California, USA) in the presence of deoxynucleoside triphosphates, fluorescence-tagged terminating analogues, DNA polymerase, $MgCl_2$ and the appropriate buffer , 2 µl of 5X sequencing buffer, 2 µl cleaned PCR product and 4 µl was electrophoresed on GeneAmp 9700 sequencing machine (Applied Biosystems, California, USA) .The sequencing temperatures conditions were 96 ^oC for 5 minutes, and 96 ^oC for 30 seconds, 55 ^oC for 15 seconds and 60 ^oC for 4 seconds for 30 cycles. Exon 18 reverse primer: 5´-GTA ATG GGC AGG AGA TAG AAC AGA-3´ was used to confirm the mutations. PCR templates were cleaned-up inorder to remove excess nucleotides and primers from PCR reactions by using exonuclease 1 to removes primers and shrimp alkaline phosphatase (ExoSap) degraded the dNTPs in the reaction. Each reaction mixture contained 2 U of exonuclease, 1 U shrimp alkaline phosphatase (Fermentas), 13.9 µl water and 5µl of PCR product. The samples were then incubated at 37 °C for 15 minutes and at 75°C for 1hour in GeneAmp® PCR System 9700 (Life technologies New York, USA).

Cleaned PCR product were sequenced with reaction contained 0.5 µM primer, 1 µl of Terminator mix Cycle Sequencing Kit (ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, California, USA) containing optimized concentrations of deoxynucleoside triphosphates, fluorescence-tagged terminating analogues, DNA polymerase, $MgCl_2$ and the appropriate buffer , 2 µl of 5X sequencing buffer, 2 µl cleaned PCR product and 4 µl water run on GeneAmp 9700 sequencing machine (Applied Biosystems) with temperature conditions 96 ^oC for 5 min, followed by 30 cycles at 96 ^oC for 30 seconds, 55 ^oC for 15 seconds and 60 ^oC for 4 minutes. Sequencing reaction cleanup was performed using ethanol/sodium acetate (NaAc). Capillary electrophoresis for sequencing reactions were run on an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems) and analyzed using sequencing Analysis Software v5.2 (Applied Biosystems).

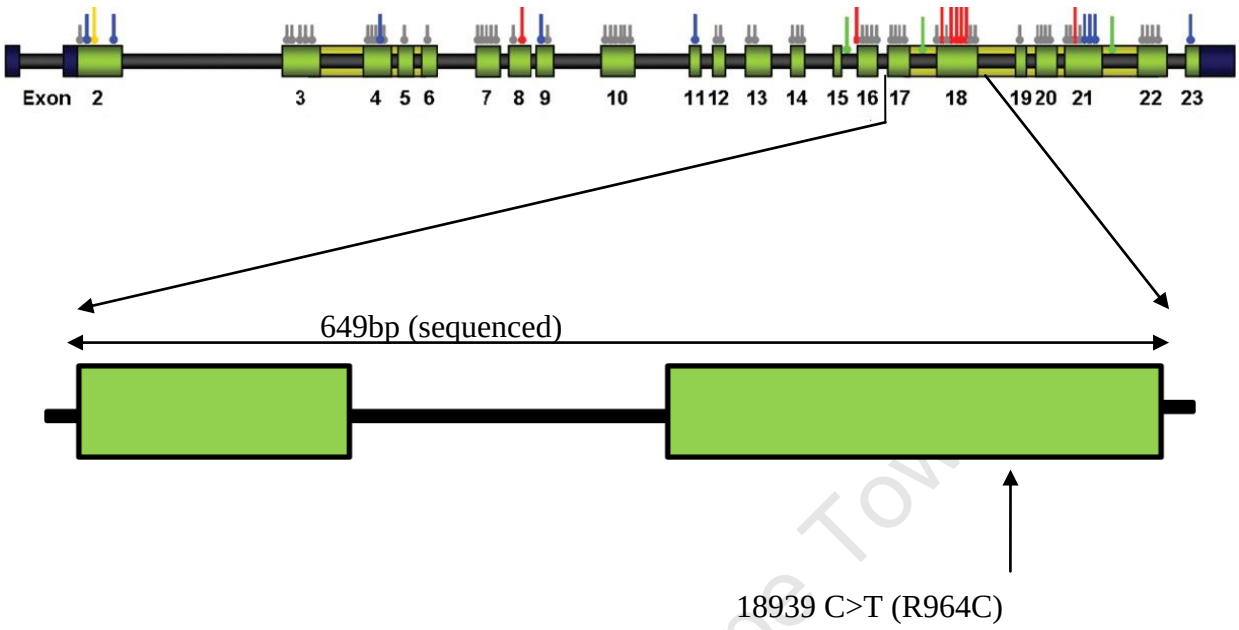

Figure 8.1: Schematic representation of human mitochondrial polymerase gamma gene on chromosome 15q25.

(A) Shows 22 exons (2-23) with corresponding introns and their positions (numbers below). Red and grey markers indicate positions of reported dominant and recessive mutations respectively (B) A schematic representation of polymerase gamma showing sequenced region. Figure adapted from Hudson and Chinnery [4]

8.1.3. Data analysis

The partially sequenced fragments of POLG-γ from position 18487 – 19136 (649bps) were assembled and aligned to NC_000015.9 reference gene using Lasergene 10 Core Suite software (DNASTAR, Wisconsin, USA). Sequences were compared and mutations (polymorphisms) were determined as nucleotide differences with the reference sequence gene. Using Stata software version SE/11 (Stata Corp; Texas, USA) and Shesis software [5] were used to determine genotype and allele frequencies. Linkage disequilibrium (LD) analysis for determined mutations was calculated and furthermore LD was measured by Lewontin's coefficient (D') and squared correlation coefficient (r^2) with range from zero from independence to 1for complete co-inheritance were carried using SHEsis [5]. Hardy Weinberg Equilibrium (HWE) analysis of genotype distribution was carried out by Chi- square goodness of fit with one degree of freedom.

Mann Whitney test was used to compare allele frequencies between Malawi population and other populations (Sub-Sahara Africa, European and Asian). Chi-square was used to determine whether the frequencies of genotypes were associated with demographic information and clinical parameters (peripheral neuropathy, lipodystrophy, lactate, CD4 cell count, viral load, gender, age, body mass index and duration on treatment). Furthermore, genotypes were compared with mtDNA levels.

8.2. Results

8.2.1. POL-γ genotype frequencies

Results on the patient's characteristic have been detailed in chapter 3. Of 215 patients' DNA samples available, 166 (77%) samples were successfully sequenced for the polymerase gamma amplicon. A total of 4 positions showed polymorphic variation, rs2307431 (19011 C>T) in exon 18 and rs58584025 (18709 indel AGGT), rs41544115 3 (18773 C>T) and rs2307449 (19103 A>C) in intron 17 and intron 18 with the reference sequence NC_000015.9. The frequencies of the minor alleles were as follows; 19011T (24%), 18709 indel (5%), 18773T (4%) and 19103C (18%). The distribution pattern of the variants in the studied population was as indicated in Table 8.1. The genotypes for all the SNPs conformed to Hardy Weinberg equilibrium. Allele and genotype frequencies were compared to other populations in Sub-Sahara Africa (Yoruba), European and, Asian taking the data from HapMap and dbSNP databases (Table 8.2). No differences were observed with respect to allele frequencies within Sub-Sahara African populations. However, rs2307431 (19011 C>T) mutation among Malawians was significantly different when compared to those for European and Asian populations (P = 0.001; P = 0.0003, respectively). We observed a high prevalence of the rs2307449 (19103 C/C) genotype in our study population (82%) when compared to Europeans (41%) and Asians (31%).

Table 8.1: Distribution of alleles in the Malawi population.

Genotype	Frequencies = (%)
18709 indel AGGT	
-/-	8 (0.048)
-/AGGT	158 (0.952)
AGGT/AGGT	-
18773 C>T	
C/ C	153 (0.956)
C /T	7 (0.0438)
T/T	-
19011 C>T	
C/ C	100 (0.758)
C /T	27 (0.204)
T /T	5 (0.038)
19103 A>C	
A/ A	23 (0.184)
C/ C	102 (0.816)
C/A	

Table 8.2: Comparison of allele frequencies between our study (Malawi) population and other world populations

Genotype	Current Study	Sub-Sahara Africa N(P)	Europeans (CEU) N(P)	Asians N(P)
18709 indel AGGT (rs58584025)	166	NA	NA	NA
-	0.048			
AGGT	0.952			
18773 C>T (rs41544115)	160	118 (0.711)	2	
C	0.978	0.975	1.00	NA
T	0.022	0.025	0.00	
19011C>T (rs2307431)	132	226 (0.891)	**220 (0.001)**	90 **(0.0003)**
C	0.860	0.858	0.995	1.00
T	0.140	0.142	0.005	0.00
19103 A>C (rs2307449)	125	**226 (0.007)**	**226 (0.001)**	**86 (0.001)**
A	0.184	0.319	0.587	0.686
C	0.816	0.681	0.413	0.314

*indel = insertion; **Sub-Sahara Africa samples from Yoruba of Ibadan; ***NA, Data not available

8.2.2. Linkage disequilibrium and haplotype analysis

Linkage disequilibrium (LD) analysis for the POLG-γ 4 SNPs obtained in our study population was performed in order to determine the pattern of inheritance by correlation coefficient r^2 using SHEsis software [5]. The strongest pairwise linkage disequilibrium (D') and coefficient (r^2) were observed between 18709insA, 18710G, 18711G and 18712T (D'= 0.99, r^2 = 1) see Linkage disequilibrium (LD) plot of POLG variants. Figure 8.2.

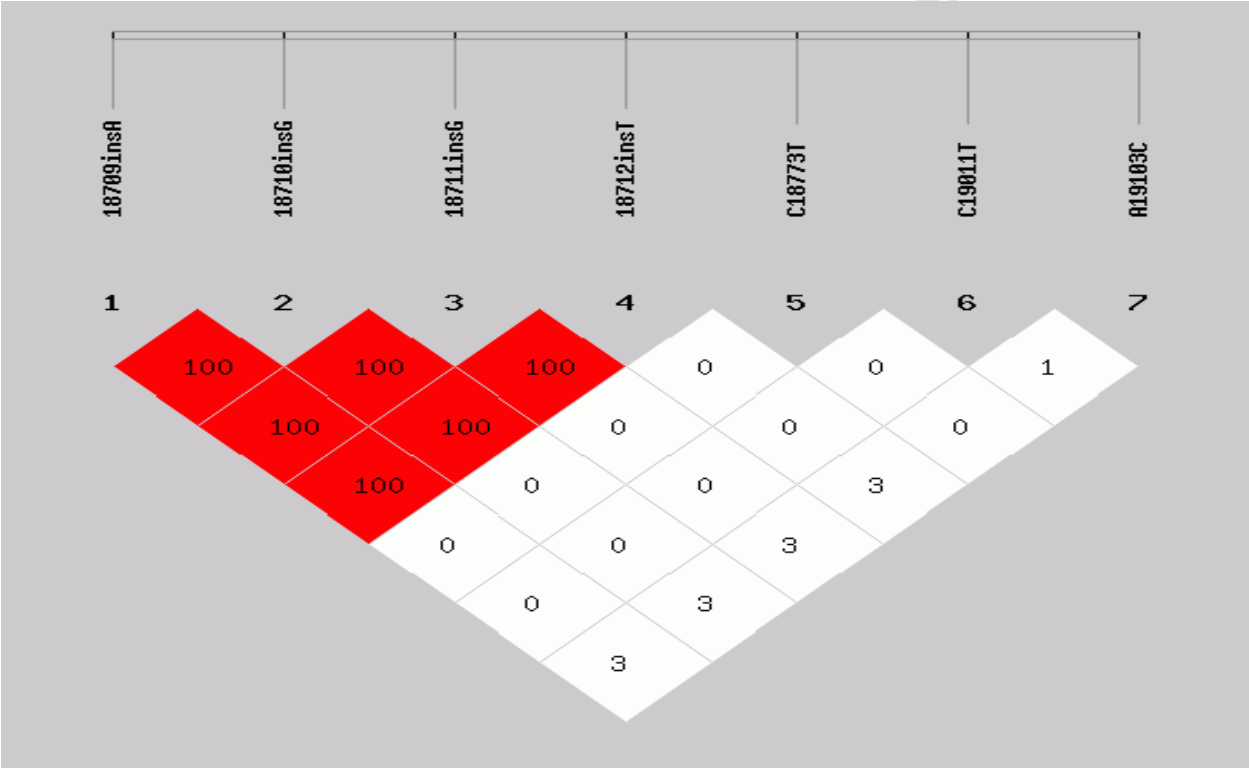

Figure 8.2: Linkage disequilibrium (LD) plot of POLG variants.
Correlation coefficient (r^2) between SNPs in POLG with bright red boxes indicating high correlation.

8.2.3. POLG mutations in association with mitochondrial toxicities

None of the demographic parameters was associated with any of the genotypes (Table 8.3) The distribution of mutations in individuals with different levels of CD4 cell count was not different. Viral suppression was not associated with any of the identified mutations. Heterozygous minor alleles identified on the mitochondrial polymerase gamma were not associated with peripheral neuropathy, lipodystrophy and hyperlactatemia in samples. In a separate analysis homozygous 19011T/T was not significantly associated with mitochondrial associated toxicities.

Table 8.3: POL-γ genotypes and association with patient features

Phenotypes	Genotypes (MAF)							
	18709-/-		**18773T**		**19011 T**		**19103 A**	
	N (freq)	**P-value**	**N (freq)**	**P-value**	**N (freq)**	**P-value**	**N (freq)**	**P-value**
Gender								
Female	5 (0.63)	0964	6 (0.86)	0.205	12 (0.37)	0.797	19 (0.83)	0.068
Male	3 (0.37)		1 (0.14)		20 (0.63)		4 (0.17)	
Age categories								
<40 years	6 (0.75)	0.282	3 (0.43)	0.465	16 (0.50)	0.371	10 (0.43)	0.976
>40 years	2 (0.25)		4 (0.57)		16 (0.50)		13 (0.56)	
BMI categories								
Underweight (<18.5 kg/m^2)	1 (0.15)	0.065	0 (0)	0.315	0 (0)	0.559	0 (0)	0.737
Normal (18-25 kg/m^2)	7 (0.87		3 (0.43)		27 (0.84)		19 (0.83)	
Overweight (>25 kg/m^2)	0 (0)		4 (0.57)		5 (0.16)		4 (0.17)	
CD4 (IQR) cells/µL	290 (191 - 435)	0.334	451 (167 – 484)	0.861	384 (317 – 503)	0.305	412 (276 – 534)	0.566
Viral Load (CPs/ml)								
<400	5 (0.63)	0.061	7 (1.00)	0.255	28 (0.87)	0.969	16 (0.76)	0.084
>400	3 (0.37)		0 (0.0)		4 (0.13)		5 (0.24)	
Duration on ART(IQR) (months)	25 (23 – 29)	0.753	24 (12 – 46)	0.958	24 (23 – 29)	0.472	25 (23 – 33)	0.333
PN								
Yes	0 (0.0)	0.102	3 (0.43)	0.244	8 (0.25)	0.724	5 (0.22)	0.986
No	8 (1.00)		4 (0.57)		24 (0.75)		18 (0.78)	
LD								
Yes	1 (0.13)	0.779	1 (0.14)	0.699	4 (0.13)	0.226	0 (0.0)	0.117
No	7 (0.87)		6 (0.86)		28 (0.87)		23 (1.00)	
LAC >2.5U/L								
Yes	2 (0.33)	0.543	1 (0.33)	0.674	2 (0.20)	0.791	2 (0.20)	0.765
No	4 (0.67)		2 (0.67)		8 (0.80)		8 (0.80)	

PN, peripheral neuropathy; LD, lipodystrophy; LAC, lactate >2.5U/L

8.2.4. POL-γ mutations and mtDNA depletion

Relative mtDNA/nDNA ratios between the wildtype genotype and variants were compared and results were as shown in Figure 8.3. Mitochondrial DNA/nDNA ratios' between samples with variant 18709 indel AGGT and those without an insertion were not significantly different (P = 0.1556). Although mtDNA/nDNA ratio were higher in the samples with genotype 18773 T/T compared to 18773 C/C this was not statistically significant (P = 0.2791). Comparison of mtDNA/nDNA ratios between genotypes 19011C/C with the heterozygous genotype, 19011C/T and homozygous 19011T/T were not statistically significant (P = 0.8496; P= 1.00 respectively). Low mtDNA/nDNA ratios were observed in the individuals with 19103 A/A genotype when compared to individual with the19103C/C genotype, however this difference was not statistically significant (P = 0.5364).

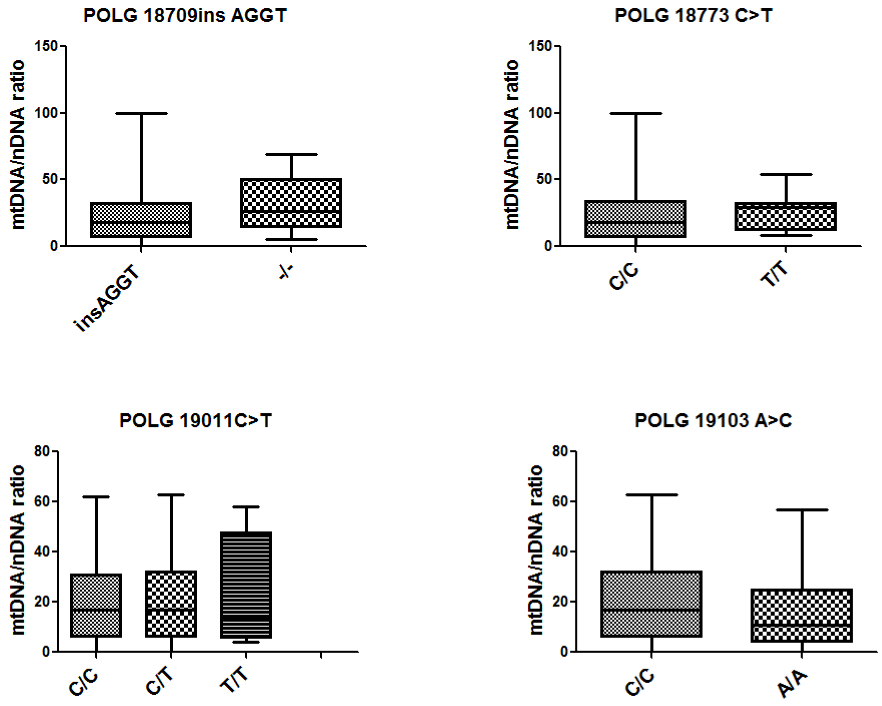

Figure 8.3: POLG-γ variants in association with mtDNA/nDNA ratio.

8.3. Discussion

Partially sequenced region in the active domain of polymerase gamma was selected based on the fact that it consists of highly conserved O helix that directly interacts with bound nucleotides. Secondly previous studies had shown that the selected region is highly polymorphic (http://tools.niehs.nih.gov/polg/) encompassing the mutation R964 which has been associated with stavudine associated lactic acidosis [1]. Secondly whole polymerase gamma was sequenced on 10 samples from the study where no known pathogenic mutations were identified [6]

Prevalence of variant 18709 indel AGGT has not been reported in other populations according to HapMAp, and we report it for the first time with highest prevalence of 95% in our population. Further analysis of the mutation in African population may provide information on the distribution and base composition. Allele frequencies for 18773T (2.2%) and 19011T (14%) in our study population were not different from frequencies in Sub-Sahara African populations , although differences were observed with other populations from other regions suggesting genetic drift could have played a role. The variation observed with respect to 19103A allele frequencies when the Malawian population is compared to other populations is further testimony of the genetic diversity of African populations [7].

Mutations in POLG have been associated with stavudine induced mitochondrial toxicities. In separate studies among French And Thai [8,9] mutations in the polymerase gamma active domain, 2890 T>C and 3428 A>G, were associated with susceptibility to lactic acidosis and lipodystrophy, respectively, whilst 2684 A>G (E895) and 2864 A>G (Y955C) amino acid substitutions have been reported to induce a higher discrimination against triphosphoralyted -d4T that prevents their incorporation into DNA [10]. Analysis of genotypes with anthropometric measurements and clinical manifestations such as peripheral neuropathy, lipodystrophy and hyperlactatemia in HIV/AIDs patients on stavudine did not show any association. In spite of having one patient who presented with lactic acidosis we did not find any of the mutations reported before to be associated with lactic acidosis to support the report by Chinnery et al [7] who suggest that variation to response to stavudine basing on mutations is differential. The identified mutations 18709ins AGGT, 18773 C>T, 19011C>T and 19103A>C have been reported to be nonpathogenic according to the PubMed database http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2307449 and absence of pathogenic

mutations are similar to our earlier study results [6]. A change at position 19103T>G (rs2307449) has been reported to be associated with aging in women of other populations of Chinese origin [11,12]. No T/G base pair substitution was observed at this position in our samples.

Polymerase gamma's main role is to replicate and repair damaged mitochondrial DNA [13] and mutations such as the R964C have been associated with decreased activities of POLG which may result in decreased mtDNA content [1,8]. No significant difference in mitochondrial level between individuals with identified variants and those without was observed possibly suggesting that these mutations may not have effects the amount of mitochondria in the cell but function. Although in a separate analysis we observed that clinical parameters such as peripheral neuropathy and hyperlactatemia were associated with decreases in mitochondrial levels, none of these adverse effects was identified with the known polymerase gamma pathogen mutation. These findings, may support a role for d4T in mtDNA depletion by inhibition of mitochondrial DNA replication [8] and this could be either through POLG activity inhibition or directly [8,14,15]. We however acknowledge that our study has several limitations and these include the fact that only a portion of the mtDNA polymerase gamma gene was screened.

8.4. References

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9. CHAPTER 9: DISCUSSION AND CONCLUSION

This section gives a summary of the overall findings, recommendations emerging from the study, limitations and conclusions. Demographic parameters of the HIV/AIDS patients as well as ethnic groups were correlated with genetic variation in mtDNA and polymerase gamma. The observations in this Malawi population were compared to other world populations. The availability of highly active antiretroviral therapy (HAART) for HIV-1 infection has led to significant decrease in mortality and disease progression, a situation that has converted HIV/AIDS from a death sentence into a chronic disease. Favorable HAART-associated virological and immunological successes observed in this study (***Outlined in chapter 3***) are consistent with findings from other studies [1,2], which have demonstrated how HAART has led to decreased disease progression.

Variations observed in patients in their response to stavudine-containing therapy in this study support the hypothesis that different individuals respond to the same treatment differently. Observation of higher CD4 cell counts in females compared to males is a confirmation of earlier reports in the Malawi population [3] which may point to gender differences in the handling of ARV drugs and CD4 recovery. The gender differences in CD4 cell counts have been observed in other populations within Sub-Sahara Africa, [4] and it has been suggested that women may naturally have high CD4 counts despite HIV status. Factors such as BMI and age >40 years have proven to be associated with risk of stavudine associated adverse effects [2,5,6,7,8,9]. The most possible mechanisms could be through increased reactive oxygen species production due to synergistic effect of both age and NRTIs [10,11]. Besides nucleoside analogue reverse-transcriptase inhibitors (NRTIs) being part of the backbone of HAART, their effectiveness is hampered by side effects [12] .

In this study observed stavudine- associated toxicities included were peripheral neuropathy and lipodystrophy in confirmation of earlier reports [13]. Although some studies have suggested that host mtDNA haplogroups and polymorphisms in the poly – γ influence the risk of stavudine associated toxicities during antiretroviral therapy treatment [14,15]; we did not observe much influence from polymerase gamma gene variation. However, this could be due to the fact that we only sequenced a portion of the polymerase gamma gene; it is possible that causative mutations

could be in the unsequenced region. We postulate that mutations in polymerase gamma could result in reduced mtDNA replication efficiency. This would result the dysfunction of mitochondrial DNA leading to insufficient ATP production, a factor that causes tissue and organ malfunctioning and ultimately present as mitochondrial toxicity [10]. That is why we investigated variation in mtDNA and polymerase gamma and their role on the response of adult Malawian HIV/AIDS patients to stavudine- containing ARTs.

Genetic diversity of the Malawi population

The genetic characteristics of the Malawi population based on the matrilineal inherited mitochondrial DNA had not been characterised before. Generally, studies on mtDNA have helped in demonstrating the African origin of humans and the relationship between various populations. Most studies on African mtDNA haplogroup lineages and their phylogenetic relationships have been based on polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) haplotyping of mtDNA control regions only and recently have started incorporating whole-mtDNA sequencing. For example, the current study is one of the few that have sequenced almost whole mtDNA in search of variation among African populations. Haplotyping only control region raises a problem when one wants to compare findings across different studies as the genotyped regions often do not overlap. The PCR-RFLP method focuses on known mutations (mutation hotspots) whilst sequencing gives you a glimpse of the variation across the sequenced region [16]. Rosa and Brehm [17] indicated that most mtDNA haplogroups on African populations show variations in distribution and sub-structuring when geographical and ethnolinguistic affiliations are considered. We investigated the mtDNA variation in the seven Malawi ethnic groups by sequencing the whole mtDNA coding region, leaving out a small portion of the control region.

We report on 184 mtDNA mutations in the Malawian population (***As described in Chapter 4)*** and these mutations were used to construct a phylogenetic tree. The constructed phylogenetic network of haplotypes represents mtDNA L lineage haplogroups (L0, L1, L2, L3) and is similar to what is expected in Sub-Sahara African populations (***Appendix D***). The structure shows the relationship between different subhaplogroups and haplogroups. The observation is in support of the fact that the current Sub-Sahara African mtDNA gene pool is a mixture of L lineage

subhaplogroups found at varying frequencies throughout the continent [18].The grouping together of sequences with similar haplotypes in the network points to a possible same maternal ancestor.

Furthermore, the absence of haplogroup L4, L5 and L6, in the Malawi population confirms earlier reports of these haplogroups being confined to West and East African populations [19,20]. The observed subhaplogroup variations between the Malawi population with other African populations confirms what earlier observations of subhaplogroups co-segregating geographically as reported by Gonder et al [16]. Earlier studies reported of high prevalence of haplogroup L0 and subhaplogroup L0a in Southern and Southeast African populations (especially Mozambique) when compared to North and West Africa [19,20], and this is consistent with our observations with haplogroup L0 having frequencies of as high as 48% in the Malawian population group. While earlier studies have reported the subhaplogroups L0d and L0k to be found almost exclusively among southern African Khoisan (SAK) speakers, apart from L0d being present among the Turkana of Kenya and populations in Mozambique [20,21,22] presence of these subhaplogroups in the Malawian ethnic groups point to possible population admixture with the Khoisan and possibly through a dispersal wave from East to Southern Africa during which some pre-Bantu speaker remnants could have settled within the country [18]. The L0f subhaplogroup in the Malawian population could be compared to that of East Africa populations of North Sudan, Ethiopia, Kenya, and Tanzania with Uganda presenting with highest frequencies different to what is observed among populations of Zimbabwe and Mozambique, where the subhaplogroup is rarely reported. Presence of the L0f subhaplogroup among Malawian ethnic groups could be due to intermarriages between different ethnic groups from east Africa.

Presence of subhaplogroup L2a which has been reported to be the most frequent in Central Africa occurs at frequency of 25% , in the Malawi population which supports the thinking that the subhaplogroup has undergone dramatic demographic expansion in the Southeastern African region [20,23]. Although studies have postulated L1c subhaplogroup to have originated in Central Africa, this subhaplogroup was observed to be present in the Malawi population at higher frequency (13%) than other Southeastern Africa populations [19,20,23] further suggesting

western Bantu migration towards Southeast Africa. However, there is need to further investigate the genetic characteristics of L1c identified in Malawi population wand compare it with that of other African populations in order to establish its originality. Whilst the most common subhaplogroup in Africa is L3e, its presence among the Malawi population could point to possible have maternal links between the Malawians with other Bantu speakers both in the Central and east Africa.

Distribution of subhaplogroups within the Malawi ethnic groups could suggest population admixture, with the Chewa and Sena being the only ethnic groups specifically associated with subhaplogroup L2a and L1c respectively, while Lhomwe, Ngoni, Tonga, Tumbuka and Yao could not be differentiated into specific subhaplogroups. Genetic differences observed between the Sena and Chewa could be a result of different cultural practices which could have prevented them from intermarriages. The genetic admixture observed among the Lhomwe, Ngoni, Tonga, Tumbuka and Yao, could possibly be due to their culture similarities as well common shared languages [24] which could have made it difficult for participants to indicate their real ethnicities. For example, the Tumbuka language is common and spoken by over 60% percent of people in the Northern region of Malawi, thereby, masking other ethnicities whose languages are least spoken making it difficult to differentiate different ethnic groups.

Secondly intermarriages are very common between Tumbuka and Ngoni from the Northern parts of Malawi such that most Ngoni are masked under the Tumbuka and these groups both have patriarchal cultures where ethnicity is defined by the male lineage. Migration and demographic factors (urbanization due industrialization) in the Southern Region of Malawi, an area which is mainly dominated by Yao and Lhomwe, might have also contributed to genetic admixture which might have contributed to failure to assign the tribes into specific haplogroups because more people of different ethnic groups moved and settled in the Lhomwe and Yao settlements when early industrialization was taking place. The slave trade is also thought to have contributed to some ethnic admixture with the Yao tribe acting as the intermediary between inhabitant worker migrants and the incoming Swahili and Arab traders [25].

African populations have been associated with high genetic-diversity and our study results further confirm these findings [20,26,27]. The observed intra- and interpopulation sequence divergences within and between ethnic groups with lower *F*ST estimates (*F*ST = 0.001– 0.152) compared to global *F*ST estimates based on variation in mtDNA (*F*ST = 0.24–0.27) [28] indicates low genetic differentiation between Malawian ethnic groups in support of close genetic relatedness as depicted by the neighbor-joining tree. However, clustering of Chewa, Yao, Tumbuka, Tonga, Lhomwe and Ngoni, may support the population admixture phenomenal where populations descended from recent mixture of groups that have long been separated with short branches and observed to be located centrally in inferred neighbor-joining trees [29].

Genetic structure relationship between Malawi ethnic groups and other Sub-Sahara Africa ethnic groups

Current studies on evolutionary relationships based on uniparental transmitted polymorphisms have primarily focused on the mtDNA genes and not on population history [17]. However complex African ethno-linguistic context must be taken into account, considering where societies are deeply structured by cultural beliefs and patterns of admixture which strongly influence genetic inheritance pattern [17]. Therefore, we further investigated the genetic structure of Malawi ethnic groups in comparison to those of other Sub-Sahara African ethnics groups (see chapter 4). Comparing the subhaplogroup frequencies among Malawi populations with populations from Mozambique and Zimbabwe which are all in Southeast Africa shows variation in the distribution of most of these subhaplogroups. Although three of the largest ethnic groups in Malawi (Lhomwe, Sena, Yao) migrated from Mozambique and share language similarities, a comparison of the distribution of major subhaplogroups L0a, L1c, L2a and L3e at country population level, the Malawian population gladiates more towards similarities with the Zimbabwe population while sort of drifting away from the Mozambican population. This could be due to the fact that we are looking at mtDNA which does not give a complete genetic picture. It could be possible if we were to investigate Y-chromosome DNA variation, the Malawian and Mozambican populations would share more resemblance and the Zimbabwean population be a little bit distant or more separated.

On the other hand, the similarities between Malawians and Zimbabweans could be a recent phenomenon, being a reflection of colonialism and the slave trade [23,30,31]. Mozambique was colonized by the Portuguese, whereas both Zimbabwe and Malawi were under British rule, thus their inhabitants were more likely to mix under the Federation of Rhodesia and Nyasaland. In terms of slave trade, Mozambique provided the link to the ocean, thus, inland countries mostly affected by slave trade are likely to have contributed to the genetic admixture in Mozambique. Therefore, further work to determine how genetically these populations are structured is needed.

Unlike non-African populations, African populations are characterised by greater levels of genetic diversity, population substructure variations, and less linkage disequilibrium (LD) among loci [32]. Using haplogroups frequencies to determine the genetic structure of different ethnic groups in Sub-Sahara Africa, a principal component analysis (PCA) demonstrated clustering of ethnic groups according to geographical region an observation also demonstrated by other studies [19,33]. This observation is supported by hypothesis that distribution pattern of mtDNA subhaplogroups is geographical-area and ethnic-group structured [34,35].

The Malawi ethnic groups genetically appear to be more closely related to Southeastern and Southern African ethnic groups. Clustering of the Malawi Yao and Kaskazi-speaking Mozambique Yao is consistent with language, these ethnic groups speak same language and also could support their common origins [36,37]. However, further analysis should be considered in order to investigate their genetic relatedness as well as to groups from East Africa who speak similar languages [37]. Similarly, the Chewa ethnic group speaks the same language as the Nyanja of Mozambique and is very close to other Bantu speakers of Southeastern and Central Africa than East Africa possibly signifying closeness in their genetic relationship but further studies are required to determine the true genetic relatedness. Interestingly, the Ngoni, Tonga, Tumbuka from Malawi cluster close to the Southern African Bantus, especially the Zulu/Xhosa, Swazi, Sotho and Makonde of South Africa, Swaziland and Mozambique.

Taking into the account gene variation, presence of the haplogroup L0 among Southeastern African ethnic groups may signify their genetic closeness to the Southern African ethnic groups [16,20,38]. Population admixture could have occurred in some ethnic groups for example the Lhomwe of Malawi and Mozambique as they are not seen close to each other in support of the

ethnologue information [36]. This observation defeats the use of language to solely characterise populations, but calls for a comprehensive molecular evolutionary analysis of the ethnic groups in Southeast Africa focusing on more markers including Y-chromosome DNA. While most Malawi ethnic groups seem close to each other using our mtDNA variation data, exceptions are observed with the Sena who appear to be closer to East African ethnic groups as depicted in the PCA (***See Chapter 4***). The Malawi Sena ethnic group which is believed to have originated from Mozambique was also clearly separated from Mozambican Sena who belongs to Kusi-speaking and are scattered across Southeast Africa. Historically, Sena is an umbrella name for sub-ethnic groups of the major Kusi-speaking groups of Makua, some Shona groups, Rabu and Phodzo and other Southeast Bantu [37,24]. Therefore, future studies should consider investigating the evolutionary relatedness between Malawi Sena with Kusi-speakers who are distributed throughout Southeast Africa [37] and their East African counterparts.

On the other hand, it is suggested that one must take precaution when predicting genetic similarity from language because during population dispersal there were tribal wars and conquering victors often imposed their language on the losers. Clustering of ethnic groups on PCA may support the hypothesis of the split been West and East Africa populations suggesting co-evolution of L0-L6 lineages in a African populations Behar et al [18]. Unlike other populations within the region, data on the mtDNA landscape of Malawi has not been available up to this point and this is a contribution towards unraveling the deep-rootedness of mtDNA lineages in Southeastern African populations. However, a full understanding of the genetic landscape and demography of the Malawian ethnic groups in relation to other Sub-Sahara Africa ethnic groups should be considered especially once other populations start also to have whole mtDNA sequence data as opposed to the current targeted SNP data. A part from population studies, mtDNA variation has been used in predicting susceptibility to drug induced toxicities [39]. Variations in mtDNA has been associated with differential susceptibility to neurodegerative and metabolic diseases as well as progression to diseases state[39] as will be discussed in the next section.

Mitochondrial DNA haplogroups and susceptibility to stavudine induced adverse effects

Susceptibility to diseases shows interindividual and interethnic differences and this has been mapped to both the nuclear and mitochondrial genome. For example, the mtDNA mutation m.3243 A>G in the tRNA (Leu(uur) gene has been associated with dysfunctional β cells in the pancreas leading to impaired insulin secretion a situation which is associated hyperglycemia [40,41]. However, in some individuals, the same mutation, m.3243 A>G has been associated with myopathy when occurring at certain high mutation loads in the skeletal muscle compared to blood [42]. The differential outcome could also be at population level, such that, different populations characterised by different mitochondrial lineage (haplogroups) may exhibit different phenotypes. This has been observed in some studies that populations characterised with haplogroup J of European descent carrying mutation m.10398 A>G are associated with longevity, while the same mutation has been associated with increased ROS and insulin resistance, a risk factor for type 2 diabetes in Northern Indians [43,44]. Also, the 9-bp deletion found in COII/$tRNA^{Ly}$ has been reported to be associated with MELAS or MERRF in individuals of Taiwanese origin [45] while this mutation has been identified at high prevalence in subhaplogroup L0a2 in Africans [20,46] as well as in this study 45% but not reported in association to disease. Furthermore, many mitochondrial dysfunction symptoms manifest when the bearer is exposed to environmental toxins. For example, studies have shown that mitochondrial subhaplogroup L1c is associated with peripheral neuropathy in HIV/AIDS patients on stavudine containing regimen [14]. Using SNPs and subhaplogroups, we report on the association of L3e with reduced risk for lipodystrophy, the association of L0a2 with increased risk of developing peripheral neuropathy and L2a with reduced risk of developing peripheral neuropathy (***As described in Chapters 5 and 6***)

Among the Malawians ethnic groups, subhaplohagroups L0a2 and L2a seem to be the important markers in susceptiblity to peripheral neuropathy which is contrary to observations among other African population group[47] where subhaplogroup L1c was reported among blacks of African origin to associate with increased susceptibility of developing stavudine associated peripheral neuropathy. These findings are a clear indication that African subhaplogroups could be viewed as having adaptive features in support of Holzinger's findings [48] suggesting that further

investigations on environmental factors and mtDNA dysfunction in the genetically diverse African population should be carried out

It has been postulated that mtDNA haplotypes may modulate oxidative phosphorylation, thus influencing the overall physiology of individuals thereby predisposing them to, or protecting them from, certain diseases [49]. The possible ways through which subhaplohagroup L0a2 associates with peripheral neuropathy could be through changes in the protein structure because of non-synonymous mutations. Within L0a2 subhaplogroup, the most significant mt DNA mutation to be associated with peripheral neuorpathy is m.5711 A>G (OR = 2.59, CI 95% = 1.15 – 5.85, P = 0.021) . This mutation is found in the mitochondrial tRNA asparagine gene which transfers the amino acid asparagine to a growing polypeptide chain at the ribosome site of protein synthesis in the mitochondria. The mutation m.5711 A>G is non-synonymous and result in amino acid change, tRNAAsn: p.Asn18Ser. It is possible that the changes may affect the structure and functional activity of the mitochondrial encoded tRNA asparagine which could result in an improper balance of amino acids in nerve tissue resulting in the development of peripheral neuropathy. Another mutation in the mitochondrial tRNA asparagine (m.5669 G>A) has been associated with myopathy [50]. The mechanisms by which stavudine alters the functional activity of mitochondrial tRNA asparagine due to the m.5711 A>G mutation is not known. It has been reported that mitochondrial mutations can be protective as well, therefore, significant reduced risk of peripheral neuropathy in subhaplogroup L2a supports that mtDNA mutations may play protective roles in certain individuals and this is confirmation of the findings by Wallace et al [11] among Europeans where the mutation m.14798 C>T in cytochrome b was associated with neuro-protection among haplogroups J1 and Uk carriers. This could be applicable to subhaplogroup L2a, with mutations m.7175 T>C and m.7274 C>T in the cytochrome c oxidase subunit I which are associated with reduced risk of developing peripheral neuropathy (OR = 0.074, CI 95% = 0.01 – 0.55, P = 0.011; OR = 0.06, CI 95% = 0.01 – 0.04, P = 0.007), respectively.

Variation in response to stavudine associated lipodystrophy dictated by subhaplogroups in our study supports the hypothesis that mtDNA variations play a role in the functional differences

between population subhaplogroups and may influence the susceptibility to environmental toxins such as drugs. The absence of lipodystrophy in subhaplogroup L3e is further proof that mtDNA variation can predispose individuals to differential susceptibility to toxicity which is in agreement to the observations in a South African cohort [51]. These differences in responses have been also observed in other populations of European origin, for example haplogroup T appeared protective against lipodystrophy [39,52]. This suggests that individuals in the same population will respond to same treatment differently, therefore it is important have more pharmacogenomics studies hence supporting precision medicine [53], at the same time increasing the health and safety of individuals of at risk [54]. Mutations in the mitochondrial respiratory genes though synonymous could as well have altered functional role of mitochondrial encoded genes by either increasing or reducing expression, through a phenomenon commonly referred to as codon-usage. Therefore the effect of these synonymous mitochondrial DNA mutations with regard to the functional structure of the gene should be evaluated.

Our study support the long observed association between age and developing lipodystrophy [55]. Age is associated with reduced expression of mtDNA as well as accumulation of ROS in muscles and brain. Therefore, a decline in mitochondria due to age may contribute to subcutaneous adipocytes tissue apoptosis and chronic mitochondrial dysfunction in adipose tissue which may induce adipocyte metabolic dysfunction which are characterised by decreased capacity for triglyceride storage and synthesis [56]. Antiretroviral therapy triggers mitochondrial dysfunction and oxidative stress, which leads to cellular senescence more in aged people compared to the young [57] a factor that may influence lipodystrophy. The decline in mtDNA expression affects fuctions of mitochondria a circumstance that may result in increased lipodystrophy. Mitochondrial DNA mutations have been associated with longevity and disease in certain populations [11]. However mitochondrial diseases have been related to molecular defects in both mtDNA and nuclear genes that code for mitochondrial components [58,59]

While the mitochondrial haplogroup/haplotypes are associated with the risk of developing stavudine- associated mitochondrial toxicities, it has been shown that mutations in polymerase

gamma may also influence the activity of the enzyme in presence of NRTIs. For example, it has been reported that the mutation R964C in the polymerase gamma, decreases the selectivity natural dNTP over d4TP an effect which has been associated with lactic acidosis [60] . According to literature, most polymerase gamma mutations are population specific and environmentally influenced [53] hence the reason for us to pursue this study among Malawian participants on stavudine.

In the sequencing of a portion of the polymerase gamma gene, non-pathological mutations were observed, further supporting the hypothesis that presence of POL- γ mutations are geographically and population structured [61]. For those mutations we observed among Malawian population, their frequencies were not statistically significantly different to Sub-Sahara African populations but significant differences were observed when compared to those in other world population (chapter 8). For the observed mutations, we also correlated them to mtDNA/nDNA levels and no significant results were observed. However, our study has the limitation that only a portion of the polymerase gamma was sequenced, thus, causative mutations could still be in the unsequenced region.

Effect of stavudine containing ART on mitochondrial levels

The distribution of mitochondrial varies among individuals and between different organs, tissues and cells depending on each's energy needs [62,63]. Mitochondrial DNA replication failure affects the quantity of mitochondria in an organ and may have pathological relevance as it is associated with respiratory chain defects [62]. Our observations of significantly decreased mitochondrial content in patients on ART when compared health controls is in agreement to observations from other studies which have shown that NRTIs are associated with mitochondria depletion [64]. Observed low mtDNA levels in patients on ART presenting peripheral neuropathy in this study confirms earlier reports of decreased mitochondria in patients with peripheral neuropathy on NTRIs containing therapy [13,65]. .

Age which was associated with the risk of developing lipodystrophy in this study it was not associated with low mitochondrial levels. Pathophysiology of aging has been linked with increase in the reactive oxygen species which increases cell loss as well as depletes mitochondrial, a mechanism which is similar to effects of stavudine resulting in drug induced toxicities [66,67].

Previous studies which reported elevated lactate levels (asymptomatic hyperlactatemia) in association with low mtDNA/nDNA ratios due to NRTI (stavudine) inhibition effect on mtDNA proliferation [68] are in agreement with present study. In addition, when compared within groups, patients with elevated lactate levels (>2.5U/L) presented with significantly lower mtDNA content when compared to patients with normal lactate levels, further confirming previous observations [68]. Accordingly, abnormal functioning of the mitochondria will result in a shift towards anaerobic oxidation of pyruvate and the formation of lactate [69,70,71]. Although in our analysis hyperlactatemia could not be associated with peripheral neuropathy and lipodystrophy (see chapter 3), this seems to be conflicting with earlier studies including one in the same population. However, many studies including our earlier study [72,73,74], have identified that there is a relationship and further suggest that hyperlactatemia may reflect the underlying pathophysiology of peripheral neuropathy and lipodystrophy. Therefore, mtDNA subhaplogroups and levels of lactate could be used as markers for patients who are at risk of developing stavudine associated toxicities such as peripheral neuropathy and hypertension and lipodystrophy.

However, a large numbers of patients in resource limited settings are still receiving stavudine containing regimen despite WHO recommendations of withdraw of the drug. Therefore quantitative evaluation of mtDNA in the peripheral blood of individual on stavudine may be a useful first step in identifying patients that may develop stavudine induced-metabolic disorders. However, it will be important to establish the expected normal mtDNA levels between different age groups and gender within the specified population to improve our understanding of the meaning of these findings ***(As described in chapters 7 and 8)***. Furthermore a prospective study

would be an ideal in order to establish the relationship of mtDNA levels and associated drug toxicities.

Conclusion

Stavudine containing therapy has proven to be effective as evidenced in this study when one considers virological success and increased immunological response in participants. Despite its effectiveness stavudine still remains an unsafe drug manifested by its increased rate of toxicities which include peripheral neuropathy, lipodystrophy and hyperlactatemia. Older age appears to be the common risk factor to susceptibility to stavudine associated toxicities; therefore it will be important in future to further consider looking at the drug metabolism profiles basing on categories of adult age groups when carrying up clinical trials. In addition we recommend close monitoring of long term toxicities in patients at high risk (>40 years) with NRTIs.

Drug associated toxicities presented by patients on stavudine containing therapy are characteristically associated with mitochondrial dysfunction. Differences observed in response to treatment by different individuals groups from this study demonstrate that mtDNA subhaplogroup may be an important factor in the development of stavudine associated toxicities for example peripheral neuropathy and lipodystrophy. Therefore, it is important to begin to understand the genetic structure of each population in order to effectively tailor prescription of drugs. Differences in mtDNA genetic variation observed within the Malawian ethnic groups and between Malawians and other African populations further strengthen the call for genetic characterization of all African populations in order to come up with specific biomarkers of both disease susceptibility and drug response.

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APPENDICES

Appendix A: Mitochondrial PCR primers and annealing temperatures

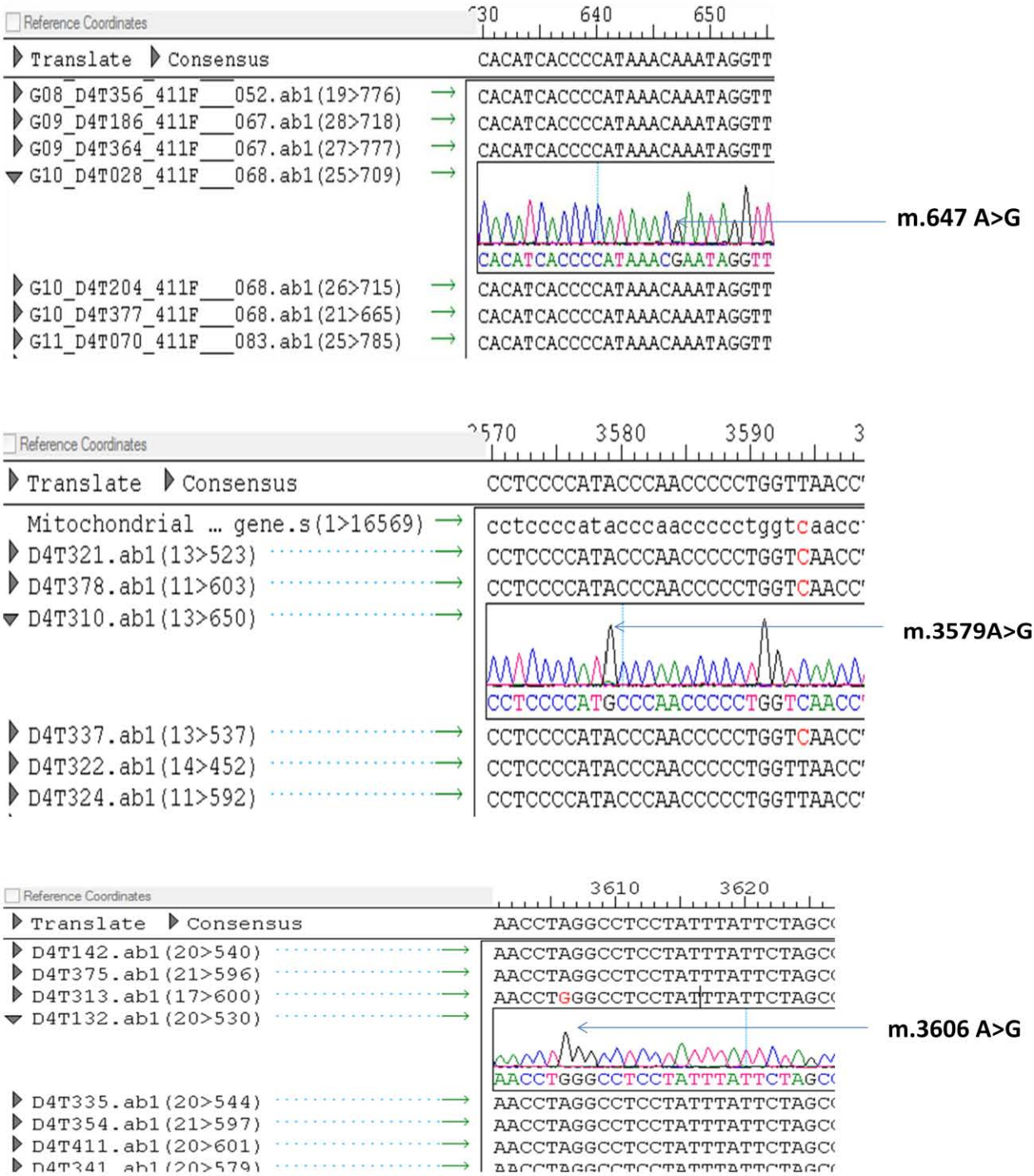
Primer Name	PCR primer sequences (5' - 3')	Amplicon size (bp)	Annealing temperature (^{0}C)
14898for 151rev	TAGCCATGCACTACTCACCAGA GGATGAGGCAGGAATCAAAGAC	1822	61
16488for 1677rev	CTGTATCCGACATCTGGTTCCT GTTTAGCTCAGAGCGGTCAAGT	1758	61
1404for 3947rev	ACTTAAGGGTCGAAGGTGGATT TCGATGTTGAAGCCTGAGACTA	2543	60
3734for 6739rev	AAGTCACCCTAGCCATCATTCTA GATATCATAGCTCAGACCATACC	3005	61
6520for 9184rev	CTGCTGGCATCACTATACTACTA GATTGGTGGGTCATTATGTGTTG	2709	60
8910for 10648rev	CTTACCACAAGGCACACCTACA GGCACAATATTGGCTAAGAGGG	1738	65
10360for 12226rev	GTCTGGCCTATGAGTGACTACA CAGTTCTTGTGAGCTTTCTCGG	1866	64.4
11977for 13830rev	CTCCCTCTACATATTTACCACAAC AAGTCCTAGGAAAGTGACAGCGA	1853	60
13477for 15349rev	GCAGGAATACCTTTCCTCACAG GTGCAAGAATAGGAGGTGGAGT	1872	60

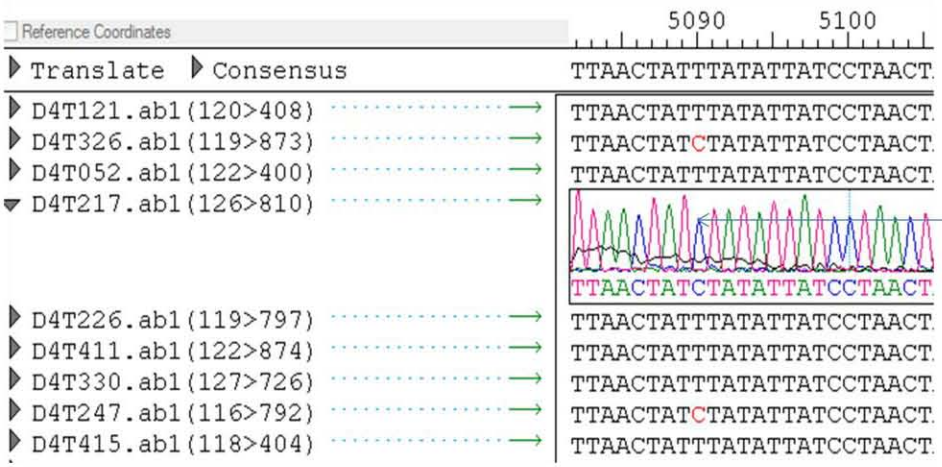
The primer name is primers' starting binding position according to Cambridge Reference Sequence. Primers adapted from Ramos et. al. [26] with adjusted annealing temperatures.

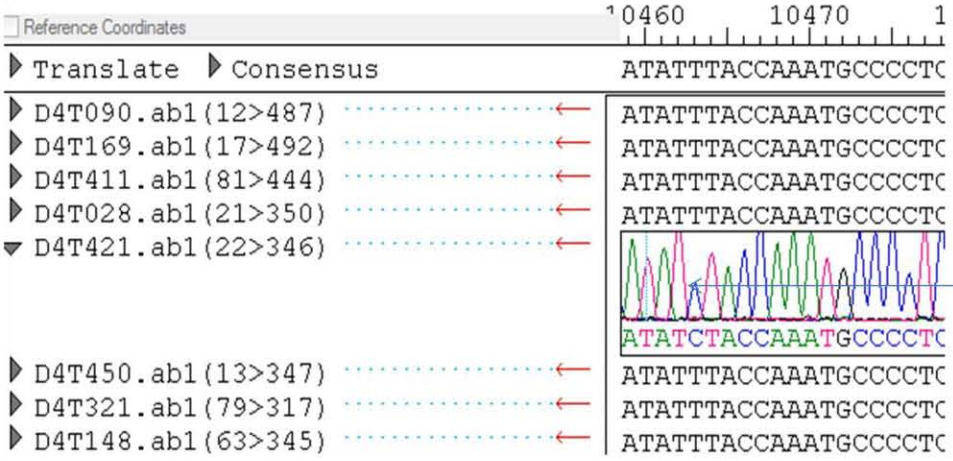
Appendix B: Table of forward and reverse sequencing primers for mtDNA coding region

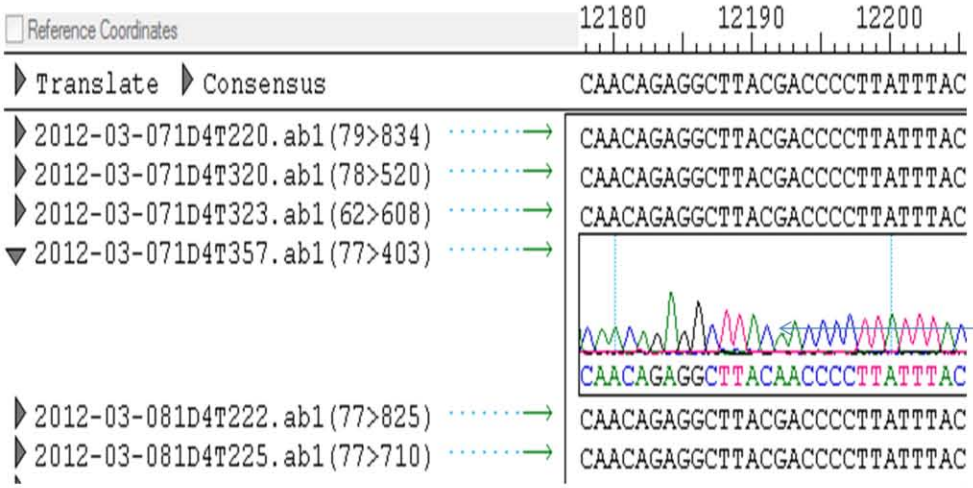
Primer Name	Sequencing Primer (5' - 3')	Annealing temperature (^{0}C)	Confirmatory Primer Name	Sequencing Primer (3' –5')
411for	CGGTATGCACTTTTAACAGTC	50	1159rev	TAAGCTGTGGCTCGTAGTGT
1404for	ACTTAAGGGTCGAAGGTGGATT	50	1677rev	GTTTAGCTCAGAGCGGTCAAGT
2028for	GATAGAATCTTAGTTCAACTT	50	2801rev	TAATGCAGGTTTGGTAGTTTA
2646for	GGTTCAGCTGTCTCTTACTTT	50	3382rev	TTCGTTCGGTAAGCATTAGGA
3239for	GCAGAGCCCGGTAATCGCATA	50	3947rev	TCGATGTTGAAGCCTGAGACTA
4346for	GAACCCATCCCTGAGAATCCA	50	5571rev	AAGTATTGCAACTTACTGAGG
4896for	TACCAAATCTCTCCCTCACTA	50	5571rev	AAGTATTGCAACTTACTGAGG
5468for	CACGCTACTCCTACCTATCTC	50	6154rev	GGAACTAGTCAGTTGCCAAAG
6520for	CTGCTGGCATCACTATACTACTA	50	7359rev	CTACTATTAGGACTTTTCGCT
7111for	ACACCCTAGACCAAACCTACG	55	8000rev	CAACGTCAAGGAGTCGCAGGT
7713for	TCCTAACACTCACAACAAAAC	55	8600rev	AGAATGATCAGTACTGCGGCG
8910for	CTTACCACAAGGCACACCTACA	50	9647rev	AGCTCAGGTGATTGATACTCC
9393for	CGAGAAAGCACATACCAAGGC	50	10154rev	TTCTATGTAGCCGTTGAGTTG
9874for	TAATATTTCACTTTACATCCA	50	10648rev	GGCACAATATTGGCTAAGAGGG
10360for	GTCTGGCCTATGAGTGACTACA	50	11163rev	CGGGTGATGATAGCCAAGGTG
10892for	ATCAACAACAACCTATTTAGC	50	11673rev	GTTTGGATGAGAATGGCTGTT
11461for	ACTCTTAAAACTAGGCGGCTA	50	12226rev	CAGTTCTTGTGAGCTTTCTCGG
11977for	CTCCCTCTACATATTTACCACAAC	50	12763rev	CGATGAACAGTTGGAATAGGT
12500for	TGTGCCTAGACCAAGAAGTTA	50	13297rev	GGTTGATGCCGATTGTAACTA
12988for	CTAGCAGCAGCAGGCAAATCA	50	13830rev	AAGTCCTAGGAAAGTGACAGCGA
13477for	GCAGGAATACCTTTCCTCACAG	50	14325REV	AACTTTAATAGTGTAGGAAGC
13950for	CTATCTAGGCCTTCTTACGAG	50	14838rev	CATCATGCGGAGATGTTGGAT
14898for	TAGCCATGCACTACTCACCAGA	50	15825rev	GTGAAGTATAGTACGGATGCT

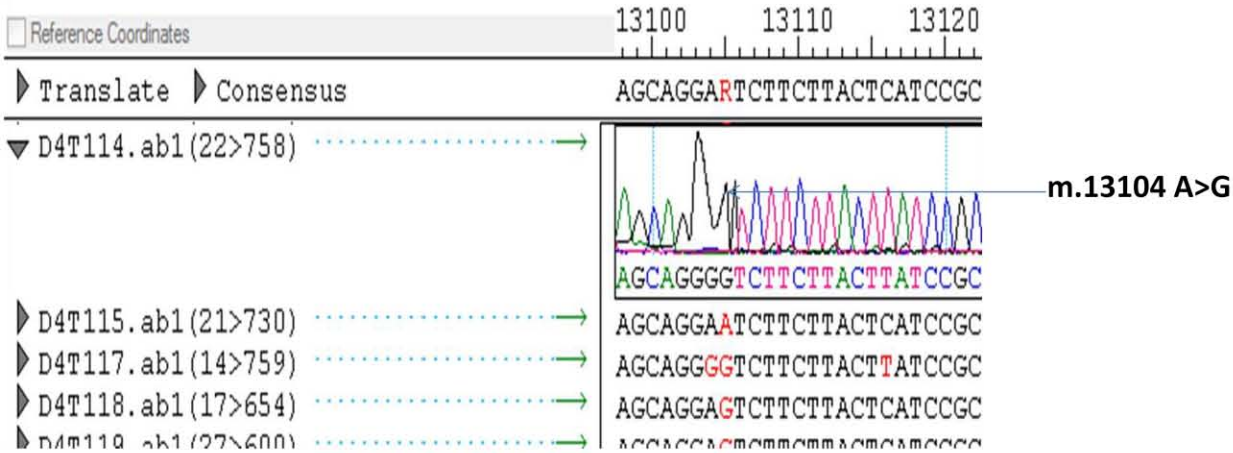
Appendix C: Examples of novel mutations and mutations not previously reported in African populations

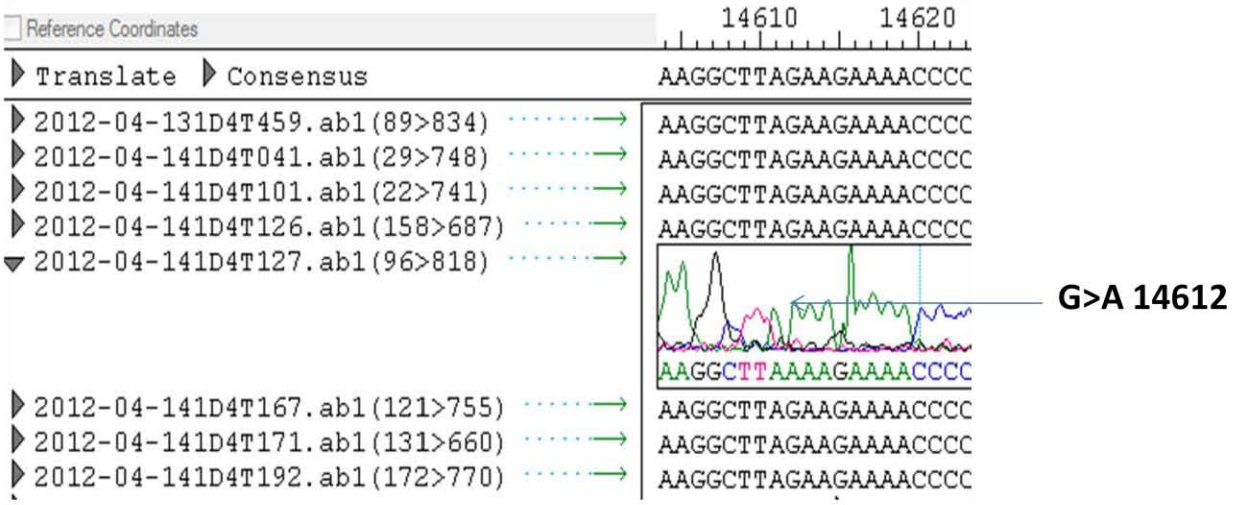


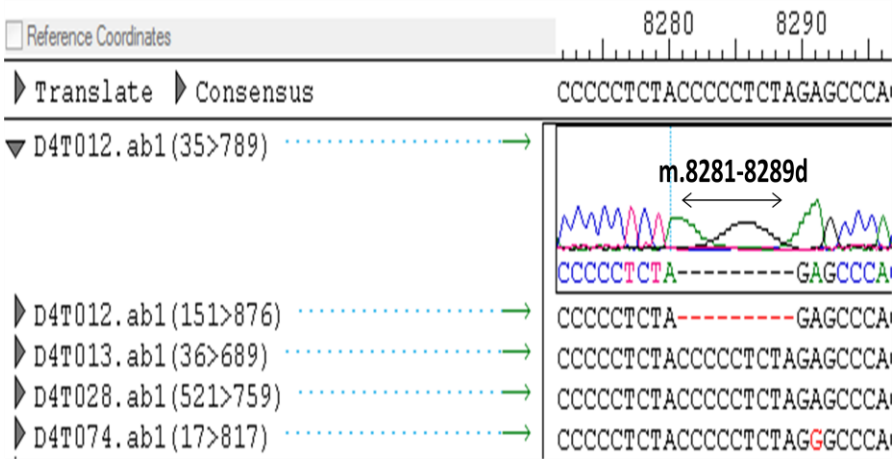

Reference Coordinates
5090
5100
Translate
Consensus
TTAACTATTTATATTATCCTAACT
D4T121.ab1(120>408)
D4T326.ab1(119>873)
D4T052.ab1(122>400)
D4T217.ab1(126>810)
D4T226.ab1(119>797)
D4T411.ab1(122>874)
D4T330.ab1(127>726)
D4T247.ab1(116>792)
D4T415.ab1(118>404)
m.5090 T>C


Reference Coordinates
10460
10470
Translate
Consensus
ATATTTACCAAATGCCCCTC
D4T090.ab1(12>487)
D4T169.ab1(17>492)
D4T411.ab1(81>444)
D4T028.ab1(21>350)
D4T421.ab1(22>346)
D4T450.ab1(13>347)
D4T321.ab1(79>317)
D4T148.ab1(63>345)
m.10463 T>C


Reference Coordinates
12180
12190
12200
Translate
Consensus
CAACAGAGGCTTACGACCCCTTATTTAC
2012-03-071D4T220.ab1(79>834)
2012-03-071D4T320.ab1(78>520)
2012-03-071D4T323.ab1(62>608)
2012-03-071D4T357.ab1(77>403)
2012-03-081D4T222.ab1(77>825)
2012-03-081D4T225.ab1(77>710)
m.12192 G>A

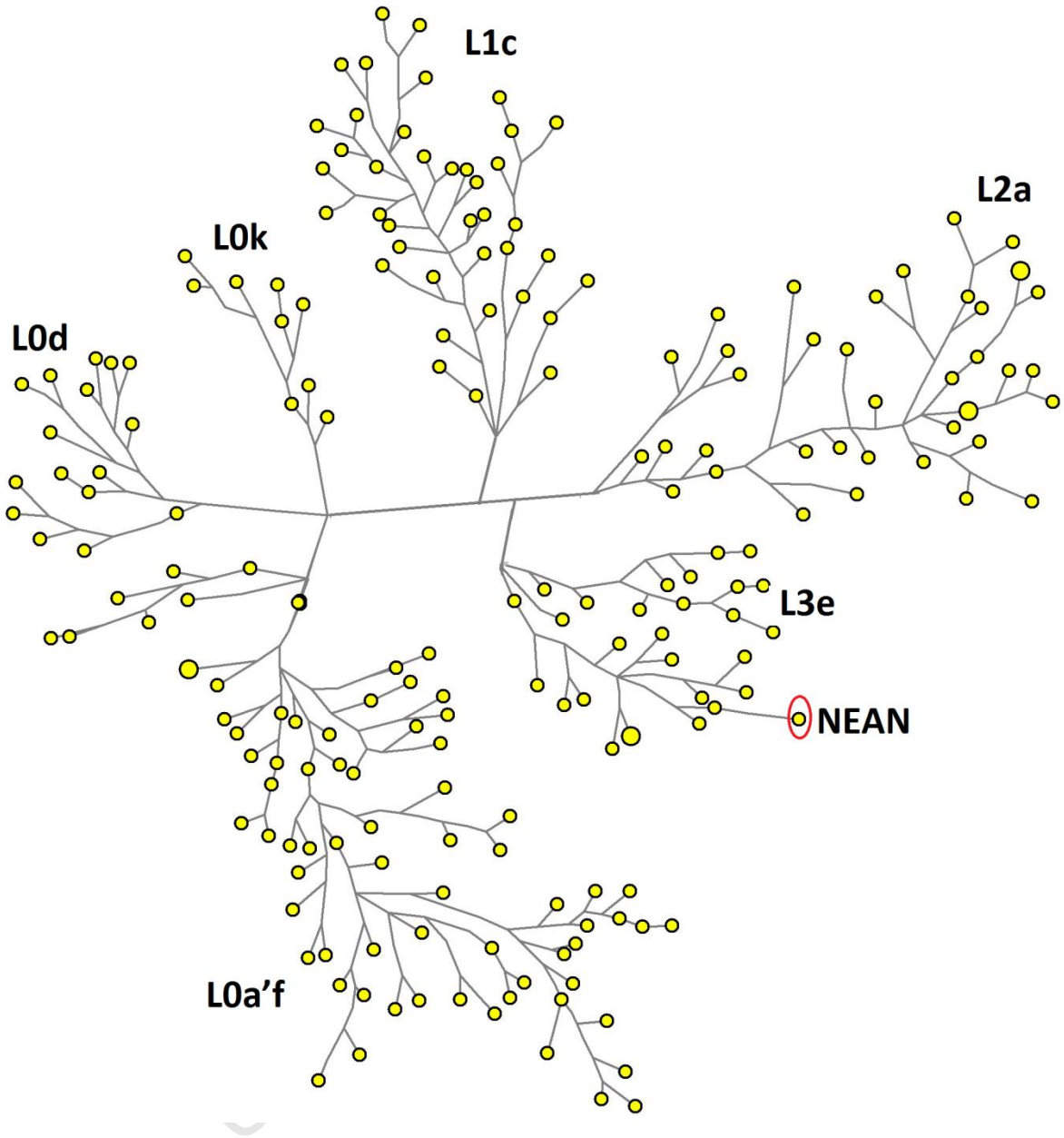

Reference Coordinates
13100
13110
13120
Translate
Consensus
AGCAGGARTCTTCTTACTCATCCGC
D4T114.ab1(22>758)
AGCAGGGGTCTTCTTACTTATCCGC
m.13104 A>G
D4T115.ab1(21>730)
AGCAGGAATCTTCTTACTCATCCGC
D4T117.ab1(14>759)
AGCAGGGGTCTTCTTACTTATCCGC
D4T118.ab1(17>654)
AGCAGGAGTCTTCTTACTCATCCGC


Reference Coordinates
14610
14620
Translate
Consensus
AAGGCTTAGAAGAAAACCCC
2012-04-131D4T459.ab1(89>834)
2012-04-141D4T041.ab1(29>748)
2012-04-141D4T101.ab1(22>741)
2012-04-141D4T126.ab1(158>687)
2012-04-141D4T127.ab1(96>818)
AAGGCTTAAAAGAAAACCCC
G>A 14612
2012-04-141D4T167.ab1(121>755)
2012-04-141D4T171.ab1(131>660)
2012-04-141D4T192.ab1(172>770)



Sequence showing known 9 base pair deletion on COX II gene

m.3579 A>G (m.3606 A>G, m.5090 T>C , m.647 A>G, m.10463 T>C, m.12192 G>A and m.13104 A>G have been reported in other populations of non Africans while known mutations while m.14612 G>A is reported for the first time.



Appendix D: Phylogenetic tree

Median joining network representing L subhaplogroups of the 7 ethnic groups of Malawi.
The tree was rooted with Neanderthal (NEAN) Genebank NC_011137 [75]. Circles represent haplotypes.
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Appendix E: List of haplotypes and haplogroups in 215 individuals from 7 Malawi ethnic groups

L0a1a A2245G C3594T T4586C T5096C C5603T T6185C C7256T C9042T G10589A C10664T G10688A T10810C T10915C G11176A G11914A G12007A T14308C

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Appendix F: Ethical approval College of Medicine (COMREC)

UNIVERSITY OF MALAWI

Principal
Prof. R.L. Broadhead, MBBS, FRCP, FRCPCH, DCH

Our Ref.:
Your Ref.: P.02/10/861

College of Medicine
Private Bag 360
Chichiri
Blantyre 3
Malawi
Telephone: 07 246
07 291
Fax: 64 700
Telex: 43744

10th May 2010

Mrs. E. Kampira
College of Medicine
P/Bag 360
Chichiri
Blantyre 3

Dear Mrs Kampira

RE: P.02/10/861 – The Role of Pharmacogenetics in Patients' Response to Antiretroviral Drugs (Efavirenz, Stavudine and Lopinavir) among Malawians

I write to inform you that COMREC reviewed your proposal mentioned above which you resubmitted. I am pleased to inform you that your proposal was approved after considering that you addressed all the queries which were raised in an earlier review.

As you proceed with the implementation of your study we would like you to take note that all requirements by the college are followed as indicated on the attached page.

Yours Sincerely,

Dr. S.Kamiza
For: CHAIRMAN - COMREC
/Kkk

Appendix G: Ethical approval University of Cape Town