PLASMA CYTOCHROME C LEVEL AS BIOMARKER FOR MONITORING ART-INDUCED MITOCHONDRIAL TOXICITY

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JULY, 2016
DECLARATION

I, Eric Akpanja Mensah, do hereby declare that except for references to other people’s work, for which I have acknowledged, this thesis/dissertation is the product of my own research carried out at the Department of Biochemistry, Cell and Molecular Biology and Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, under the supervision of Drs. Augustine Ocloo, Bismark Sarfo and Evelyn Y. Bonney.

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DEDICATION

This thesis is dedicated to the Almighty God for his protection, favour and strength.
ACKNOWLEDGMENT

I am very grateful to God for seeing me through this course and for all the good things he has done for me.

I would like to say a very big thank you to Dr. Augustine Ocloo who has always been the brain behind this work, and without him this work wouldn’t have been completed.

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# TABLE OF CONTENT

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>i</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENT</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LISTS OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS AND ACRONYMS</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiii</td>
</tr>
<tr>
<td>CHAPTER ONE</td>
<td>1</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Background</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Problem Statement</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Justification</td>
<td>4</td>
</tr>
<tr>
<td>1.4 Hypothesis</td>
<td>5</td>
</tr>
<tr>
<td>1.5 Aim of Study</td>
<td>5</td>
</tr>
<tr>
<td>1.6 Specific Objectives</td>
<td>5</td>
</tr>
<tr>
<td>CHAPTER TWO</td>
<td>7</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>7</td>
</tr>
<tr>
<td>2.1 The Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS)</td>
<td>7</td>
</tr>
<tr>
<td>2.1.1 Brief History</td>
<td>7</td>
</tr>
<tr>
<td>2.1.2 The Human Immunodeficiency Virus</td>
<td>8</td>
</tr>
<tr>
<td>2.1.3 Immunology of HIV Infection</td>
<td>11</td>
</tr>
<tr>
<td>2.1.4 Mechanism of HIV infection</td>
<td>13</td>
</tr>
<tr>
<td>2.2 Antiretroviral Therapy (ART)</td>
<td>14</td>
</tr>
<tr>
<td>2.3 The Mitochondrion: Structure and Function</td>
<td>22</td>
</tr>
<tr>
<td>2.3.1 Structure</td>
<td>22</td>
</tr>
<tr>
<td>2.3.2 Function</td>
<td>24</td>
</tr>
</tbody>
</table>
2.4 Mitochondria and Programmed Cell Death (Apoptosis) ............................................................ 27

2.5 The Role of Mitochondria in Drug-Induced Toxicity ................................................................ 30
  2.5.1 ART-induced toxicity ............................................................................................................. 31

2.6 Cytochrome c as Biomarker for ART-Induced Mitochondrial Toxicity ..................................... 34

CHAPTER THREE ............................................................................................................................ 37

MATERIALS AND METHODS ........................................................................................................ 37

3.1 Materials ........................................................................................................................................ 37

3.2 Methods ......................................................................................................................................... 37
  3.2.1 Study sites .............................................................................................................................. 37
  3.2.2 Study population .................................................................................................................. 37
  3.2.3 Ethical clearance .................................................................................................................. 38
  3.2.4 Extraction of patients’ data ................................................................................................. 38
  3.2.5 Sample collection ................................................................................................................ 38
  3.2.6 Plasma and serum preparation ............................................................................................. 38
  3.2.7 Screening for symptoms of ART-induced toxicity ............................................................. 39
  3.2.8 Plasma Cyt-c assay .............................................................................................................. 39
  3.2.9 Statistical analysis ............................................................................................................... 41

CHAPTER FOUR .............................................................................................................................. 42

RESULTS ......................................................................................................................................... 42

4.1 Demographic Characteristics of the Study Participants .............................................................. 42

4.2 Clinical Stages of HIV Infection in the Study Participants .......................................................... 44

4.3 Duration of HIV Infection and Duration of ART ....................................................................... 44

4.4 Levels of CD4+ T Cell Counts in the Study Participants .............................................................. 46

4.5 Antiretroviral Drug Combinations Among Study Participants .................................................... 46

4.6 Classification of Study Participants based on Duration of ART .................................................. 48

4.7 Results of Screening of Participants for Symptoms of ART Toxicity ......................................... 49
  4.7.1 Levels of serum creatine kinase ....................................................................................... 50

4.7.2 Levels of serum amylase ...................................................................................................... 51
4.7.3 Levels of serum cholesterol and triglyceride ........................................................... 52
4.8 Incidence of ART-Induced Toxicity in the Study Participants ................................. 53
4.9 Plasma Cyt-c Levels .................................................................................................. 54
  4.9.1 Plasma Cyt-c levels among participants with and without ART-induced toxicity . 54
  4.9.2 Plasma Cyt-c levels among participants with ART-induced toxicity .................. 55
  4.9.3 Plasma Cyt-c levels between participant on ART for less or more than 12 months. 56
4.10 Association Between Plasma Cyt-c and Patients’ Characteristics ............................... 57

CHAPTER FIVE ..................................................................................................................... 60
DISCUSSION, CONCLUSION AND RECOMMENDATIONS ........................................... 60
  5.1 Participant Characteristics .......................................................................................... 60
  5.2 Incidence of ART-Induced Toxicity Among Participants ........................................ 63
  5.3 Plasma Cyt-c Levels ................................................................................................ 65
  5.4 Association Between Plasma Cyt-c and Patients Characteristics ............................ 68
  5.5 Conclusion ............................................................................................................. 68
  5.6 Limitations ............................................................................................................ 69
  5.7 Recommendations ................................................................................................ 69

REFERENCES ................................................................................................................... 70
APPENDICES .................................................................................................................. 80
Appendix I: Patient Consent Form .................................................................................. 80
Appendix II: Abstraction Questionnaire ......................................................................... 84
Appendix III: Cyt-c ELISA Reagents Preparation ............................................................... 91
LIST OF TABLES

Table 2.1: WHO Recommended antiretroviral drug combinations .......................................... 16
Table 4.1: Demographic characteristics of study participants .................................................. 43
Table 4.2: WHO Clinical Stage of participants .................................................................... 45
Table 4.3: Duration of HIV infection and ART ..................................................................... 45
Table 4.4: CD4+ T cell counts of participants ..................................................................... 47
Table 4.5: Antiretroviral drug combinations among study participants .............................. 47
Table 4.6: Participants on ART for more and less than 12 months ..................................... 49
Table 4.7: Participants screened for ART toxicity ................................................................. 50
LISTS OF FIGURES

Fig. 2.1: HIV Structure and Mechanism of infection ............................................................. 10
Fig. 2.2: HIV/AIDS disease progression ............................................................................. 12
Fig. 2.3: Nucleoside/tide analogue reverse transcriptase inhibitors ...................................... 17
Fig. 2.4: Non-nucleoside analogue reverse transcriptase inhibitors ..................................... 18
Fig. 2.5: Protease inhibitors ............................................................................................... 19
Fig. 2.6: Structure of Bevirimat, an HIV maturation inhibitor ............................................. 21
Fig. 2.7: First and second-line antiretroviral drug combinations recommended by WHO ..... 21
Fig. 2.8: Mitochondrion Structure ..................................................................................... 23
Fig. 2.9: Schematic view of the Electron Transport Chain ................................................... 23
Fig. 2.10: Mechanism of apoptosis ..................................................................................... 29
Fig. 2.11: Oxidative stress and subsequent cell death induced by AZT, Dox and cisplatin .... 31
Fig. 2.12: Enzymatic activation of nucleoside/tide reverse transcriptase inhibitors .......... 34
Fig. 2.13: Structure of Cyt-c showing the haem group in the middle .................................... 35
Fig. 3.1: Serial dilution of 10 ng/ml standard Cyt-c on microwell plate ................................. 41
Fig. 4.1: Serum levels of creatine kinase among participants screened ............................... 51
Fig. 4.2: Serum levels of amylase among participants screened ........................................... 52
Fig. 4.3: Serum levels of cholesterol and triglyceride among participants screened .......... 53
Fig. 4.4: ART toxicity among the 60 participants screened .................................................. 54
Fig. 4.5: Plasma Cyt-c levels in participants with and without symptoms of ART toxicity .... 55
Fig. 4.6: Plasma Cyt-c levels among participants with ART toxicity ................................... 56
Fig. 4.7: Plasma Cyt-c level between participants on ART for less or more than 12 months ... 57
Fig. 4.8: Relationship between plasma Cyt-c and duration of ART ..................................... 58
Fig. 4.9: Relationship between plasma Cyt-c and duration of HIV infection ....................... 58
Fig. 4.10: Association between plasma Cyt-c and age of study participants ....................... 59
Fig. 4.11: Association between plasma Cyt-c and CD4 count of participants ....................... 59
LIST OF ABBREVIATIONS AND ACRONYMS

3TC: Lamivudine
ABC: Abacavir
ADP: Adenosine diphosphate
AIDS: Acquired Immunodeficiency Syndrome
AIF: Apoptosis-inducing factor
AMP: Adenosine monophosphate
ANT: Adenine nucleotide translocator
APAF-1: Apoptosis protease-activating factor 1
APV: Amprenavir
ART: Antiretroviral therapy
ATP: Adenosine triphosphate
ATPase: Adenosine triphosphate synthase
ATZ: Atazanavir
AZT: Zidovudine
CCCP: Carbonyl cyanide m-chlorophenylhydrazone
CCR5: C-C Chemokine receptor 5
CD4: Cluster of differentiation 4
CK: Creatine kinase
CO₂: Carbon dioxide
CoA: Coenzyme A
CoQ: Ubiquinone
CoQH₂: Ubiquinol
COX: Cytochrome c oxidase
CXCR4: Chemokine receptor type 4
Cyt-c: Cytochrome c
d4T: Stavudine
ddC: Zalcitabine
ddi: Didanosine
DLV: Delavirdine
DNP: 2,4-dinitrophenol
DRV: Darunavir
EFV: Efavirenz
ETC: Electron Transport Chain
ETV: Etravirine
FADH$_2$: Flavin adenine dinucleotide (reduced)
FCCP: carbonyl cyanide $p$-trifluoromethoxy-phenylhydrazone
FeS: Iron Sulphur
FI: Fusion inhibitor
FMN: Flavin mononucleotide
FPV: Fosamprenavir
FTC: Emtricitabine
gp: Glycoprotein
Gpx 1: Glutathione peroxidase-1
Gpx 4: Glutathione peroxidase-4
GSH: glutathione
H$_2$O: Water
H$_2$O$_2$: Hydrogen peroxide
HAART: Highly Active Antiretroviral therapy
HIV: Human Immunodeficiency Virus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>IDV</td>
<td>Indinavir</td>
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<tr>
<td>II</td>
<td>Integrase inhibitor</td>
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<tr>
<td>kD</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LPV</td>
<td>Lopinavir</td>
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<tr>
<td>MI</td>
<td>Maturation inhibitor</td>
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<tr>
<td>MMP</td>
<td>Mitochondrial Membrane Permeability</td>
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<td>MnSOD</td>
<td>Manganese Superoxide Dismutase</td>
</tr>
<tr>
<td>mt</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide (oxidized)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NFV</td>
<td>Nelfinavir</td>
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<tr>
<td>NMIMR</td>
<td>Noguchi Memorial Institute for Medical Research</td>
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<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NVP</td>
<td>Nevirapine</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide ion</td>
</tr>
<tr>
<td>OH⁻</td>
<td>Hydroxide ion</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate Dehydrogenase</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>Prx3</td>
<td>Peroxiredoxin-3</td>
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<tr>
<td>PTP</td>
<td>Permeability Transition Pores</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RPV</td>
<td>Rilpivirine</td>
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<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
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<tr>
<td>RTV</td>
<td>Ritonavir</td>
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<tr>
<td>SQV</td>
<td>Saquinavir</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglyceride</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid</td>
</tr>
<tr>
<td>TDF</td>
<td>Tenofovir</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TPV</td>
<td>Tripranavir</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>Zn/CuSOD</td>
<td>Zinc or Copper Superoxide Dismutase</td>
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ABSTRACT

HIV/AIDS is a major public health issue, having killed several millions of people, with sub-Saharan Africa mostly affected. Management of HIV infection, over the past decades, has been carried out using antiretroviral (ARV) drugs. Although antiretroviral therapy (ART) has decreased morbidity and mortality from HIV, long term therapy is associated with toxicity and severe side effects. These side effects have been attributed in part to ART-induced mitochondrial toxicity due to mitochondrial enzyme inhibition and induction of apoptosis. Diagnosing ART toxicity is, however, challenging as there is no standard test due to absence of reliable diagnostic biomarker. Cytochrome c (Cyt-c), a pro-apoptotic protein, has been suggested as a potential biomarker for monitoring ART-induced toxicity in a pilot study in African-Americans. However, for plasma Cyt-c to be used as a universal biomarker for ART toxicity, the study must be repeated among different populations. The present study therefore aimed at determining whether plasma Cyt-c levels correlate with duration of ART in Ghanaian HIV patients. Ghanaian HIV patients (n = 80) on ART were recruited and 60 were screened for symptoms of ART toxicity. Plasma levels of Cyt-c were measured. Out of the 60 participants, 11 (18.3%) were found with symptom of myopathy, 12 (20%) with pancreatitis, 21 (35%) with hyperlipidaemia and 36 (60%) with at least one of the symptoms. In general, 60% was observed to show toxicity and 40% to be without toxicity. Concentration of plasma Cyt-c was higher (0.122 ng/ml) in patients with toxicity than in those without toxicity (0.05 ng/ml) though the difference was not statistically significant (p = 0.148). No correlation was found between plasma Cyt-c level and duration of ART, age and CD4 counts. Plasma Cyt-c level could be used as an alternative approach to investigate toxicity associated with ART but this requires further studies.
CHAPTER ONE
INTRODUCTION

1.1 Background

In an attempt to manage human immunodeficiency virus (HIV) infection, multiple classes of antiretroviral drugs which act on different viral targets are used (Anochiplt et al., 2015). The use of multiple antiretroviral drugs, also known as antiretroviral therapy (ART) or highly active antiretroviral therapy (HAART), to control HIV replication has proven to be very successful (Maagaard and Kvale, 2009) by reducing morbidity and mortality due to HIV infection over the past two decades (Anderson et al., 2004).

However, studies have shown that long term use of antiretroviral drugs causes mitochondrial toxicity (Kakuda, 2000), which leads to severe side effects resulting in several clinical manifestations (Langs-Barlow et al., 2015) such as pancreatitis, insulin resistance, liver failure, lipodystrophy and lactic acidosis (Anderson et al., 2004). Furthermore, many of these clinical manifestations have been reported to resemble those of mitochondrial (mt) diseases, with histological evidence revealing abnormal mitochondria and/or reduction in mitochondrial DNA content (Anderson et al., 2004). Mitochondrial toxicity, otherwise referred to as mitochondrial dysfunction, is associated with symptoms such as myopathy, peripheral neuropathy, pancreatitis, hyperlipidaemia, lipodystrophy and lactic acidosis (Dykens and Will, 2007; Hamilton et al., 2008). Mitochondrial changes have also been associated with insulin resistance and diabetes in HIV patients (Bissuel et al., 1994; Fleischman et al., 2007).

The mechanism underlying ART-induced mitochondrial toxicity includes competitive inhibition of polymerase gamma, an important enzyme responsible for mitochondrial DNA
synthesis. Nucleoside-/nucleotide-analogue reverse transcriptase inhibitors (NRTIs/NtRTIs) are the major antiretroviral agents that target mitochondrial polymerase gamma (Kohler and Lewis, 2007; Dykens and Will, 2007). This is because of the ability of the phosphorylated forms of NRTIs to accumulate in the mitochondrial matrix due to their high affinity for mitochondrial nucleotide carrier proteins (Dolce et al., 2001). In addition, mitochondrial compartments have several kinases that phosphorylate NRTIs into toxic triphosphate forms (Anderson et al., 2004).

Inhibition of mitochondrial polymerase gamma results in reduction in mtDNA content, reduction in electron transport chain (ETC) proteins, reduction in ATP production, increased oxidative stress, mitochondrial DNA mutation, mitochondrial failure, reduced efficiency of cellular metabolism, tissue failure and hence toxicity (Apostolova et al., 2011; Maagaard and Kvale, 2009; Langs-Barlow et al., 2015; Anderson et al., 2004). NRTIs such as zidovudine (AZT) directly promotes oxidative stress, inhibit electron transport chain and also inhibit several other mitochondrial enzymes (Apostolova et al., 2011). In vitro studies have also revealed ART-induced impairment of mitochondrial adenylate kinase, an enzyme responsible for intracellular energy homeostasis [ATP + AMP = 2ADP] (Kakuda, 2000). There is also the impairment of adenosine diphosphate/adenosine triphosphate translocator by these nucleoside inhibitors (Kakuda, 2000). Outside the mitochondria, NRTIs impair nuclear DNA repair by inhibiting DNA polymerase beta (Kakuda, 2000).

A direct relationship exists between HIV infection and mitochondrial dysfunction (Cossarizza et al., 2011). Some HIV gene products such as Tat, Env and protease interact with pro-apoptotic proteins to induce mitochondrial membrane permeability (Maagaard and Kvale, 2009). This consequently results in cytochrome c (Cyt-c) release and destruction of
mitochondrial membrane potential which affect mitochondrial function. Also, patients with advanced HIV disease (Acquired Immunodeficiency Syndrome - AIDS) often have overexpression of cytokines, elevated cell activation, increased nucleic acid synthesis and up-regulated production of kinases that phosphorylate NRTIs (Anderson et al., 2004).

1.2 Problem Statement

Although HAART has significantly reduced mortality due to HIV infection, persistent morbidity due to the development of chronic ART-associated complications and toxicities such as diabetes, myopathy, lipodystrophy and liver dysfunction in HIV patients is a major global concern (Dyken and Will, 2007). These complications, however, are attributed to a number of factors such as chronic immune activation and inflammation, toxicity due to ART and inadequate clinical and immunological monitoring of patients (Anderson et al., 2004; Cossarizza et al., 2011).

In spite of the revelation of the role of mitochondrial toxicity in HIV and HAART complications for several years, not only is the mechanism underlying the pathophysiology of these complications poorly understood, but there is also lack of simple and reliable biomarkers for monitoring ART-related toxicity. In addition, current clinical approach for diagnosing or assessing ART-induced mitochondrial toxicity, which combines clinical signs, laboratory analysis and imaging examinations for investigating specific tissue dysfunction, is expensive and often misleading (Langs-Barlow et al., 2015).

In addition to lack of biomarkers for ART toxicity monitoring, there is also inadequate data on ART-induced mitochondrial toxicity among Ghanaian HIV patients. Most Ghanaian HIV patients are not monitored for toxicity that comes with ART. There is therefore minimum
clinical or laboratory evidence revealing symptoms of ART-induced mitochondrial toxicity in this population.

A strong positive correlation between plasma Cyt-c levels and duration of ART was recently observed in a pilot study in predominantly African-American HIV patients on ART (Langs-Barlow et al., 2015). The study led to the proposal that plasma Cyt-c could be a potential biological marker to monitor ART-induced mitochondrial toxicity and to guide treatment decisions during therapy.

1.3 Justification

Side effects and toxicity have posed a threat to positive contribution of ART to the effort at managing HIV infection and AIDS. The search for remedy to drug induced mitochondrial toxicity has been widely reported (Hamilton et al., 2008). Plasma level of Cyt-c for investigating ART-related mitochondrial toxicity is gradually gaining popularity due to its strong association with apoptosis. Interestingly, assaying this biomarker is simple, direct and cost effective as compared to current clinical approach for investigating toxicity (Langs-Barlow et al., 2015).

A better understanding of the mechanism behind mitochondrial toxicity in patients on ART is very relevant for future management of HIV infection especially in Sub-Saharan Africa. Nonetheless, early detection of mitochondrial toxicity in HIV patients on ART, especially NRTIs and PIs, could prevent the development of chronic complications such as diabetes and liver failure. Obtaining a simple, direct and cost effective biomarker for proper monitoring of patients on ART will inform treatment decisions and also help prevent the side effects associated with ART.
A pilot study by Langs-Barlow et al., (2015), which was the first study to assess plasma level of Cyt-c as a biological marker of ART-induced mitochondrial toxicity, was conducted in a population which was predominantly African-American. However, in order to establish plasma Cyt-c level as a universal biological marker for assessing ART-induced mitochondrial toxicity, it is imperative that diverse clinical studies on plasma Cyt-c in HIV patients on ART are carried out across different populations especially in Sub-Saharan Africa where HIV/AIDS is most prevalent.

1.4 Hypothesis
Plasma Cyt-c level in HIV patients with ART-induced mitochondrial toxicity is higher than their counterparts without ART-induced mitochondrial toxicity.

1.5 Aim of Study
The overall objective is to determine whether plasma Cyt-c levels correlate with duration of ART in Ghanaian HIV patients.

1.6 Specific Objectives
- To screen Ghanaian HIV patients on ART for symptoms of mitochondrial toxicity (pancreatitis, myopathy and hyperlipidaemia)
- To compare plasma Cyt-c levels between patients with symptoms of mitochondrial toxicity and patients without toxicity
- To compare plasma Cyt-c levels between patients on ART for less than twelve (12) months and patients on ART for more than 12 months
- To determine the relationship between plasma Cyt-c levels and duration of ART in Ghanaian HIV patients
- To determine the association between certain patients characteristics (age, CD4 count and duration of HIV infection) and plasma Cyt-c concentrations
CHAPTER TWO
LITERATURE REVIEW

2.1 The Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS)

2.1.1 Brief History

The Human immunodeficiency virus (HIV) is the pathogen responsible for the acquired immunodeficiency syndrome (AIDS) disease (Fanales-Belasio et al., 2010; Anochie et al., 2015). This disease has become a global public health issue over the past two decades, and has claimed the lives of millions of people across the globe (Girard, 2006). According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), HIV has so far killed more than 34 million people worldwide, and about 36.7 million people were found to be living with the disease at the end of 2015 (UNAIDS, 2015). Sub-Saharan Africa is known to be mostly affected with HIV infection and it accounts for about 70% of the global total HIV infections (UNAIDS, 2015).

HIV infection was first recognized in 1959, when blood samples collected from the Democratic Republic of Congo were found to contain the virus (Pence, 2008; Zhu et al., 1998). Subsequently in 1978, some gays in the United States of America developed Kaposí sarcoma, Pneumocystis carinii pneumonia and other AIDS symptoms, and this disease condition became known as a ‘gay-related infectious disease (Pence, 2008).

In 1983, Luc Montagnier, a researcher at Pasteur Institute in France, published in his paper a novel retrovirus which he isolated from a non-AIDS patient with enlarged lymph nodes (Gallo, 2015). Later in 1984, Robert Gallo, a scientist at the National Cancer Institute in the
US, officially reported novel viruses, which were isolated from AIDS or pre-AIDS patients, as the causative agent of AIDS (Gallo, 2015). These novel viruses were named HIV. Gallo and colleagues subsequently developed the antibody diagnostic test for HIV detection (Gallo, 2015). Between 1985 and 1986, a group of dedicated scientist tested several compounds for anti-HIV activity in Robert Gallo’s laboratory, where the virus was isolated. Zidovudine (AZT) was found to inhibit HIV reverse transcriptase, and that ignited the search for other potential inhibitors of the virus (Gallo, 2015).

2.1.2 The Human Immunodeficiency Virus

HIV is a member of the genus *Lentivirus* and belongs to the family *Retroviridae* (Anochie et al., 2015). Current HIV isolates are classified into two types, HIV-type 1 (HIV-1) and HIV-type 2 (HIV-2) (Fanales-Belasio et al., 2010). Though both groups have similar structure, HIV-1 and HIV-2 differ from each other by the differences in the organization of their genome. Aside differences in genome organization, both viruses have the same structural and functional genes just as all members of the retrovirus family. HIV-1 is the worldwide causative agent of AIDS and it is considered to be more virulent than HIV-2 (Pence, 2008). HIV-1 is sub-grouped into HIV-1-M, HIV-1-N and HIV-1-O, with HIV-1-M being the most prevalent (Fischer and Madden, 2011). HIV-2 on the other hand is found only in restricted regions of Western and Central Africa (Pence, 2008). It is less virulent and takes much longer time to progress to AIDS. The viral particle is roughly spherical and has a diameter of around 100 nm (Fanales-Belasio et al., 2010).

The structure of HIV, as shown in Figure 2.1A, reveals the presence of two identical copies of single-stranded positive-sense RNA molecules that constitute the HIV genome, and surrounded by a conical capsid protein p24 (Turner and Summers, 1999). The viral genome,
like all retroviruses, is characterized by the presence of three structural genes: *gag*, *pol* and *env* genes. In addition to these structural genes, HIV-1 and HIV-2 have several other accessory genes such as *vif*, *vpx*, *vpr*, *nef*, *rev*, and *tat* (Turner and Summers, 1999). Each of the single-stranded RNA molecules is tightly bound by nucleocapsid proteins p7. The capsid protein also houses enzymes such as reverse transcriptase and integrase. HIV reverse transcriptase is responsible for reverse transcribing the single stranded HIV RNA molecules into double stranded DNAs, which are then incorporated into the host genome by integrase. Around the capsid is a matrix, made up p17 viral protein, which ensures viral integrity and houses HIV protease responsible for posttranslational modification of expressed viral proteins into functional proteins. Surrounding the matrix is a phospholipid bilayer envelope acquired from host cell when a new viral particle buds from the cell. Embedded in the viral envelop are numerous host and viral proteins/receptors that the virus uses to attach and enter host cells. The most outstanding of these receptors are the heterodimer *Env* glycoprotein complexes called gp160, each of which consist of an outer trimer glycoproteins gp120 and a transmembrane glycoprotein gp41. These two glycoproteins, gp120 and gp41, are coded by the *Env* gene. Embedded proteins which are acquired from the host during budding are the human leucocyte antigen (HLA) class I and II proteins and ICAM-I adhesion proteins, which promote attachment of viral particle to the host cells (Fanales-Belasio et al., 2010).
Fig. 2.1: HIV Structure and Mechanism of infection.
(A) Structure of HIV showing the core capsid and its contents, matrix and the phospholipid bilayer envelope. (B) Two groups of HIV-1: M-trophic and T-trophic HIV and the types of co-receptors required for host entry. (C) Mechanism of HIV infection and replication cycle. (Fanales-Belasio et al., 2010)
2.1.3 Immunology of HIV Infection

HIV targets and weakens the immune system of infected individuals by destroying and impairing the functions of the immune cells (Paranjape, 2005). Thus, patients infected with HIV gradually become immunodeficient and susceptible to infections from other pathogens. HIV infects mainly CD4 T-cells of the immune system and causes a progressive reduction in CD4 T-cell count (Badley et al., 2000). CD4 T-cells are a group of T-cells that upon activation produce numerous cytokines that activate other immune cells such as B-cells, phagocytes and cytotoxic T-cells to execute the function of the immune system. Destruction of CD4 T-cells, however, results in the disruption of the function of the immune system. Progression of HIV infection can be measured using several parameters such as CD4 count, plasma viral load and plasma HIV RNA levels (Paranjape, 2005).

The acute phase of HIV infection, as shown in Figure 2.2, is characterized by gradual reduction in CD4 T-cells but gradual increase in HIV antibodies, increase in HIV plasma RNA and increase in HIV cytotoxic T lymphocytes (CTL) (Paranjape, 2005). The asymptomatic phase is characterized by high levels of HIV CTL and HIV antibodies which are indications of high viral load. CD4 T-cells level continue to decrease in asymptomatic phase until the onset of AIDS (Paranjape, 2005).

HIV-1 infects CD4+ T-cells, macrophages and dendritic cells through the interaction between the viral gp120 proteins and host CD4 molecules/chemokine receptors (Sluis-cremer, 2014). CD4 molecules act as co-receptors of the major histocompatibility complex II (MHC II) during antigen detection. They are expressed on the surface of numerous immune cells including 60% of circulating T-lymphocytes, T-cell precursors in bone marrow and thymus, monocytes and macrophages, eosinophils, dendritic cell and microglial cells (Fanales-Belasio
et al., 2010). Depending on the type of chemokine receptor required for entry, HIV-1 are classified into groups. Monocyte/Macrophage-tropic (M-tropic) non-syncytium-inducing (NSI) HIV-1 uses the β-chemokine receptor CCR5 and CD4 molecules for entry into host. CCR5 is found on immune cells such as monocytes, macrophages, dendritic cells and activated T-cells (Fanales-Belasio et al., 2010). M-tropic HIV-1 is also referred to as R5 strains and they infect and replicate in macrophages, dendritic cells and CD4\(^+\) T cells. However, T-lymphocyte-tropic (T-tropic) syncytium-inducing (SI) HIV-1 uses the α-chemokine receptor CXCR4 and CD4 molecules for host entry, and are also referred to as X4 strains. T-tropic HIV-1 infects mainly CD4\(^+\) T-cell and not necessarily macrophages (Yi et al., 1999; Fanales-Belasio et al., 2010). Some HIV-1 isolates, called dual tropic or X4R5, are able to interact with both CCR5 and CXCR4 receptors (Yi et al., 1999).

Fig. 2.2: HIV/AIDS disease progression (Paranjape 2005)
2.1.4 Mechanism of HIV infection

Mechanism of HIV infection and replication cycle can be summarized into six steps as shown in Figure 2.1C. The first step involves binding, fusion and entry of the viral particle into the host cell. Binding of the viral gp120 protein with the host surface CD4 molecule leads to a structural or conformational change in the virus gp160 complex and a consequent exposure of a specific domain in gp120 (Hara and Olson, 2002). The exposed gp120 domain binds to one chemokine receptors (CCR5 or CXCR4) of the host cell (Turner and Summers, 1999). This results in a double binding of gp120 to both CD4 and a chemokine receptor, and this strong interaction facilitates the penetration of gp41 into the host cell membrane. Some domain repeats in the gp41 protein form into a hairpin which allows fusion of the virus with the cell membrane of the host and subsequent release of viral capsid into the host cell. The gp120 protein facilitates binding of HIV to host cell whereas gp41 facilitates fusion and entry of the virus with the plasma membrane of the host (Fanales-Belasio et al., 2010).

The second step involves uncoating of the virus capsid and release of the positive sense single-stranded RNA molecules into the cytosol. In the third step, the single-stranded RNA molecule is reverse transcribed into RNA/DNA hybrid double helix by the HIV reverse transcriptase (Sluis-cremer, 2014). The RNA strand of the double helix is degraded by the ribonuclease H site of the HIV reverse transcriptase whilst the polymerase site converts the single stranded viral DNA into a proviral double stranded DNA. In the fourth step, the viral double stranded DNA molecule is transported into the nucleus and incorporated into the host genome by the virus integrase. Upon host cell activation, the proviral DNA is transcribed into messenger RNA molecules which migrate into the cytoplasm for viral protein synthesis. The gag gene codes for the structural proteins p24 of capsid, p7 of nucleocapsid and p17 of matrix. The env gene codes for the gp160 complex protein, whilst the pol gene codes for the
replication enzymes which include reverse transcriptase, integrase and protease (Fanales-Belasio et al., 2010).

Expressed HIV proteins undergo posttranslational modification mainly via proteolysis by the enzyme protease (Turner and Summers, 1999). For example, long chain env glycoprotein precursor is cleaved into gp120 and gp41 by protease. HIV protease also cleaves a large 160 kD precursor protein molecule into p24, p17, p9 and p7. This proteolytic cleavage of nascent proteins leads to the production of functional proteins and the generation of infectious viral particles. Expressed functional replication enzymes are packaged together with two copies of RNA molecules and nucleocapsid p7 and surrounded by the gag proteins to form the core of a maturing HIV particle. In T-lymphocytes, newly formed viral particles move to the cytosolic surface of the host cell membrane and bud through it acquiring a new phospholipid bilayer envelope with gp160 proteins forming spikes in the envelope. In monocytes and macrophages, virions are packed into vacuoles and released into the cytoplasm where they bud from the vacuoles to acquire envelope (Fanales-Belasio et al., 2010).

2.2 Antiretroviral Therapy (ART)

HIV/AIDS has no cure, and currently no vaccine exists for its prevention (Cohen et al., 2007). However, in order to manage or control the rate of HIV infection and replication, multiple classes of antiretroviral drugs that act on different viral targets are used (Anochie et al., 2015). Over the past decades, the use of antiretroviral therapy as a way of managing HIV/AIDS has proven to be very successful by significantly reducing mortality and morbidity caused by HIV (Langs-Barlow et al., 2015; Anderson et al., 2004).
The 2012 UNAIDS reports on access to antiretroviral therapy (ART) in Africa emphasize the need to initiate ART for all adult HIV patients whose CD4 count is below or equal to 500 cells per mm$^3$ (UNAIDS, 2012). The reports also highlight ART initiation in all HIV positive adults with active tuberculosis (TB) and active hepatitis B co-infection and severe liver disorders irrespective of their CD4 counts. The use of ART helps to reduce circulating blood HIV RNA which subsequently prevents sexual transmission of HIV-1 (Cohen et al., 2007). The use of ART to prevent sexual transmission of HIV could be achieved through effective treatment of infected persons, non-occupational post-exposure prophylaxis and pre-exposure prophylaxis (Cohen et al., 2007).

The use of multiple classes of antiretroviral drugs is generally referred to as highly active antiretroviral therapy (HAART) or simply ART (Anochie et al., 2015; Sendi et al., 2001). These drugs suppress HIV replication in immune cells and bring down viral load to undetectable levels. The various classes of antiretroviral drugs used are nucleoside- and nucleotide-analogue reverse transcriptase inhibitors (NRTIs/NtRTIs), non-nucleoside- and nucleotide-analogue reverse transcriptase inhibitors (NNRTIs), Protease inhibitors (PIs), Fusion inhibitors (FI), Integrase inhibitors (II), Entry inhibitors and Maturation inhibitors (MI) (Anochie et al., 2015). Triple combination therapy (Table 2.1) is the standard and the most recommended medication by the World Health Organization. This normally consists of two NRTIs and one inhibitor from the other classes (Sendi et al., 2001).
### Table 2.1: WHO Recommended antiretroviral drug combinations

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<tr>
<th>1st Drug</th>
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Triple drug combination among HIV naïve patients. The first two of the drugs are always NRTIs and the one coming from the other classes.

NRTIs/NtRTIs form the backbone of HAART and they are included in all combinations. This class of drugs functions as competitive inhibitors of HIV reverse transcriptase. They are nucleotide analogues lacking 3'-OH group of the ribose component necessary for chain elongation (Singh et al., 2014). These inhibitors block HIV DNA chain elongation once they are incorporated into the growing chain and are therefore referred to as DNA chain terminators (Singh et al., 2014). Examples of these inhibitors approved for use include Zidovudine (AZT, Azidothymidine), Stavudine (d4T), Emtricitabine (FTC), Zalcitabine (ddC), Didanosine (ddl), Lamivudine (3TC), Abacavir (ABC) and Tenofovir (TDF) (Sendi et al., 2001). The chemical structures of these inhibitors are shown in Figure 2.3.

NNRTIs inhibit HIV-1 reverse transcriptase by binding directly to the enzyme and distorting its RNA/DNA binding capacity (Sluis-cremer, 2014). The aromatic structures of this group of inhibitors bind to specific hydrophobic regions close to the DNA polymerase active site of the enzyme. So long as the inhibitor is bound to the enzyme, the enzyme is unable to bind HIV RNA or DNA molecule due to steric hindrance introduced by the inhibitor. Examples of
these NNRTIs include Nevirapine (NVP), Efavirenz (EFV), Delavirdine (DLV), Etravirine (ETV) and Rilpivirine (RPV), the structures of which are shown in Figure 2.4 (Sendi et al., 2001).

(a) zidovudine (AZT)          (b) didanosine (ddI)          (c) stavudine (d4T)

(d) zalcitabine (ddC)          (e) lamivudine (3TC)

(f) abacavir (ABC)             (g) emtricitabine (FTC)          (h) tenofovir (TDF)

Fig. 2. 3: Nucleoside/tide analogue reverse transcriptase inhibitors. (Singh et al., 2014)
HIV protease inhibitors (PIs) target and suppress the assembly of new viral particles by disrupting posttranslational modification of nascent polyproteins (Mahdi et al., 2015). These inhibitors bind and inhibit HIV protease which is the enzyme responsible for cleavage of \textit{gag} and \textit{gag-pol} polyprotein precursors into functional proteins or enzymes for package into a new virion (Pokorna et al., 2009). The inhibitors bind competitively to the protease binding site and reduce the virus’ ability to bind and cleave polypeptide chains. Viral maturation and subsequently the production of infective viral particles is therefore curtailed (Pokorna et al., 2009). Currently approved HIV protease inhibitors include Nelfinavir (NFV), Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Amprenavir (APV), Lopinavir/ritonavir
(LPV/RTV), Fosamprenavir (FPV), Atazanavir (ATV), Tripranavir (TPV) and Darunavir (DRV) with some of the structures shown in Figure 2.5 (Mahdi et al., 2015; Anochie et al., 2015).

Fig. 2.5: Protease inhibitors (Pokorna et al., 2009).
Fusion inhibitors are a class of antiretroviral drugs that block fusion of HIV particle with the cell membrane of host to prevent capsid release into host cell (Pang et al., 2009). Following gp120-CD4 interaction is a conformational change that allows gp41 to form a pore in the host plasma membrane to facilitate viral fusion. Interaction between two heptad repeat regions in gp41 facilitates the fusion process. Fusion inhibitors, such as Enfuvirtide, bind heptad repeat 1 (HR1) and disrupt it from interacting with heptad repeat 2 (HR2), which then prevents the formation of the hairpin structure of gp41 necessary for fusion (Boyd and Pett, 2008). Several other peptide HIV fusion inhibitors have recently been developed (Pang et al., 2009).

Integrase inhibitors, which include Raltegravir, inhibit HIV integrase and block the incorporation of HIV double-stranded DNA into host genome (Anochie et al., 2015).

Entry inhibitors are designed to bind co-receptors or chemokine receptors, such as CCR5 and CXCR4, of host cells. This prevents subsequent interaction of HIV gp-120 with the host chemokine receptors. This inhibition however blocks the entry of HIV gp-41 protein into host cell membrane, thus, preventing entry of the virus. Examples of HIV entry inhibitors include AMD 3100, SCH-C, PRO 140, T-20, T-1249, BMS 806 and PRO 542 (Hara and Olson, 2002).

Maturation inhibitors block the production of functional HIV capsid protein which houses the viral genome. Expression of gag proteins leads to the production of a polyprotein capsid which needs to be taken through posttranslational modification via proteolytic cleavage. Maturation inhibitors block cleavage of polyprotein capsids, thus, preventing the formation of functional capsids (Anochie et al., 2015). Some maturation inhibitors include Bevirimat (Fig. 2.6) and Vivecom (Martin et al., 2008). Figure 2.7 summarizes WHO’s recommended first- and second-line antiretroviral drug combinations (Gilks et al., 2006).
Fig. 2. 6: Structure of Bevirimat, an HIV maturation inhibitor (Martin et al., 2008)

Fig. 2. 7: First and second-line antiretroviral drug combinations recommended by WHO. (Gilks et al., 2006).
2.3 The Mitochondrion: Structure and Function

2.3.1 Structure

Mitochondria are organelles present in all eukaryotic cells except red blood cells. These organelles are morphologically similar in structure and function between species, but they vary in number from cell to cell (Nunnari and Suomalainen, 2012). They have an oblong or sausage shape and are about 1-10 µm in diameter, which is about the size of a bacterium (Mulchandani, 2010). They are believed to have originated from proteobacteria which were captured by protoeukaryotic cells by endocytosis (Nunnari and Suomalainen, 2012). The serial endosymbiont theory explains the symbiotic relationship and the subsequent loss of redundant genes and the transfer of genes from the endosymbiotic bacteria to the nucleus of the eukaryotic cells (Scheffler, 2000).

The mitochondrion, as shown in Figure 2.8, is bounded by two membrane systems; the outer and inner membranes (Scheffler, 2000). The outer membrane is assumed to be relatively permeable to smaller molecules and ionic species, but the inner membrane is largely impermeable. The space between the two membranes, called intermembrane space, serves as a microenvironment containing several proteins essential in cell physiology, mitochondrial energetics and cell death (Foster et al., 2006). The inner mitochondrial membrane is folded into cristae and contains a variety of mitochondrial membrane bound enzyme systems such as the respiratory or electron transport chain (ETC) and the adenosine triphosphate synthase (ATPase) (Fig. 2.9) (Duchen, 2004b). The ETC and ATPase work together to accomplish one of the major functions of mitochondria, which is ATP production (Nunnari and Suomalainen, 2012; Duchen, 2004a).
Inside mitochondrial matrix are circular DNA molecules made up of 37 genes that code for 22 types of transfer RNAs (tRNA), 2 types of ribosomal RNAs (rRNA) and 13 polypeptide subunits which are components of ETC system (Benkhalifa et al., 2014). Because mitochondria lack several of DNA repair enzymes compared to the nucleus, mitochondrial DNA (mtDNA) is particularly susceptible to mutation (Graziewicz et al., 2006).

**Fig. 2. 8: Mitochondrion Structure**
(A) Structure of mitochondrion as viewed by electron tomography showing the outer membrane (dark shade) and inner membrane (lighter shade). The inner membrane is extended into cristae in the matrix. (B) Schematic drawing of mitochondrion showing general features. (Scheffler, 2000; Foster et al., 2006)

**Fig. 2. 9: Schematic view of the Electron Transport Chain**
The electron transport chain consists of complexes I, II, III, IV and ATP synthase found in the inner mitochondrial membrane (Duchen, 2004b).
2.3.2 Function

Mitochondria are generally described as the ‘powerhouses’ of the cell (Pagliarini and Rutter, 2013). They produce energy in the form of ATP which is essential in almost all metabolic activities. Cellular metabolic activities are completely shuttered when ATP supply is curtailed. This ultimately results in organ failure and subsequent death of the organism. Aside ATP production, several other metabolic reactions also take place in the mitochondria. These include Tricarboxylic Acid (TCA) Cycle, urea cycle, steroid hormone and porphyrin synthesis, lipid metabolism (β-oxidation), xenobiotic metabolism, amino acid inter-conversions, glucose sensing/insulin regulation and cellular calcium (Ca$^{2+}$) homeostasis (Amaral et al., 2008; Duchen et al., 2008).

Cellular energy production is accomplished through glycolysis, β-oxidation and mitochondrial oxidative phosphorylation (OXPHOS) (Wallace and Fan, 2010). The OXPHOS system consists of the ETC and ATP synthase. Glucose from digested food substances is metabolized in the cytosol of a cell into pyruvate via glycolysis. Pyruvate enters mitochondrion where it is converted by pyruvate dehydrogenase (PDH) to produce acetyl-CoA, carbon dioxide (CO$_2$) and a reducing equivalent NADH + H$^+$.

Fatty acids from food also enter mitochondrion as fatty acyl-CoA and oxidized by β-oxidation to generate acetyl-CoA, NADH + H$^+$ and FADH$_2$. Acetyl-CoA generated from both glucose and fatty acids enters the TCA cycle to generate more reducing equivalents (NADH + H$^+$ and FADH$_2$) for ATP production in the OXPHOS (Wallace and Fan, 2010).

Mitochondrial OXPHOS system is organized into five main complexes (I, II, III, IV and V) as shown in Fig. 2.9. They are assembled from different polypeptide subunits encoded in both nuclear and mitochondrial genes (Wallace and Fan, 2010). The polypeptide components of
complexes I, II, III and IV act as electron carriers to form the electron transport chain (ETC). Complex V is the ATP synthase responsible for adenosine diphosphate (ADP) phosphorylation into adenosine triphosphate (ATP).

Electrons are passed from NADH + H⁺ and FADH₂ to complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) respectively. Complex I has a Flavin mononucleotide (FMN) cofactor which accepts electrons and pass them through a chain of seven Iron Sulphur (FeS) centres to the ubiquinone (CoQ) binding site. Electrons from both complexes are then passed to ubisemiquinone (Coenzyme Q₁₀, CoQ) to form ubiquinol (CoQH₂). Electrons from CoQH₂ are transferred successively to complex III (bc₁ complex) and then to Cyt-c. Cyt-c then carries the electrons to complex IV (Cyt-c oxidase, COX) and finally to oxygen (1/2O₂) to form water (H₂O). The energy released as electrons move down the ETC is used to pump protons across the inner membrane via complex I, III and IV out of the matrix. This leads to a build-up of proton electrochemical gradient (proton motive force) in the mitochondrial intermembrane space which could be used to import proteins and calcium into the mitochondrial matrix, to produce heat and also to synthesize ATP (Wallace and Fan, 2010). Under ideal conditions protons pumped into the intermembrane space are pumped back into the matrix via ATP synthase to produce a drive for ADP phosphorylation into ATP.

Studies have shown that not all protons pumped into the intermembrane space via ETC are pumped back into the matrix via ATP synthase. That is, ETC and ATP synthase are not 100% coupled. A phenomenon called proton leak describes proton transfer from mitochondrial intermembrane space into the matrix without passing through ATP synthase. Proton leak reduces ATP production but helps generate heat and minimizes production of reactive oxygen
species. Uncoupling proteins (UCP-1, -2, -3, -4, -5) and adenine nucleotide translocator (ANT) are known to leak protons into the matrix (Mailloux et al., 2013). Proton leak can be described as basal when mediated by ANT, and as inducible when mediated by UCPs and other synthetic compounds such as carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP), carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP) (Mailloux et al., 2013).

Mitochondria are sources of much of the endogenous reactive oxygen species (ROS) of the cell (Murphy, 2009). ROS are produced as by-products of OXPHOS. Excessive production of NADH + H⁺ and FADH₂ results in over reduction of the ETC, increased ATP/ADP ratio and high electrochemical potential difference across the inner mitochondrial membrane. Complexes I and III are the initial sites for mitochondrial ROS (superoxide, O₂⁻) production (Murphy, 2009; Foster et al., 2006). In complex I, high NADH/NAD⁺ ratio causes full reduction of FMN cofactor which reacts directly with oxygen (O₂) to produce O₂⁻. Under high CoQH₂/CoQ and significant proton-motive force, electrons are forced back from CoQH₂ to complex I through the CoQ binding site. This leads to further reduction of FMN and its one electron reduction of O₂ to O₂⁻ (Murphy, 2009). Complex III, which is made up of 11 polypeptides, three haems and one FeS center, transfers electrons from CoQH₂ to Cyt-c during the Q-cycle. Inhibition of the Q₁ site leads to the production of O₂⁻ from the reaction between O₂ and ubisemiquinone at the Q₀ site (Foster et al., 2006).

The O₂⁻ from complex I is released into the mitochondrial matrix and converted to hydrogen peroxide (H₂O₂) by the manganese dependent superoxide dismutase (MnSOD), whereas the superoxide generated from complex III is released in the intermembrane space and converted to hydrogen peroxide by zinc or copper dependent superoxide dismutase (Zn/CuSOD).
Hydrogen peroxide can diffuse into the cytosol and nucleus, and can be reduced to hydroxyl radical (OH), which is the most reactive of all the ROS (Fariss et al., 2005). In addition to inhibition of respiratory complexes, inhibition of proton pump into mitochondrial matrix via ATP synthase increase ROS production (Mailloux et al., 2013). Excessive mitochondrial ROS production causes oxidative stress and can cause damage to cellular proteins, lipids and nucleic acids via oxidation, and can ultimately destroy the cell (Mailloux et al., 2013). Endogenous antioxidants play significant role in mopping ROS generated by mitochondria. Peroxiredoxin-3 and -5 (Prx3 and Prx5) and glutathione (GSH)/glutathione peroxidase-1 and -4 (Gpx1 and Gpx4) systems are responsible for H$_2$O$_2$ sequestration and degradation in the mitochondria (Mailloux et al., 2013). Gpx1 degrades matrix H$_2$O$_2$ whilst Gpx4 degrades inter-membrane space H$_2$O$_2$.

Factors that affect mitochondrial function such as aging, infections and antiretroviral drugs, ultimately cause increased cellular levels of ROS (Perez-Manute et al., 2013). HIV-infected patients have been observed to have high levels of tumor necrosis factor (TNFα) which is known to affect mitochondrial function (Perez-Manute et al., 2013). Recent studies have also showed that patients infected with various classes of RNA viruses, such as HIV, human influenza virus and Hepatitis c virus, are more likely to have oxidative stress which could lead to uncontrolled cell death in specific tissues like adipose tissues causing weight loss (Reshi et al., 2014).

### 2.4 Mitochondria and Programmed Cell Death (Apoptosis)

Mitochondria play a central regulatory role in programmed cell death, otherwise called apoptosis (Fig. 2.10). Apoptosis forms an important part of normal growth of an organism (Elmore, 2007; Grossmann, 2002; Majno and Joris, 1995). It is a genetically predetermined
process that eventually leads to the elimination of superfluous, ectopic, damaged or mutated
cells to allow for normal growth and maintenance of tissue homeostasis (Kroemer et al.,
2007; Kroemer and Reed, 2000). Inhibition of apoptosis causes tumour pathogenesis (cancer)
and excessive cell death could also lead to acute and chronic complications such as diabetes
(Hassan et al., 2014).

The mechanism of apoptosis is broadly classified into extrinsic and intrinsic pathways
(Hongmei, 2012). Both pathways lead to the activation of cysteine-dependent aspartate-
directed proteases (caspases) (McIlwain et al., 2013) which cleave specific substrates and
ultimately leads to chromatin condensation, nuclear fragmentation, cell shrinkage and the
exposure of phosphatidylserine on the surface of the plasma membrane. The intrinsic
pathway of apoptosis, also referred to as mitochondrial pathway, occurs via an intracellular
cascade of events in which mitochondria play a very significant role (Hongmei, 2012;
Elmore, 2007). The induction of mitochondrial membrane permeability (MMP) by
intracellular stress (DNA damage, endoplasmic reticulum stress) leads to the release of
proapoptotic factors, specifically Cyt-c from the mitochondrial intermembrane space. Cyt-c
when present in the cytosol, induces the formation of a hydrolytic molecular platform called
apoptosome via the assembly of apoptosis protease-activating factor 1 (APAF-1) and
ATP/dATP (Elmore, 2007; Duchen, 2004a). Apoptosome promotes proteolytic maturation of
caspase-9, which in turn, cleaves and activates effector caspase-3, -6 and -7. MMP alone,
without caspases activation, can cause cell death via several means.

MMP can cause irreversible mitochondrial dysfunction via disruption of mitochondrial
membrane potential and ATP production. Additionally, MMP leads to the release of caspase-
independent death effectors such as apoptosis-inducing factor (AIF) and endonuclease G
(EndoG) into the cytosol (Fulda and Debatin, 2006). AIF and EndoG then translocate into the nucleus where they facilitate DNA fragmentation and chromatin condensation leading to the morphological features of apoptosis. Consequently, most apoptosis in vertebrates occurs mainly via the intrinsic pathway (Green and Kroemer, 2004).

The induction of MMP is via opening of mitochondrial permeability transition pores (mtPTP) which consists of adenine nucleotide translocator (ANT), voltage-dependent anion channel (VDAC) and CPD (Foster et al., 2006). Conditions such as elevated mitochondrial Ca$^{2+}$ and ROS facilitate opening of the mPTP (Duchen et al., 2008). This, inadvertently, causes the impermeable inner mitochondrial membrane to become permeable and allowing exit of mitochondrial contents.

**Fig. 2. 10: Mechanism of apoptosis.**
Left: extrinsic pathway activated by death receptors and dependency receptors on plasma membrane; Right: intrinsic pathway mediated by mitochondrial release of cytochrome c following mitochondrial membrane permeabilization (Kroemer et al., 2007).
2.5 The Role of Mitochondria in Drug-Induced Toxicity

Drugs and many other chemical substances ingested for nutritional and therapeutic effects end up altering mitochondrial function (Beeson et al., 2010). These substances are considered toxic mainly because they target the mitochondria and induce their dysfunction. Mitochondrial dysfunction, also called mitochondrial toxicity, affect cellular metabolism and result in cell death and/or tissue failure. Cell death and subsequent tissue failure may lead to development of chronic complications such as diabetes.

The delicate and highly sensitive nature makes mitochondria a reservoir for several pharmaceutical and environmental chemicals which interfere with mitochondrial bioenergetics (Wallace and Starkov, 2000). These xenobiotics that accumulate in the mitochondrial matrix inhibit and uncouple mitochondrial respiratory chain; inhibit tricarboxylic acid cycle, fatty acid oxidation and mtDNA and mitochondrial protein synthesis resulting in the production of ROS (Meyer et al., 2013). Increased generation of ROS overshadows cellular antioxidant capacity and cause harm to DNA, proteins/enzymes and lipids. Drug-induced interference of mitochondrial energy production and metabolic activities coupled with increased ROS production, as observed in drugs like Adriamycin, cause cell death and tissue dysfunction (Meyer et al., 2013). Cellular drug metabolism leads to the production of very reactive intermediates that can directly reduce oxygen to generate ROS. Chlorpromazine, an antipsychotic drug for treating psychotic disorder, nausea, vomiting, hiccups and others, is converted into an excited compound under photodechlorination which subsequently transfers energy to oxygen to generate superoxide ions (Deavall et al., 2012). Many other drugs such as AZT, Dox (a phsychedelic amphetamine) and Cisplatin (an anti-cancer drug) increase ROS production resulting in mitochondrial dysfunction with clinical
manifestations such as myopathy, cardiotoxicity and ototoxicity respectively (Deavall et al., 2012) (Fig. 2.11).

2.5.1 ART-induced toxicity

In spite of the positive impacts of ART in reducing mortality and morbidity due to HIV, long-term therapy has been found to be associated with severe side effects and development of chronic complications. Clinical manifestations of these complications include lactic acidosis and hyperlactatemia, lipodystrophy, peripheral neuropathy, myopathy, pancreatitis and many others (Langs-Barlow et al., 2015; Anderson et al., 2004). NRTIs are the major class of antiretroviral agents responsible for many of these complications (Anderson et al., 2004).
These complications, however, may occur due to a number of factors such as chronic immune activation and inflammation, toxicity due to ART and inadequate clinical monitoring of HIV patients (Maagaard and Kvale, 2009).

ART-induced toxicity is largely caused by their effect on the mitochondria. Side effects that come with chemotherapy are basically as a result of the impact of the drugs on mitochondrial function. Studies have shown that many antiretroviral drugs, especially NRTIs, directly affect the mitochondria by inhibiting several of its essential enzymes (Kohler and Lewis, 2007). Histological studies on affected tissues, as evidence of NRTI toxicity, show a change in mitochondrial morphology and/or depletion of mitochondrial DNA (Anderson et al., 2004).

NRTIs require intracellular phosphorylation by endogenous kinase into active triphosphate forms for anti-HIV activity as shown in Figure 2.12. Triphosphate forms of NRTIs have high affinity for mitochondrial nucleotide carrier proteins which are responsible for shuttling endogenous nucleotides into mitochondria from the cytosol for mitochondrial DNA synthesis (Dolce et al., 2001). Consequently, activated forms of NRTIs accumulate in the mitochondrial matrix at a faster rate than endogenous nucleotides. NRTIs not only inhibit HIV reverse transcriptase, but also inhibit mitochondrial DNA polymerase gamma, an enzyme responsible for mitochondrial DNA synthesis (Langs-Barlow et al., 2015; Anderson et al., 2004; Maagaard and Kvale, 2009). Competitive inhibition of mitochondrial DNA polymerase gamma by NRTIs leads to disruption of mitochondrial DNA synthesis and reduced mitochondrial DNA content in specific tissues (Kohler and Lewis, 2007). This leads to reduced expression of proteins essential for mitochondrial metabolic activities and hence causing mitochondrial dysfunction/toxicity. Mitochondria’s loss of function affects cell survival and eventually leads to cell death and tissue impairment.
NRTIs also disrupt cellular energy homeostasis. They do so by inhibiting mitochondrial adenylate kinase (Kakuda, 2000), an enzyme responsible for converting excess ATP into ADP in order to balance the energy needs of the cell. By inhibiting adenylate kinase, these inhibitors are able to curtail the supply of ADP and thus halting further synthesis of ATP. These inhibitors also impair ADP/ATP translocator, an enzyme responsible for importing ADP from cytosol into mitochondrial matrix, and ATP from mitochondrial matrix into cytosol (Kakuda, 2000). Apart from mitochondria, NRTIs have been found to disrupt nuclear DNA repair by inhibiting DNA polymerase beta, an enzyme responsible for nuclear DNA repair (Kakuda, 2000).

NRTIs, NNRTIs and PIs negatively influence mitochondrial function in diverse ways. Aside inhibition of polymerase gamma, NRTIs such as zidovudine (AZT) also promotes oxidative stress and inhibits ATP production (Apostolova et al., 2011). Studies on isolated mitochondria revealed inhibition of mitochondrial adenosine nucleotide translocator (ANT) and also inhibition of ETC by AZT (Apostolova et al., 2011). Inhibition of ANT disrupts ADP/ATP shuttle between mitochondria and cytosol. Combined inhibition of ETC and ANT curtail ATP production.

Furthermore, antiretroviral drugs have been found to upsurge apoptosis in specific tissues (Langs-Barlow et al., 2015; Apostolova et al., 2010). Although the precise mechanism has not been fully established, it has been suggested that these drugs cause apoptosis by the intrinsic pathway (Maagaard and Kvale, 2009). That is, by inducing mitochondrial toxicity, these drugs cause the release of Cyt-c from the inter-membrane space into the cytosol after inducing mitochondrial membrane permeability. The presence of Cyt-c in the cytosol initiates the formation of an apoptosis platform (apoptosome) that facilitates the activation of caspase-
9 and subsequent activation of effector caspases-3, -6 and -7 and finally leading to the apoptotic phenotype (Kroemer et al., 2007).

However, not all HIV patients on ART experience toxicity. Some few patients are able to use antiretroviral drugs for a long period of time without clinical signs of toxicity (Bailey and Anderson, 2010).

**Fig. 2. 12: Enzymatic activation of nucleoside/tide reverse transcriptase inhibitors.**

Specific kinases are responsible for converting each agent into triphosphate forms (Anderson et al., 2004). In the first part ZDV is phosphorylated into ZDV monophosphate by thymidine kinases, and then to ZDV diphosphate by thymidylate kinase. The diphosphate is further converted to ZDV triphosphate by yet another kinase. These reactions occur in the cytosol but can also occur in the mitochondria.

### 2.6 Cytochrome c as Biomarker for ART-Induced Mitochondrial Toxicity

Cyt-c is a water soluble haem protein (Fig. 2.13) having a net positive charge (Babbitt et al., 2015) and found predominantly in the mitochondrial intermembrane space (Soltys et al., 2001). This protein is highly conserved and consists of a single polypeptide chain with about 105 amino acids (Soltys et al., 2001). It has a molecular mass of approximately 12 kDa and an isoelectric point (pI) of 10-10.5 (Babbitt et al., 2015; Soltys et al., 2001). Cyt-c, like many
mitochondrial proteins, is encoded by nuclear gene and synthesized as a cytoplasmic apocytochrome c precursor and then imported into the mitochondrial intermembrane space.

Cyt-c is widely known to mediate a very important role especially in ETC and apoptosis (Ow et al., 2008). As a universal catalyst of respiration, Cyt-c undertakes one electron transfer from Cyt-c reductase in complex III to cytochrome oxidase in complex IV, and it does that via transition between the ferrous and ferric states.

The process of programed cell death during development or in response to infections or DNA damage forms part of normal cellular metabolic activities. The role of Cyt-c in the apoptotic pathway is well identified. When cells receive apoptotic stimulus, Bax and Bak which are pro-apoptotic proteins belonging to the B-cell lymphoma protein-2 (Bcl-2) form a complex with voltage-dependent anion channel component of the permeability transition pore (PTP) of the outer mitochondrial membrane (Kroemer et al., 2007). The complex formed facilitates permeability of the outer membrane. The permeability state of the outer membrane facilitates Cyt-c release from the intermembrane space into the cytosol upon calcium release.

![Structure of Cyt-c showing the haem group in the middle](http://www.genome.jp/dbget-bin/www_bget?cpd:C00524)

**Fig. 2.13: Structure of Cyt-c showing the haem group in the middle**

(http://www.genome.jp/dbget-bin/www_bget?cpd:C00524)
Cytosolic or released Cyt-c associates with inositol-3-phosphate receptor (IP3 receptor) on endoplasmic reticulum (ER), causing calcium release from ER (Duchen et al., 2008). Increase in cytosolic calcium levels, however, triggers massive Cyt-c release from the mitochondrial intermembrane space. Cytosolic Cyt-c triggers the formation of apoptosome and subsequent activation of caspase-9 which also activates caspases-3, 6 and 7. Activated caspase-3, 6 and 7 destroy the cell from within via degradation of essential enzymes systems and DNA fragmentation (Kroemer et al., 2007).

Levels of Cyt-c released into the cytosol can be detected by selective cell lysis. Blood levels of Cyt-c are extremely low in healthy individuals. High levels of serum or plasma Cyt-c may be attributed to several factors such as disease conditions, aging and drugs. Serum levels of Cyt-c have been found to be very high in patients receiving cancer chemotherapy (Renz et al., 2001). Plasma levels of Cyt-c have also been shown to be high in HIV patients on ART who are experiencing drug toxicity (Langs-Barlow et al., 2015). These studies therefore confirm the release of Cyt-c into the cytosol and then into blood circulation. Detection and measurement of Cyt-c in serum and plasma is done by simple ELISA technique (Langs-Barlow et al., 2015; Renz et al., 2001).
CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

Human Cyt-c Platinum ELISA kit was obtained from Bender Medsystems (Vienna, Austria). BTS 350 Semi-Automated Analyzer was used to measure serum creatine kinase and amylase while serum levels of triacylglyceride (TAG) and cholesterol were measured using A15 Random Access Analyzer from BioSystems (Barcelona, Spain).

3.2 Methods

3.2.1 Study sites

HIV-infected patients on ART were enrolled into the study from Pantang Hospital ART Centre and Korle-Bu Fevers Unit. Pantang ART Centre is a clinical facility which is part of the Pantang Psychiatric Hospital located at Pantang in the La-Nkwantanang Madina District, Greater Accra Region. Korle-Bu Fevers Unit is a facility, which is part of the Korle-Bu Teaching Hospital (University of Ghana) located at Korle-Bu in the Greater Accra Region. Although these two facilities are dedicated to treating HIV-infected patients, they also attend to patients with other infectious diseases such as malaria, chicken pox, rabies and many others. After giving their consent, whole blood samples were collected from all participants for serum and plasma preparation.

3.2.2 Study population

The study was conducted among Ghanaian HIV-infected patients receiving antiretroviral treatment especially with NRTI-based medications. Eighty (80) patients consisting of 16 male and 64 female adults between the ages of 20 and 60 years and were on ART were recruited
into the study. The patients were then categorized into two groups of less than 12 months ART experience and more than 12 months ART experience.

3.2.3 Ethical clearance

Ethical approval was received from the Ethical Committee of the College of Basic and Applied Sciences, University of Ghana, Legon, and also from Ghana Health Service.

3.2.4 Extraction of patients’ data

Extraction of participants’ data was done using questionnaires after the participants had given their consent to the study. The extraction was done from the participants’ hospital folders with the help of their care providers. Information that could not be obtained from folders was obtained directly from participants.

3.2.5 Sample collection

Whole blood samples were collected with the help of a phlebotomist. Each participant donated 10 ml whole blood, with 5 ml in EDTA tubes and 5 ml in serum separator tubes. EDTA tubes containing blood samples were inverted 10 times to thoroughly mix blood and anticoagulant. Samples in serum separator tubes were allowed to clot prior to serum preparation.

3.2.6 Plasma and serum preparation

Samples were kept at room temperature after collection prior to centrifugation in order to obtain either the serum or plasma samples. In preparing plasma, samples in EDTA tubes were centrifuged at 3000 rpm for 10 min. For serum, samples in serum separator tubes were
centrifuged at 2500 rpm for 10 min. Supernatants were aliquoted into well labelled cryovials using sterile transfer pipettes. Plasma and serum were stored at −80 °C until use.

3.2.7 Screening for symptoms of ART-induced toxicity

Patients recruited into the study did not have documented clinical or laboratory evidence of ART-induced toxicity. They were therefore screened for symptoms of toxicity. Clinical symptoms of myopathy, pancreatitis and hyperlipidaemia, which are all symptoms of ART-induced mitochondrial toxicity (Brinkman and Copeland, 2000) were investigated by measuring the levels of serum creatine kinase (CK), amylase and cholesterol and triglycerides respectively. Patients with abnormal levels of at least one of the markers were classified as having ART-induced toxicity.

Serum levels of CK and amylase were measured by BTS 350 Semi-Automated Analyser using LED configurations of 340 and 405 respectively. Triglyceride and cholesterol levels were measured by A15 Random Access Analyser. These analyses were carried out at MDS-Lancet Laboratories Ghana Limited, East Legon, Accra.

3.2.8 Plasma Cyt-c assay

Plasma levels of Cyt-c were assayed using a method described by Langs-Barlow et al. (2015). Concentration of plasma Cyt-c was examined using Human Cyt-c ELISA kit from Bender MedSystems (Vienna, Austria). The Human Cyt-c standard that came with the kit was reconstituted by adding 250 μl distilled water to obtain a 10 ng/ml Cyt-c solution as instructed by the manufacturer. It was gently swirled to ensure complete solubilisation and allowed to stand for about 30 minutes before use. All plasma samples were prediluted (1:2) with assay buffer by mixing 150 μl of each sample with 150 μl assay buffer.
Microwell strips were washed twice with 400 µl wash buffer per well. The wells were labelled as standard, blank and sample wells. Serial dilutions were then prepared from the 10 ng/ml Cyt-c standard. Dilution of the standard Cyt-c was done directly on the microwell plates. When carrying out the serial dilution, 100 µl of assay buffer was added in duplicate to all standard wells. An aliquot of 100 µl of the 10 ng/ml standard was added in duplicate into wells A1 and A2 and mixed thoroughly by repeated aspiration and ejection to obtain a concentration of 5 ng/ml. An aliquot of 100 µl was transferred from wells A1 and A2 into wells B1 and B2 respectively to obtain 2.5 ng/ml concentrations. The transfer continued 5 more times until two rows of Cyt-c standard dilutions were obtained, with concentration ranging from 5.00 to 0.08 ng/ml. A volume of 100 µl diluted standard was discarded from the last well (G1 and G2). A summary of the serial dilution of the Cyt-c standard is shown in Figure 3.1.

Aliquots of 100 µl prediluted plasma samples were added to designated sample wells in duplicate, and 100 µl assay buffer was added to blank wells in duplicate. A volume of 50 µl biotin-conjugate was added to all wells. The strips were covered and incubated for two hours at 25 ºC. At the end of the two hours, the microwell strips were emptied and washed three times with wash buffer. An aliquot of 100 µl Streptavidin-HRP was added to all wells, covered and incubated for an hour at 25 ºC. After, the strips were emptied and washed three times with Wash Buffer. A volume of 100 µl tetramethyl-benzidine (TMB) Substrate Solution was added to all wells and incubated for 10 minutes at 25 ºC. Aliquot of 100 µl Stop Solution was added to all wells and absorbance read at 450 nm using a microplate reader. The ELISA kit had a limit of detection of 0.05 ng/ml.
3.2.9 Statistical analysis

The relationship between patient characteristics and plasma Cyt-c concentration was determined using Spearman correlation coefficients. Two-sample tests, one-sample t-test of proportion and Wilcoxon sum-rank test were used to determine the differences between groups.
CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics of the Study Participants

Eighty (80) Ghanaian HIV-infected patients, who were on NRTI antiretroviral drugs, were recruited into this study. The participants consisted of 80% females; and majority, about 96%, were between the ages of 25 and 54 years. More than half of the participants (53.7%) were married. In terms of level of education, many of the participants, 37.5%, had Junior High School education and only 10% had no formal education at all. The study participants consisted of people who were self-employed (65%), full time workers (17.5%), unemployed (16.3%) or working part-time (1.3%). Majority of the participants (93.7%) were Christians. Details of the demographic characteristics of the participants can be found in Table 4.1.
Table 4.1: Demographic characteristics of study participants

<table>
<thead>
<tr>
<th>Variables</th>
<th>Participants (n = 80)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16 (20.0)</td>
</tr>
<tr>
<td>Female</td>
<td>64 (80.0)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>25-34</td>
<td>30 (37.5)</td>
</tr>
<tr>
<td>35-44</td>
<td>30 (37.5)</td>
</tr>
<tr>
<td>45-54</td>
<td>17 (21.3)</td>
</tr>
<tr>
<td>55-64</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td><strong>Marital status</strong></td>
<td></td>
</tr>
<tr>
<td>Separated</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>Widow</td>
<td>3 (3.7)</td>
</tr>
<tr>
<td>Divorced</td>
<td>6 (7.5)</td>
</tr>
<tr>
<td>Single</td>
<td>13 (16.3)</td>
</tr>
<tr>
<td>Cohabiting</td>
<td>13 (16.3)</td>
</tr>
<tr>
<td>Married</td>
<td>43 (53.7)</td>
</tr>
<tr>
<td><strong>Educational level</strong></td>
<td></td>
</tr>
<tr>
<td>Tertiary</td>
<td>4 (5.0)</td>
</tr>
<tr>
<td>Nil</td>
<td>8 (10.0)</td>
</tr>
<tr>
<td>MS</td>
<td>10 (12.5)</td>
</tr>
<tr>
<td>Primary</td>
<td>13 (16.3)</td>
</tr>
<tr>
<td>SHS</td>
<td>15 (18.7)</td>
</tr>
<tr>
<td>JHS</td>
<td>30 (37.5)</td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
<td></td>
</tr>
<tr>
<td>Part time</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>Unemployed</td>
<td>13 (16.3)</td>
</tr>
<tr>
<td>Full time</td>
<td>14 (17.5)</td>
</tr>
<tr>
<td>Self employed</td>
<td>52 (65.0)</td>
</tr>
<tr>
<td><strong>Religion</strong></td>
<td></td>
</tr>
<tr>
<td>Muslim</td>
<td>5 (6.3)</td>
</tr>
<tr>
<td>Christian</td>
<td>75 (93.7)</td>
</tr>
</tbody>
</table>

Characteristics of the 80 Ghanaian HIV+ patients on ART recruited into this study. They were categorized according to gender, age, marital status, educational level, occupation and religion. MS: Middle School; SHS: Senior High School; JHS: Junior High School
4.2 Clinical Stages of HIV Infection in the Study Participants

The clinical stages of the study participants were assessed based on the World Health Organisation (WHO) guidelines (Table 4.2). Half of the participants (50%) did not have written documents showing their clinical stages of HIV infection. However, documented clinical examinations such as body weights, temperature, CD4 counts, opportunistic infections and others suggested that majority of the participants with unknown clinical stage were unlikely to be in Stage 4. Many of those with known clinical stages belonged to Stage 1 (12.5%), Stage 2 (18.9%) and Stage 3 (13.7%) with only 5% in Stage 4 which is the most advanced stage of HIV infection.

4.3 Duration of HIV Infection and Duration of ART

Duration of HIV infection among participants was calculated using participants’ dates of HIV positive test result or period after first HIV+ diagnosis. Again, more than half of the participants (67.5%) had been diagnosed with HIV for more than 12 months with the remaining 32.5% having been diagnosed with HIV for less than 12 months (Table 4.3). Duration of ART was easier to calculate since the exact ART starting dates were clearly stated in participants’ folders. More than half of the participants (58.7%) had been on ART for more than 12 months and 41.3% had been on therapy for less than 12 months.
### Table 4.2: WHO Clinical Stage of participants

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>Participants (n = 80)</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>10 (12.5)</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>15 (18.8)</td>
<td></td>
</tr>
<tr>
<td>Stage 3</td>
<td>11 (13.7)</td>
<td></td>
</tr>
<tr>
<td>Stage 4</td>
<td>4 (5.0)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>40 (50.0)</td>
<td></td>
</tr>
</tbody>
</table>

Patients recruited into this study were categorized into their respective clinical stages. Stage 1: asymptomatic; Stage 2: moderate weight loss, respiratory tract infections; Stage 3: severe weight loss, persistent fever, chronic diarrhoea, anaemia; Stage 4: wasting syndrome, recurring severe bacterial pneumonia and oesophageal candidiasis (WHO, 2005).

### Table 4.3: Duration of HIV infection and ART

<table>
<thead>
<tr>
<th>Duration (months)</th>
<th>HIV infection</th>
<th>ART</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Participants (n = 80)</td>
<td>Participants (n = 80)</td>
</tr>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td>1-3</td>
<td>8 (10.0)</td>
<td>13 (16.3)</td>
</tr>
<tr>
<td>4-6</td>
<td>9 (11.3)</td>
<td>10 (12.5)</td>
</tr>
<tr>
<td>7-9</td>
<td>6 (7.5)</td>
<td>7 (8.8)</td>
</tr>
<tr>
<td>10-12</td>
<td>3 (3.7)</td>
<td>3 (3.7)</td>
</tr>
<tr>
<td>&gt;12</td>
<td>54 (67.5)</td>
<td>47 (58.7)</td>
</tr>
</tbody>
</table>

Participants were classified, based on their duration of HIV infection and duration of therapy, into ranges of 1-3, 3-6, 6-9, 9-12 and >12 months. Duration of HIV infection was defined by period after first HIV+ diagnosis.
4.4 Levels of CD4+ T Cell Counts in the Study Participants

All the study participants had at least one test result showing their CD4+ T cell counts. The most recent CD4+ T cell count of participants were used in this study. As shown in Table 4.4, 28 out of the 80 participants representing 35% had CD4 counts of more than 500 cells/mm³. The highest CD4 count observed among the study participants was 1371 cells/mm³. Majority (55.1%) had CD4 counts between 200 and 500 cells/mm³. Few of the participants (10%) had developed AIDS with CD4 counts falling below 200 cells/mm³. The lowest CD4 count observed among the study participants was 6 cells/mm³.

4.5 Antiretroviral Drug Combinations Among Study Participants

The various drug combinations among study participants, according to recommendations by WHO and the National HIV/AIDS/STI Control Programme guidelines (NACP) for ART in Ghana, were also assessed and recorded in Table 4.5. All the participants were taking three different antiretroviral drugs, two of which were nucleoside/tide analogues and one being either an NNRTI or PI. The nucleoside/tide analogues used by the participants were Zidovudine (AZT), Tenofovir disoproxil (TDF), Lamivudine (3TC) and Emtricitabine (FTC). Efavirenz (EFV) and Nevirapine (NVP) were the only NNRTIs used by the participants. Lopinavir/ritonavir (LPV/r) which is a PI was used by only two of the study participants. More than half of the participants (77.5%) were using the combination of Tenofovir disoproxil + Lamivudine + Efavirenz, and 11.2% using Tenofovir disoproxil + Emtricitabine + Efavirenz. Zidovudine was used by 6% of the study participants.
Table 4.4: CD4+ T cell counts of participants

<table>
<thead>
<tr>
<th>CD4+ count (cell/mm³)</th>
<th>Participants (n = 80)</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;200</td>
<td></td>
<td>8 (10.0)</td>
</tr>
<tr>
<td>200-349</td>
<td></td>
<td>19 (23.8)</td>
</tr>
<tr>
<td>350-499</td>
<td></td>
<td>25 (31.3)</td>
</tr>
<tr>
<td>≥500</td>
<td></td>
<td>28 (35.0)</td>
</tr>
</tbody>
</table>

Participants were grouped based on their CD4+ T cell counts. CD4+ T cell count <200 cells/mm³ means patient has developed the acquired immunodeficiency syndrome AIDS. With CD4 count between 200 and 500 cells/mm³, a patient is recommended for ART. Above 500 cells/mm³ is normal (Gilks et al., 2006).

Table 4.5: Antiretroviral drug combinations among study participants

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Participants (n = 80)</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT+3TC+EFV</td>
<td></td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>TDF+FTC+NVP</td>
<td></td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>TDF+3TC+LPV/r</td>
<td></td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>AZT+3TC+NVP</td>
<td></td>
<td>5 (6.2)</td>
</tr>
<tr>
<td>TDF+FTC+EFV</td>
<td></td>
<td>9 (11.2)</td>
</tr>
<tr>
<td>TDF+3TC+EFV</td>
<td></td>
<td>62 (77.5)</td>
</tr>
</tbody>
</table>

Triple drug combinations among the patients recruited into the study. Nucleoside/tide analogues: zidovudine (AZT), lamivudine (3TC), tenofovir (TDF) and emtricitabine (FTC); NNRTIs: efavirenz (EFV) and nevirapine (NVP); Protease inhibitor: lopinavir/ritonavir (LPV/r)
4.6 Classification of Study Participants based on Duration of ART

The study participants were classified into two groups based on the duration of ART. These consisted of those who had been on ART for less than 12 (<12) months and those who had been on ART for more than 12 (>12) months. About 41.3% of the study participants had been on ART for less than 12 months whilst 58.7% had been on ART for more than 12 months (Table 4.6).

Certain patient characteristics were compared between the two groups. There was no significant difference between the two groups in terms of age ($p = 0.63$) and CD4 count ($p = 0.06$). The average duration of HIV infection and average duration of ART were about 100 fold higher in patients on ART for > 12 months than in patients on ART for < 12 months. All the participants in both groups were under treatment with nucleoside/tide analogues. Apart from the two participants who were taking protease inhibitor among those on ART for <12 months, all the other participants in both groups were taking NNRTIs.
Table 4.6: Participants on ART for more and less than 12 months

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients on ART for &lt; 12 months (n=33)</th>
<th>Patients on ART for &gt;12 months (n=47)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of HIV (years)</td>
<td>1.2 (± 2.44)</td>
<td>4.1 (± 2.22)</td>
<td>0.01</td>
</tr>
<tr>
<td>Duration of ART (years)</td>
<td>0.3 (± 0.24)</td>
<td>3.9 (± 1.73)</td>
<td>0.01</td>
</tr>
<tr>
<td>CD4+ T cell counts</td>
<td>403.6 (± 246.03)</td>
<td>514.5 (± 267.52)</td>
<td>0.06</td>
</tr>
<tr>
<td>Nucleoside/tide exposure</td>
<td>100.0%</td>
<td>100.0%</td>
<td>1.00</td>
</tr>
<tr>
<td>NNRTIs exposure</td>
<td>93.9%</td>
<td>100%</td>
<td>0.09</td>
</tr>
<tr>
<td>Protease inhibitor</td>
<td>6.1%</td>
<td>0.0%</td>
<td></td>
</tr>
<tr>
<td>Integrase inhibitor</td>
<td>0.0%</td>
<td>0.0%</td>
<td></td>
</tr>
</tbody>
</table>

Comparison of some parameters between participants on therapy for less and those on it for more than 12 months. For continuous variables, P values were calculated using Two-sample t test with equal variance, and Two-sample test of proportion for categorical variables. Duration of HIV, duration of ART and CD4+ T cell counts are stated as Mean (±SD).

4.7 Results of Screening of Participants for Symptoms of ART Toxicity

Out of the eighty (80) participants recruited into this study, sixty (60) were screened for clinical symptoms of ART toxicity by assessing the levels of creatine kinase, amylase, cholesterol and triacylglycerides. About 67% of the participants that were screened for toxicity had been on ART for >12 months and 33% had been on it for <12 months (Table 4.7). All (100%) of the participants screened were using nucleoside/tide analogues, 98.3% using NNRTIs and 1.7% using PI.
Table 4.7: Participants screened for ART toxicity

<table>
<thead>
<tr>
<th>Variables</th>
<th>Participants (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
</tr>
<tr>
<td><strong>Duration of HIV</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;12 months</td>
<td>14 (23.3%)</td>
</tr>
<tr>
<td>&gt;12 months</td>
<td>46 (76.7%)</td>
</tr>
<tr>
<td><strong>Duration of ART</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;12 months</td>
<td>20 (33.3%)</td>
</tr>
<tr>
<td>&gt;12 months</td>
<td>40 (66.7%)</td>
</tr>
<tr>
<td>Nucleoside/tide analogues</td>
<td>60 (100%)</td>
</tr>
<tr>
<td>NNRTIs</td>
<td>59 (98.3%)</td>
</tr>
<tr>
<td>PI</td>
<td>1 (1.7%)</td>
</tr>
</tbody>
</table>

A brief characterization of participants screened for symptoms of toxicity, showing how many had been diagnosed with HIV, and how many had been of ART for < and > 12 months; and also the proportion treated with the various inhibitors.

4.7.1 Levels of serum creatine kinase

According to MDS-Lancet Laboratories Ghana Limited, serum levels of CK is high when above 190 IU/L, and is an indication of muscle or/and brain damage (Cinamon, 2004). In this study, serum levels of CK was measured in all the 60 participants of which 11, representing 18.3%, had CK greater than 190 IU/L and with a mean CK ± SD of 270.2 ± 87.07 IU/L (Fig. 4.1A). The remaining 49, representing 81.7%, had normal CK levels of less than 190 IU/L, with a mean CK of 118.2 ±38.49 IU/L. Out of the 11 participants with CK>190 IU/L, 6 (54.5%) had been on ART for less than 12 months and 5 (45.5%) had been on ART for more than 12 months (Fig.4.1B). Out of the 11 participants with CK>190 IU/L, 4 were being treated with AZT+3TC+NVP, 6 with TDF+3TC+EFV and 1 with TDF+FTC+EFV.
**Fig. 4.1: Serum levels of creatine kinase among participants screened**

(A) Participants with CK levels greater than 190 IU/L (18.3%) and those with CK less than 190 IU/L (81.7 IU/L). (B) Participants with CK>190 IU/L and had been on ART for less than 12 months (54.5%) and those with CK >190 IU/L and had been on ART for more than 12 months (45.5%). \( P \) value calculated using one-sample t-test of proportion.

### 4.7.2 Levels of serum amylase

Serum amylase, according to MDS-Lancet Laboratories Ghana Limited, is high when >125 IU/L. Out of the 60 participants, 12 (20%) had serum amylase levels greater than 125 IU/L and with a mean amylase of 207.1 ± 108.21 IU/L (Fig. 4.2A). The remaining 48 (60%) had amylase levels less than 125 IU/L and a mean amylase of 82.7 ± 25.06 IU/L. Among the 12 participants with amylase >125 IU/L, 7 representing 58.3% had been on ART for less than 12 months and 5 representing 41.7% had been on ART for more than 12 months (Fig. 4.2B). Among the 12 participants with amylase > 125 IU/L, 10 were treated with TDF+3TC+EFV and 2 with TDF+FTC+EFV.
Fig. 4.2: Serum levels of amylase among participants screened
(A) Participants with amylase levels greater than 125 IU/L (20%) and those with amylase less than 125 IU/L (80%). (B) Participants with amylase >125 IU/L and had been on ART for less than 12 months (58.3%) and those with amylase >125 IU/L and had been on ART for more than 12 months (41.7%). *P* value calculated using one-sample t-test of proportion.

### 4.7.3 Levels of serum cholesterol and triglyceride

A fasting blood test was carried out to assess participants’ blood levels of cholesterol and TAG. Serum cholesterol and TAG, according to MDS-Lancet Laboratories Ghana Limited, are high when above 5.2 mmol/L and >1.7 mmol/L respectively. Out of the 60 participants, 21 representing 35% had Cholesterol greater than 5.2 mmol/L and TAG greater than 1.7 mmol/L (Fig. 4.3A). The remaining 39 representing 65% had Cholesterol less than 5.2 mmol/L and TAG less than 1.7 mmol/L. Out of the 21 with Cholesterol >5.2 mmol/L and TAG>1.7 mmol/L, 7 (33.3%) had been on ART for less than 12 months and 14 (66.6%) had been on ART for more than 12 months (Fig 4.3B). Among the 21 participants with elevated cholesterol and TAG, 18 were being treated with TDF+3TC+EFV and one person each with TDF+FTC+EFV, AZT+3TC+NVP and TDF+3TC+LPV/r.
Fig. 4.3: Serum levels of cholesterol and triglyceride among participants screened

(A) Participants with cholesterol and TAG greater than 5.2 mmol/L and 1.7 mmol/L respectively (35%) and those with cholesterol and TAG less than 5.2 mmol/L and 1.7 mmol/L respectively (65%).

(B) Participants with cholesterol >5.2 and TAG >1.7 mmol/L and had been on ART for less than 12 months (33.3%), and those with cholesterol >5.2 and TAG >1.7 mmol/L and had been on ART for more than 12 months. $P$ value, calculated using one-sample t-test of proportion, was statistically significant.

4.8 Incidence of ART-Induced Toxicity in the Study Participants

After assessing serum levels of the aforementioned biomarkers, the 60 participants were classified as those with or without symptoms of ART toxicity. Participants with abnormal level of at least one of the serum biomarkers were classified as having ART-induced toxicity with symptoms of myopathy (CK >190 IU/L), pancreatitis (amylase >125 IU/L), and hyperlipidaemia (cholesterol >5.2 and TAG >1.7 mmol/L). Out of the 60 participants, 36 (60%) had at least one of the symptoms screened and were classified as having ART-induced toxicity (Fig. 4.4A). The remaining 24 (40%) did not have any of the symptoms screened and were classified as having no ART toxicity. Among the 36 participants classified as having ART toxicity, 15 (41.7%) had been on ART for less than 12 months and 21 (58.3%) had been on ART for more than 12 months (Fig. 4.4B). Overall, 13.3% of the 60 participants screened had more than one of the symptoms screened.
Fig. 4.4: ART toxicity among the 60 participants screened
(A) Participants with symptoms of ART toxicity (60%) and those with no symptom of toxicity (40%).
(B) Participants with ART toxicity and had been on ART for less than 12 months (41.7%) and more than 12 months (58.3%). P value, calculated using one-sample t-test of proportion, was statistically significant.

4.9 Plasma Cyt-c Levels
Concentration of plasma Cyt-c was measured for all the 80 participants recruited into the study and several comparisons were made. Plasma Cyt-c levels were compared between participants classified as having ART toxicity and those classified as not having toxicity; and also between participants who have been on ART for more than 12 months and those for less than 12 months.

4.9.1 Plasma Cyt-c levels among participants with and without ART-induced toxicity
Plasma Cyt-c concentration was compared between participants with ART-induced toxicity and participants without toxicity. A dot-plot of the plasma Cyt-c levels among participants screened for symptoms of ART toxicity is shown in Figure 4.5. The median plasma Cyt-c level among participants classified without ART toxicity (labelled 0; n=24) was lower (0.05 ng/ml, range <0.05 – 0.72 ng/ml) than those with symptoms of ART toxicity (labelled 1, n=36) (0.12 ng/ml, range <0.05 – 1.22 ng/ml). Although the median plasma Cyt-c for
participants with ART toxicity was higher than those with no toxicity, the difference was not statistically significant \( (p = 0.148) \). Plasma Cyt-c levels of two ART-naïve HIV patients (labelled 2) were measured and found to be <0.05 ng/ml.

**Fig. 4.5: Plasma Cyt-c levels in participants with and without symptoms of ART toxicity.**
The red horizontal dot lines represent the median Cyt-c concentrations. \( P \) value was calculated using Wilcoxon sum-rank test.

### 4.9.2 Plasma Cyt-c levels among participants with ART-induced toxicity

As indicated earlier in Fig. 4.4B, some of the participants with toxicity had been on ART for less than or more than 12 months. Plasma Cyt-c levels were therefore compared between the two groups of participants. As shown in Fig. 4.6, the median plasma Cyt-c in those who had been on ART for less than 12 months (labelled 1) was 0.13 ng/ml and that for those who had been on ART for more than 12 months (labelled 2) was 0.12 ng/ml. The difference in median plasma Cyt-c concentration between the two groups was not statistically significant \( (p = 0.974) \). When the single outlier in those on ART for less than 12 months was removed, \( p \) was 0.748.
Fig. 4.6: Plasma Cyt-c levels among participants with ART toxicity. The red horizontal dot lines represent the median Cyt-c concentrations. *P* value was calculated using Wilcoxon sum-rank test.

### 4.9.3 Plasma Cyt-c levels between participant on ART for less or more than 12 months

Plasma Cyt-c levels were examined among the entire (80) participants recruited into the study. Concentration of plasma Cyt-c was compared between participants who had been on ART for less than 12 months (*n* = 33) and those for more than 12 months (*n* = 47). From Fig. 4.7, the median plasma Cyt-c for participants on ART for less than 12 months (labelled 1) was 0.11 ng/ml (range <0.05 – 1.22 ng/ml) and that for those on ART for more than 12 months (labelled 2) was 0.06 ng/ml (range <0.05 – 0.79 ng/ml). However, the difference in the median plasma Cyt-c was not statistically significant between the two groups (*p* = 0.876). When the single outlier in those on ART for less than 12 months was removed, *p* was 0.587.
Fig. 4.7: Plasma Cyt-c level between participants on ART for less or more than 12 months.
The red horizontal dot lines represent the median Cyt-c concentrations. $P$ value was calculated using Wilcoxon sum-rank test.

4.10 Association Between Plasma Cyt-c and Patients’ Characteristics

The relationship between plasma Cyt-c and some patients’ characteristics were also determined. Scatter plots showing the various associations are shown in Fig 4.8. There were no correlations between plasma Cyt-c levels and duration of ART (Spearman rho = 0.02, $p = 0.89$) (Fig. 4.8), plasma Cyt-c levels and duration of HIV infection (Spearman rho = 0.06, $p = 0.61$) (Fig. 4.9), plasma Cyt-c levels and age (Spearman rho = 0.11, $p = 0.32$) (Fig. 4.10) and plasma Cyt-c level and CD4 count (Spearman rho = -0.14, $p = 0.23$) (Fig. 4.11).
Fig. 4.8: Relationship between plasma Cyt-c and duration of ART
Spearman rho = 0.02, $p = 0.89$.

Fig. 4.9: Relationship between plasma Cyt-c and duration of HIV infection
Spearman rho = 0.06, $p = 0.61$. 
Fig. 4.10: Association between plasma Cyt-c and age of study participants
Spearman rho = 0.11, $p = 0.32$.

Fig. 4.11: Association between plasma Cyt-c and CD4 count of participants
Spearman rho = -0.14, $p = 0.23$. 
CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Participant Characteristics

Out of the 80 participants recruited into this study, 16 (20%) were males with the remaining 64 (80%) being females (Table 4.1). The lower number of male participants as compared to female participants could be due to several reasons. First of all, Ghanaian pregnant women receiving antenatal care are mandated to screen for HIV and those who test positive are put on ART purposely for prevention of mother to child transmission (PMTCT); which is in accordance with the declaration by the United Nations General Assembly Special Session to reduce infants infected with HIV. Secondly, HIV care providers at the study sites explained that most men do not show interest in ART after being diagnosed with HIV. As a result of these, ART centres are dominated by female HIV patients. Consequently, the use of convenient sampling to obtain participants resulted in the higher number of females compared to males. There was no significant difference between the male and female participants in terms of CD4+ T cell counts, plasma Cyt-c level, serum cholesterol/TAG and amylase. However, HIV-infected patients on ART recruited into some previous studies were largely males. For example, a study on the prevalence of pancreatitis in HIV patients on therapy consisted of 70% males (Moore et. al., 2001), and a recent case-control study on circulating plasma Cyt-c consisted of only 38% females with the 62% being males (Langs-Barlow et al., 2015).

More than half (67.5%) of the study participants had been diagnosed with HIV for > 12 months, and with an average duration of HIV infection of about 4 years (Table 4.3). The rest (32.5%) had been diagnosed for < 12 months with an average duration of about 1 year. Durations of HIV infection among the participants were calculated using period after first
HIV+ diagnosis. This is because determining the exact duration of HIV infection among HIV patients is very tedious and often impossible as it is almost unlikely to determine the exact time a patient contracted the virus. For the purpose of research, duration of HIV infection is always an estimation that takes into consideration the time of HIV+ diagnosis (Langs-Barlow et al., 2015). The duration of HIV infection observed among the study participants is lower as compared to a recent case-control study by Langs-Barlow et al. (2015) where the average duration of HIV infection for cases was 8.4 years and that for controls was 15.4 years.

CD4 count of participants on ART for > 12 months was found to be normal (500 cell/mm$^3$ and above) whilst those on ART for < 12 months had CD4 count less than normal value (Table 4.6). Analysis of past CD4 tests results of participants on ART for > 12 months were lower than normal prior to ART and during the initial period of the therapy but gradually increased as therapy continued. This observation is consistent with previous reports that HIV infection results in reduction in CD4+ T cells (Paranjape, 2005) and that ART helps to improve and prolong the lives of HIV infected persons (Cohen et al., 2007) by restoring and maintaining CD4 levels (National HIV/AIDS/STI Control Programme, 2010).

All the participants recruited into this study were on NRTI-based antiretroviral drugs (Table 4.5) regardless of their clinical stage (Table 4.2) or CD4 counts (Table 4.4). This treatment regime observed among the patients in the present study was in accordance with the World Health Organization’s recent recommendations for ART, which directs that initiation of ART should be irrespective of the clinical stage or CD4 count of an HIV positive patient (WHO, 2015). Some of the participants had started ART not too long ago even though they had been diagnosed with HIV for a relatively longer period.
Furthermore, all the participants, except two, were on first-line treatment with majority using the less toxic NRTIs (TDF and 3TC) and a few using AZT which is known to be more toxic (Deavall et al., 2012; Gilks et al., 2006). In Ghana, only three (3) classes of antiretroviral drugs (NRTIs, NNRTIs and PIs) are approved for HIV treatment. The recommended nucleoside/tide analogues for ART are AZT, TDF, 3TC, ABC and FTC (National HIV/AIDS/STI Control Programme, 2010). The non-nucleotide inhibitors used are NVP and EFV, and the protease inhibitors used are LPV/r and ATV/r. According to the National HIV/AIDS/STI Control Programme guidelines for ART in Ghana, antiretroviral drugs for ART-naïve persons are classified into first-line and second-line drugs. The first-line drugs, which are the preferred start-up drugs for HIV treatment in ART-naïve patients, are classified into either first-choice drugs or second-choice drugs with each further categorized into first-option and second-option. We found that majority (88.8%) of the participants recruited into this study were being treated with second-choice drugs in the second-option category and were taking the triple drug combination of TDF+3TC/FTC+EFV (tenofovir + lamivudine or emtricitabine + efavirenz) (Table 4.5). One participant that was being treated with second-choice drugs was found in the first-option category and was taking the combination of TDF+FTC+NVP (tenofovir + emtricitabine + nevirapine).

Some of the participants (6.3%) were being treated with first-choice drugs in the first-option category where they were taking the combination of AZT+3TC+NVP (zidovudine + lamivudine + nevirapine). Only one participant was being treated with first-choice drugs in the second-option category with the combination of AZT+3TC+EFV (zidovudine + lamivudine+efavirenz). Second-line drugs, which are also grouped into first and second alternatives, are used when first-line treatment fails to improve CD4 counts or fails to suppress viral replication. Two out of the 80 participants were found being treated with second-line
drugs in the first alternative consisting of TDF+3TC+LPV/r (tenofovir + lamivudine + lopinavir/ritonavir) (Table 4.5). The various drug combinations among all the participants recruited into this study were consistent with recommendations by the National HIV/AIDS/STI Control Programme guidelines for ART in Ghana and also consistent with the guidelines by the WHO.

5.2 Incidence of ART-Induced Toxicity Among Participants

The laboratory/biochemical analyses conducted in this study established some level of ART toxicity among the patients, which was irrespective of the duration of therapy (Fig. 4.4). The duration of ART, which will cause toxicity is not well established as different patients may respond differently to therapy and may experience side effects at different times.

Although identifying patients with ART toxicity are usually achieved via clinical and/or laboratory analysis (Langs-Barlow et al., 2015), several studies have used laboratory analysis to identify ART toxicity among HIV-infected patients (Brinkman and Copeland, 2000). Levels of biomarkers suggestive of myopathy (Fig. 4.1), pancreatitis (Fig. 4.2) and hyperlipidaemia (Fig. 4.3) were found in both participants who had been on ART for < 12 and > 12 months.

Out of the 60 participants screened for toxicity, 11 (18.3%) had CK levels greater than normal (Fig. 4.1) which is an indication of muscle weakness or myopathy. Among the 11 participants with elevated CK levels, 4 were being treated with AZT+3TC+NVP (zidovudine + lamivudine + nevirapine), 6 with TDF+3TC+EFV (tenofovir + lamivudine + efavirenz) and 1 with TDF+FTC+EFV (tenofovir + emtricitabine + efavirenz). Studies have shown that NRTIs are the major causes of myopathy among HIV patients on treatment (Nikhil and
Santosh, 2015; Brinkman and Copeland, 2000). Myopathy among HIV patients is strongly associated with AZT and its prevalence was said to be very high in the past when AZT was taken in high dose (Nikhil and Santosh, 2015). However, myopathy has been observed in HIV patients treated with efavirenz (an NNRTI), stavudine and didanosine (NRTIs) (Simpson et al., 2004). Although, tenofovir and emtricitabine, which are NRTIs, have not been associated with myopathy (Nikhil and Santosh, 2015), the present study observed myopathy in some HIV infected patients, who were being treated with tenofovir, emtricitabine and lamivudine. Myopathy among HIV patients could be caused by the HIV itself, by HIV wasting syndrome, opportunistic infections or by tumoral infiltration of muscles (Chariot and Gherardi, 1995).

Among the 60 participants screened for toxicity, 12 (20%) had elevated levels of serum amylase (Fig. 4.2), which suggests inflammation of the pancreas. Out of the 12 participants with high serum amylase, 10 were being treated with TDF+3TC+EFV (tenofovir + lamivudine + efavirenz) and 2 with TDF+FTC+EFV (tenofovir + emtricitabine + efavirenz). The incidence of pancreatitis among HIV patients on therapy has been observed in other studies in which patients were being treated with didanosine (ddI), stavudine (d4T) and zidovudine (AZT) and were found to have elevated serum amylase in addition to other biomarkers (Reisler et al., 2005). Furthermore, the use of didanosine in combination with hydroxyurea has also been found to increase the risk of pancreatitis in HIV patients (Moore et al., 2001). Even though participants in the present study were not treated with didanosine or stavudine, elevated levels of amylase under the treatment of tenofovir, lamivudine and emtricitabine were observed.
In addition to the aforementioned toxicities, 21 (35%) out of the 60 participants had hyperlipidaemia with elevated serum levels of cholesterol and TAG (Fig. 4.3). Among the 21, 18 were on TDF+3TC+EFV (tenofovir + lamivudine + efavirenz) and one person each was on AZT+3TC+NVP (zidovudine + lamivudine + nevirapine), TDF+FTC+EFV (tenofovir + emtricitabine + efavirenz) and TDF+3TC+LPV/r (tenofovir + lamivudine + lopinavir/ritonavir). Previous studies have reported hyperlipidaemia in HIV patients treated with AZT+3TC+LPV/r or Nelf or EFV (Carpentier et al., 2005). However, HIV-infected patients are known to have high serum levels of cholesterol and TAG prior to initiation of ART due to HIV-induced reduction in cellular catabolism and the levels continue to increase during the period of ART (Oh and Hegele, 2007).

The 60 participants, who were assessed for toxicity were classified into two groups (those with and those without toxicity) based on the presence of abnormal level of at least one of the biomarkers analysed as carried out previously by Langs-Barlow et al. (2015). More than half (60%) of the participants showed ART toxicity, with some having been on ART for <12 months and some for >12 months (Fig 4.4). The occurrence of toxicity among HIV patients on therapy has been observed in several studies. A clinical study by Kowalska et al. (2016) found toxicity among HIV patients on ART, and also demonstrated that toxicity associated with ART is a major factor responsible for change/modification in medication.

5.3 Plasma Cyt-c Levels

As indicated above, the study generally classified the patients into two groups in terms of toxicity; those with and those without toxicity. The concentration of plasma Cyt-c in patients with toxicity was higher than in those without toxicity (Fig 4.5). However, the difference was
not statistically significant. The present study therefore demonstrated for the first time in Ghanaian HIV patients that ART toxicity influences plasma levels of Cyt-c.

Conversely, a pilot case-control study in predominantly African-American HIV patients on ART demonstrated a significantly higher plasma Cyt-c levels in participants with toxicity than those without toxicity (Langs-Barlow et al., 2015). In addition, plasma Cyt-c levels observed in the present study were lower than those reported by Langs-Barlow et al. (2015). The median plasma Cyt-c level of 0.64 ng/ml (range <0.04 to 3.85) were observed in cases and <0.04 ng/ml (range <0.04-0.95) in controls in the study by Langs-Barlow et al. (2015) in which cases were defined as HIV patients on ART for > 12 months and with at least one laboratory and/or clinical evidence of ART toxicity and controls as HIV patients on ART for > 12 months and without ART toxicity. The levels of plasma Cyt-c in the present study ranged from <0.05 to 1.22 ng/ml.

An examination of patients characteristic in the previous study revealed that the average duration of HIV infection (case: 8.4, control: 15.4 years) and average duration of ART (case: 7.2, control: 7.9 years) were higher than those of the participants used in the present study, where the average duration of ART in years was $2.4 \pm 2.2$. Furthermore, quite a good number of the cases and controls were being treated with protease and integrase inhibitors as compared to the present study where only two participants, with plasma Cyt-c level of <0.05 and 0.11 ng/ml respectively, were taking protease inhibitors with none taking integrase inhibitors. Protease inhibitors are strongly linked to increased cell death (Apostolova et al., 2010) and hence increased levels of Cyt-c in circulation. The relatively low duration of HIV infection, low duration of ART exposure and the absence of protease and integrase inhibitors could be factors responsible for the low levels of Cyt-c measured in this study.
Additionally, it is possible that some of the participants who were classified as not having toxicity actually had toxicity but could not be identified due to the small number of biomarkers screened in the present study. These unidentified groups could be having other common ART toxicities such as lactic acidosis, peripheral neuropathy, liver disorders, lipodystrophy, anaemia, thrombocytopenia, pancytopenia, high blood levels of urea/creatinine and several others which were identified in the participants in the case-control study by Langs-Barlow et al. (2015).

Furthermore, plasma Cyt-c levels were observed to be independent of duration of ART in participants with toxicity. Plasma Cyt-c levels were rather higher in the participants on ART for less than 12 months than those who had been on ART for more than 12 months (Fig. 4.6) although the difference was not statistically significant. This suggests that the observed ART toxicity, which resulted in elevated circulating Cyt-c, occurs irrespective of duration of ART. However, this requires further studies, preferably a longitudinal one, that monitor Cyt-c levels of patients from the onset of ART.

Indeed, the same trend was observed in the study population in general. The plasma Cyt-c levels were observed to be generally higher in participants who had been on ART for < 12 months than that of those who had been on ART for > 12 months though the difference was not statistically significant (Fig. 4.7) indicating that the plasma Cyt-c levels in the study population was higher during the early stage of ART and declined as therapy continues. Although, no significant correlation was observed between plasma Cyt-c levels and duration of ART (Fig. 4.4), the positive spearman rho indicates that there was a non-significant positive correlation between plasma Cyt-c level and duration of ART. This is consistent with other research findings that long term exposure to antiretroviral drugs is linked to toxicity.
(Gardner et al., 2013) and hence upsurges Cyt-c levels in circulation (Langs-Barlow et al., 2015). It is also important to note that aside ART, HIV infection itself has the potential to induce mitochondrial dysfunction, via excessive oxidative stress (Perez-Matute et al., 2013), and apoptosis (Garg et al., 2012) which could lead to Cyt-c release into circulation.

5.4 Association Between Plasma Cyt-c and Patients Characteristics

This study has demonstrated no significant correlations between plasma Cyt-c and duration of HIV infection (Fig. 4.9), age (Fig. 4.10) and CD4 counts (Fig. 4.11) of participants although Spearman rho was positive for the correlations between plasma Cyt-c and duration of HIV infection and age. This suggests that concentration of plasma Cyt-c increases with increasing duration of HIV infection and age of participants. This also agrees with the fact that HIV has the potential to induce apoptosis which could increase circulating Cyt-c levels. Aside medication, age is one of the factors that induce apoptosis via accumulated free radical damage (Elmore, 2007) and hence increases Cyt-c levels in circulation. Spearman rho for the association between plasma Cyt-c and CD4 count was negative which also suggests that plasma Cyt-c increases with decreasing CD4 counts. This can be explained by the fact that low CD4 count is associated with faster disease progression, increased immune suppression and the development of wasting syndrome (Gilks et al., 2006). Wasting syndrome is characterized by increased cell death and hence increases level of Cyt-c in circulation.

5.5 Conclusion

This study demonstrated that some Ghanaian HIV patients on ART, who are predominantly females and treated with first-line medications regardless of their CD4 counts and clinical stages, have symptoms of myopathy, pancreatitis and hyperlipidaemia which are all
symptoms consistent with ART-induced mitochondrial toxicity. These symptoms of ART toxicity among Ghanaian HIV patients occur irrespective of the duration exposed to therapy.

The study has also shown that ART influences plasma levels of Cyt-c; and patients with ART-induced toxicity have higher levels of plasma Cyt-c. However, concentration of plasma Cyt-c may not depend on the duration of ART and HIV infection, age or CD4 count of HIV-infected patients on therapy. Assaying for plasma Cyt-c was found to be simple, less invasive and reliable and could be an alternative approach to diagnosing ART toxicity.

5.6 Limitations
Only three (3) out of the numerous symptoms of ART toxicity were assessed among participants in this study. Therefore, some participants that were classified as not having toxicity might be wrongly identified. Also, not all the participants recruited into this study were screened for symptoms of toxicity due to inadequate funds.

5.7 Recommendations
- More symptoms of ART toxicity such as lactic acidosis, lipodystrophy, peripheral neuropathy, liver dysfunction, anaemia, thrombocytopenia, pancytopenia and many others should be looked at in Ghanaian HIV patients on ART in subsequent studies.
- Plasma Cyt-c levels of patients who have been on ART for a relatively longer period should be assessed.
- A longitudinal study should be carried out to monitor the trend (rise/fall) of plasma Cyt-c in Ghanaian HIV patients on therapy.
REFERENCES


WHO (2015). Guidelines on when to start antiretroviral therapy and on pre-exposure prophylaxis for HIV. Switzerland.


APPENDICES

Appendix I: Patient Consent Form

Title: Plasma cytochrome c level as biomarker for monitoring ART-induced mitochondrial toxicity

Investigator: Eric Akpanja Mensah

Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon

General Information about the Research
This study seeks to ascertain the relationship between plasma cytochrome c levels and mitochondrial toxicity due to the antiretroviral therapy (ART) among Ghanaian HIV patients. Although ART has decreased ill-health and death from HIV, long term therapy is associated with toxicity and severe side effects. These side effects if detected early through proper monitoring can be managed and the individual could be protected from developing complications. However, the side effect of ART caused by its toxicity to the mitochondria which are tiny bodies (organelles) in all our cells which provide us with energy are difficult to monitor because of lack of reliable monitoring tool (biomarker).

Cytochrome c, a tiny lipid soluble molecule which does not normally occur in the blood but is rather located in the mitochondrial membrane and are important for the proper functioning of the mitochondria could under certain conditions such as the effects caused by certain drugs be released into the blood. Under this condition, the mitochondria become dysfunctional and the released cytochrome c initiates a process called programmed cell death (apoptosis). The uncontrolled cell death and mitochondrial dysfunction are known to be responsible for some of the side effects associated with the antiretroviral therapy. Therefore measurement of
plasma cytochrome c level could serve as a monitoring tool for ART-induced mitochondrial toxicity.

The study therefore seeks to understand whether plasma cytochrome c levels are higher in HIV patients on ART, who are showing clinical signs of mitochondrial toxicity.

**Possible Risks and Discomforts**

This study will require you to provide about 10ml of blood. The blood will be drawn by a trained medical laboratory personnel. There is some amount of discomfort associated with the process but this will be the same as what you will normally feel during routine blood collection in the laboratory for your regular checkups. Your blood sample will be collected only once in this study.

**Possible Benefits**

The direct benefit of this study to you is that the laboratory results will be made available to you on request and you will also know whether you have developed or in the process of developing mitochondrial toxicity due to your medication. The results from the study will also provide background information which will contribute to identifying a factor (biomarker) for monitoring ART-induced mitochondrial toxicity.

**Confidentiality**

Data shall be well secured and protected from any other person apart from the supervisors and the investigator. All information gathered will be treated with strict confidentiality.
Compensation

Because of lack of funds, there is no compensation for participation, however patients who need to travel from far could be assisted with transportation.

Additional Cost

This study will be carried out at no cost to participants.

Voluntary Participation and Right to Leave the Research

Your participation in this study is absolutely voluntary and you are free to withdraw.

Notification of Significant New Findings

The results of the study will be made available to participants on request.

Contacts for Additional Information

If you have further questions on the study you can contact:

Dr. Augustine Ocloo (0242005428) of College of Basic and Applied Sciences, UG, Dr. Bismark Sarfo (0244244892) of School of Public Health, CHS or Dr. Evelyn Y. Bonney (0244785677) of Noguchi Memorial Institute for Medical Research, CHS, UG

Your rights as a Participant

This research has been reviewed and approved by the Ethical Review Committee of the College of Basic and Applied Sciences (ECBAS), University of Ghana. If you have any questions about your rights as a research participant you can contact the Administrator of ECBAS, Mr. Sampson Addo between the hours of 8am-5pm on 0244 692 728 or email addresses: saddo@staff.ug.edu.gh
VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title (*name of research*) has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

...........................................  .................................................................
Date  Name and signature or mark of volunteer

**If volunteers cannot read the form themselves, a witness must sign here:**

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

...........................................  .................................................................
Date  Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

...........................................  .................................................................
Date  Name Signature of Person Who Obtained Consent
Appendix II: Abstraction Questionnaire

ID: …………………………………

Abstraction date: …………………………………

Patient Identification

1. Patient’s ID code: ………………………………………………………………………

2. Date of Birth: ………………………………………………………………………

3. Age: ………………………

4. Gender:
   - Male
   - Female

5. Marital Status
   - Married
   - Single
   - Divorced
   - Separated
   - Widow
   - Cohabiting

6. Educational Level
   - Nil
   - Primary
   - JHS
   - SHS/Tech
   - MSLC
7. Occupation
   - Full time
   - Part time
   - Self employed
   - Unemployed

8. Religion
   - Muslim
   - Christian
   - Traditional
   - Other
   - None

9. Referral Information
   - Diagnostic testing
   - Walk-in VCT site
   - PMTCT Program
   - Old patient
   - Transfer in on ART
   - Transfer in from pediatric
   - From TB program
   - STI testing

10. Funding Option
    - Out of pocket
    - Medical insurance
    - Special project
Clinical Care

11. Date of HIV+ test: .................................................

12. HIV type
   - HIV I
   - HIV II
   - HIV I & HIV II

13. TB screening
   - Yes
   - No

14. If yes for 13…TB result
   - Positive
   - Negative

15. TB treatment initiated
   - Yes
   - No

16. Presence of opportunistic infections
   - Positive
   - Negative

17. Current opportunistic infection medication
   - Cotrimoxazole
   - Fluconazole
   - TB treatment

18. Medication for other conditions
   - Yes
19. Medication Name: .................................................................

20. Past ARV experience
   o Yes
   o No

21. If yes in 20 Drugs and Dates
   i. Drug: ..........................................................Duration: .........................
   ii. Drug: ..........................................................Duration: .........................
   iii. Drug: ..........................................................Duration: .........................

Pregnancy information

22. Pregnant? .................................................................

23. Duration: .............................................

24. Expected delivery date: .........................

25. ARV prophylaxis for PMTCT
   o Yes
   o No

26. If yes in 25, Date started .........................

27. (If sexually active) Disclosure to sexual partner?
   o Yes
   o No

28. (If sexually active) Regular condom use?
   o Yes
   o No

29. Symptom Screen (eg. Jaundice)
   i. Symptom ....................................................

87
ii. Symptom ........................................

iii. Symptom ........................................

iv. Symptom ........................................

**CD4 test information**

30. Baseline CD4 count: ..............................

31. Baseline test date: .................................

32. Second CD4 count: .................................

33. Date of second CD4 count: ......................

34. Third CD4 count: .................................

35. Date of third CD4 count: .......................

36. Fourth CD4 count: .................................

37. Date of fourth CD4 count: ......................

38. Most recent CD4 count: .........................

39. Date of most recent CD4 count: ...............  

40. WHO clinical stage of patient
   - Stage 1
   - Stage 2
   - Stage 3
   - Stage 4

41. Is patient recommended for ART treatment
   - Yes
   - No

42. If yes for 41, was adherence counselling done?
   - Yes
   - No
43. Is patient on ART?
   - Yes
   - No

**ARV treatment and Adherence**

44. Date of last visit: ……………………………..

45. Number of ARV missed

**ARV status of patient**

46. Start date: …………………………………………

47. Patient length of time on continuous use of ART
   - Between 1 to 3 months
   - Between 3 to 6 months
   - Between 6 to 9 months
   - Between 9 to 12 months
   - For more than 12 months
   - Discontinued ART completely

48. If ART discontinued, what was the reason?
   ……………………………………………………………………………………………
   ……………………………………………………………………………………………

**ARV treatment prescribed**

49. First line ARV drugs

   **First choice**
   - AZT+3TC+NVP
   - AZT+3TC+EFV
Second choice

- AZT+3TC+NVP
- TDF+3TC+EFV
- TDF+FTC+NVP
- TDF+FTC+EFV

50. Second line ARV drugs

First choice

- TDF+FTC+LPV/r
- TDF+3TC+LPV/r
- TDF+FTC+ATV/r
- DF+3TC+ATV/r

Second choice

- AZT+3TC+LPV/r
- AZT+3TC+ATV/r
# Appendix III: Cyt-c ELISA Reagents Preparation

## (A) Wash Buffer

<table>
<thead>
<tr>
<th>Number of Strips</th>
<th>Wash Buffer Concentrate (ml)</th>
<th>Distilled Water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 6</td>
<td>25</td>
<td>475</td>
</tr>
<tr>
<td>1 - 12</td>
<td>50</td>
<td>950</td>
</tr>
</tbody>
</table>

## (B) Assay Buffer

<table>
<thead>
<tr>
<th>Number of Strips</th>
<th>Assay Buffer Concentrate (ml)</th>
<th>Distilled Water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 6</td>
<td>2.5</td>
<td>47.5</td>
</tr>
<tr>
<td>1 - 12</td>
<td>5.0</td>
<td>95.0</td>
</tr>
</tbody>
</table>

## (C) Biotin-Conjugate

<table>
<thead>
<tr>
<th>Number of Strips</th>
<th>Biotin-Conjugate (ml)</th>
<th>Assay Buffer (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 6</td>
<td>0.03</td>
<td>2.97</td>
</tr>
<tr>
<td>1 - 12</td>
<td>0.06</td>
<td>5.94</td>
</tr>
</tbody>
</table>

## (D) Streptavidin-HRP

<table>
<thead>
<tr>
<th>Number of Strips</th>
<th>Streptavidin-HRP (ml)</th>
<th>Assay Buffer (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 6</td>
<td>0.03</td>
<td>5.97</td>
</tr>
<tr>
<td>1 - 12</td>
<td>0.03</td>
<td>11.94</td>
</tr>
</tbody>
</table>