The effect of Tenofovir on renal function and immunological response in HIV-positive patients in Lesotho

ELTONY MUGOMERI

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Supervisor: Dr. D. Olivier (DTech)
Co-supervisor: Prof. WMJ vd Heever-Kriek (PhD)

BLOEMFONTEIN

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DECLARATION WITH REGARD TO INDEPENDENT WORK

I, ELTONY MUGOMERI, identity number- ███████ and student number-210028203, do hereby declare that this research project submitted to the Central University of Technology, Free State for the Degree MAGISTER TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY, is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

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DEDICATION

This work is dedicated to my mum and my late father who taught me to have a positive attitude and to work hard all the time when duty calls.

ACKNOWLEDGEMENTS

SUMMARY

INTRODUCTION: The renal effects of Tenofovir Disoproxil Fumarate (TDF) and antiretroviral treatment (ART) outcomes remain under-reported in African settings. The study sought to assess immunological outcomes and to compare renal function outcomes between patients exposed to TDF and unexposed patient group.

METHODS: Phase 1 of the study was a retrospective case control analysis of serum creatinine data for 312 ART naïve adult patients exposed to TDF and 173 unexposed patients enrolled on ART between Dec 2006 and Jan 2011 at Roma Health Service Area in Lesotho. Sub-optimal renal function outcomes were serum creatinine clearance values <50 ml/min calculated using the Cockcroft-Gault equation. Phase 2 was based on re-sampling of the study population and analysis of CD4 counts of 516 adult naïve HIV-positive patients. Univariate logistic regression (p<0.1) and multivariate analyses (p<0.05) were performed using STATA$^®$ version 11 software.

RESULTS: Overall, 153 (31.5%) patients had moderate baseline (30-60 ml/min) renal insufficiency. Renal function improved by +2 ml/min at 24 months. Almost 18% (n=312) of the patients on TDF were erroneously put on TDF. The use of TDF was a marginally significant factor (p=0.054) associated with CrCl<50 ml/min outcomes in univariate analysis but was insignificant (p=0.122) in multivariate analysis. Female gender (p=0.016), high blood pressure (p=0.009), ages over 60 (p=0.004), and underweight (p<0.001) were significantly associated with CrCl<50 ml/min outcomes. The proportion of patients who developed immunological failure in this study was low (6.8%, n=516). The mean CD4 count increased significantly after treatment (p<0.001). Baseline CD4 count below 50 cells/mm^3 (p=0.049) and male gender (p=0.005) were significantly associated with sub-optimal immunological outcomes.

CONCLUSIONS: TDF is a weak contributing factor associated with renal impairment outcomes compared to other variables such as hypertension, older age, underweight and female gender. More research on long term effects of TDF is recommended. Baseline renal function screening should be improved to minimise leakages of patients contraindicated of TDF. Although the patients' immunological status generally improved, males and patients with low baseline CD4 counts should be monitored closely while on ART.

Key words: *Tenofovir; antiretroviral treatment; CD4 count; immunological failure*

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
3TC	Lamivudine
ABC	Abacavir
ACE	Angiotensin converting enzyme
ADA	American diabetes association
AIDS	Acquired Immunodeficiency Syndrome
AIF	Apoptosis-inducing factor
ALP	Alkaline phosphatase
ALT	Alanine transaminase
anti-HBc	Antibodies to Hepatitis B core antigens
ART	Antiretroviral treatment
ARV	Antiretroviral
ASAT	Aspartate aminotransferase
AST	Aspartate transaminase
ATP	Adenosine triphosphate
ATV/r	Atazanavir boosted with Ritonavir
AZT	Zidovudine
BL	Baseline
BMI	Body mass index
BNF	British national formulary
BP	Bloood Pressure

BUN	Blood urea nitrogen
CA	Capsid (protein)
CCR5	Chemokine co-receptor 5 (Sub-family CC)
CI	Confidence interval
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology
CrCl	Serum creatinine clearance
^{51}Cr-EDTA	Ethylenediaminetetraacetic acid radiolabeled with Chromium-51
CRP	C-reactive protein
CxCR4	Chemokine co-receptor 4 (Sub-family Cx)
CytC	Cytochrome C
D4T	Stavudine
Da	Daltons
dATP	Deoxyadenosine triphosphate
ddATP	Dideoxyadenosine triphosphate
DDI	Didanosine
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbant assay
Env	HIV "Envelope" gene which codes for gp160
EPO	Erythropoietin
ESRD	End-stage renal disease
FAD	Flavin adenine dinucleotide
FDA	Food and drug administration

fl	Femtolitre (1 fl = 10^{-18} litres)
FTC	Emtricitabine
g/dl	Grammes per decilitre (1 deci-litre = 0.1 litres)
Gag	Group specific antigens
GFR	Glomerular filtration rate
GGT	Gamma glutamyl transpeptidase
GoB	Government of Botswana
GoL	Government of Lesotho
GoS	Government of Swaziland
gp	Glycoprotein
H^+	Hydrogen ions
Hb	Haemoglobin
HBeAg	Hepatitis e antigen
HBsAg	Hepatitis B surface antigens
HBV	*Hepatitis B Virus*
HCT	Haematocrit
HDL	High density lipoproteins
HDL-C	High density lipoproteins cholesterol
HIV	*Human immunodeficiency virus*
HLA	Human leukocyte antigen
*h*OAT	Human organic ion transporter
HTN	Hypertension
IgM	Immunoglobulin M
IMF	Immunological failure

IQR	Inter-quartile range
IU/l	International units per litre
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LDL-C	Low density lipoproteins cholesterol
LFT	Liver function test
LPV/r	Lopinavir boosted with Ritonavir
LTR	Long terminal repeat
M	Main (HIV variant)
MA	Matrix (protein)
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
MDRD	Modification of diet in renal disease
MEIA	Microparticle enzyme immunoassay
mg/dl	milligrammes per decilitre
MHCc	Major histocompatibility complex
MHC	Mean haemoglobin concentration
ml/min	Millilitres per minute
mm^3	Cubic millimetre (1 mm^3 = 10^{-9} m^3)
mmHg	Millimetre mercury
mmol/l	Millimoles per litre
MRP	Apical membrane transporter
mtDNA	Mitochondrial DNA

N	Non-M and non-O (HIV variant)
NASBA	Nucleic acid sequence based amplification
Nef	Negative regulatory factor
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
O	Outlier (HIV variant)
OAT	Organic ion transporter
OR	Odds ratio
PCR	Polymerase chain reaction
PEPFAR	President's emergency plan for AIDS relief
pg	Picogramme (1 pg = 10^{-15} kilogrammes)
PGE2	Prostaglandin E2
P-gp	Permeability glycoprotein
PI	Protease inhibitor
PIs	Protease inhibitors
Pol	HIV gene that encodes for reverse transcriptase, integrase, and protease
POLG	Mitochondrial DNA polymerase gamma (γ)
PR	Pyrophosphate
PTH	Parathyroid hormone
RBC	Red blood cell
RDW	Red blood cell distribution width
Rev	Regulator of expression of virion proteins
RHSA	Roma health service area
RNA	Ribonucleic acid

RT	Reverse Transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
T helper	A sub-group of lymphocytes which mature in the thymus
Tat	Trans-activator of transcription
TB	Tuberculosis
Tc-DTPA	Diethylene triamine pentacaetic acid radiolabeled with Technetium-99
TDF	Tenofovir Disoproxil Fumarate
TG	Triglycerides
Tm	Trans-membrane
μ	Micro (10^{-6})
UDP-	Uridine diphospho-
μmol/l	Micromoles per litre
Vif	Viral infectivity factor
VL	Viral load
VLDL	Very low density lipoproteins
VLF	Virological failure
Vpr	Viral protein R
Vpu	Viral protein unique
WHO	World health organisation
γ	Gamma

CHAPTER 1:   INTRODUCTION

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1.1 Background

Human immunodeficiency virus (HIV) is a lentivirus (slowly replicating retrovirus) that causes acquired immunodeficiency syndrome (AIDS) (Cochrane, 2011). According to a report by the Government of Lesotho (GoL), Lesotho has an adult HIV and AIDS prevalence rate of 23% (GoL, 2012). The country ranks third highest in the world after Botswana and Swaziland which have prevalence rates of 25% and 26% respectively (Government of Botswana (GoB), 2012; Government of Swaziland (GoS), 2012). The Government of Lesotho launched the decentralised antiretroviral treatment (ART) programmes as part of the effort to mitigate the impact of HIV and AIDS. Nonetheless, some of the challenges facing the decentralised ART programmes include limited access to specialised laboratory tests such as viral load tests required to check for emergence of HIV drug resistance and a limited number of first-line and second-line ART regimens.

Tenofovir Disoproxil Fumarate (TDF) was introduced at Roma Health Service Area in 2008. The assessment of renal function in patients on TDF-based ART regimens is important because TDF has been associated with renal toxicity (Young, Buchacz, Baker, Moorman, Wood, Chmiel & Brooks, 2007). Renal function outcomes following the use of TDF have not been reported at Roma Health Service since TDF was introduced. The assessment of TDF renal toxicity is also of particular importance in Lesotho because the success of current ART programmes in Lesotho depends so much on the performance of the drug TDF, which currently forms the backbone of first-line ART drugs.

In the first reports on TDF-associated renal outcomes at Scott Hospital in Lesotho by Bygrave, Kranzer, Hilderbrand, Jouquet, Goemaere, Vlahakis, Triviño, Makakole & Ford (2011a) in 2008 and Bygrave, Ford, van Cutsem, Hilderbrand, Jouquet, Goemaere, Vlahakis, Triviño, Makakole & Kranzer (2011b) in 2009, TDF showed favourable outcomes with rare cases of TDF-associated renal toxicity. However, the

follow up time was limited to 24 months. The prevalence of sub-optimal renal function outcomes in HIV patients on TDF need to be investigated in different cohorts preferably with longer duration of treatment to establish the trend of TDF-associated renal function outcome in Lesotho. Moreover, data on TDF-associated renal function outcomes compared to renal function outcomes in HIV patients on non-TDF-based regimens in Lesotho are scarce.

The assessment of treatment outcomes in terms of immunological outcomes as assessed by clusters of differentiation 4 (CD4) counts in Lesotho is another important issue where there is a gap. It is not known if the introduction of TDF at Roma Health Service Area has resulted in improved treatment outcomes. ART outcomes in Lesotho are still measured by CD4 counts despite the limited utility of CD4 counts in detecting HIV treatment failure (Kanapathipillai, McGuire, Mogha, Szumilin, Heinzelmann & Pujades-Rodriguez, 2011). According to Tiam (2008), Lesotho may be a fertile ground for the emergence of drug resistance and treatment failure mainly due to poor adherence to antiretroviral drugs. Summarily, the study had two main aims. Firstly, to assess renal function outcomes and secondly, to assess immunological outcomes of patients and compare the outcomes of the TDF-exposed patients to a control group of TDF-unexposed patients.

1.2 Problem Statement

Although numerous studies report that TDF is generally safe to use with respect to nephrotoxicity, other studies have found evidence of TDF-associated renal toxicity. The most common conclusions from the studies on TDF safety stated that TDF-associated nephrotoxicity is a rare event and that most patients who develop severe nephrotoxicity on exposure to TDF have pre-existing renal disorders. The studies include Reid, Stöhr, Walker, Williams, Kityo, Hughes, Kambugu, Gilks, Mugyenyi, Munderi, Hakim & Gibb (2008) in Zimbabwe; Brennan, Evans, Maskew, Naicker, Ive, Sanne, Maotoe & Fox (2011) in South Africa; Gérard, Chazallon, Taburet, Girard, Aboulker & Piketty (2007) in France; and O'Donnell, Scarsi, Darin, Gerzenshtein, Postelnick & Palella (2011) in the USA.

Contrarily, various studies have found evidence of TDF nephrotoxicity in non-predisposed patients. In one African setting (Senegal), De Beaudrap, Diallo, Landman, Guèye, Ndiaye, Diouf, Kane, Etard, Girard, Sow & Delaporte (2010), reported that patients on TDF had higher rates of transition from mild to moderate renal insufficiency. Young *et al.* (2007) in the USA and Manosuthi, Mankatitham, Lueangniyomkul, Prasithsirikul, Tantanathip, Suntisuklappon, Narkksoksung, Nilkamhang & Sungkanuparph (2010b) in Thailand found that TDF-exposed groups had significantly higher incidences of renal disorder compared to TDF-unexposed groups. The use of TDF in HIV treatment therefore remains controversial with respect to renal safety. The controversy underpins the need to monitor patients on TDF-based ART.

Besides TDF-associated nephrotoxicity, data on immunological outcomes are lacking. This problem is not unique to Lesotho only, but generally to many African settings. The evaluation of immunological outcomes is important in many ways. Firstly, the evaluation of immunological outcomes may highlight the overall performance of the ART programme in meeting the goals of ART. Secondly, the evaluation of immunological outcomes may show the effectiveness of ART drugs in use as well as the recently introduced drug TDF.

1.3 Aims and Objectives

1.3.1 Aims

The study had two main aims. The first aim was to determine the extent to which TDF may cause renal toxicity by comparing the differences in renal function outcomes between patients who are on TDF-based ART regimens and the patients on non-TDF-based ART regimens. The second aim was to determine the incidence of sub-optimal immunological outcomes based on CD4 counts.

1.3.2   Study Objectives

The objectives of the study were:

i. To determine incidence of renal function insufficiency at baseline and variables associated with renal insufficiency function at baseline;

ii. To determine general renal function outcomes and the variables associated with the development of sub-optimal serum creatinine clearance (CrCl) outcomes (CrCl<50 ml/min);

iii. To determine general immunological outcomes based on CD4 counts and the proportion of patients with sub-optimal immunological outcomes (immunological failure);

iv. To identify risk factors associated with sub-optimal immunological outcomes based on CD4 counts.

1.4 Dissertation Outline

Chapter 1 outlines the research problem concerning TDF-associated renal toxicity and immunological outcomes. Chapter 2 reviews the literature pertinent to the understanding of the principles of antiretroviral therapy, renal function, and immunological response. The literature reviewed in chapter 2 formed the basis of the research design outlined in chapter 3. Chapter 3 outlines how data for renal outcomes based on serum creatinine data and immunological outcomes based on CD4 counts, were collected and analysed. Chapter 4 presents results, beginning with renal outcomes and then the immunological outcomes. Chapter 5 discusses the results. Chapter 6 wraps up the study with an analogical account of the implications of the results obtained as well as giving recommendations in light of the current literature on TDF-associated nephrotoxicity and immunological outcomes. Chapter 7 lists the references cited in the study. Chapter 8 (Appendices) comprises ethical clearance documents and more detailed technical methodologies used in data analysis.

CHAPTER 2: LITERATURE REVIEW

CHAPTER 2:   LITERATURE REVIEW

The chapter summarised the impact of HIV and AIDS on the health sector in Lesotho; the challenges facing the current ART regimens in Lesotho; principles of antiretroviral therapy, immunological response, and renal function; as well as the laboratory tests required for initiating ART and monitoring patients while on ARVs. The chapter also critically assessed the evidence on TDF renal toxicity and weighed the effect of other risk factors for developing renal abnormalities against the threat from TDF. Other pertinent themes central to the study objectives or informative to the subjects in focus were also included.

2.1 Background Information on HIV and AIDS in Lesotho and Current ART Practice

2.1.1   Impact of HIV and AIDS on the health sector, intervention programmes; and the challenges

Lesotho, which is located in the eastern part of Southern Africa, is a landlocked country completely surrounded by the Republic of South Africa. According to a report by the Government of Lesotho, Lesotho has an adult HIV and AIDS prevalence rate of 23% (GoL, 2012). The country ranks third highest in the world after Botswana and Swaziland which have the prevalence rates of 25% and 26% respectively (GoB, 2012; GoS, 2012).

The disease HIV and AIDS has had a devastating impact on the economy particularly the health sector. For instance, HIV and AIDS alone has caused a drop in life expectancy over the past two decades from over 50 years to the current estimate of 41 years. In 2011 alone, official figures showed that 8 500 people (close to 0.5% of the entire population) died from HIV and AIDS related illnesses (GoL, 2012). According to 2011 estimates, 289 841 adults and children (about 15% of the population) were living with HIV and AIDS. Out of the number living with HIV and AIDS, 123 187 or 42% were in immediate need of antiretroviral therapy. Moreover, 19% of the total demand for ART was by children under the age of 15. According to GoL (2012), the total 2010 annual investment in HIV and AIDS intervention programmes was about eight hundred and thirteen million Maloti in Lesotho

currency (M 813 million) which was equivalent to one hundred and eight million United States Dollars (USD 110 million). The total annual expenditure on HIV and AIDS was 12.6% of the national budget. HIV and AIDS is therefore, a significant burden on the national budget.

This monetary budget could only meet 70% of the total cost estimates and the other 30% was supplemented by development partners such as Global Fund, President's Emergency Plan For AIDS Relief (PEPFAR), Millennium Challenge Account, Clinton Health Access Initiative, World Bank, European Union, Irish Aid, Baylor International Paediatric AIDS Initiative, the Kellogg Foundation among others (GoL, 2012). The large number of development partners involved indicates that the disease HIV and AIDS poses a huge financial challenge to Lesotho.

The Government of Lesotho has made great efforts to mitigate the impact of HIV and AIDS. Some of the intervening steps taken by the Government include increasing the budget allocation for HIV and AIDS and decentralising the provision of ART at the health center level (GoL, 2012). However, the decentralised ART programmes are facing many challenges which include limited resources to perform basic and specialised laboratory tests such as viral load tests, and lack of adherence to ART by some patient groups (Tiam, 2008).

Following recommendations by the WHO, the Government of Lesotho switched patients on Stavudine-based antiretroviral drug regimens to TDF-based regimens (WHO, 2010). Since then, TDF has become the cornerstone of antiretroviral treatment of HIV and AIDS in Lesotho. The switching from Stavudine was in principle based on the premised survival rates of patients taking TDF-based regimens at higher CD4 count thresholds of 350 cells/mm^3 as compared to Stavudine-based regimens (Figure 2.1). Moreover, the WHO recommendations seemed to be based on recommendations by some key researchers on antiretroviral drugs who postulated that TDF may even be considered for the prophylaxis of HIV infections (De Clercq, 2009; WHO; 2009).
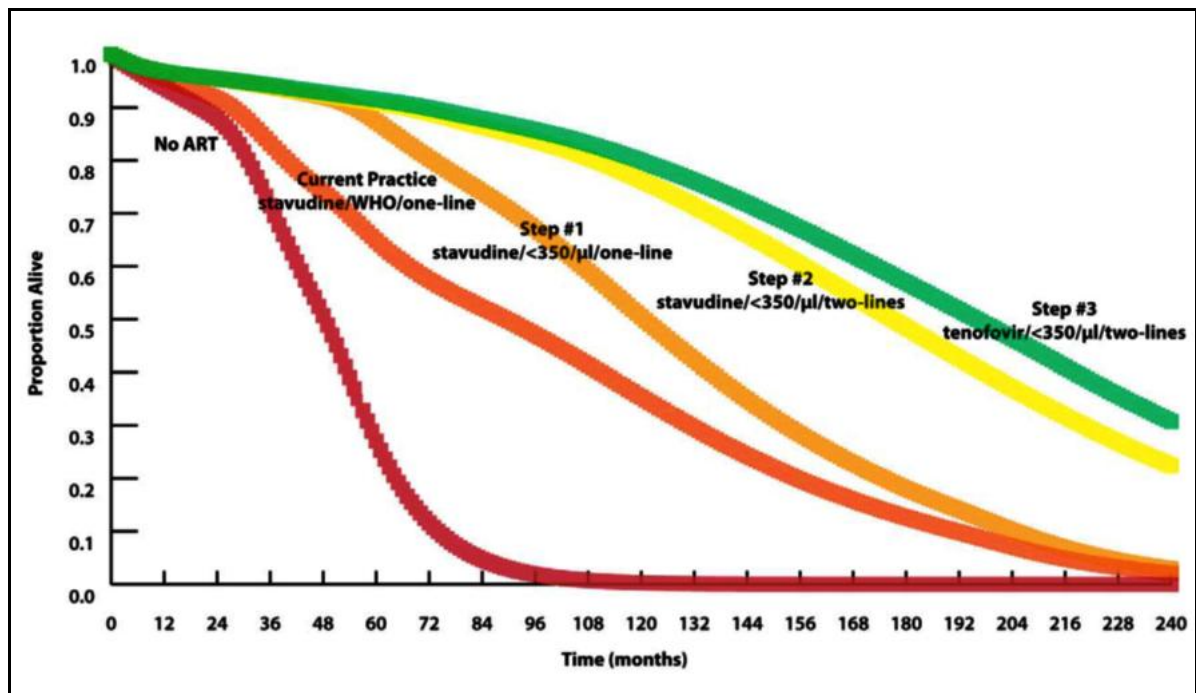
Figure 2.1: Projected survival curves for the different ART treatment models.

(Walensky, Wood, Ciaranello, Paltiel, Lorenzana, Anglaret, Stoler & Freedberg, 2010).

The use of TDF-based antiretroviral drugs has been associated with renal toxicity. The similarity among TDF and structurally related nephrotoxic drugs is what brought about the concerns of TDF safety in the first place (Fernandez-Fernandez, Montoya-Ferrer, Sanz, Sanchez-Niño, Izquierdo, Poveda, Sainz-Prestel, Ortiz-Martin, Parra-Rodriguez, Selgas, Ruiz-Ortega, Egido & Ortiz, 2011). Researchers then began TDF safety trials on laboratory animals and human beings. Unfortunately, while the debate on renal safety of TDF still rages on, numerous HIV patients in many countries, including Lesotho, are already taking the drug. Therefore, despite the survival benefits attributable to the use of TDF, TDF may pose a risk of renal toxicity. This review begins by looking at current antiretroviral drug repertoire and regimens combinations used in Lesotho before moving on to review the profiles of the drug regimens.

2.1.2   The rationale of use of ART regimens in Lesotho and the challenges

The current practice in Lesotho recommends the use of three ARV drugs given as a combination. In HIV patients who have never been on HIV treatment before (treatment naïve), the first line treatment consists of two nucleoside reverse

transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) selected from the recommended drugs shown in Table 2.1 (GoL, 2010). The use of regimens based on at least two drug classes is done to minimise emergence of drug resistance among the patients taking the antiretroviral drugs. NRTIs currently in use include TDF as shown in Table 2.1.

Table 2.1: Current first line and second line ART drugs recommended for adults in Lesotho.

First line drugs		Second line drugs	
NRTIs	NNRTIs	NRTIs	Protease inhibitors (PIs)
Lamivudine (3TC)	Nevirapine (NVP)	Didanosine (DDI)*	Lopinavir/r (LPV/r)
Tenofovir Disoproxil Fumarate (TDF)	Efavirenz (EFV)	Abacavir (ABC)	Atazanavir/r (ATV/r)[*]
Zidovudine (AZT)			

NRTI = Nucleoside reverse transcriptase inhibitor; * = drug not available; NNRTI = non-nucleoside reverse transcriptase inhibitor (GoL, 2010).

The number of alternative ART regimens is severely limited. For example, in the second line NRTI category, DDI is no longer being supplied. Atazanavir co-formulated with a dosage booster drug, Ritonavir (ATV/r) is also not widely available in Lesotho. The limited number of possible combinations for the currently available ART regimens is further limited by the existence of drug to drug interactions, adverse drug reactions and patient conditions such as comorbidities and pregnancy (See Table 2.2). For example, in HIV patients on TB treatment that includes Rifampicin, the number of ART combinations is even more limited. Rifampicin reduces concentrations of NNRTIs (especially Nevirapine) and some Protease inhibitors (PIs) because Rifampicin induces the cytochrome P450 enzymes and a drug efflux pump called permeability glycoprotein (P-gp) (Wilson, Cotton, Bekker, Meyers, Venter & Maartens, 2008). HIV patients co-infected with TB therefore may need to be given a PI drug, Lopinavir boosted with Ritonavir (LPV/r) at an added expense.

Table 2.2: Current guidelines on ART practice in Lesotho.

Target population	Preferred option	Alternative	Indications and contraindications criteria
Adults and Adolescents (>12 years and >35 kg)	TDF+3TC+EFV	AZT+NVP	TDF+3TC+EFV is the preferred 1st-line regimen. May use ABC if TDF is contraindicated. Avoid TDF if BL CrCl is <50 ml/min.
Pregnant women	TDF+3TC+EFV	TDF+NVP or AZT+NVP	Use NVP in women who expect to become pregnant. Do not initiate EFV in the first trimester. Avoid AZT in patients with Hb<8mg/dl (use TDF). NVP in women with BL CD4 between 250 and 350 may cause hepatotoxicity.
HIV with TB comorbidity	TDF+3TC+EFV	AZT or boosted LPV/r	Initiate ART as soon as possible within the first 8 weeks. Avoid NVP in patients on TB treatment. Use AZT+3TC+EFV. Use LPV/r if Efavirenz is contra-indicated.
HIV with Hepatitis comorbidity	TDF+3TC+EFV	NVP	Do HBsAg screening at BL. Use two ARVs with anti-HBV activity

AZT = Zidovudine; 3TC = Lamivudine; EFV = Efavirenz; LPV/r = Lopinavir/Ritonavir; NVP = Nevirapine; TDF = Tenofovir; ABC = Abacavir; CrCl = serum creatinine clearance (GoL, 2010).

Stavudine is no longer used and has been phased out due to adverse drug reactions as recommended by the World Health Organisation (WHO, 2010). The phasing out of Stavudine presented a challenge to ART programmes in Lesotho because a smaller number of drug combinations remained. Furthermore, the available ART drug combinations in Lesotho may pose even a greater challenge to ART programmes in future if serious cases of drug resistance emerge.

To illustrate the predicament that the limited ART regimens pose, a pregnant female in the first trimester with baseline renal insufficiency and tuberculosis-HIV (TB/HIV) co-infection (not an uncommon event in Lesotho) would be contra-indicated for TDF, NVP, and EFV (Table 2.2), meaning that such a patient would be treated with

Abacavir and probably LPV/r, drugs which happen to be in the second line. Moreover, according to Lesotho guidelines, TDF should be ceased if calculated creatinine clearance is less than 50 ml/min (GoL, 2010). In cases where patients are anaemic and at the same time cannot be treated with TDF due to renal insufficiency, AZT and TDF are ruled out leaving the clinicians with no other first line drug combination (GoL, 2010). Putting some patients in the second line because of the limited first line drug combinations might compromise the national arsenal of drugs for future use against emerging HIV drug resistant strains.

Switching to second line is recommended in cases where treatment failure as assessed by clinical and CD4 count is suspected or confirmed by viral load tests. However, both access to viral load tests and access to second line ARVs in Lesotho is limited (GoL, 2010). The limitations imply that the capacity to prove explicitly that a patient needs second line therapy as well as the capacity to supply more efficacious but expensive ARVs is greatly curtailed.

Notwithstanding the proven efficacy that TDF has shown against HIV and Hepatitis (De Clercq, 2009), over-dependence on one drug such as TDF might result in the emergence of transmitted HIV drug resistance cases. Transmitted HIV drug resistance may occur if individuals with acquired drug resistance, probably due to lack of adherence to TDF pass the infection to ART naïve individuals (Clavel & Hance, 2004; Gianella & Richman, 2010).

Therefore, the Lesotho national guidelines on HIV-AIDS care and treatment have TDF as one drug of choice for first line treatment. The main challenges facing the ART programmes in Lesotho include a limited number of ARV combinations, limited access to viral load tests and overdependence on one drug TDF.

2.2 Nephrotoxicity of TDF: The Evidence; the Controversy; and the Discrepancies in the Studies on TDF Nephrotoxicity

2.2.1 The evidence and the controversy

Tenofovir disoproxil fumarate (TDF) has been generally approved to be a safe drug for HIV-1 treatment (Gallant & Deresinski, 2003). In 2009, the WHO recommended that TDF and Zidovudine replace Stavudine. However, animal studies (Lebrecht,

Venhoff, Kirschner, Wiech, Venhoff & Walker, 2009), case reports (Herlitz, Mohan, Stokes, Radhakrishnan, D'Agati & Markowitz, 2010) and observational studies (Wood, Shah, Steenhoff, Meyers, Kaplan & Rutstein, 2009; Agarwala, Mohan, Herlitz & Cheng, 2010; Soler-Palacín, Melendo, Noguera-Julian, Fortuny, Navarro, Mellado, Garcia, Uriona, Martín-Nalda & Figueras, 2011) have shown that high doses of TDF can cause nephrotoxicity.

Fewer studies have been done on the African continent compared to other continents such as Europe. A study carried out in Johannesburg, South Africa by Brennan, Evans, Maskew, Naicker, Ive, Sanne, Maotoe & Fox (2011), reported that TDF may exacerbate pre-existing renal disorders but may not be responsible for initiating renal dysfunction in patients with baseline CrCl>50 ml/min. The study however recommended screening renal function before the administration of TDF and dose adjustments in patients with CrCl<50 ml/min.

Another study done in Senegal by De Beaudrap *et al.* (2010) had a sample of 40 patients exposed to TDF. Patients on TDF experienced a higher rate of transition from mild to moderate renal impairment when compared with patients on non-TDF regimens. In a study carried out in Zimbabwe, by Reid *et al.* (2008), the researchers reported that renal impairment in patients on ART was evidently related to intercurrent or concomitant disease. The same study did not find any significant differences among ART regimens with respect to renal function. Reid *et al.* (2008) were however cautious. They recommended that studies on long term use of ART may be necessary.

High doses of TDF have been observed to cause nephrotoxicity in some animal studies (Van Rompay, Brignolo, Meyer, Jerome, Tarara, Spinner, Hamilton, Hirst, Bennett, Canfield, Dearman, Von Morgenland, Allen, Valverde, Castillo, Martin, Samii, Bendele, Desjardins, Marthas, Pedersen & Bischofberger, 2004). Cases of severe renal dysfunction and significant reductions in creatinine clearance among TDF-treated patients were reported after the drug was certified for public use (Antoniou, Raboud, Chirhin, Yoong, Govan, Gough, Rachlis & Loutfy, 2005; Winston, Amin, Mallon, Marriott, Carr, Cooper & Emery, 2006). Current international guidelines contraindicate use of TDF when creatinine clearance falls below 50

ml/min but some exceptions can be made if dosages of TDF are sufficiently reduced (Antoniou *et al.*, 2005).

However, TDF was not associated with significant renal toxicity or changes in glomerular filtration rate (GFR) in clinical trials of patients with baseline CrCl>50 ml/min (Schooley, Ruane, Myers, Beall, Lampiris, Berger, Chen, Miller, Isaacson & Cheng, (2002); Izzedine Hulot, Vittecoq, Gallant, Staszewski, Launay-Vacher, Cheng & Deray, 2005). In a study carried out in the United Kingdom with a relatively large sample size of 10 343 adults who were all exposed to TDF-containing ART, 51 patients developed serious renal disorders and 227 developed increased serum creatinine (Nelson, Katlama, Montaner, Cooper, Gazzard, Clotet, Lazzarin, Schewe, Lange, Wyatt, Curtis, Chen, Smith, Bischofberger & Rooney, 2007). Baseline risk factors for the development of increased serum creatinine were elevated serum creatinine, concomitant nephrotoxic medications, underweight, advanced age, and lower CD4 count.

In other studies conducted in the USA by Gallant, Winston, DeJesus, Pozniak, Chen, Cheng & Enejosa (2008) and in France by Izzedine *et al.* (2005), the authors concluded otherwise. The USA study included 556 on TDF and 555 control patients on Stavudine or Zidovudine. The France study included 299 on TDF and 301 on Stavudine. Both studies had a similar duration of 144 weeks. Only small clinically insignificant differences in GFR over 144 weeks were noticed. The France study even reported that TDF and Stavudine had similar renal safety profiles in treatment-naive HIV-infected patients who had the baseline CrCl>50 ml/min.

The effect of TDF is likely to become clearer with time as many more patients continue to take the drug. In a study conducted by Crum-Cianflone, Ganesan, Teneza-Mora, Riddle, Medina, Barahona & Brodine (2010), renal dysfunction was associated with duration of tenofovir use, older age and lower CD4 nadir where CD4 nadir refers to lowest CD4 cell count measured after HIV infection. Long term use of TDF therefore is a major concern.

Very few studies attempted to establish empirical evidence of TDF on renal tubules or mitochondria in renal tubular cells. Maggi, Montinaro, Bellacosa, Pietanza, Volpe, Graziano, Strippoli & Angarano (2012) attempted to demonstrate TDF-associated

mitochondrial damage and tubular cell damage by measuring urinary cytochrome C and alpha-glutathione S-transferase (α-glutathione S-transferase) respectively. Mitochondrial damage was confirmed by moderate increases in urinary cytochrome C but tubular cell damage could not be confirmed.

Since 2005, many prospective and retrospective studies, including case reports, have been published. The studies do not agree on the renal safety of TDF. A summary of studies on the link between use of TDF and development of renal disorders is given in Table 2.3. The differences in conclusions by different researchers may indicate the need for more research in different settings and patients' characteristics to ascertain the renal outcomes of patients on TDF.

2.2.2   Methodology discrepancies in the studies on renal safety of TDF

According to Young *et al.* (2007), the apparent differences in findings outlined in Table 2.3 may stem from methodologies employed by different studies on the renal safety of TDF. Intervention studies or clinical trials, cohort studies, case control studies and a fourth type of study called case series were the main methodologies used in the studies on renal safety of TDF. However, the majority of the studies were cohort and case control studies. Comparing the studies is compounded by the the fact that each study had its own unique objectives which required specific methodologies to be followed. This section begins by defining and outlining the limitations of cohort, case control, clinical trials, and case studies; and goes on to point out some of the major differences, similarities, and gaps in the studies on renal toxicity of TDF cited in Table 2.3.

As is noticeable in Table 2.3, the number of clinical trials reported on TDF is relatively small when compared to other types of studies. Apparently, conclusions by the two clinical trials included in Table 2.3 concurred to a large extent with minor but suspicion-rising conclusions. While Izzedine *et al.* (2005), concluded that TDF had similar renal safety profile to Stavudine, Gallant *et al.* (2008), noted that there were small differences in GFR between groups on TDF and the group on Stavudine and Zidovudine although the differences were not clinically significant. Conclusions from the two studies failed to allay the concerns on TDF renal safety by other researchers, hence the debate on TDF renal safety continues.

Table 2.3: A summary of studies on TDF-associated renal injury.

Study, Place	Study design	Measure of renal clearance	Main Findings
Antoniou *et al.* (2005), Canada	Prospective cohort of 172 patients exposed to TDF; observed over 25 months	Serum creatinine data	Slight increases in serum creatinine occurred with TDF. Significant nephrotoxicity was rare.
Izzedine *et al.* (2005), France	Double-blind clinical trial over 36 months; 299 patients exposed to TDF *vs* 301 patients on D4T-based regimens	Serum creatinine data	TDF and Stavudine, had similar renal safety profile in treatment-naive HIV-infected patients with normal renal function at baseline
Padilla *et al.* (2005), Spain	Retrospective case-control study over 12 months; 122 on TDF + Lopinavir/ ritonavir *vs* 194 unexposed	Serum creatinine data	5 patients in the TDF group versus 1 in the control group developed grade 1 or higher serum creatinine elevations (p=0.018).
Gérard *et al.* (2007), France	Prospective cohort over 12 months; 53 patients on TDF + Atazanavir/ ritonavir	Serum creatinine data	TDF dosage was not associated with changes in CrCl. Severe TDF-related nephrotoxicity was a rare event.
Nelson *et al.* (2007), UK	Prospective cohort over 48 months in 10343 adults exposed to TDF-containing regimens	Serum creatinine data	51 developed serious renal disorder. 227 developed elevated serum creatinine. Baseline risk factors were elevated serum creatinine, concomitant nephrotoxic medications, low body weight, advanced age, and lower CD4 count.
Young *et al.* (2007), USA	Prospective cohort over 36 months in 593 patients exposed to TDF *vs* 521 unexposed to	Cockcroft-Gault	Renal disease was diagnosed in 7 TDF-exposed and 3 TDF-unexposed patients.

	TDF		
Gallant *et al.* **(2008), USA**	Randomised - controlled clinical trial over 36 months; 556 on TDF-based regimens vs 555 patients on D4T or AZT-based regimens	Cockcroft-Gault	Although small differences in GFR over time were noted, there was no clinically relevant renal disease or adverse event recorded in antiretroviral-naive patients treated with TDF.
Reid *et al.* **(2008), Zimbabwe**	Prospective study over 24 months in 3 316 patients; TDF-exposed *vs* TDF-unexposed	Cockcroft-Gault	Severe eGFR impairment was infrequent regardless of ART regimen and was generally related to intercurrent disease.
Wood *et al.* **(2009), USA**	Case report: Two adolescents perinatally infected by HIV; patients exposed to TDF-containing regimens.	Serum Creatinine data	There was an abrupt rise in serum creatinine while on TDF. One patient had an underlying kidney disease. The other patient on TDF developed rickets and hypophosphataemia.
Agarwala *et al.* **(2010), USA**	Case report : 41 year old patient exposed to TDF-containing regimen	Urine protein, creatinine	The patient developed proximal tubular dysfunction consistent with TDF toxicity.
Chaisiri *et al.* **(2010), Thailand**	Retrospective study with 21 months follow-up in 405 adults with low body weight; patients exposed to TDF-containing regimens	Serum creatinine data	The study revealed high incidence of TDF-associated renal function decline among patients with low-body weight and BMI
Crum-Cianflone *et al.* **(2010), USA**	Retrospective study over 24 months in 717 adult patients; TDF-exposed *vs* TDF-unexposed group	MDRD	Renal dysfunction was associated with duration of TDF use, lower CD4 nadir and old age.
De Beaudrap *et al.* **(2010), Senegal**	Prospective cohort over 42 months; 40 patients on TDF *vs* 388 patients	Cockcroft-Gault and MDRD	After 12 months, patients on TDF experienced a higher rate of transition from mild to moderate renal impairment when compared

	unexposed to TDF		with patients not on TDF.
Manosuthi *et al.* (2010b), Thailand	Prospective cohort; 28 patients on TDF + EFV *vs* 34 on TDF + NVP group; observed over 6 months	MDRD	TDF-associated renal impairment was higher in the group receiving TDF with Nevirapine compared to TDF with Efavirenz.
Patel *et al.* (2010), India	Observational longitudinal cohort of 1271 adults over 42 months; all patients exposed to TDF-containing regimens	Cockcroft-Gault	79 developed impaired serum creatinine and 5 developed Fanconi's syndrome. All the patients' serum creatinine levels normalised after stopping TDF.
Post *et al.* (2010), UK	Prospective cohort over 12 months in 385 patients; Abacavir+3TC, TDF+EFV, and FTC + EFV	Urinary excretion of retinol-binding protein and β_2-microglobulin	Patients in the TDF with Efavirenz group had higher levels of retinol-binding protein and β_2-microglobulin. No significant difference in GFR between the test groups.
Brennan *et al.* (2011), South Africa	Retrospective cohort over 48 months in 890 adults; all patients exposed to TDF-containing regimens	Serum creatinine data	Renal dysfunction in TDF patients is likely related to pre-existing renal disorder. TDF exacerbates pre-existing renal disorder.
Calza *et al.* (2011), Italy	Retrospective case-control study over 24 months; 201 TDF-exposed *vs* 123 TDF-unexposed to TDF	MDRD	The TDF-exposed group had a greater decline in GFR through 24 months. Reduced GFR was significantly associated with older age, diabetes, hypertension and concomitant therapy with a protease inhibitor.
Manosuthi *et al.* (2011), Thailand	Retrospective over 6 months; 130 adults exposed to TDF-	Serum creatinine data	Incidence of acute renal failure was 0.26 per 100 person-months. Renal function progressed to irreversible damage in one

	containing ART formula		patient.
Nishijima *et al.* (2011), Japan	Retrospective cohort of 495 patients exposed to TDF-containing regimens	MDRD. Renal dysfunction defined as $\geq$25% in CrCl from baseline	97 (19.6%) patients had TDF-associated renal dysfunction. Small body weight was a significant risk while small body mass index had marginal significance (p=0.058).
O'Donnell *et al.* (2011), USA	A retrospective cohort over 72 months in 514 patients; TDF-exposed vs TDF-unexposed group	Serum creatinine data	Renal impairment occurred in 14% of the cohort and was not correlated with exposure to TDF but rather to chronic comorbidity conditions such as diabetes, old age, weight loss and low endpoint CD4 counts.
Pavie *et al.* (2011), France	Case report: one adult male patient exposed to TDF-containing regimen	-	The patient developed acute renal failure 2 weeks after introduction of TDF based ART. Renal impairment was not reversed two years after switching to non-TDF formula.
Soler-Palacín *et al.* (2011), Spain	Prospective cohort over 77 months; 40 infants and adolescents exposed to TDF-containing regimens for at least six months	Serum creatinine, urine protein, serum phosphate and potassium.	TDF use showed a significant association with renal tubular dysfunction in HIV-infected paediatric patients.
Tordato *et al.* (2011), Italy	Retrospective case-control in 1 505 patients; TDF-exposed *vs* TDF-unexposed group	Cockcroft-Gault	TDF and protease inhibitors were associated with a greater risk of decreased renal function as measured by estimated GFR. There was a relatively high rate of mild renal dysfunction in the absence of ART.
Viganò *et al.* (2011), Italy	Prospective cohort over 60 months in 26 infants	Serum creatinine and phosphate,	TDF had an excellent renal safety profile in HIV-infected children,

	and adolescents; Baseline: D4T and a protease inhibitor (PI). D4T switched with TDF and the PI switched with EFV	urine protein, urine glucose, and urine α-1-microglobulin.	adolescents and young adults regularly followed up for 60 months.
Chua *et al*. (2012), Singapore	Retrospective cohort: 226 patients on TDF-based ART	Cockroft-Gault. Serum creatinine	7.9% of patients had renal impairment outcomes (CrCl<50 ml/min). Renal toxicity was rare and transient among the patients
Maggi *et al*. (2012), Italy	Prospective cohort: 73 patients on TDF/FTC-based regimen vs 28 patients on ABC/3TC-based regimens	MDRD. Serum creatinine, uric acid, cytochrome C, α-glutathione S-transferase	Urinary excretion of phosphate and uric acid significantly increased in patients on TDF compared to those on Abacavir. Moderate increases in α-glutathione S-transferase indicated low-level mitochondrial damage.

TDF = Tenofovir Disoproxil Fumarate; EFV = Efavirenz; NVP = Nevirapine; FTC = Emtricitabine; 3TC = Lamivudine; D4T = Stavudine; ABC = Abacavir; BMI = Body Mass Index; MDRD = Modification of Diet in Renal Disease; CrCl = Creatinine Clearance; *vs* = versus.

According to Wassertheil-Smoller (2004), each of the two study types has limitations. For example, case control studies may be said to be liable to sampling bias especially in the selection of control cases where the selected controls may not be a good representative sample of the reference population. Another drawback for case control studies is that they are usually done retrospectively whilst cohort studies are usually done prospectively. Cohort studies are therefore less liable to bias because the cases are detected and defined as they occur during the study. However, cohort studies need longer study times and may not be practical in situations where observation time is limited. Hence, in principle, the studies on renal toxicity of TDF are fundamentally different and the reported results may need to be compared with the methodological differences in mind.

With reference to Table 2.3, the majority of the studies were cohort and case control studies. Although the majority of cohort studies were done prospectively, some of the cohort studies were done retrospectively. There are several documented limitations of using retrospective data obtained from medical records (Chua, Llorin, Lai, Cavailler & Law, 2012). Limitations include incomplete data, difficulty in verifying the data, and variability in the quality of documentation among physicians. Therefore, it may not be surprising that the differences in the conclusions given by the studies in Table 2.3 may have emanated from the differences in the methodologies used in the studies.

There were differences in methods used for estimation of GFR and in the criteria for defining renal dysfunction. Others used the Cockcroft-Gault formula (Gallant *et al.*, 2008; Patel, Patel, Ranjan, Patel & Patel, 2010) to calculate renal clearance while some studies used modification of diet in renal disease (MDRD) formula (Crum-Cianflone *et al.*, 2010; Calza, Trapani, Tedeschi, Piergentili, Manfredi, Colangeli & Viale, 2011) for GFR. Some studies did not indicate the estimation equations and cut-off they used to evaluate and define renal dysfunction. Serum creatinine is known to be affected by demographic factors such as age, gender, ethnic group, muscle mass and diet (Harmoinen, Lehtimaki, Korpela, Turjanmaa & Saha, 2003). Use of different methodologies with respect to GFR estimation may have contributed to the variation in the results obtained.

The other major discrepancy among the studies was the sample size. The smallest sample in Table 2.3 was 26 (Viganò, Bedogni, Manfredini, Giacomet, Cerini, di Nello, Penagini, Caprio & Zuccotti, 2011) and the largest was 10 343 (Nelson *et al.*, 2007). The disparity in sample size may complicate the comparability of the studies.

According to Young *et al.* (2007), the criteria for defining renal dysfunction differ among different studies. The lack of uniformity in defining the sub-optimal threshold of renal function may be one reason why different studies concluded differently on the renal safety of TDF. The other disparity in study methodology was observed in residual confounding with respect to concomitant therapies for intercurrent disease such as TB, diabetes and hypertension (Young *et al.*, 2007). The clinical and socio-demographic characteristics of the study populations are also strikingly different.

The differences in the demographic characteristics make the comparison of different studies difficult.

Another major discrepancy was variation in the duration of the studies. The duration of the studies excluding case reports and clinical trials ranged from six months up to 77 months. The median duration of the studies excluding clinical trials was 24 months. Studies with longer duration are therefore still needed to allow better comparisons of the studies on renal toxicity of TDF.

2.3 Mechanisms and the Risk Factors of Renal Disease

2.3.1   General risk factors for renal disease

The process of ascertaining causes of renal disease in HIV patients is complicated by the numerous possible causes of renal disease, including HIV itself, which has been associated with renal impairment on its own (Sakka, Bakoyannis, Chini, Gargalianos, Sambatakou, Antoniadou, Chrysos, Paparizos, Daikos, Katsarou, Touloumi & Lazanas, 2012). According to Farag, Kari & Singh (2012), diabetes, obesity and hypertension are the main threats in some countries such as Saudi Arabia. In a cohort of HIV-positive Greek patients, older age and female gender were significant determinant variables for chronic kidney disease (Sakka *et al*., 2012).

In another cohort study in Iran (Tohidi, Hasheminia, Mohebi, Khalili, Hosseinpanah, Yazdani, Nasiri, Azizi & Hadaegh, 2012), female gender was also a significant determinant. Other variables that were significantly associated with the development of chronic kidney disease included age, family history, low baseline GFR, diabetes mellitus and hypertension. However, Tohidi *et al*. (2012), reported that there were clear-cut differences between male and female gender. The same researchers recommended that investigations of risk factors for developing chronic kidney disease be done separately between males and females.

In one study done in the USA, African-American ethnicity, Hepatitis C co-infection, low CD4 cell count and high viral load while on ART were associated with higher risk of chronic kidney disease (Kalayjian, Lau, Mechekano, Crane, Rodriguez,

Salata, Krishnasami, Willig, Martin, Moore, Eron & Kitahata, 2012). Use of TDF and a Ritonavir-boosted protease inhibitor was also associated with higher CKD risk.

2.3.2 Mechanisms of drug-induced nephrotoxicity

The major function of the kidney is to concentrate and excrete toxic metabolites and drugs. The kidneys are therefore a frequent site of drug toxicity. Drugs may damage the kidney through several mechanisms. Tubular cell toxicity is one of the mechanisms by which drugs may cause renal injury. The role of the proximal tubule in concentrating and reabsorbing the glomerular filtrate makes it vulnerable to toxic injury (Schetz, Dasta, Goldstein & Golper, 2005). Tubular toxicity is, at least in part, dose-dependent and is the cause of kidney injury associated with aminoglycosides, amphotericins, antiviral drugs such as Foscarnet, Cidofovir and antiretroviral drugs such as Tenofovir.

Interstitial nephritis, characterised by inflammation of the renal interstitial tissue and tubules has been associated with antibiotics such as beta-lactams (β-lactams) and quinolones especially Ciprofloxacin, Rifampicin, Macrolides, Sulfonamides, Tetracyclines; diuretics such as Thiazides, Allopurinol; antiviral drugs such as Acyclovir and Indinavir; and non-steroidal anti-inflammatory drugs (NSAIDs) (Schetz *et al.*, 2005; Loh & Cohen, 2009). A number of drugs may cause interstitial nephritis. It appears most antibiotics belonging to the β-lactams quinolone groups may cause interstitial nephritis.

Nephrotoxicity of NSAIDs can inhibit renal prostaglandin synthesis, resulting in unbalanced vasodilation and vasoconstriction forces. Moreover, antibiotics and analgesics, including NSAIDs, commonly cause tubulo-interstitial nephritis probably because they are widely used throughout the world (Loh & Cohen, 2009).

Another mechanism through which drugs may cause glomerular injury is immune complex deposition. Immune complex deposition on the glomerular membrane may result in membranous glomerulonephritis. Though membranous glomerulonephritis looks simple to understand, the exact mechanism and threshold of immune complex deposits required before pathological manifestations are noticed, remains elusive to

researchers. Numerous drugs, including NSAIDs, Captopril and Probenecid, are associated with glomerulonephritis (Loh & Cohen, 2009).

The precipitation of crystals (nephrolithiasis) in distal tubular lumens depends on pH and hydration status which explains why precipitation of crystals is triggered by drugs such as Acyclovir, Sulfonamide, Methotrexate, Indinavir and Triamterene (Schetz *et al.*, 2005; Loh & Cohen, 2009) and other drugs such as alcohol, thiazide diuretics, Cyclosporine, Furosemide and Cisplatin (Loh & Cohen, 2009). Hypercalcaemia due to excess vitamin D may result in calcium phosphate deposition. Consuming foods with high vitamin C content may also promote the formation of calcium oxalate crystals.

Some drugs such as Indinavir, Cyclosporine and Quinine are known to cause thrombotic microangiopathy which is characterised by findings typical of haemolytic uraemic syndrome (Schetz *et al.*, 2005). Osmotic nephrosis, on the other hand, is the mechanism of nephrotoxicity associated with high doses of sucrose-rich intravenous plasma volume expanders such as sucrose, dextrans, and starches (Schetz *et al.*, 2005; Loh & Cohen, 2009).

Therefore, numerous drugs are associated with renal toxicity. As would be expected, the use of different drugs such as is the case in HIV treatment, increases the vulnerability of the kidney. These drugs however will not cause disease in every patient who takes them. A number of risk factors have been identified which predisposes certain groups of individuals.

2.3.3 Risk factors for drug-induced nephrotoxicity

The risk of drug-induced nephrotoxicity increases with age probably due to reduction of GFR which occurs in old age (Thomson, 1995; Naughton, 2008; Muhlberg & Platt, 1999). Biological differences between men and women have also been found to contribute to different responses to nephrotoxic drugs (Schwartz, 2003).

Patients with pre-existing renal insufficiency have a higher risk of developing drug-induced nephrotoxicity and chronic kidney disease (Harbarth *et al.*, 2001; Naughton, 2008) and diabetes (Blackshear, Davidman & Stillman, 1983; Naughton, 2008). In

addition, patients with sepsis (Schrier & Wang, 2004), sodium depletion and patients on diuretics (Blackshear Davidman & Stillman, 1983; Schetz *et al.*, 2005) are at risk of developing drug-induced nephrotoxicity.

According to the explanations given by Schetz *et al.* (2005) and Naughton, (2008), sepsis increases the risk of nephrotoxicity due to the associated systemic and renal haemodynamic alterations. The use of angiotensin converting enzyme (ACE) inhibitors, and angiotensin receptor blockers (ARB) may result in GFR decrease because the kidney relies on efferent vasoconstriction for the maintenance of glomerular filtration pressure. Sodium-depleted patients also have impaired renal haemodynamics and impaired renin–angiotensin system. Use of diuretics is associated with higher risk of nephrotoxicity because diuretics can reduce circulating volume and increase renal excretion of sodium (Schetz *et al.*, 2005).

Other factors that may increase risk of drug-nephrotoxicity include higher drug dosages, using nephrotoxic drugs for long time, frequent use of nephrotoxic drugs, parenteral route of drug administration, and drug formulation type with lipid formulations having less risk (Schetz *et al.*, 2005).

2.3.4   Weighing the effect of TDF versus other risk factors

The evidence linking the use of TDF and the development of renal disease has always been controversial. Most methodologies that have been used to rule out the effect of other factors such as demographic factors and comorbidities have largely relied on multivariate analysis. Empirical evidence linking the use of TDF and renal tubular cell or mitochondrial damage is very scarce. However, the effect of confounders can never be ruled out with certainty even when multivariate logistic regression analysis is used.

Several studies have identified possible factors that may increase the risk of TDF nephrotoxicity among patients taking the drug. It is interesting to compare the effect that TDF might have on renal function relative to other factors. In most cases, researchers have listed several factors associated with renal impairment. For example, a study by Crum-Cianflone *et al.* (2010) reported that use of TDF, female gender, older age, African-American ethnicity and lower CD4 count were some of

the factors associated with renal impairment. Nelson *et al.* (2007) identified elevated serum creatinine, concomitant nephrotoxic medications and low body weight as additional risk factors associated with developing TDF-linked renal disorders besides older age and lower CD4 count. Such long lists of factors imply that the effect of TDF alone is unlikely to be isolated without some form of empirical evidence given.

Hypertension and diabetes are conditions frequently mentioned in the literature as some of the major risk factors of developing TDF-associated nephrotoxicity (Young *et al*., 2007; Calza *et al.,* 2011). Obesity is another factor which may contribute to renal disease (Tesauro, Mascali, Franzese, Cipriani, Cardillo & Di Daniele, 2012). Hypertension is cited in the literature as both a potential cause and consequence of chronic renal disease (Dworkin & Shemin, 1999). Therefore, it is important to understand how hypertension, diabetes, and obesity are linked to renal disease. It is also important to know why hypertension, diabetes and obesity are important factors of renal disease in African settings.

The link between hypertension and chronic renal disease seems to emanate from the disrupted renal function particularly the gromerulus and the ill-controlled activation of the renin-angiotensin system. Initially, hypertension increases glomerular capillary pressure (Dworkin & Shemin, 1999). The immediate consequence of the impaired glomerular capillary pressure seems to be increased protein filtration and endothelial damage resulting in increased release of cytokines that promote fibrosis of kidney tissue. According to the literature, one of the most common kidney diseases associated with hypertension is chronic glomerulonephritis (Chobanian, Bakris, Black, Cushman, Green, Izzo, Jones, Materson, Oparil, Wright & Roccella, 2003). Therefore, hypertension is an important confounding factor in the analysis of TDF-associated renal toxicity.

The prevalence of hypertension in African settings may probably give an indication of the general trend that TDF-associated nephrotoxicity is likely to take. On the other hand, the prevalence of hypertension may be inextricably intertwined with the prevalence of a host of other co-morbidities such as diabetes, pregnancy, hormonal imbalances and other factors implicated in hypertensive disorders (Sutters, 2009).

Whereas the prevalence of hypertension is estimated to be about 10-15% among white Americans, the prevalence of hypertension is estimated to be twice as high among African-Americans (Naicker, 2003; Sutters, 2009). In a recent study by Hendriks, Wit, Roos, Brewster, Akande, de Beer, Mfinanga, Kahwa, Gatongi, Van Rooy, Janssens, Lammers, Kramer, Bonfrer, Gaeb, van der Gaag, Rinke, Lange & Schultsz (2012), the prevalence of hypertension was as high as 38% in one urban Namibian cohort. Hendriks *et al*. (2012) also reported hypertension prevalences of at least 20% in Kenya, Tanzania and Nigeria. Alarmingly enough, at least one-third of the individuals with hypertension had grade II ($\geq$160/100 mmHg) or grade III hypertension ($\geq$180/110 mmHg). In one Nigerian cohort study, 43.3% of the individuals with hypertension had grade II or III hypertension (Hendriks *et al*., 2012).

Patients suffering from both hypertension and diabetes need special attention due to poor prognosis when the two conditions occur concurrently and the increased risk of developing diabetic nephropathy. According to the guidelines by the British National Formulary (BNF, 2006), patients with both hypertension and diabetes should be given ACE inhibitors or angiotensin-II receptor antagonists while having closer blood pressure monitoring (BNF, 2006). However, in the case that the patient has renal impairment in addition to hypertension, ACE inhibitors should be used with caution. Maintaining a strict drug regime as recommended is still a challenge in African settings due to limited drug choices and laboratory tests.

Many cases of hypertension and diabetes in remote African settings may remain undiagnosed due to the intricate procedures for diagnosing hypertension and diabetes as discussed in section 2.5.4 and 2.5.5 respectively. For instance, according to Sutters (2009), an HIV patient with an elevated blood pressure reading would be required to visit the hospital for a second blood pressure measurement within one month. In most cases, this protocol is not followed, resulting in a wrong diagnosis. Due to the high prevalence of hypertension in African settings, investigations for TDF-associated renal disease in African settings need to rule out hypertension as the primary cause of renal disease despite the challenges.

According to Tesauro *et al*. (2012), obesity and hypertension are some of the critical factors that can accelerate the progression of kidney disease. The search for the chemical factors or hormones linking obesity, hypertension and chronic nephropathy

has been on-going for years. Recently, some possible links between obesity, hypertension and kidney disease have been reported. Dipocytokines such as leptin and adiponectin are some of the potential leads. Leptin precipitates hypertension possibly through increasing serum catecholamine levels which are linked with hypertension when sustained for extended periods. In addition to increasing blood pressure, leptin has been associated with glomerulosclerosis through induction of type I collagen synthesis in mesangial cells and type IV collagen in glomerular endothelial cells (Ballerman, 1999). In contrast to leptin, it is rather the lack of adiponectin which is associated with hypertension and consequently, kidney disease.

Some genetic lineages expressing certain genetic polymorphisms in the gene encoding for adiponectin have been associated with obesity and therefore, increased risk of developing kidney disease (Iwashima, Katsuya, Ishikawa, Ouchi, Ohishi, Sugimoto, Fu, Motone, Yamamoto, Matsuo, Ohashi, Kihara, Funahashi, Rakugi, Matsuzawa & Ogihara, 2004). These genetic disorders could be the reason why obesity has been linked to family history. For example, Iwashima *et al.* (2004) described the link between arterial hypertension in Japanese individuals and genetic polymorphism for the gene encoding adiponectin. In addition to genetic polymorphisms, the links between obesity and kidney disease have also been attributed to metabolic syndrome. Metabolic syndrome is a risk factor for hypertension and diabetes (Bakris, 2007).

Another risk factor for developing kidney disease is smoking. Smoking has been linked to microvasculature disturbance which means that smoking may damage small vessels in the circulatory system resulting in increased risk of atherosclerosis (Odden, Tager, Gansevoort, Bakker, Fried, Newman, Katz, Satterfield, Harris, Sarnak, Siscovick & Shlipak, 2013).

Small body weight due to small stature is a potential risk factor especially in African settings. Nishijima, Komatsu, Gatanaga, Aoki, Watanabe, Kinai, Honda, Tanuma, Yazaki, Tsukada, Honda, Teruya, Kikuchi & Oka (2011), reported that small body weight is associated with a higher risk of developing TDF-associated renal impairment. According to Nishijima *et al.* (2011), patients of Japanese descent have smaller stature on average compared to White Americans and African-Americans.

Patients of African descent are smaller in stature on average than people of European and American descent. Therefore Africans may also be at higher risk of TDF-associated renal impairment compared to other races with bigger stature.

Other risk factors include exposure to high doses of TDF, pre-existing renal disorders and extensive pre-treatment with nucleoside reverse transcriptase inhibitors (Noah, 2012). The use of Atazanavir/ ritonavir (Gérard *et al*., 2007) concurrently with TDF is associated with a higher risk of developing renal disorder. The results of the study by Gérard *et al*. (2007) also augmented the theory that concomitant nephrotoxic medications may increase the risk of developing TDF-associated renal disorder. The renal disorder is believed to emanate from the interaction between the protease inhibitors with the renal transport of organic ions which leads to accumulation of TDF in the proximal tubules.

There is need despite the challenges, to control for a host of other risk factors that may cause or worsen renal impairment besides TDF. It seems the high prevalence of hypertension and diabetes coupled with lack of resources to diagnoses and manage these two disease conditions in African settings poses a greater challenge than the threat that TDF poses to renal function. More efforts are therefore warranted in strengthening laboratory systems in African settings.

2.4 Profiles of Antiretroviral Drugs: Rationale of Use; Mechanisms of Action; and Adverse Reactions

According to Wilson *et al.* (2008), only three classes of ARVs are commonly available in Southern Africa. The other drug classes such as integrase inhibitors and entry inhibitors are not available due to resource limitations. The three classes of ARVs available are nucleoside reverse transcriptase inhibitors (NRTIs); non-nucleoside reverse transcriptase inhibitors (NNRTIs); and protease inhibitors (PIs). NRTIs and NNRTIs are drugs that inhibit HIV reverse transcriptase. PIs are drugs that interfere with HIV protease. Other classes of antiretroviral drugs such as entry inhibitors are not available due to costs associated with acquiring and monitoring the use of the drugs. The profiles of the different ARV drug classes available in Southern Africa, including molecular structures, mechanisms of action and adverse-effects of the drugs, are outlined in Section 2.4.1.

2.4.1   Profile of Tenofovir Disoproxil Fumarate

2.4.1.1 Structure and mechanism of action

TDF (structure shown in Figure 2.2) targets HIV reverse transcriptase and acts as a chain terminator, following intracellular phosphorylation to the diphosphate form (See Figure 2.3). TDF is a drug synthesized by modifying the organic chemical Tenofovir. Tenofovir disoproxil fumarate is rapidly absorbed and converted to tenofovir following oral administration. Structurally, TDF is slightly different from other nucleoside reverse transcriptase inhibitors (NRTIs) in that it contains only one phosphate group (Fernandez-Fernandez *et al.*, 2011).
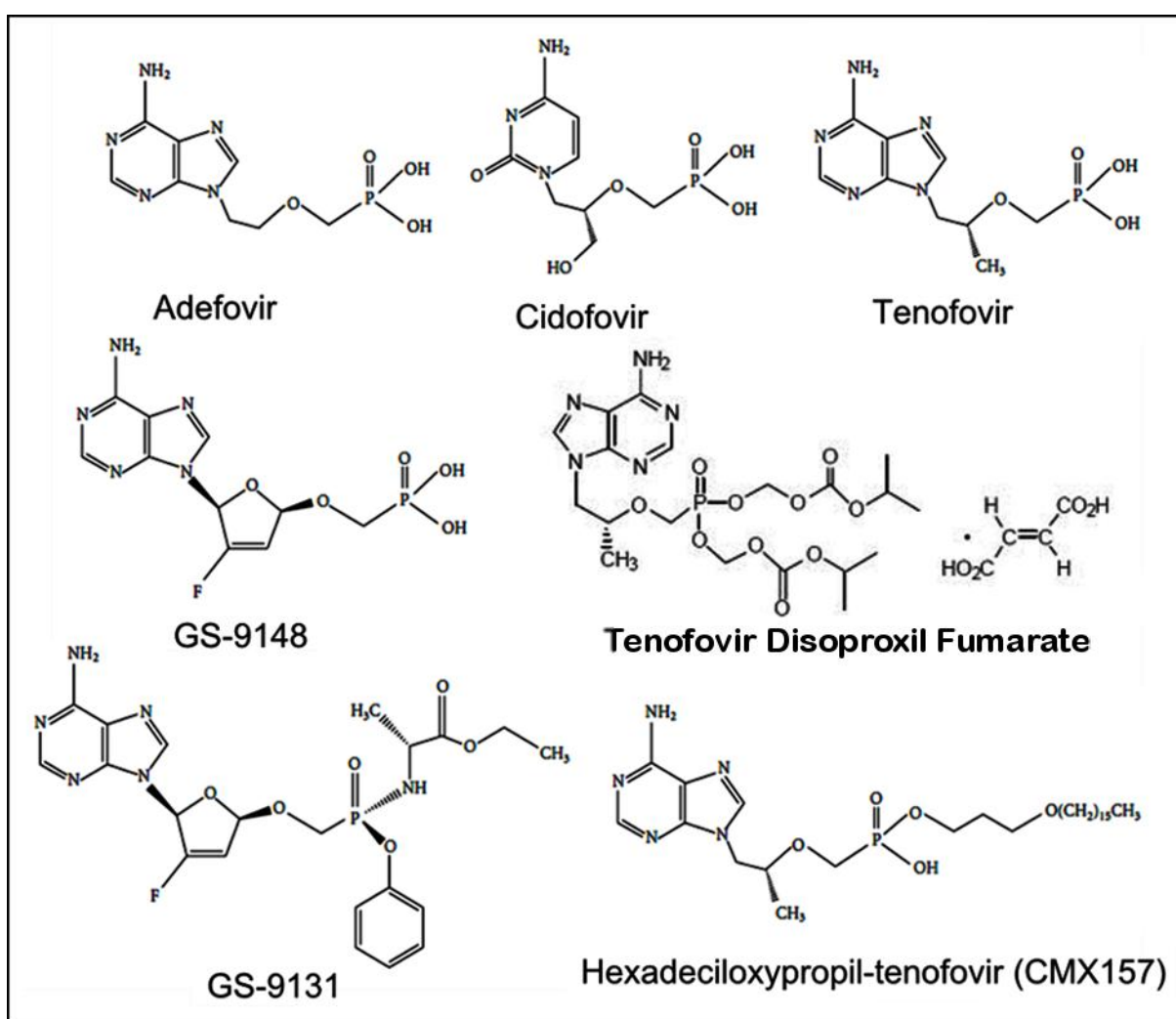
Figure 2.2: Chemical structure of Adefovir, Cidofovir and Tenofovir:

(Fernandez-Fernandez *et al.*, 2011).
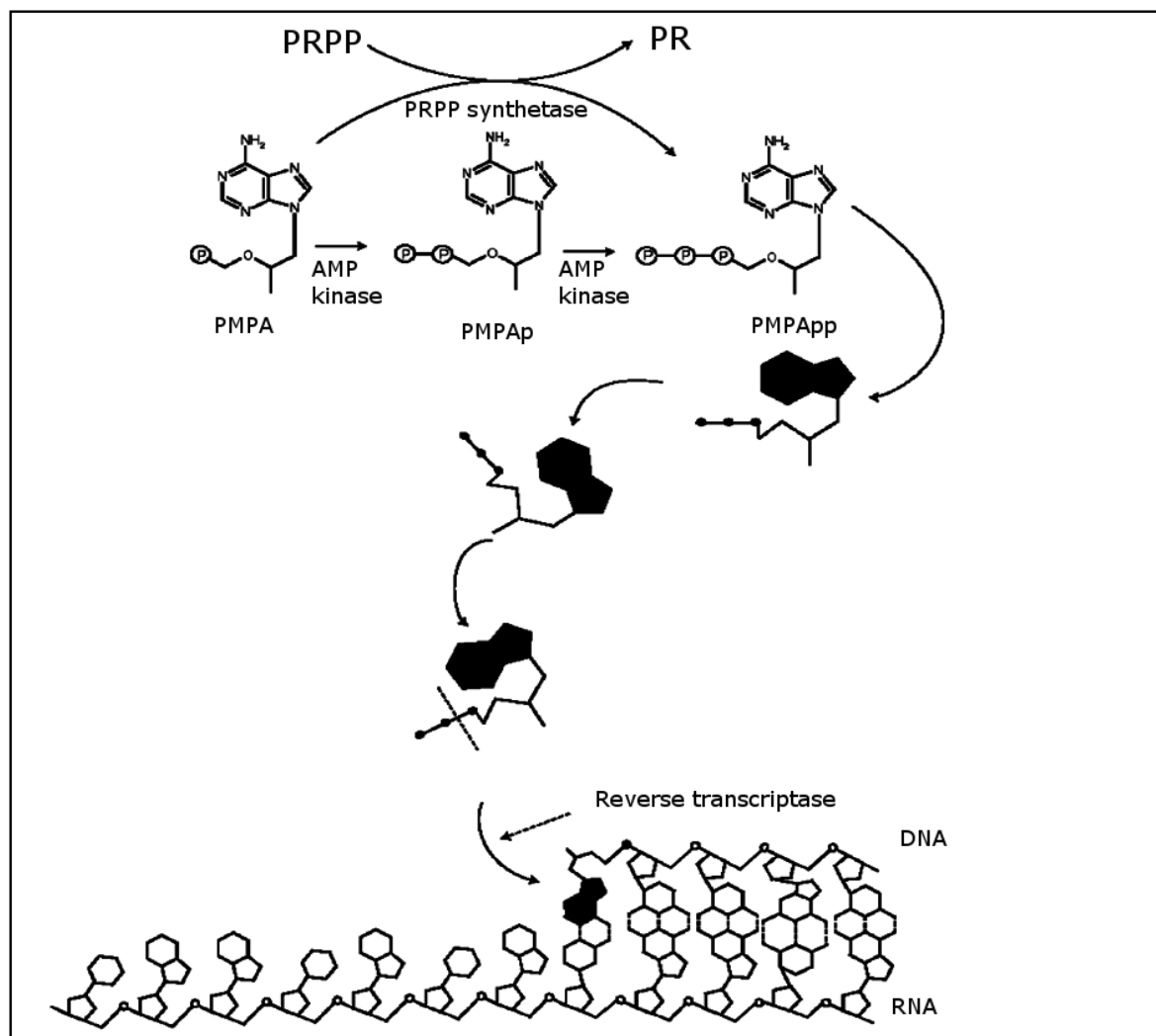
Figure 2.3: Mechanism of antiviral action of Tenofovir.

PR = Pyrophosphate; PRPP = Phosphoribosyl Pyrophosphate; PMPA = Phosphonylmethoxy-propyl adenine; PMPAp = Phosphonylmethoxy-propyl adenine monophosphate; PMPApp = Phosphonylmethoxy-propyl adenine diphosphate; RNA = Ribonucleic Acid; DNA = Deoxyribonucleic acid; AMP = Adenosine Monophosphate, (De Clercq, 2004).

TDF is found in Atripla® tablets made by Bristol-Myers Squibb & Gilead Sciences; Truvada® and Viread® which are made by Gilead Sciences. Whereas Atripla® tablets contain 600mg Efavirenz, 200mg Emtricitabine and 300mg TDF; Truvada® contains 200mg Emtricitabine and 300mg TDF; and Viread® contains 300mg TDF (Noah, 2012).

As mentioned in Section 2.1.1, the structural similarity between Tenofovir, Adefovir and Cidofovir (Figure 2.2) is what brought about the concerns about TDF's renal safety in the first place (Fernandez-Fernandez *et al.*, 2011). After renal toxicity

concerns were confirmed in patients taking TDF, researchers focused on ways of reducing uptake by renal proximal tubular cells.

Two methods to reduce uptake by renal proximal tubular cells were identified (Fernandez-Fernandez *et al.*, 2011). The methods included esterifying the TDF compound with an alkoxyalkyl group and ribose-modification. Esterifying TDF compound resulted in a TDF derivative which could be disguised as lysophospholipids. One such compound was hexadecyloxypropyl-Tenofovir and is shown in Figure 2.2.

The second method of ribose-modification resulted in a chemical similar to the usual substrate for viral ribonucleic acid (RNA)-directed deoxyribonucleic acid (DNA) polymerase and therefore may have less renal toxicity. However, success with the derivatives of TDF is not yet reported.

2.4.1.2 Mechanisms of TDF nephrotoxicity; clinical effects; and reversibility of TDF nephrotoxicity

The proposed mechanisms by which TDF causes renal toxicity are associated with renal proximal tubule injury and glomerular toxicity. Renal proximal tubules are known to be involved in the excretion of drugs such as TDF (Kohler, Hosseini, Green, Abuin, Ludaway, Russ, Santoianni & Lewis, 2011). Studies have unraveled possible mechanisms by which TDF damages renal proximal tubules. The first proposed mechanism is premised on the possible interaction between TDF and organic anion transporters, code-named human organic ion transporter 1 and 3 (*h*OAT1 and *h*OAT3*)*, which transport ions across renal tubular cells (See Figure 2.4). Researchers have come to realise that TDF affects *h*OAT1 more than it does on *OAT3* (Herlitz, Mohan, Stokes, Radhakrishnan, D'Agati & Markowitz, 2010).

The second proposed mechanism is based on the possible interaction between TDF and mitochondrial DNA polymerase γ. Due to the abundance of mitochondria in renal tubular cells and proximity of the mitochondria to the organic anion transporters, TDF is likely to enter mitochondria and cause damage. Tenofovir may decrease mtDNA content by inhibiting mitochondrial DNA polymerase gamma (γ) (POLG in Figure 2.4) resulting in mitochondrial injury which may lead to premature death of the tubular cell (apoptosis).
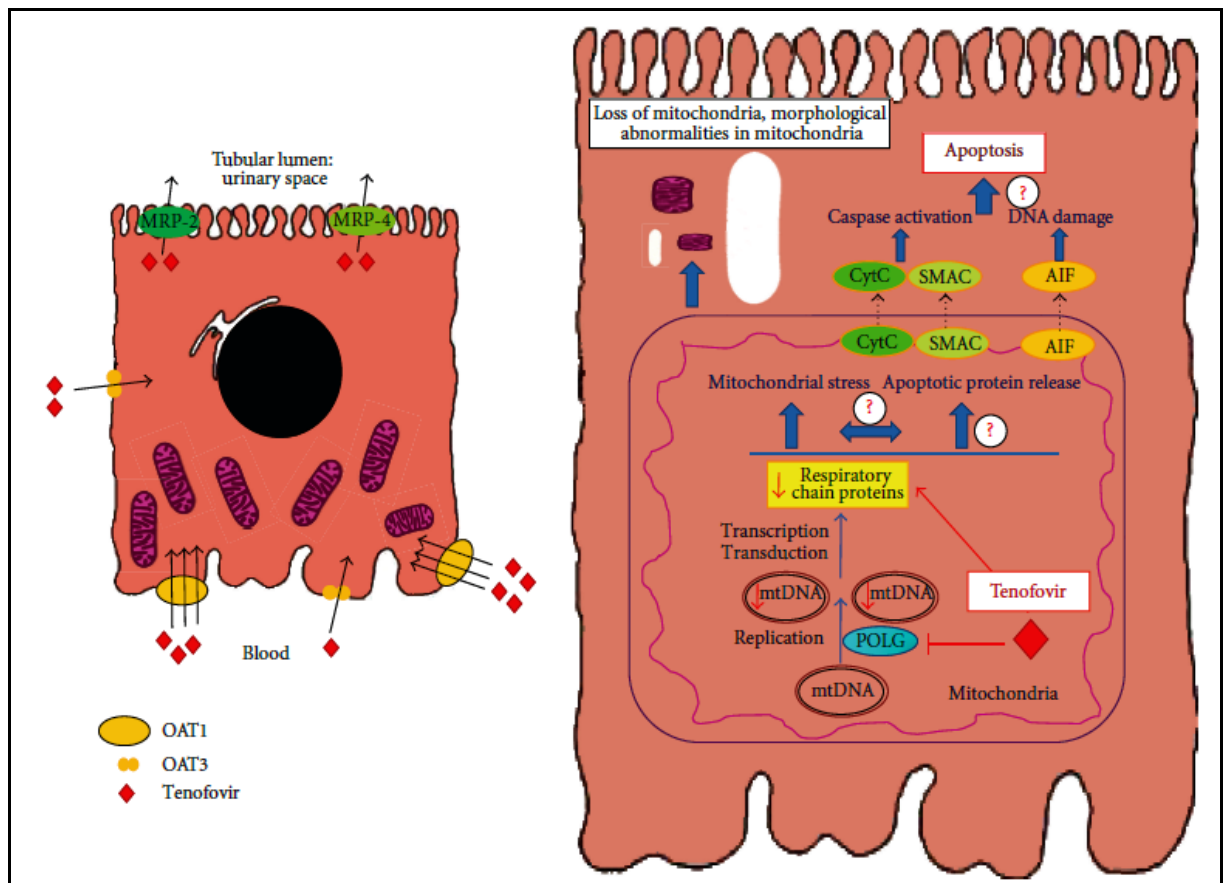
Figure 2.4: Proposed mechanism of TDF renal toxicity.

OAT = organic ion transporter; POLG = mitochondrial DNA polymerase γ; CytC = Cytochrome C; mtDNA = mitochondrial DNA; AIF = Apoptosis-inducing factor (Fernandez-Fernandez *et al.*, 2011).

The resultant apoptosis is thought to be mediated by enzymes of the respiratory chain such as cytochrome C which are released in response to mitochondrial stress. Cytochrome C is believed to trigger pro-apoptotic events when released into the cytosol (Ow, Green, Hao & Mak, 2008). Following the release of cytochrome C, some form of diffuse cytoplasmic injury is noticeable on lysosomes and other organelles. The alpha-glutathione S-transferase (α-glutathione S-transferase) enzyme has also been put forward as a potential biomarker of the diffuse cytoplasmic injury on renal tubular cells (Maggi *et al.,* 2012)

Measuring cytochrome C has been postulated as a plausible means of measuring mitochondrial damage (Zager, Johnsos & Hanson, 2004). Following mitochondrial damage, levels of urinary cytochrome C increases and may save as a marker of mitochondrial damage. Maggi *et al.* (2012) recently demonstrated the concept of

detecting mitochondrial damage and tubular cell necrosis by measuring urinary cytochrome C and α-glutathione S-transferase respectively. Although no decisive pattern could be observed for α-glutathione S-transferase, patients on TDF-based ART had a low-level increase in cytochrome C after 12 months.

The other problem which exacerbates TDF's renal toxicity is that there are several drugs which interfere with both organic anion transporters and apical membrane transporters. The interaction with the transporters results in accumulation of TDF in the renal tubular cells. Drugs that interact with the TDF transporters and their effects are shown in Table 2.4.

Given that proximal tubules contribute to drug excretion from the body and that TDF interferes with ion transport across renal tubular cells, it is highly likely that TDF can lead to the development of renal toxicity. Didanosine and Ritonavir are also known to interfere with transport of ions across the tubular cell membranes. As a result, TDF taken concomitantly with Didanosine or Ritonavir can result in worse renal damage.

Although the exact intracellular targets in renal tubular cells are not clear, the clinical effects of TDF are now better understood. Some authors believe that the depletion of mitochondrial DNA results in a compromised respiratory chain and consequently dysfunctional metabolic pathways. For instance, impaired respiratory chain results in less consumption of reduced adenosine triphosphate (NAD) and reduced flavin adenine dinucleotide (FAD). NAD and FAD are end-products of fatty acid oxidation. Reduced consumption of NAD and FAD may result in negative feedback and accumulation of intracellular triglycerides.

Typical clinical results of mitochondrial toxicity include liver damage, impaired fat distribution, skeletal muscle weakness (myopathy), poly-neuropathy, hypo-phosphataemia due to a diminished renal phosphate re-absorption and Fanconi's syndrome due to impaired re-absorption of proteins and solutes (Fernandez-Fernandez *et al.*, 2011). Depending on the affected tissue or organ, mitochondrial toxicity may result in deposition of lipids in liver tissues (steatohepatitis). Steatohepatitis may result in liver failure and lactic acidosis.

Table 2.4: Drugs that interfere with TDF transporters and the effects of the drugs.

Transporter	Drug interaction	Effect
Organic anion transporter-1 (*h*OAT1)	Probenecid inhibits *h*OAT1	Probenecid might decrease the renal toxicity by TDF as it is known to decrease the renal toxicity of Cidofovir
	NSAIDs inhibit *h*OAT1	NSAIDs such as aspirin and Ibuprofen are associated with Tenofovir nephrotoxicity
	Acyclovir competes with TDF	Acyclovir increases serum concentrations of Tenofovir
	Didanosine (DDI) competes with Tenofovir Disoproxil Fumarate (TDF)	Tenofovir increases DDI levels
Apical membrane transporter (MRP-4)	Probenecid, Dipyridamole and NSAIDs inhibit MRP-4	NSAIDs associated with Tenofovir nephrotoxicity
	Acyclovir and other related drugs such as Cidofovir inhibit MRP-4	Acyclovir increases serum concentrations of Tenofovir
Apical membrane transporter (MRP-2)	Ritonavir is transported by MRP-2 and therefore competes with TDF	Ritonavir increases Tenofovir concentration and has been associated with Tenofovir nephrotoxicity

NSAIDs = non-steroidal anti-inflammatory drugs; TDF = Tenofovir Disoproxil Fumarate; DDI = Didanosine; *h*OAT1 = human organic ion transporter (Fernandez-Fernandez *et al*., 2011).

It is important to note that Fanconi's syndrome is one of the most common side effects of TDF (Izzedine, Thibault, Valantin, Peytavin, Schneider & Benhamou, 2010). Classically, Fanconi's syndrome is characterized by an increased amount of phosphate, amino acids and glucose in the urine, and low phosphate levels in the blood (Noah, 2012). The urinary loss may manifest clinically in the form of increased urination, thirst, tiredness, bone pain or weakness. However, not every decrease in blood phosphate levels may be linked to Fanconi's syndrome as there are other causes as well.

TDF is also associated with loss of bone mineral density. A two-year follow-up study by Luetkemeyer, Havlir & Currier (2010), identified a high rate of pathological bone loss over time and a high prevalence of Vitamin D deficiency in the TDF cohort.

Concerning reversibility of TDF nephrotoxicity, Patel *et al.* (2010) reported that TDF-associated renal impairment was reversible in all the patients who had developed impaired serum creatinine after taking TDF-containing ART when the patients stopped taking TDF. In contrast, a study by Wever, van Agtmael & Carr (2010) concluded that TDF-related renal toxicity is not reversible.

Some researchers believe that TDF-associated renal injury may only be reversed by using drug interventions. Recently, some studies on Rosiglitazone, a drug that may reverse renal toxicity of TDF have been conducted in rats (Libório, Andrade, Pereira, Sanches, Shimizu & Seguro, 2008). Rosiglitazone is known to induce expression of ion transporters, thereby rejuvenating the lost renal function in patients with damaged kidneys. However, use of the drug in human beings was terminated in European markets shortly after the drug was licensed due to the association of the drug with cardiovascular problems (Blind, Dunder, De Graeff & Abadie, 2011).

Another drug which has been tried in patients with TDF-associated renal injury is Probenecid (Izzedine *et al.*, 2010). Probenecid might decrease the renal toxicity by TDF as it is known to decrease the renal toxicity of Cidofovir, a drug closely related to TDF (See Table 2.5). Use of Probenecid in reducing TDF nephrotoxicity is however limited due to adverse effects of Probenecid (Lalezari, Stagg, Kuppermann, Holland, Kramer, Ives, Youle, Robinson, Drew & Jaffe, 1997). The search for drug interventions against TDF nephrotoxicity therefore continues.

2.4.2 Profiles of nucleoside reverse transcriptase inhibitors (NRTIs) other than TDF

Zidovudine (AZT), Lamivudine (3TC), Stavudine (D4T), Emtricitabine (FTC), Diadnosine (DDI) and Abacavir (ABC) belong to the nucleoside analogue reverse-transcriptase inhibitor (NRTI) class of antiretroviral drugs (De Clercq, 2004). The most important functional group in the structures of AZT, 3TC, ABC, D4T, and FTC (See section A in Figure 2.5), is the organic hydroxyl group which can easily be

phosphorylated resulting in triphosphate analogues of DNA building blocks such as thymidine. Section B in Figure 2.5 shows the structures of non-nucleoside reverse transcriptase inhibitors that are reviewed in section 2.4.3. The phosphorylated metabolites of the drugs are therefore the active forms of the drugs.

A. Nucleoside reverse transcriptase inhibitors (NRTIs):

Zidovudine   Lamivudine   Emtricitabine   Stavudine

Abacavir   Didanosine

B. Non-nucleoside reverse transcriptase inhibitors (NNRTIs):

Nevirapine   Efavirenz
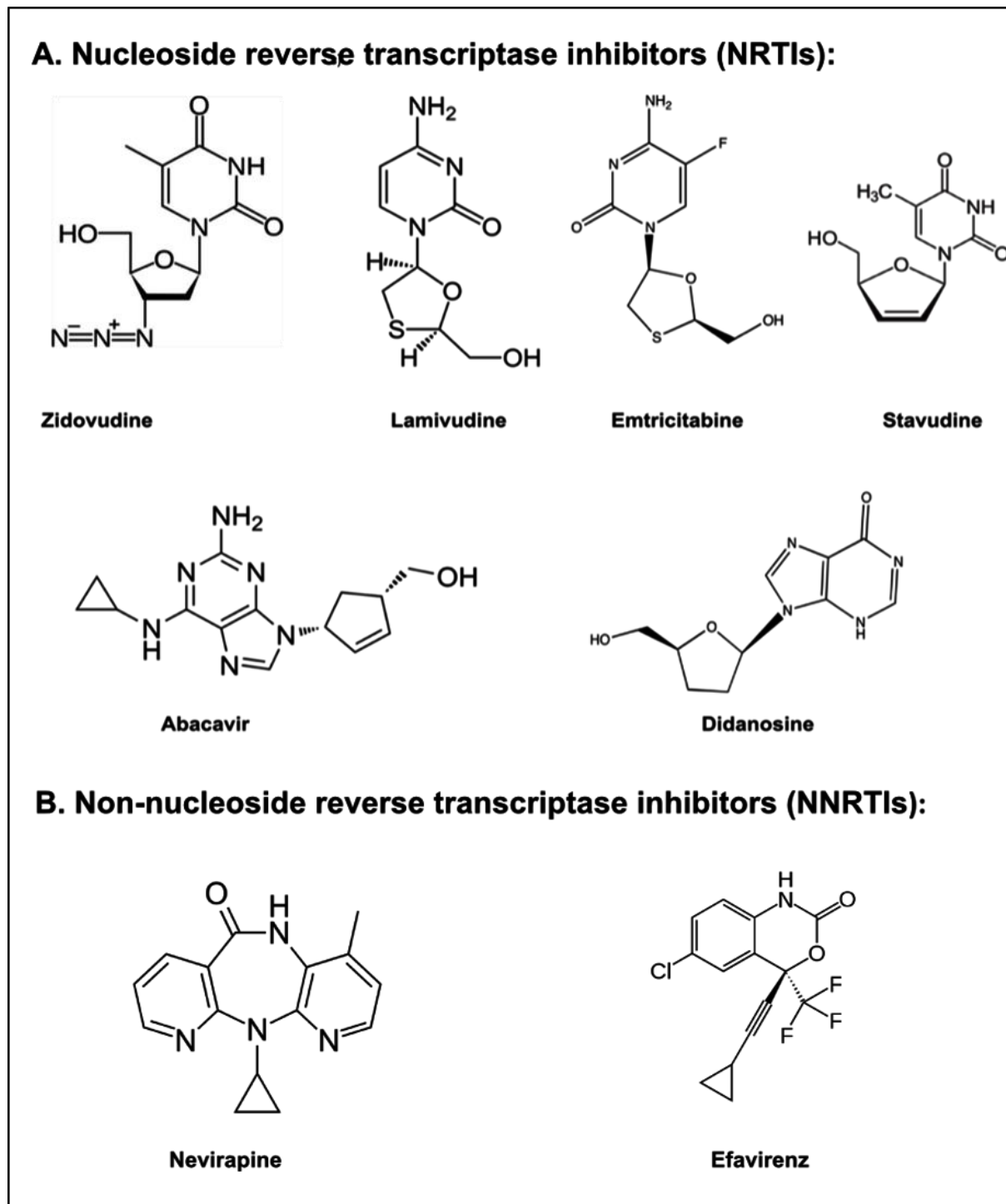
Figure 2.5: Structure of nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs).

(De Clercq, 2004).

Zidovudine (AZT) is an analogue of thymidine. AZT inhibits HIV's reverse transcriptase, the enzyme that the virus uses to copy RNA into DNA (De Clercq, 2004). NRTIs such as AZT, being structurally similar to the natural nucleotide building blocks of DNA, can block the conversion of viral RNA into proviral DNA. The NRTIs however, cannot work unless they are tri-phosphorylated first inside the cells (Figure 2.6).
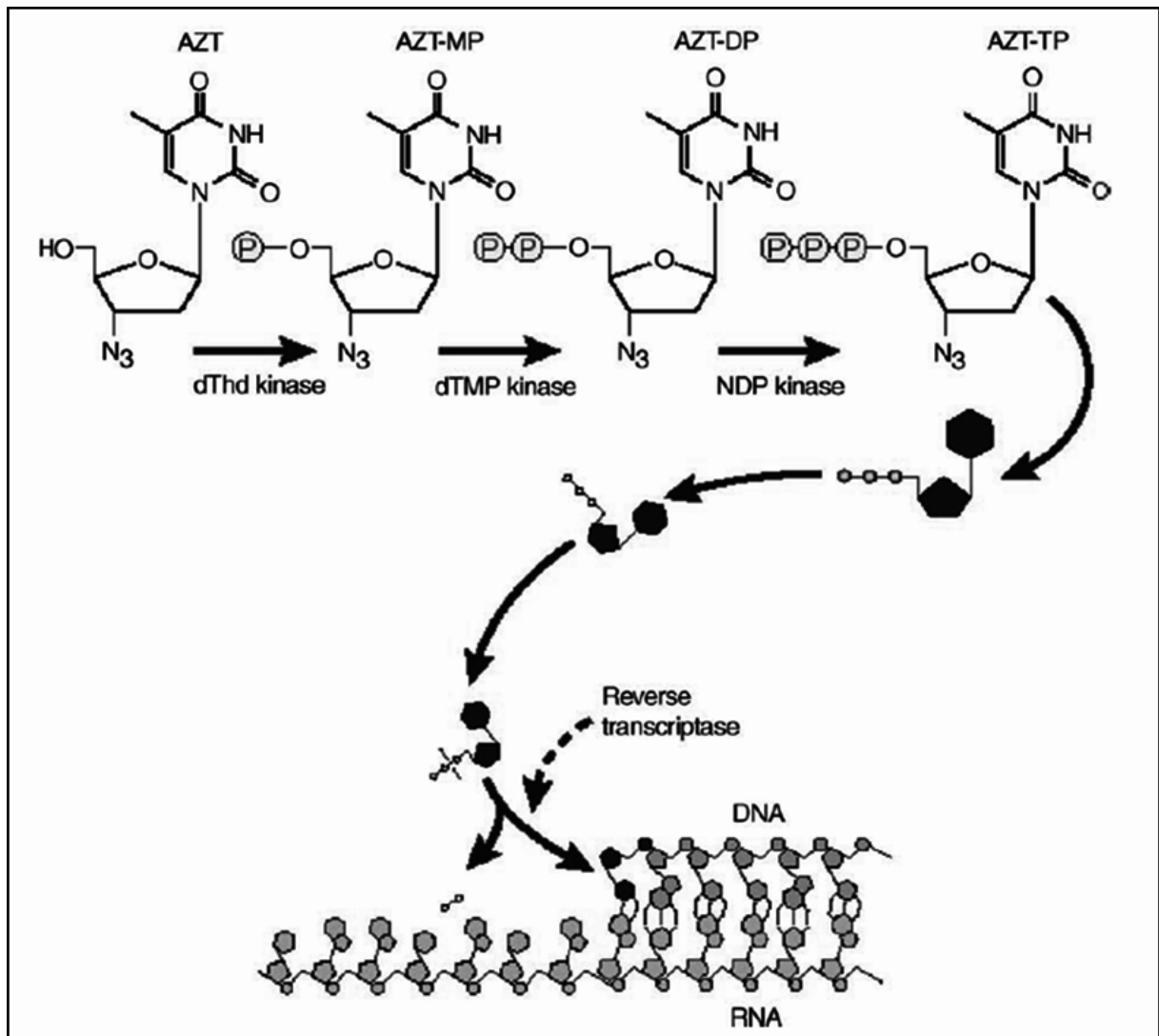
Figure 2.6: Mechanism of action of Zidovudine.
(De Clercq, 2002).

The major drawback associated with NRTIs is that the NRTIs also inhibit mitochondrial DNA polymerase which results in adverse effects of NRTIs such as hyperlactaemia and steatohepatitis. Didanosine and Stavudine are the common drugs known to impair mitochondrial function which explains why Stavudine has

been discontinued in many parts of the world, including Lesotho (Wilson *et al.*, 2008).

Selectivity of AZT emanates from the fact that AZT has more affinity for HIV viral DNA polymerase than for human DNA polymerase and also from the fact that human cells have the ability to quickly repair their own DNA chains if they are broken by AZT whereas HIV does not have that ability (Tomas & Adrian, 2010). Moreover, the azido group of AZT which is lipophilic (See Figure 2.6) enables the drug to permeate cell membranes of infected cells faster than other ARVs (Zimmerman, Mahony & Prus, 1987). Table 2.5 summarises adverse drug reactions for AZT and other NRTI drugs; and adverse drug reactions of protease inhibitors which are reviewed in section 2.4.4. AZT has to be used in combination with the other NRTIs to reduce the chances of drug resistance development. HIV has the potential to gain resistance to AZT by mutation of its reverse transcriptase (Re, Bon, Monari, Gibellini, Schiavone, Vitone, Chiodo & La Placa, 2003). AZT, like most ART drugs, has adverse-effects.

Lamivudine (3TC) is an analogue of cytidine. It can inhibit HIV-1 or HIV-2 reverse transcriptase and also the reverse transcriptase of *Hepatitis B virus*. The mechanism of action for 3TC is similar to that of AZT (Figure 2.6). The active forms of 3TC are phosphorylated metabolites that compete for incorporation into HIV viral DNA where they competitively inhibit HIV reverse transcriptase enzyme and terminate DNA synthesis by blocking the formation of the 5' to 3' phosphodiester bond essential for elongation of the DNA chain (De Clercq, 2004). The development of resistance to 3TC is due to mutations in the reverse transcriptase gene of HIV (Re *et al.*, 2003).

Stavudine (D4T) is an analogue of thymidine. The active form of the drug, Stavudine triphosphate is formed through a process of phosphorylation (De Clercq, 2002). Stavudine triphosphate competitively inhibits HIV reverse transcriptase which results in termination of DNA synthesis. The most common side effects of D4T are peripheral neuropathy, lipoatrophy and lipodystrophy (Table 2.5). In 2009, the WHO recommended that countries phase out the use of D4T, and switch to AZT, TDF or ABC because of the long-term, irreversible adverse effects of D4T (WHO, 2010).

Abacavir (ABC) targets HIV reverse transcriptase enzyme where it acts as a chain terminator, following intracellular phosphorylation (De Clercq, 2004). ABC is the drug of choice in patients with resistance to AZT (Hawkins, 2010). Prior to initiation of ABC, it is recommended that patients be tested for human leukocyte antigen (HLA) typing to rule out the likelihood of the patients developing hypersensitivity reaction commonly associated with the B*5701 allele (Zolopa & Katz, 2009). The main side effect of ABC, therefore, is severe hypersensitivity and the risk of heart attack which in some studies has been reported to be increased by nearly 90% (See Table 2.5). ABC may also aggravate liver condition in patients with compromised liver function.

Emtricitabine (FTC) is an analogue of cytidine (De Clercq, 2004). The Food and Drug Administration (FDA) agency of the United States approved the use of Truvada$^©$ which contains FTC and TDF and also Atripla$^©$ which contains Efavirenz, FTC and TDF. FTC also has activity against Hepatitis B virus (De Clercq, 2004).

Skin discolouration is the only side-effect which is more common among people taking FTC compared with other antiretroviral drugs in clinical trials (Hawkins, 2010). FTC is excreted via the kidneys. Abnormal kidney function has been reported in a few people receiving FTC. However, there is no significant interaction between the FTC and Tenofovir.

Didanosine (DDI) is a nucleoside analogue of guanosine and is a reverse transcriptase inhibitor. The presence of hypoxanthine component attached to the sugar ring and not the usual bases used in the synthesis of DNA is what differentiates DDI from nucleoside analogues (De Clercq, 2004). Inside the cell, DDI is phosphorylated to dideoxyadenosine triphosphate (ddATP) which is the active form of the drug. It terminates DNA synthesis by competing with natural deoxyadenosine triphosphate (dATP) required for viral reverse transcription (De Clercq, 2004). DDI is mainly eliminated through the kidneys (Zapor, 2004). DDI interacts with Allopurinol, Indinavir, Ketoconazole, Itraconazole, Ciprofloxacin and Delavirdine which explains why co-administration of these drugs is contra-indicated.

Table 2.5: Adverse effects associated with antiretroviral drugs

ARV class	ARV Drugs	Adverse drug reactions
NRTIs	Zidovudine (AZT)	Anaemia, elevated MCV, neutropaenia, nausea, headache, myalgia, myopathy, hyperlactaemia, steatohepatitis, lipoatrophy
	Didanosine (DDI)	Peripheral neuropathy, pancreatitis, nausea, diarrhoea, hyperlactaemia, steatohepatitis.
	Lamivudine (3TC)	Hyperlactaemia, steatohepatitis, Hepatitis B flare if discontinued, red blood cell aplasia
	Stavudine (D4T)	Peripheral neuropathy, elevated MCV, hypertriglyceridaemia, lipoatrophy, hyperlactaemia and steatohepatitis.
	Abacavir (ABC)	Systemic hypersensitivity reaction, hyperlactaemia, steatohepatitis
	Tenofovir (TDF)	Renal failure, decreased mineral density, hyperlactaemia, steatohepatitis, hepatitis flare if discontinued.
	Emtricitabine (FTC)	Headache, nausea, hyperpigmentation, hyperlactaemia, steatohepatitis, hepatitis flares if discontinued.
NNRTIs	Nevirapine (NVP)	Rash, hepatitis
	Efavirenz (EFV)	Rash, neurosychiatric symptoms, hepatitis, dyslipidaemia
PIs	Nelfinavir	Diarrhoea, insulin resistance, dyslipidaemia, hepatitis
	Indinavir	Kidney stones, unconjugated hyperbilirubinaemia, gastrointestinal (GI) upset, hair loss, insulin resistance, dyslipidaemia
	Ritonavir	Poorly tolerated and rarely used as sole PI in adults, GI upset, hepatitis, taste perversion, insulin resistance, and dyslipidaemia.
	Saquinavir	Mild GI upset, headache, hepatitis, insulin resistance, dyslipidaemia
	Atazanavir	Unconjugated hyperbilirubinaemia, GI upset, insulin resistance, dyslipidaemia,
	Fosamprenavir	Rash, GI upset, hepatitis, insulin resistance, dyslipidaemia
	Lopinavir/ritonavir	GI upset, hepatitis, insulin resistance, dyslipidaemia.

MCV = Mean Cell Volume, GI = Gastrointestinal (Wilson *et al.*, 2008).

2.4.3   Profiles of non-nucleoside reverse transcriptase inhibitors (NNRTIs)

Nevirapine (NVP) and Efavirenz (EFV) are non-nucleoside reverse transcriptase inhibitors (See Figure 2.5) that inhibit the same enzyme target, the reverse transcriptase (De Clercq, 2004). The main difference between the nucleoside and the non-nucleoside reverse transcriptase inhibitors is that unlike NRTIs, which bind at the enzyme's active site, NNRTIs bind at another site away from the active site generally known as the NNRTI pocket (De Clercq, 2004). NVP and EFV are not effective against HIV-2, because the pocket of HIV-2 reverse transcriptase has a different structure. As with other ART drugs, HIV quickly develops drug resistance if NVP is not used in combinations with three or more antiretroviral drugs.

NVP and EFV are widely available in Southern Africa. Whereas, NVP and EFV commonly cause hypersensitivity rashes and hepatitis, NVP causes more severe rashes and hepatitis. EFV is well known to cause neuropsychiatric symptoms such as insomnia and dizziness. Moreover, EFV is thought to have teratogenic potential which may cause congenital deformities such as neural tube defects (Wilson *et al.*, 2008).

Anti-tuberculosis drugs especially Rifampicin significantly lower Nevirapine levels (Niemi, Backman, Fromm, Neuvonen & Kivistö, 2003). Rifampicin and NVP can induce the activity of cytochrome P450 enzymes in the liver (Niemi *et al.*, 2003). The two drugs should therefore not be administered concurrently. Manosuthi, Tantanathip, Chimsuntorn, Eampokarap, Thongyen, Nilkamhang & Sungkanuparph (2010a), however recommended that NVP-based ART is an option for HIV-infected patients who receive Rifampicin in resource limited countries especially those who cannot tolerate EFV.

The World Health Organisation approved the use of Nevirapine prophylaxis in many developing countries as a way of reducing mother-to-child transmission of HIV (Guay, Musoke, Fleming, Bagenda, Allen, Nakabiito, Sherman, Bakaki, Ducar, Deseyve, Emel, Mirochnick, Fowler, Mofenson, Miotti, Dransfield, Bray, Mmiro & Jackson, 1999). According to Guay *et al.* (1999), Nevirapine can reduce the rate of HIV transmission by almost 50% compared with a short course of Zidovudine (AZT) prophylaxis. However, some researchers are concerned that NNRTI resistance,

commonly observed in both mothers and infants after they are given Nevirapine, may compromise the response to future regimens containing NNRTIs (Eshleman, Mracna, Guay, Deseyve, Cunningham, Mirochnick, Musoke, Fleming, Glenn Fowler, Mofenson, Mmiro & Jackson, 2001).

EFV in combination with TDF/ 3TC or AZT/ 3TC is recommended in treatment-naïve patients in adults and adolescents (De Clercq, 2004). EFV is metabolised by the liver, and is both a substrate and inducer of the cytochrome P450 system (Zapor, 2004). EFV may therefore interact with other drugs metabolised in the liver. For example, EFV lowers blood levels of most protease inhibitors such as Amprenavir, Atazanavir, Indinavir or Saquinavir. This can result in incomplete inhibition of viral replication, which can allow multi-drug resistant viruses to evolve. The other NNRTI, Delavirdine, is rarely used due to its comparatively less potency and inconvenient dosage requirements (Zolopa & Katz, 2009).

It is important to understand the role played by cytochrome 450 in drug metabolism because the cytochrome P450 enzymes are central to drug metabolism (Guengerich, 2008). The cytochrome P450 enzymes are haemo-protein enzymes because they contain a haeme iron centre. The cytochrome P450 enzymes catalyse the oxidation of organic substances such as lipids, steroidal hormones, and drugs.

Drugs may induce cytochrome P450 enzymes through the biosynthesis of an iso-enzyme or inhibit cytochrome P450 enzymes by direct enzyme inhibition (Guengerich, 2008). It is important to note that this is a major source of adverse drug interactions, since changes in cytochrome P450 enzyme activity may affect the metabolism and hence the clearance of drugs. Typically, if one drug inhibits the cytochrome P450 enzyme-mediated metabolism of one drug, the second drug may accumulate within the body resulting in toxicity.

2.4.4   Profiles of Protease Inhibitors

HIV-1 protease is an aspartic acid protease (Collier & Squires, 2003). HIV protease is an enzyme that consists of two 99 amino acid chains non-covalently bound together. The HIV-1 protease plays a crucial role in cleaving small proteins from the HIV-1's polyprotein precursor known as group-specific antigens (*Gag)*. This cleaving

step allows the creation of structural and enzymatic proteins such as p24 and p17 which are crucial for the formation of mature virions (Collier & Squires, 2003).

Most HIV-1 PIs (see Figure 2.7) are transition state analogues of HIV-1 protease meaning that they are structurally similar to substrate molecules normally acted upon by HIV-1 protease.
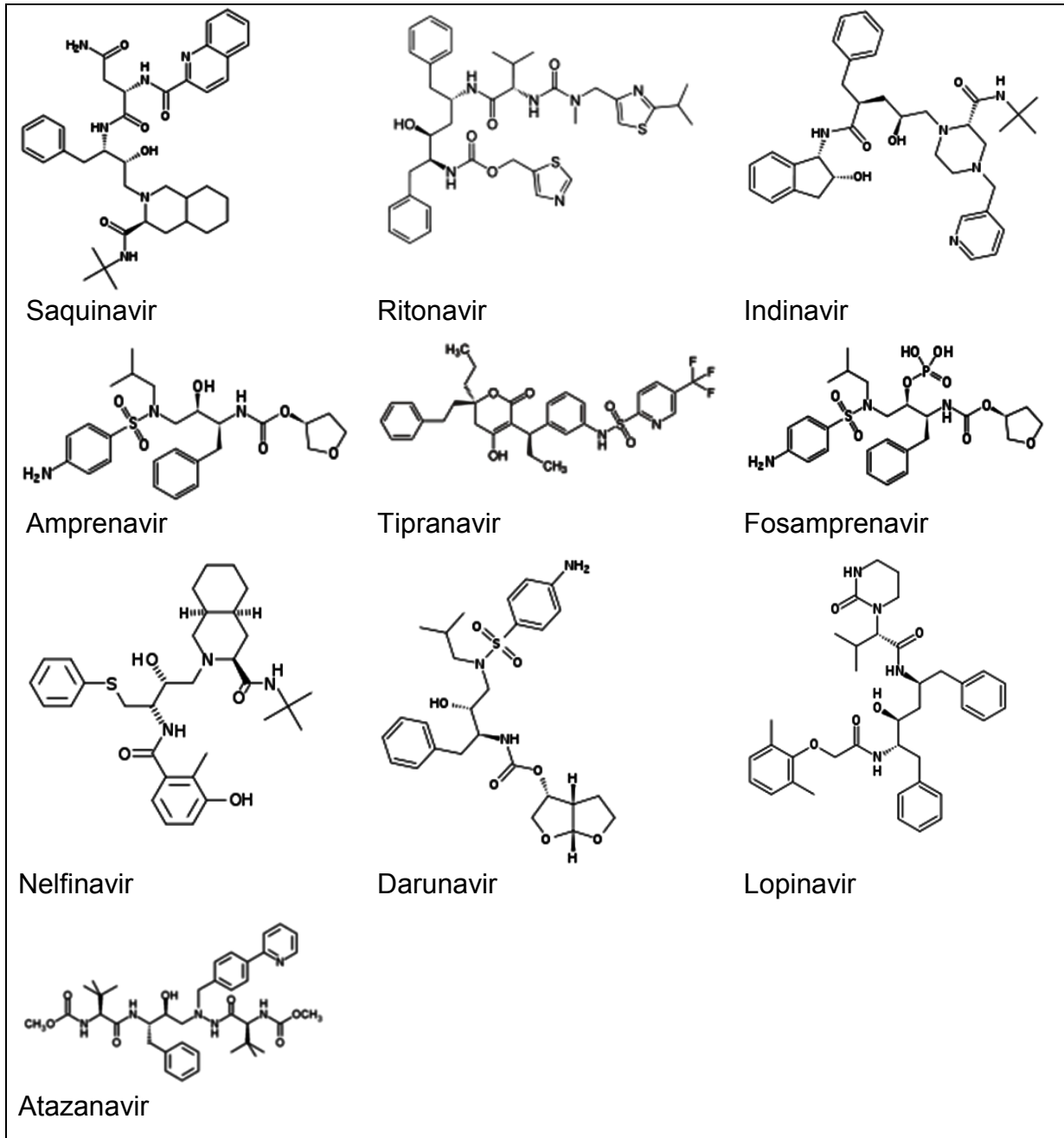
Figure 2.7: Structures of Protease Inhibitors.

(De Clercq, 2004).

Besides Tipranavir, all PIs are peptidomimetic which means that PIs contain hydroxyethylene scaffolds which can mimic the normal peptide linkage that is normally cleaved by the HIV protease. If the HIV-1 protease is inhibited, ineffective viral proteins are produced (De Clercq, 2009).

Saquinavir is a hydroxyethylamine transition state analogue of HIV-1 protease (Collier & Squires, 2003). Saquinavir can act against HIV-1 and HIV-2 (De Clercq, 2004). Interestingly, most PIs, including Saquinavir, are metabolised by the cytochrome P450 system which means besides inhibiting and inducing various cytochrome P450 enzymes, the PIs are also associated with various drug-interactions (Zolopa & Katz, 2009). Detailed information on adverse effects commonly associated with PIs is shown in Table 2.5. The most common adverse effects of protease inhibitors (PIs) are insulin resistance, dyslipidaemia, and gastrointestinal disturbances usually associated with nausea, vomiting and diarrhoea. Increased risk of myocardial infarction has also been noted among patients on PIs (Wilson *et al.*, 2008).

Ritonavir's mechanism of action is similar to Saquinavir. However, Ritonavir is less effective against HIV-2 (Danner, 2003). Significant gastrointestinal adverse effects and the requirement for multiple dosages are the main drawbacks of Ritonavir. However, being a strong inhibitor of the cytochrome P450 enzymes, Ritonavir is usually used in a combination with other protease inhibitors such as Indinavir and Saquinavir for pharmacokinetic boosting (Danner, 2003). Small doses of Ritonavir increase plasma concentration and prolong half-lives of other PIs. For this reason, Ritonavir is normally combined in formulations with other PIs such as in Lopinavir/ritonavir formulations. The Lopinavir/ritonavir formulation is available for second line therapy in Lesotho. Unfortunately, Ritonavir-boosting of PIs increases the risk of adverse effects such as insulin resistance and dyslipidaemia (Wilson *et al.*, 2008).

Indinavir, a hydroxyethylene HIV-1 protease inhibitor, is a molecular analogue of phenylalanine-proline cleavage sites of HIV-1 *Gag*-polyproteins. Indinavir is normally given in combination with Ritonavir (Gulick, 2003). Indinavir causes nephrotoxicity and is also known to cause kidney stones (Zolopa & Katz, 2009). Due to these reasons, the drug has since been discontinued in many parts of the world.

Nelfinavir was one of the first protease inhibitors to be used for treatment of pediatric HIV infection (Haubrich & Havlir, 2003). Another protease inhibitor, Amprenavir, has fewer chiral centres and therefore is easier to synthesise and is more soluble in aqueous solvents giving it a better oral bioavailability. However, Amprenavir has since been replaced by its prodrug, Fosamprenavir, because Fosamprenavir is rapidly and extensively metabolised and has an even better solubility and bioavailability than Amprenavir (Brunton LL, Parker KL, Blumenthal DK, & Buxton, 2008). Darunavir is a non-peptidic analogue of Amprenavir, with a change in the terminal tetrahydrofuran group. Darunavir is more effective in binding with the HIV protease (Murphy, 2003).

Lopinavir is structurally identical to Ritonavir. Lopinavir is normally co-formulated with Ritonavir under the trade name Kaletra. Lopinavir is known for its activity against Ritonavir resistant isolates HIV-1 (Johnson & Kuritzkes, 2003)

Atazanavir's mechanism of action is similar to Saquinavir (De Clercq, 2004). Atazanavir has better drug resistance profile than the other HIV protease inhibitors. However, Atazanavir has been associated with the development of kidney stones (Chan-Tack, Struble & Birnkrant, 2007; Gérard *et al.*, 2007). The kidney stones may result from the fact that about 7% of Atazanavir is normally excreted unchanged in urine (Chan-Tack *et al.*, 2007). Atazanavir combined with Ritonavir is one other second line ARV formulation available in Lesotho.

The other PI, Tipranavir, is a non-peptidic HIV-1 protease inhibitor whose activity is based on a different principle termed —coumarin scaffold" (De Clercq, 2009). Tipranavir has broad antiretroviral activity against HIV-1 strains, including resistant strains (De Clercq, 2009).

2.4.5   Profiles of other antiretroviral drugs

Apart from the ART drugs mentioned and profiled so far, numerous other drugs which still fall in the classic categories of NRTI, NNRTI, PIs, integrase inhibitors such as Raltegravir and entry inhibitors which include fusion inhibitors and chemokine co-receptor 5 (CCR5) antagonists are in development. However, despite the impressive dynamics of antiretroviral drug research and experimentation in more

affluent countries, the number of PIs used in resource limited countries such as Lesotho remains minimal. For instance, only three PIs are available in Lesotho, which are Lopinavir and Atazanavir co-formulated with Ritonavir (Table 2.1). The reason is due to the high costs of acquisition and laboratory monitoring required for some of the PI drugs (Wilson *et al.*, 2008). For instance, Maraviroc, a CCR5 receptor antagonist can only be used after determining chemokine receptor tropism in the patient because the drug is only effective against CCR5-tropic HIV (Zolopa & Katz, 2009; Wilson *et al.*, 2008). Another entry inhibitor is Enfuvirtide, which is expensive and requires daily injections (Tortora, Funke & Case, 2012)

Maturation inhibitors inhibit the conversion of a precursor of capsid protein to mature capsid protein, resulting in a dysfunctional capsid that makes the virus non-infectious (Tortora, Funke & Case, 2012). Other potential drugs are Tetherins, which fix the newly formed virus to the cell, preventing its release and spread to other cells (Tortora, Funke & Case, 2012). It is important to note that most desirable ARVs would be drugs that can eradicate the virus in its dormant stage in the latent reservoirs.

2.5 Essential Laboratory Tests and the Associated Physiological Principles for Initiating and Monitoring Patients on ART

Monitoring treatment progress in patients on ART is a challenging task especially in areas where access to crucial laboratory tests are often very limited. Accurate interpretation of the laboratory tests required for monitoring patients on ART depends on the extent to which one understands the physiological principles associated with the tests. Therefore, it was important to review the current methods of assessing various physiological systems pertinent to the monitoring of HIV treatment such as renal, liver, and pancreatic function; the diagnosis and management of diabetes and hypertension; and lipid profile. Diagnostic methods for hypertension and diabetes, as well as treatment methods were included in this section because of their relevance to the themes of the study.

To clarify the utility and limitations of the CD4 counts, a detailed account of the immunological role played by CD4 cells and the methods of assessing CD4 counts was necessary. It was also necessary to review current perspectives on HIV's unique structure, pathogenicity, and the laboratory tests for detecting drug

resistance to ARVs because of the scale of the literature concepts pertinent to the study.

2.5.1   Full blood count and the diagnosis of haematological abnormalities

The full blood count or complete blood count directly measures four parameters: red blood cell count (RBC count), haemoglobin concentration, mean corpuscular volume (MCV) and red blood cell distribution width (RDW); and derives other parameters such as haematocrit, mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) from the parameters directly measured (Hoffbrand, Moss & Pettit, 2006). The full blood count is essential in monitoring patients on ART especially for detection of anaemia, opportunistic infections and other haematological abnormalities that are common in HIV patients. Anaemia, being the most common haematological abnormality, has to be checked often and managed if diagnosed in HIV patients (Wilson *et al.*, 2008).

Few studies from Southern Africa address the region's ethnic and geographical diversity with respect to haematological reference ranges. Table 2.6 presents haematological reference ranges for people of the Basotho lineage (Lawrie, Coetzee, Becker, Mahlangu, Stevens & Glencross, 2009). The reference ranges of platelet count are in people of Basotho lineage are still to be determined. However, according to WHO, (2008), the cutoff value of normal haemoglobin range in non-pregnant women is 11.0 x 10^{12}/l and 13.0 x 10^{12}/l for men aged 15 or older.

Anaemia type is usually diagnosed from red blood cell size or from the evaluation of red blood cell production, destruction and loss (Hoffbrand *et al*, 2006). Microcytic anaemia is indicated if red blood cells are smaller than 80 fermito-litres (fl). Normocytic anaemia is given as the diagnosis if the red blood cells are between 80 and 100 fl in size and macrocytic anaemia is indicated if red blood cells are larger than 100 fl. Causes of normocytic anaemia include acute blood loss, aplastic anaemia, anaemia of chronic disease and haemolytic anaemia (Hoffbrand *et al*, 2006).

The use of MCV to diagnose anaemia is advantagious in that it quickly exposes some of the common causes of anaemia; for instance, a microcytic anaemia is often

the result of iron deficiency (Hoffbrand *et al*, 2006). Macrocytosis, which is the result of a disruption to the division and maturing of pro-erythroblasts in the bone marrow, is usually due to vitamin B12 and folic acid deficiency (Hoffbrand *et al*, 2006). Macrocytosis is uncommon in HIV except in patients on AZT (Mitsuyasu, 2003).

Table 2.6: Haematological reference ranges for people of Basotho lineage.

Haematological parameter	Male	Female
Red blood cell count (RBC) (10^{12}/l)	4.49-5.90	3.85-5.25
Haemoglobin (HGB) (g/dl)	13.7-17.8	11.7-16.0
White blood cell count (WBC) (10^9/l)	2.53-8.43	2.90-9.10
Haematocrit (HCT) (l/l)	0.41-0.52	0.35-0.47
Mean cell volume (MCV) (fl)	81-99	81-99
Mean cell haemoglobin (MCH) (pg)	27.2-33.6	27.2-33.6
Mean cell haemoglobin concentration (MCHC) (g/dl)	32.1-35.5	32.1-35.5
Platelet concentration (10^9/l)[a]	137-373	178-400
Neutrophils (10^9/l)	0.958-6.403	0.958-6.403
Lymphocytes (10^9/l)	1.012-2.972	0.840-3.256
Monocytes (10^9/l)	0.082-0.607	0.082-0.607
Eosinophils (10^9/l)	0-0.276	0-0.276

[a] = Platelet concentration values were adapted from the South African National Health Laboratory Service; fl = femto-litre; pg = pico-gramme (Lawrie *et al.*, 2009).

A systematic approach to entangling the cause of anaemia as presented in Figure 2.8 is useful before attempts to treat anaemia are taken. Although the suggested methods of further classifying the type of anaemia and treatments look simple in Figure 2.8, many cases of anaemia in African settings are not fully investigated due to resource limitations. For example, few health centres and hospitals have laboratories with the capacity to process bone marrow aspirate biopsy and few

patients have access to hormone therapy such as erythropoietin. Low levels of vitamin B12 and folate are a common occurrence in HIV patients and giving vitamin B12 and folate supplements may correct some of the abnormalities (Wilson *et al.,* 2008).
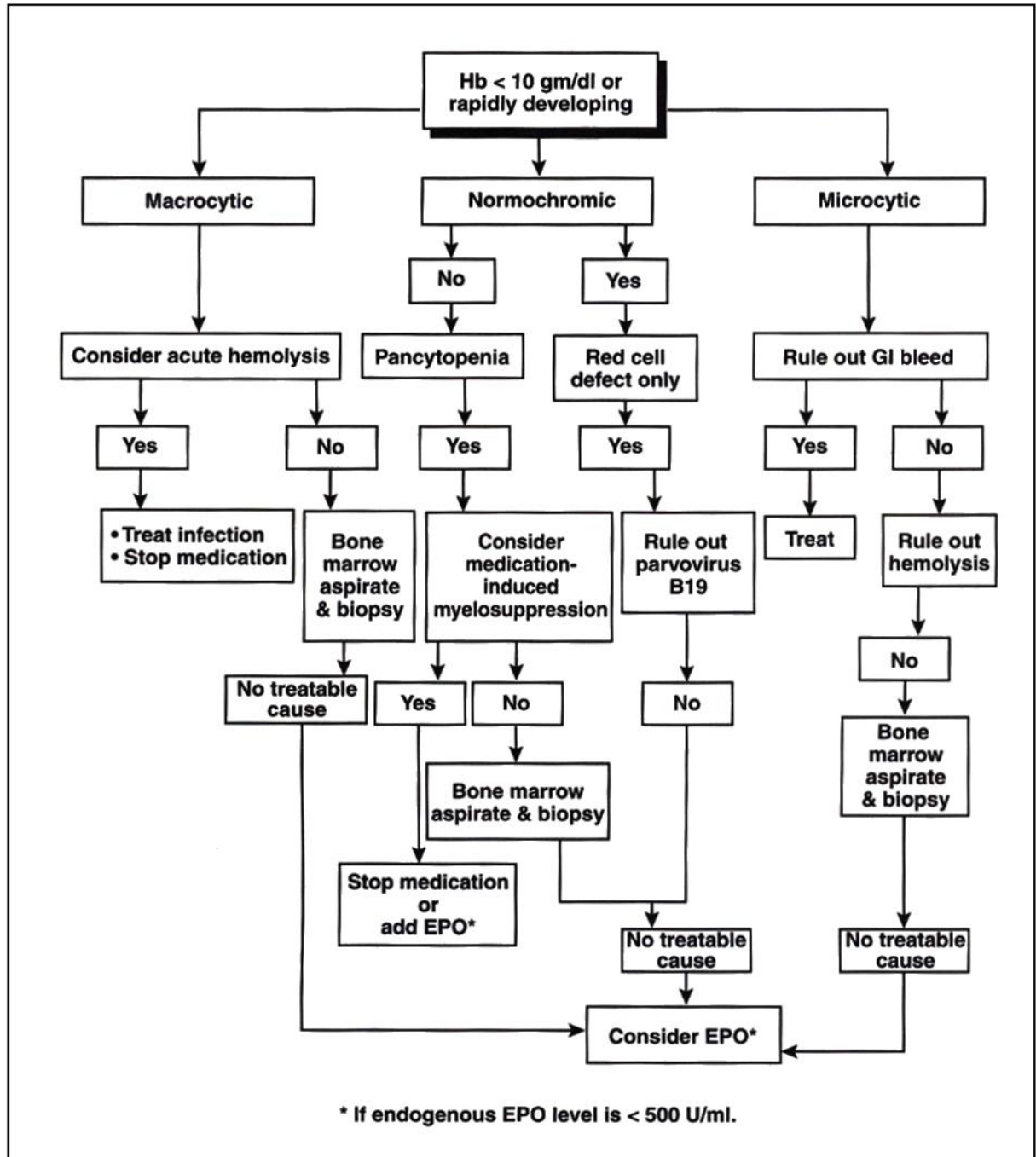
Figure 2.8: Evaluation of anaemia in HIV infection.

EPO = erythropoietin, GI = gastrointestinal, Hb = haemoglobin (Mitsuyasu, 2003).

Classifying anaemia by evaluating production, destruction or loss of red blood cells needs the reticulocyte count results. Reticulocyte count is usually used to differentiate decreased red blood cell production from increased red blood cell destruction. Increased reticulocyte count may therefore indicate haemolysis. Lactate dehydrogenase (LDH) may also indicate cell destruction or bleeding (Hoffbrand *et al*, 2006).

Anaemia in HIV is typical of the anaemia of chronic disease (ACD) phenomenon. The anaemia in HIV and AIDS is usually normochromic and normocytic with low reticulocyte count (Mitsuyasu, 2003). Chronic infections are known to result in cytokine-mediated sequestration of iron in macrophages (Wilson *et al.*, 2008). Cytokines increase the release of hepcidin from hepatocytes. The cytokines that increase hepcidin release are interleukin-1, interleukin-6 and tumour necrosis factor-α. The released hepcidin is thought to result in the destruction of a transmembrane transporter protein known as ferroportin in macrophages. The resultant limited iron availability restricts growth of microorganisms (Wilson *et al.*, 2008).

Iron studies are not readily available in resource limited settings such as Lesotho. Despite this, the mean haemoglobin concentration (MHC) can confirm iron deficiency anaemia. For example, if the MHC is very low and there are signs of cytokine-mediated weight loss, fever, and active infections, iron supplements may be given and haemoglobin response assessed after two weeks (Wilson *et al.*, 2008).

Other common findings in HIV patients include low white cell count (leucopaenia) involving neutrocytes, lymphocytes, and monocytes (Wilson *et al.*, 2008). Atypical lymphocytes may also occur. Neutropaenia is usually associated with drugs such as AZT and D4T. Severe lymphopaenia signals advanced HIV and AIDS disease stages. Low platelet count is usually characterised by platelet destruction and disseminated intravascular coagulation. Impaired making of platelets due to stem cell damage coupled with altered reticulo-endothelial dysfunction are some of the factors which are thought to contribute to the thrombopaenia (Wilson *et al.*, 2008).

2.5.2 Liver function tests and the diagnosis of liver disease

Although total liver failure is rare, liver toxicity related to the use of ARVs is a significant cause of death among HIV-infected patients (Joshi, O'Grady, Dieterich, Gazzard & Agarwal, 2011). According to Price & Thio, (2010), the prevalence of severe hepatotoxicity among HIV patients on ARVs is as high as 10% of the patients.

Hepatotoxicity may occur through several mechanisms, including hypersensitivity reaction, direct toxicity, mitochondrial toxicity and immune reconstitution. Hypersensitivity reaction has been associated with the use of NVP (Sulkowski, Thomas, Mehta, Chaisson & Moore, 2002), ABC and Maraviroc (Joshi *et al*., 2011). NNRTIs can also cause direct toxicity on the liver (Price & Thio, 2010). Nucleoside analogs may lead to mitochondrial toxicity. One other cause is immune reconstitution syndrome while on ART, is associated with increased cytolytic activity against *Hepatitis virus* infected liver cells, a decline in HIV RNA and an increase in CD4 T cell count (Price & Thio, 2010).

As a general rule, patients with pre-existing liver disease need strict monitoring when taking high risk ARVs. Risk factors for severe hepatotoxicity while on ART include elevated liver enzymes before initiating treatment, chronic Hepatitis B or C, and concomitant hepatotoxic medication. Use of protease inhibitors such as Lopinavir, Ritonavir, Indinavir, Tipranavir and Atazanavir has also been associated with hepatotoxicity (Chan-Tack, Struble & Birnkrant, 2008; Torti, Lapadula, Antinori, Quirino, Maserati, Castelnuovo, Maggiolo, De Luca, Paraninfo, Antonucci, Migliorino, Lazzarin, Di Perri, Rizzardini, Esposito & Carosi, 2009). Atazanavir and Indinavir inhibit the hepatic enzyme uridine diphospho-glucuronosyl transferase (UDP-glucuronosyl transferase), increasing the level of bilirubin in up to 50% of patients (Torti *et al*., 2009). Although, the mechanisms of hepatotoxicity for protease inhibitors such as Tipranavir boosted with Ritonavir (Tipranavir/r) are not clear, use of Tipranavir/r has been associated with transaminase elevations (Chan-Tack, Struble & Birnkrant, 2008). Other factors mentioned in the literature include older age, obesity, female gender, thrombocytopenia, high alcohol intake, increased viral load and even renal dysfunction (Servoss, Kitch, Andersen, Reisler, Chung & Robbins, 2006; Nunez & Soriano, 2005).

Liver function tests (LFTs) are tests designed to ascertain the state or cause of liver disease. LFTs can be used to monitor ART drugs because some of the ART drugs may cause liver damage. LFTs are generally indicated in HIV patients who are beginning hepatotoxic ART regimens such as Nevirapine and Stavudine-containing ART regimens. LFTs are also indicated in HIV patients with a history of liver disease and in patients with signs of hepatomegaly (Wilson *et al.*, 2008). Wilson *et al.* (2008) recommended that Hepatitis B and C should be screened before ART is initiated and may be routinely checked if resources permit.

Liver disease is classified into two categories of injury: cell necrosis and cholestasis (Burke, 2002; Chung & Sherman, 2003). Cell necrosis is further classified into acute or chronic type. Whereas viral hepatitis, toxic hepatitis, alcoholic hepatitis and ischaemic necrosis are classified under acute necrosis, chronic active hepatitis, autoimmune hepatitis and cirrhosis are classified under chronic cell necrosis. Cholestasis may be intra-hepatic or extra-hepatic.

Albumin, whose main functions are to maintain osmotic balance in the vascular space and to transport minerals in the blood such as calcium, is used to assess liver function. Elevated albumin indicates dehydration whilst low albumin indicates liver dysfunction (Burke, 2002). High concentrations of albumin in urine usually signify chronic liver diseases such as nephrotic syndrome.

Alkaline phosphatase (ALP) is a liver enzyme found primarily in liver cells particularly the liver cells lining the biliary ducts. Elevated ALP levels usually indicate cholestatic injury, bile duct obstruction or intrahepatic cholestasis (Burke, 2002).

The enzyme alanine transaminase (ALT) or alanine aminotransferase (ALAT) is primarily found in the cytoplasm of hepatocytes. ALT is regarded as the most sensitive and specific test for hepatocellular injury (Chung & Sherman, 2003). Raised ALT levels in the blood may indicate the occurrence of damaged liver tissue. Liver tissue damage may occur as a result of many causes, including drug toxicity, and obstructed bile ducts or cirrhosis of the liver. ALT should however be interpreted in conjunction with other LFTs.

Aspartate transaminase (AST) or aspartate aminotransferase (ASAT) is produced by liver parenchymal cells, muscle cells and red blood cells. Raised AST levels in

blood indicate increased red blood cell destruction, acute liver damage, and cardiac or skeletal muscle damage. AST is therefore not a specific indicator of liver damage or disease. However, using the ratio of AST to ALT may increase the diagnostic accuracy of both ALT and AST (Chung & Sherman, 2003).

The liver processes bilirubin by conjugating it and secreting it into bile, which is finally excreted into the intestines (Burke, 2002). Bilirubin, which is one of the breakdown products of haeme, is formed during the destruction of red blood cells. In liver disease bilirubin is not processed. The unprocessed bilirubin accumulates in the body, causing a condition called jaundice which is characterised by dark urine and yellowing of the skin or eyes.

If the level of unconjugated bilirubin is high, then the cause of liver disease is most likely to be located in pre-hepatic areas of the blood stream. Typical causes of high levels of unconjugated bilirubin include haemolysis, viral hepatitis, or liver cirrhosis. Conjugated bilirubin is normally elevated in cases where the liver is able to conjugate bilirubin but is not able to excrete the conjugated bilirubin due to bile duct obstruction (Burke, 2002).

Gamma glutamyl transpeptidase (GGT) is reasonably specific to the liver and is generally regarded as a better indicator of cholestatic damage than other LFTs such as ALP. High levels of GGT are synonymous with chronic alcohol toxicity and drug-induced cholestatis (Chung & Sherman, 2003). GGT may be elevated even with minor damage of the liver. GGT is also crucial in identification of the causes of elevated ALP.

Hepatitis B and C are important liver diseases in the management of HIV patients. Hepatitis B or C co-infection in HIV patients is common in Southern Africa. Whereas *Hepatitis B virus* – HIV (HBV/HIV) co-infection may be as high as 10% or more in HIV positive patients, Hepatitis C co-infection with HIV is relatively less prevalent (Kapembwa, Goldman, Lakhi, Banda, Bowa, Vermund, Mulenga, Chama & Chi, 2011; Patel, Davis, Tolle, Mabikwa & Anabwani, 2011; Mayaphi, Roussow, Masemola, Olorunju, Mphahlele & Martin, 2012). Some researchers have stated that there is a need for monitoring liver disease in HIV patients in Africa (Rockstroh,

Peters & Wedemeyer, 2011). Hence, a short review of current methods for diagnosis of viral Hepatitis infections especially Hepatitis B and C is important.

Hepatitis B infection is a serious disease caused by *Hepatitis B virus* (HBV). HBV infects the liver causing chronic liver problems. HIV/HBV co-infection complicates the management of ARVs. Diagnosis of acute HBV infection is based on the presence of immunoglobulin M (IgM) antibodies to Hepatitis B core antigens (anti-HBc) or the presence of Hepatitis B surface antigens (HBsAg). HBV can also persist as a chronic infection. Diagnosis of chronic HBV infection is based on the absence of anti-HBc IgM antibodies and the presence of HBsAg, Hepatitis e antigen (HBeAg) or HBV DNA (Pincus, Tierno, Fenelus, Bowne, Bluth, 2011).

2.5.3 The renal system and methods of assessing renal function

2.5.3.1 Structure and function of the renal system

Understanding the structure and function of the kidney gives a better understanding of the development of kidney disease. The review of the structure and function of the kidney is also crucial for clarification of how the diagnostic methods for kidney disease work.

The main function of the kidney is to regulate the volume and composition of body fluids by allowing ultra-filtration of plasma at the glomerulus and reabsorbing some of the components of the ultra-filtrate at specific points along the nephron (Barrett, Barman, Boitano & Brooks, 2010). The kidney plays a central role in the excretion of urea, ammonia and creatinine from the breakdown of protein; uric acid from nucleic acids; drugs and toxins.

The functional unit of the kidney is the nephron (Barrett *et al*., 2010). Each nephron consists of several important fuctional structures that include renal tubules, the glomerulus, and Bowman's capsule as shown in Figure 2.9. It is important to note that the kidney cannot regenerate new nephrons when damaged. Therefore, renal injury, normal ageing, or disease gradually decreases the number of nephrons. For example, the number of functioning nephrons usually decreases about 10% every 10 years after the age of 40. This means that at age 80, there may be 40% fewer nephrons than there were at age 40 (Guyton & Hall, 2011).
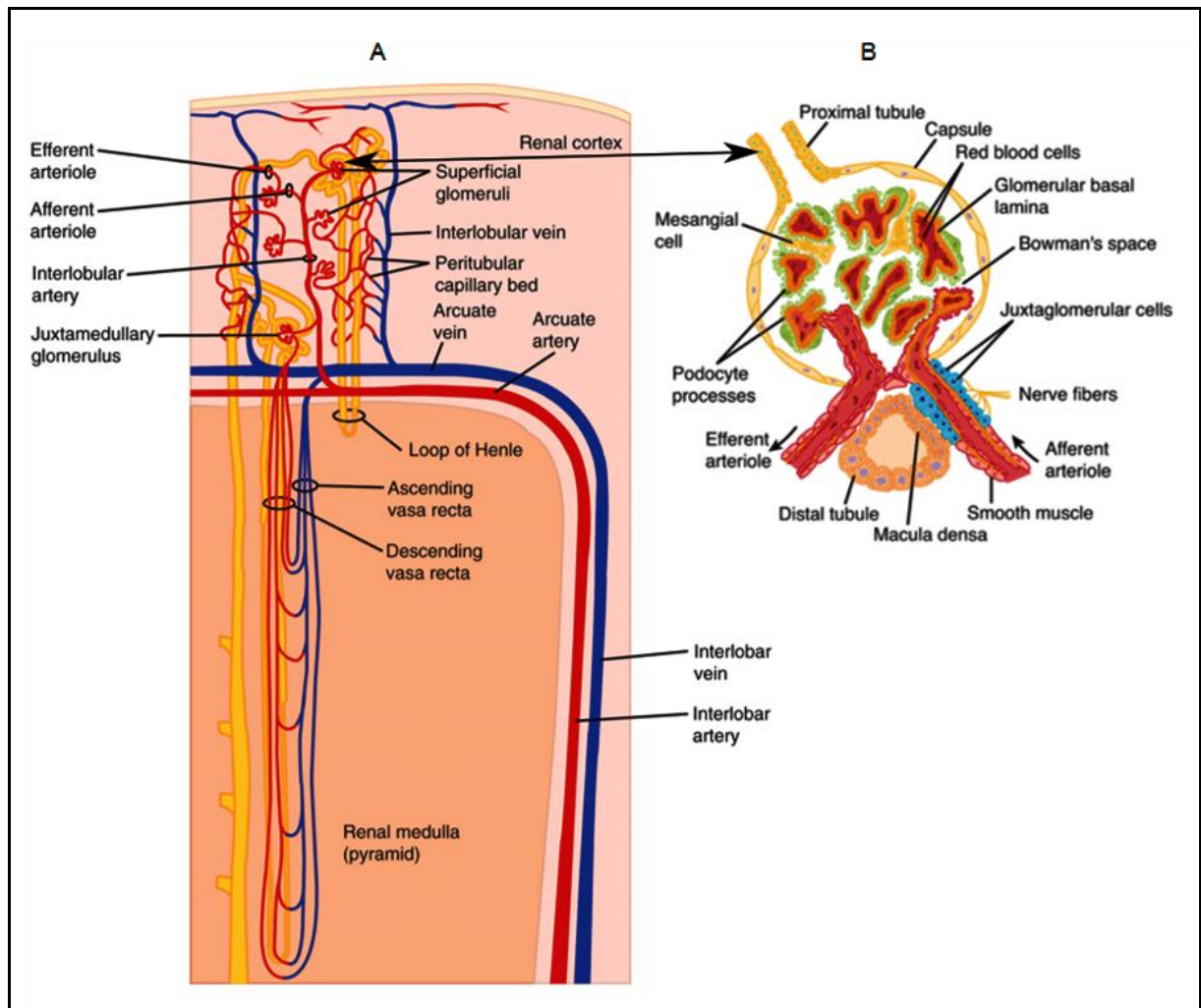
Figure 2.9: Morphology of the nephron and the associated blood supply. (Barrett *et al*., 2010).

Different parts of the kidney serve distinct functions (Barrett *et al*., 2010). The cortex produces erythropoietin in response to hypoxia. The medulla, which consists of lipid-laden interstitial cells, is important in prostaglandin production. The epithelium of the collecting ducts is made up of principal cells and intercalated cells. The principal cells are involved in sodium reabsorption and vasopressin-stimulated water reabsorption. The intercalated cells are involved in acid secretion and bicarbonate transport.

The reabsorption of some of the components of the ultra-filtrate at specific points along the nephron is a key function of the kidney. The reabsorption of sodium and

chloride ions is crucial for the control of electrolyte and water levels in the body (Guyton & Hall, 2011). The transport of sodium is coupled to the movement of H^+ ions, glucose, amino acids, organic acids, phosphate, and other electrolytes and substances across the renal tubule walls. Sodium is reabsorbed through passive diffusion and active transport by sodium-potassium-ATPase pumps located in the basolateral membranes.

Kidneys are also important for excretion of calcium and phosphate (Guyton & Hall, 2011). The most important factor controlling this reabsorption of calcium is parathyroid hormone (PTH). Renal phosphate excretion is controlled by the concentration of phosphate ions in the plasma through negative feedback mechanism and parathyroid hormone (Guyton & Hall, 2011). PTH can directly increase the renal excretion of phosphate. The kidney is also essential for vitamin D metabolism; it hydroxylates 25-hydroxycholecalciferol to the active form, 1,25-dihydroxycholecalciferol. The failure of this process contributes to the hypocalcaemia and bone disease of chronic renal failure.

A variety of hormonal and chemical factors affect kidney function. For example, adrenocortical steroid hormones such as aldosterone increase sodium reabsorption. Prostaglandins such as prostaglandin E2 (PGE2) have an opposite effect to aldosterone. PGE2 causes urinary loss of sodium. Angiotensin II acts on proximal tubules resulting in increased reabsorption of sodium and bicarbonate. Endothelial-derived nitric oxide decreases renal vascular resistance, increases renal vasodilation and therefore increases GFR. Renin, a protease enzyme, acting together with angiotensin-converting enzyme (ACE) in the kidneys convert part of the circulating angiotensin I and some of the angiotensin I synthesized in the kidneys to angiotensin II (Guyton & Hall, 2011).

Therefore, the kidney plays a central role in regulation of various plasma components. The kidney is also an important gland which produces several hormones. Understanding renal function is key to unravelling the causes of renal disease, including the effect of TDF.

2.5.3.2 Methods of assessing renal function

Renal function tests are designed to assess the state or cause of kidney disease. Glomerular filtration rate (GFR) provides a useful index for assessing renal function (Watnick & Dirkx, 2009). GFR is usually estimated by measuring the renal clearance of substances that are freely filtered through the glomerulus using the formula:

Renal clearance of substance Y = $\dfrac{U \, x \, V}{(P)}$

Where U and P represent urine and plasma concentrations of substance Y, and V represents the urine flow rate in ml/min (Watnick & Dirkx, 2009).

The direct methods for assessing renal function which happen to be the gold standard methods for measuring renal clearance use chemicals such as inulin, iohexol, ^{51}Cr-EDTA, ^{99}Tc-DTPA, and ^{125}Iodine-labelled iothalamate. These substances are expensive, require estimates of body surface area and are not readily available (Risch & Huber, 2005; Mouton & Holder, 2006). These substances also require intravenous infusion and timed urine collections over a period of several hours making it costly and cumbersome. As a result, a number of alternative measures for estimating GFR have been devised.

GFR derived from serum creatinine values is normally used to measure renal function in rural settings. Creatinine, serum electrolytes, and blood urea nitrogen (BUN) are indirect indicators of renal function. While creatinine and BUN are generally adequate in diagnosing the cause of kidney disease, BUN and creatinine may not indicate kidney disease until 50% of kidney function is impaired (Risch & Huber, 2005). Hence, the most accurate indicators of renal function such as glomerular filtration rate estimated by creatinine clearance is measured to assess renal function in most cases.

A low or decreasing GFR as estimated from serum creatinine is generally considered to be an indicator of chronic kidney disease though the utility of serum creatinine is limited by the inaccuracy of serum creatinine measurement. Serum creatinine concentration is affected by age, gender, muscle mass, diet and race (Harmoinen *et al.*, 2003; Mouton & Holder, 2006). Serum creatinine values are also

affected by haemolysis which can occur if serum is not separated from clotted venous blood for more than 16 hours (Ford & Berg, 2008). In more affluent countries, serum cystatin C has replaced serum creatinine (Risch & Huber, 2005).

The use of equations such as Cockcroft-Gault or the modification of diet in renal disease (MDRD) formulae in estimating GFR has been shown to give more valid estimates of GFR than serum creatinine alone (Froissart, Rossert, Jacquot, Paillard & Houillier, 2005). The Cockcroft-Gault formula for calculating GFR is derived from serum creatinine values as follows:

GFR (ml/min) = [(140-age) x weight (kg)]/ [serum creatinine (µmol/l) x 1.23(men) or 1.04(women)].

Therefore, in addition to patients' serum creatinine, the Cockcroft-Gault formula incorporates patients' age, gender and weight.

Looking closer at the Cockcroft-Gault equation, it becomes clear that the equation is dependent on age, weight and gender. People with low body weight, older age and female gender will have higher serum creatinine values than people with higher body weight, younger age and male gender for the same amount of serum creatinine

The most recently adopted tool for calculating the estimated GFR is the MDRD formula. The MDRD equation on the other hand incorporates age, gender and ethnicity but not weight (Froissart *et al.*, 2005). The MDRD formula in mg/dl for estimating GFR is shown below.

GFR (ml/min) = 186 X Serum Creatinine$^{-1.154}$ X Age$^{-0.203}$ X [1.212 if Black] X [0.742 if Female]

The diagnostic accuracy of the Cockcroft-Gault and MDRD equations has been found to be comparable to the accuracy of serum cystatin C (Harmoinen *et al.*, 2003). However, the MDRD equation has been demonstrated to be more accurate in patients of African descent (Levey, Bosch, Lewis, Greene, Rogers & Roth, 1999).

The chronic kidney disease epidemiology collaboration (CKD-EPI) published a new formula for estimating GFR in 2009 (Levey, Stevens, Schmid, Zhang, Castro, Feldman, Kusek, Eggers, Van Lente, Greene & Coresh, 2009) which was expected to improve accuracy in estimating GFR. According to Matsushita, Selvin, Bash, Astor & Coresh, (2010), the CKD-EPI equation performs better than the MDRD equation, especially at higher GFR.

Another indirect indicator of kidney disease is raised level of protein in urine (proteinuria). Albumin is usually the major protein found in chronic kidney disease (Mouton & Holder, 2006) although other proteins such as β_2-microglobulin (Gatanaga 2006) and retinol-binding protein (Del Palacio, Romero & Casado, 2012) may also be found. Moreover, β_2-microglobulin has been reported to be even more sensitive than serum creatinine in detecting damage of renal tubules by TDF (Gatanaga 2006).

Sodium, potassium, chloride and bicarbonate are the four serum electrolytes that are crucial in the diagnosis of renal tubular acidosis (Watnick & Dirkx, 2009). Renal tubular acidosis which has been identified as the hallmark sign of tenofovir-associated renal damage (Kohler *et al.*, 2011) is characterised by acidosis and electrolyte imbalances (Watnick & Dirkx, 2009). The acidosis and electrolyte imbalances are usually a result of impaired renal hydrogen ion excretion, impaired bicarbonate re-absorption, or rarely, a result of abnormal production of aldosterone hormone. The renal tubules also regulate potassium and sodium levels. Therefore, impaired renal tubules can be detected by sodium, potassium, chloride and bicarbonate (Watnick & Dirkx, 2009).

2.5.4 Diagnosis, treatment and classification of hypertension

Besides serum creatinine, urea and electrolytes; the measurement of blood pressure is equally essential for the diagnosis and prognosis of renal disease because hypertension is a risk factor of renal disease (Sutters, 2009). Hypertension classified into different stages depending on the level of blood pressure. According to Chobanian *et al.* (2003), hypertension stage I is when the measured blood pressure $\geq$140 mmHg systolic and/or $\geq$90 mmHg diastolic. Blood pressure values $\geq$160 mmHg systolic and/or $\geq$100 mmHg diastolic are classified as hypertension

stage II (Chobanian *et al.*, 2003). Individuals at high risk of developing hypertension (pre-hypertension) have blood pressure measurements $\geq$120 mmHg systolic and/or $\geq$80 mmHg diastolic but falling below the hypertension stage I range.

Guidelines generally recommend treatment to be initiated with the cheapest and most cost-effective drug such as thiazide diuretics unless there are contraindications. Thiazides such as Hydrochlorothiazide are examples of diuretic drugs that may be used to control hypertension. Thiazides lower blood pressure initially by decreasing plasma volume, and have also been associated with the reduction of peripheral vascular resistance in the long term (Sutters, 2009). Diuretics on their own may not be adequate to control hypertension, hence β-blockers such as Atenolol, and ACE inhibitors (angiotensin-converting enzyme inhibitors) such as Captopril may be used in combination with the diuretic drugs (British National Formulary (BNF), 2006).

ACE inhibitors primarily act by inhibiting the renin–angiotensin–aldosterone system. β-blockers (β-adrenergic blocking agents) act through decreasing the heart rate and cardiac output (Sutters, 2009). Angiotensin-II receptor antagonists may be used in cases where ACE inhibitors are contraindicated. Calcium-channel blockers such as Nifedipine may also be used where thiazides are contraindicated. In principle, calcium-channel blockers act by increasing peripheral vasodilation.

In cases where hypertension is resistant to treatment, α-blockers may also be used in combination with other antihypertensive drugs. Again in principle, α-blockers such as Prazosin reduce blood pressure by lowering peripheral vascular resistance. Antihypertensive drugs also include drugs that lower blood pressure by stimulating the α-adrenergic receptors in the central nervous system, thus reducing efferent peripheral blood flow. Examples of such drugs include Methyldopa which is also known as Aldomate. Drug treatment for hypertension has many challenges due to contraindications in many disease conditions and age restrictions. For example, thiazides are contraindicated in gout (BNF, 2006).

Treatment for hypertension is usually done by using a combination of drugs. Use of two or more drugs allows lower dosages to be used while achieving the desired effect. Low dosages minimise adverse reactions. Appropriate combinations include:

a diuretic with a β-blocker, an ACE inhibitor, or an angiotensin II receptor antagonist; and a calcium-channel blocker plus either an ACE inhibitor, an angiotensin II receptor antagonist, or a β-blocker. Alpha blockers may be used with any of the other classes but are usually reserved for third-line therapy unless specifically indicated for some reason.

Some anti-hypertensive drugs have been associated with renal toxicity. Although it is not clear whether the toxicities are dose-dependent or not, long term use of some anti-hypertensive drugs may accelerate renal damage. Typical anti-hypertensive drugs that are potentially nephrotoxic include Captopril, Atenolol, and Nifedipine. Furosemide has also been included in the list of potentially nephrotoxic drugs (BNF, 2006).

Researchers have identified various possible risk factors and causes of hypertension which may be ascertained through laboratory tests and clinical examinations. Some of the possible causes of hypertension include chronic kidney disease, Cushing syndrome and pheochromocytoma. Whereas Cushing syndrome is a disorder resulting from increased adrenocortical secretion of cortisol, pheochromocytoma is a rare catecholamine-secreting hypertension-associated tumour of the adrenal medulla. Other possible causes of hypertension include excessive secretion of aldosterone hormone, thyroid and parathyroid disease.

Hypertension may also be caused by certain drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) and chemicals such as sodium and ethanol. Laboratory tests such as electrocardiogram (ECG) and other tests for metabolic states such urinalysis, creatinine, blood glucose, including GFR, potassium and lipid profiles, are recommended before initiating anti-hypertensive drugs in order to identify the cause. Interestingly, reduced GFR indicates a poor prognosis for hypertension and has been associated with increased risk of cardiovascular disease (Mann, Gerstein, Pogue, Bosch & Yusuf, 2001).

2.5.5 Diagnosis, treatment, classification, and clinical effects of diabetes mellitus

Diabetes mellitus is a metabolic disease that may result from defects in insulin secretion or action which means diabetes mellitus may be caused by insulin

deficiency (type I diabetes) or insulin resistance (type II diabetes). Insulin deficiency has been associated with autoimmune destruction of the β-cells of the pancreas whilst insulin resistance has been associated with inadequate compensatory insulin secretory response (American Diabetes Association (ADA), 2009). Insulin is a critical hormone that regulates blood glucose levels. Hypertension occurs with twice the frequency in the diabetic compared with the non-diabetic population, and up to 50% of patients with type 2 diabetes mellitus.

Diabetes mellitus is characterised by hyperglycaemia. Therefore, hyperglycaemia in the fasting state or after a challenge with an oral glucose load is used as a diagnostic marker of diabetes mellitus. According to guidelines set by the ADA, diabetes mellitus is defined as the presence of a non-fasting blood glucose of ≥ 11.1 mmol/l, a fasting blood glucose of ≥ 7.0 mmol/l, or blood glucose of ≥ 11.1 mmol/l during an oral glucose tolerance test normally administered over two hours (ADA, 2009). Diabetes mellitus is a confounder to analysis of kidney damage associated with TDF because the disease is associated with failure of various organs, including the kidneys (ADA, 2009).

Insulin and glucagon, secreted by the pancreas, are the two important hormones that regulate blood glucose levels (Guyton & Hall, 2011). The β-cells in the pancreatic islets of Langerhans secrete insulin whilst α-cells secrete glucagon.

Insulin exerts its effect by combining with insulin receptors which trigger intracellular cascade of events. Summarily, activated insulin receptors trigger the tyrosine kinase activity, which results in phosphorylation of enzymes that catalyse glucose, fat and protein metabolism (Guyton & Hall, 2011). The end result is increased cellular uptake of glucose, accelerated protein and fat synthesis. The rate of glucose cellular uptake is organ and tissue specific. For example, muscle cells, adipose tissue and liver cells are more responsive to insulin-activated glucose intake.

Glucagon, cortisol, growth hormone, progesterone and estrogen are other hormones that may increase insulin secretion although progesterone and estrogen can do so to a lesser extent (Guyton & Hall, 2011). Therefore, these hormones may increase the risk for developing diabetes mellitus through exhaustion of the β-cells of the islets of Langerhans. Generally, glucagon opposes the effect of insulin.

Typically, glucagon increases the blood glucose concentration when the levels become too low.

The effect of hyperglycaemia is dehydration due to increased osmotic pressure in the extracellular fluids. Chronic hyperglycaemia causes tissue injury, including kidney tissues. Damage to kidney tissue usually manifests as diabetic nephropathy. Retinal damage, heart attacks, peripheral tissue damage, and peripheral nerve problems are some of the effects of hyperglycaemia. Another effect of hyperglycaemia is the depletion of the body's proteins and fat.

Treatment of diabetes mellitus type I is simply done by giving insulin. Several forms of therapeutic insulin, which differ mainly in duration of activity, are commercially available (Guyton & Hall, 2011). Some long-acting insulin forms are precipitated with zinc or protein derivatives to delay absorption and therefore have lasting effects.

In contrast to type I, type II diabetes is associated with increased plasma insulin concentration (Guyton & Hall, 2011). The hyperinsulinaemia occurs as a compensatory mechanism for insulin resistance on target cells and tissues. Obesity is the main factor which contributes to insulin resistance. Typically, insulin resistance triggers a cascade of metabolic disorders collectively known as the metabolic syndrome.

Treatment for type II diabetes may not need the use of drugs. Dieting and exercise may be adequate to induce weight loss and to reverse the insulin resistance (Guyton & Hall, 2011). However, drugs such as metformin, thiazolidinediones, and sulfonylureas, may be used to increase insulin sensitivity or to stimulate increased pancreatic secretion of insulin.

2.5.6   Lipid profile: Rationale of use in HIV treatment and methods of measurement

Lipid profile is often important in HIV-positive patients with a history of cardiovascular disease. HIV has been associated with metabolic syndrome characterised by insulin resistance, lipodystrophy and dyslipidaemia (Rasheed, Yan & Lau, 2008; Crook, 2007). Dyslipidaemia in HIV patients on ART have been associated with exposure to Abacavir and protease inhibitors such as Ritonavir-

boosted Indinavir (Young, Weber, Rickenbach, Furrer, Bernasconi, Hirschel, Tarr, Vernazza, Battegay & Bucher, 2005). Metabolic syndrome is a risk factor for hypertension and diabetes which are also risk factors for kidney disease (Bakris, 2007). Therefore, understanding the rationale of lipid profile in HIV-positive patients and the methods of measurement of lipids is crucial for monitoring patients on ART.

Cholesterol and triglycerides are the two common types of lipids in blood. Lipids are usually conjugated to lipoproteins. Lipoproteins contain globular particles known as apoproteins. The more the quantity of apoproteins a lipoprotein has, the higher the density of the lipoprotein. Conversely, triglycerides decrease the density of lipoproteins. In principle, high density lipoproteins (HDL) consist of more apoproteins and cholesterol. Low density lipoproteins (LDL) are less dense because they contain fewer apoproteins and more cholesterol. Very low density lipoproteins (VLDL) are the least in density and they mainly contain triglycerides.

Lipoproteins may be classified in increasing order of density as: Chylomicrons, VLDL, LDL, and HDL. HDL facilitates the transfer of apoproteins among lipoproteins and cholesterol into other lipoproteins or directly into the liver. In principle, the higher the level of LDL cholesterol, the greater the risk of atherosclerotic heart disease; conversely, the higher the HDL cholesterol, the lower the risk (Gazi, Tsimihodimos, Filippatos, Saougos, Bairaktari, Tselepis & Elisaf, 2006).

Triglycerides (TG), total cholesterol and HDL-cholesterol are the measured parameters and LDL-cholesterol, non-HDL-cholesterol as well as the ratio of total cholesterol: HDL-cholesterol; LDL-cholesterol: HDL-cholesterol; and TG: HDL-cholesterol. Serum TG and total cholesterol are measured by enzymatic methods. Serum HDL-cholesterol is measured following the precipitation of the apoprotein B containing chylomicrons and lipoproteins of VLDL and LDL by phosphotungstic acid in the presence of magnesium ions. Serum LDL-C is derived from total cholesterol, HDL-cholesterol and Serum TG values using various formulae. The formulae were recently evaluated by Gazi *et al*. (2006). However, the Friedewald formula remains popular despite the reported limitations of the formula in patients with metabolic syndrome (Friedewald & Levy, 1972). The Friedewald formula formula is:

$$\text{LDL-C (mg/dl)} = \text{total cholesterol} - \text{HDL-C} - (\text{TG} \times 0.2)$$

Reference ranges for lipid profiles include total cholesterol <5 mmol/l, LDL cholesterol <3 mmol/l, HDL cholesterol in males >1 mmol/l, HDL cholesterol in females >1.2 mmol/l, and triglycerides <1.7 mmol/l.

Extremely high levels of triglycerides have been associated with increased levels of chylomicrons or chylomicronaemia (Leaf, 2009). Chylomicronaemia has serious consequences, including acute pancreatitis, abdominal pain, lipaemia retinalis, and eruptive xanthomata. High levels of triglycerides have been reported in patients on protease inhibitors (Duro, Sarmento-Castro, Almeida, Medeiros & Rebelo, 2013). The report by Duro *et al*. (2013) encouraged the use of NNRTI instead of protease inhibitors because NNRTI drugs had a better lipid profile compared to protease inhibitors.

2.5.7   Pancreatic enzymes: Rationale; utility; and methods of assessment

Pancreatic disease presenting with pancreatic lesions is common in HIV-positive patients. Acute pancreatitis in HIV-positive patients attracted the attention of researchers during the early years of HIV research (Bonacini, 1991). Researchers of that time were noticing cases of pancreatitis evidenced by raised serum amylase enzyme sometimes associated with raised pancreatic lipase enzymes (Murthy DeGregorio, Oates & Blair, 1992).

Traditionally, the diagnosis of pancreatitis has been based on both clinical features such abdominal pain with vomiting and elevation of serum concentrations of pancreatic enzymes - amylase and/or lipase (Gomez, Addison, De Rosa, Brooks & Cameron, 2012). In principle, a value three times greater than normal has been a diagnostic indicator of pancreatitis. Follow-up tests which include radiological imaging and CT scans are normally required to rule out possible causes of pancreatitis such as alcohol-induced pancreatitis, dyslipidaemia, and gall stones which can obstruct the biliary ducts and cause pancreatitis. Typically, obstructed biliary ducts would be accompanied by abnormal liver function tests as well. Fortunately, modern scanning methods can pin-point the cause of pancreatitis more precisely. In some cases, C reactive protein (CRP) levels may also be measured to probe the severity of acute pancreatits (Dervenis, Johnson, Bassi, Bradley, Imrie, McMahon & Modlin, 1999).

The utility of measuring serum amylase and lipase as indicators of pancreatic disease in HIV-positive patients was demonstrated by Hancock, Smith, Hawkins, Gazzard & Ball (1997) and Gomez *et al.* (2012). Serum amylase emerged as a poorer indicator of pancreatic disease compared to lipase.

There has been controversy over the role of protease inhibitors in causing acute pancreatitis. Although some studies such as by Chapman, Woolley, Visvanathan & Korman (2007), implicated protease inhibitors with pancreatitis, Bush & Kosmiski (2003) and Riedel, Gebo, Moore & Lucas (2008) did not find the link between protease inhibitors and pancreatitis. The link between pancreatitis and protease inhibitors was pinned on the fact that protease inhibitors were known to increase levels of triglycerides. Increased levels of triglycerides had been associated with acute pancreatitis (Bush & Kosmiski, 2003). Other ART drugs that have been associated with pancreatitis include Stavudine (Riedel *et al.*, 2008). According to Riedel *et al.* (2008) TDF, Abacavir, and Efavirenz are not associated with an increased risk of pancreatitis. ART drug combination of Lamivudine/Stavudine/Indinavir has also been associated with pancreatitis (Battillocchi, Diana, Dandolo, Stefanini, D'Amore & Negro, 2002). Non-ART drugs that may cause pancreatitis include warfarin and hydrochlorothiazide (Battillocchi *et al.*, 2002).

2.5.8 HIV and CD4 cells: Rationale and utility of CD4 counts in monitoring HIV treatment

2.5.8.1 Structure of HIV and its genome

The *human immunodeficiency virus* (HIV) is a retrovirus belonging to the lentivirus family. *Lent* is a Latin prefix which signifies slow disease progression (Cochrane, 2011). Being a retrovirus, HIV has two single-stranded RNA molecules which are associated with three enzymes–namely: reverse transcriptase, integrase and protease which are necessary for viral DNA and RNA synthesis (Wood, 2006; Cochrane, 2011). The structure of HIV is shown in Figure 2.10.

The nucleocapsid which covers the viral genome consists of p24 protein in the inner layer and p17 protein in the outer layer (See nucleocapsid in Figure 2.10). The p24 is also called the viral matrix protein (MA). The p17 is also called the capsid protein

(CA). The CA may form an icosahedral or conical core, depending on the viral strain. The viral envelope mainly derived from the host cell is a lipid layer which contains spikes of gp41 (also known as the trans-membrane protein or TM) and the gp120 (also known as the external surface protein or SU). The gp120 is bound to the gp41 by non-covalent interactions (Cochrane, 2011). The gp41 protein traverses the lipid bilayer. The notation gp120 stands for a glycoprotein with a molecular weight of 120 000 Daltons (Da) (Tortora, Funke & Case, 2012).

While the reverse transcriptase, the protease, the integrase enzymes, and the envelope proteins seem to have structural roles for the virus, there are other proteins which have regulatory roles. Six of such proteins with regulatory roles are: *Vif, Vpu, Vpr, Tat, Rev*, and *Nef* (Cochrane, 2011).
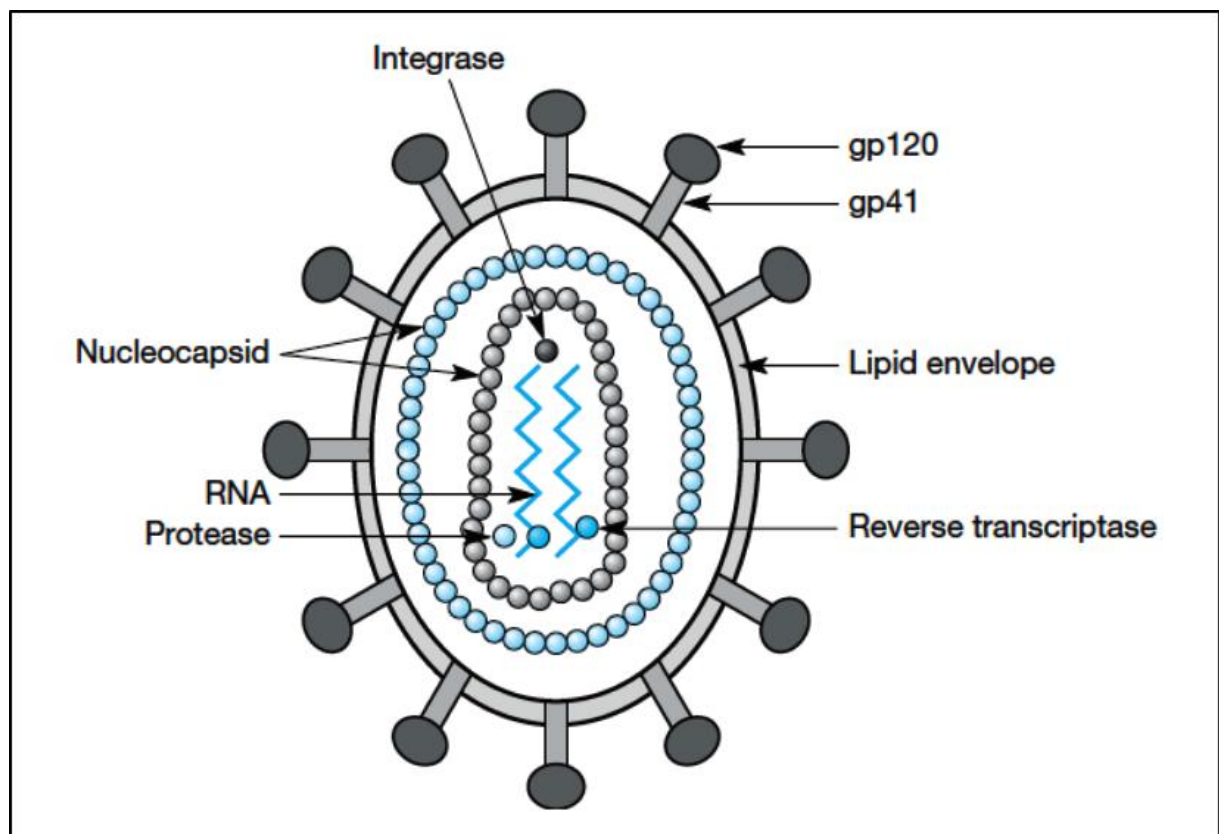
Figure 2.10: Schematic diagram of HIV.
(Wood, 2006).

Tracing the proteins back to the genes that code for them unravels more mysteries of the virus. The *gag*-gene codes for structural proteins which include the p24 proteins, the p17 proteins and other structural proteins (Virella, 2001). The *pol*-gene

codes for the reverse transcriptase. It is also believed that the *pol*-gene codes for proteins that play important roles, including the polymerase, the ribonuclease, and the endonucleases which include the integrase and the ligase. The *env*-gene codes for envelope glycoproteins. In addition, a gene located at the *gag*-pol junction codes for the protease enzyme (Cochrane, 2011). It is important to note that currently, antiretroviral drugs typically target the reverse transcriptase, the protease, the integrase enzymes as well as viral entry.

The HIV genome codes for several regulatory proteins (Virella, 2001). The genes coding for regulatory proteins include: the *tat* or transactivator of transcription gene which promotes pro-viral genome expression during transcription; the *rev*-gene which regulates expression of viral structural proteins; the *nef*-gene or negative expression factor gene which down-regulates MHC-I and CD4 expression facilitating the evasion of attack from the body's immune response. The schematic diagram of the HIV genome in Figure 2.11 depicts the specific regions of the genome and the corresponding proteins.
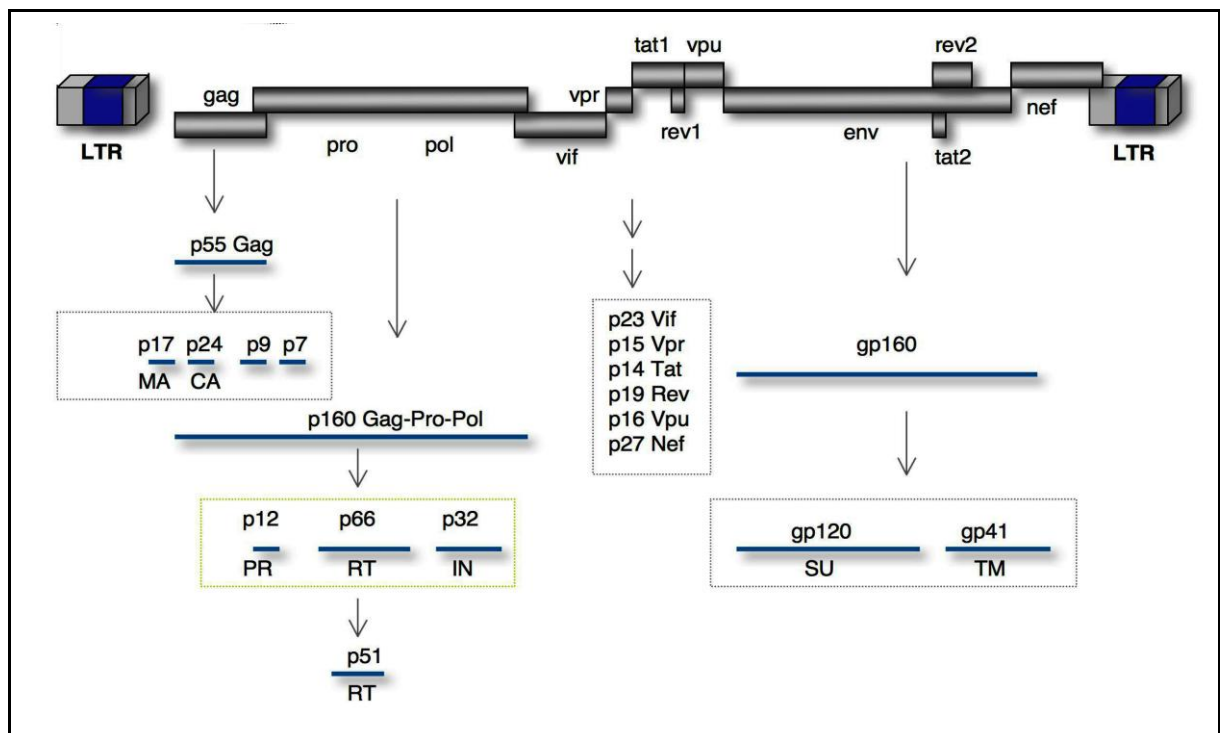
Figure 2.11: Schematic diagram of HIV-1 genome and the associated proteins.

LTR= long terminal repeat; MA= matrix; CA= capsid; PR= protease; RT= reverse transcriptase; IN = integrase; SU= surface; TM= transmembrane: Adapted from Montano & Sebastiani (2009).

2.5.8.2 Infectivity and pathogenicity of HIV

In the initial stages of HIV infection, dendritic cells are the main vehicles that pick up the virus from the blood circulation and carry it to the lymphoid organs. Apparently, dendritic cells are essential for the initiation of primary antigen-specific immune response to many infections, including HIV infection, because dendritic cells have good antigen presenting properties (Noah, 2012). In the lymphoid organs, HIV gets its first contacts with the cells of the immune system which include mostly the activated T cells, where a massive initial immune response occurs (Tortora, Funke & Case, 2012).

HIV infects cells expressing CD4 receptor molecules on the surface together with either of the two chemokine co-receptor molecules termed CCR5 and CXCR4 (Tortora, Funke & Case, 2012). It is important to note that CCR5 nomenclature is based on the beginning amino acid sequence of the proteins. For example, CCR5 means that the beginning sequence consists of cysteines, and Cx in CXCR4 indicates some other proteins. Ideally, HIV infects the T helper cell population of lymphocytes as the primary target. Unfortunately, monocytes in blood, macrophages in tissues, and dendritic cells in blood and tissues are receptive for HIV. It is now believed that many other cells that do not express the CD4 molecule can also become infected, which means that there could be some other unknown receptors for HIV (Tortora, Funke & Case, 2012). The detail of the role played by the CD4 receptor molecules and the chemokine co-receptor molecules in viral entry is outlined in section 2.5.8.5.

After viral entry, viral RNA is released and transcribed into DNA by the enzyme reverse transcriptase (Tortora, Funke & Case, 2012). The transcribed viral DNA is integrated into the chromosomal DNA of the host cell with the aid of the integrase enzyme. According to Cochrane (2011), a copy of proviral DNA is integrated into the cell genome at a random site. The integrated proviral DNA at this stage may take control the production of new viruses or may lie dormant as a provirus for many years.

The virus evades immune defenses by lying dormant or undergoing rapid antigenic changes. Retroviruses have a high mutation rate compared to DNA viruses and are

also known for lacking the corrective ―proofreading" capacity commonly noticed in DNA viruses (Tortora, Funke & Case, 2012). The mutations pause challenges especially in vaccine development, drug effectiveness and even diagnostic tests. The mutations have given rise to many variants of HIV which is broadly classified into HIV-1 and HIV-2. Researchers have since identified three HIV-1 groups– namely: main (M), outlier (O) and non-M or non-O (N). Sub-group M is responsible for more than 95% of global HIV infections (Tortora, Funke & Case, 2012). The M subtype is sub-divided into further sub-classes denoted by ―A,B, C" notation of which M subtype C is the most prevalent in the world but concentrated in India, Eastern and Southern Africa.

2.5.8.3 Stages of HIV infection and world health organization (WHO) clinical staging of HIV and AIDS disease

Generally, disease progression of HIV infection has three main stages (Tortora, Funke & Case, 2012). The first stage is characterized by viral loads of up to 10 million viral RNA/ml of blood. The immune response, led by CD8-labeled cytotoxic T cells, succeeds in reducing the number of infected CD4 cells at this point but a number of infected cells are not targeted due to viral latency. In the next phase, CD8 cells continue to keep HIV under check. However, the CD4 cells remain subdued to the extent that some opportunistic infections such as yeasts, shingles, and diarrhea may take advantage.

In the third phase, when CD4 counts drop below 350 cells/mm^3, many more opportunistic infections such as cytomegalovirus eye infections; toxoplasmosis of the brain, tuberculosis; pneumocystis, and Kaposi's sarcoma erupts (See Table 2.7).

The World Health Organization currently recommends four clinical stages according to the progression of HIV infection (WHO, 2010). The first clinical stage is generally asymptomatic although generalized lymphadenopathy may occur due to the massive initial immunological response in the lymph nodes. WHO clinical stage II is characterised by weight loss of not more than 10% and minor infections such as recurrent upper respiratory infections, and superficial fungal infections on the skin.

Manifestations of WHO clinical stage III are consistent with clinical features of declining immunity such as excessive weight loss, prolonged bacterial infections, and frequent fevers. Due to the weakening immunity, tuberculosis, oral candiasis and oral hairy leukoplakia are important signs of WHO clinical stage III. In the WHO clinical stage IV, more serious forms of infections as listed in Table 2.7 may occur.

Table 2.7: Common opportunistic infections associated with HIV infection.

Pathogen type	Disease	Disease description
Protozoa	*Cryptosporidium hominis*	Persistent diarrhoea
	Toxoplasma gondii	Encephalitis
	Isospora belli	Gastroenteritis
Viruses	Cytomegalovirus	Fever, encephalitis, blindness
	Herpes simplex virus	Vesicles of skin and mucous membranes
	Varicella-zoster virus	Shingles
Bacteria	*Mycobacterium tuberculosis*	Tuberculosis
	M. avium-intracellulare	May infect many organs; gastroenteritis and other highly variable symptoms
Fungi	*Pneumocystis jirovecii*	Life-threatening pneumonia
	Histoplasma capsulatum	Disseminated infection
	Cryptococcus neoformans	Disseminated, but especially meningitis
	Candida albicans	Overgrowth on oral and vaginal mucous membranes (stage 2 of HIV infection); Overgrowth in oesophagus, lungs (stage 2)
Cancers	Kaposi's sarcoma	Cancer of skin and blood vessels (caused by human herpes virus 8)
	Hairy leukoplakia	Whitish patches on mucous membranes; commonly considered precancerous
	Cervical dysplasia	Abnormal cervical growth

(Tortora, Funke & Case, 2012).

Staging patients according to the WHO clinical staging criteria has always been difficult and controversial without CD4 counts. CD4 counts are more definitive for

staging the progression of HIV infection. In one study conducted in Uganda by Kagaayi, Makumbi, Nakigozi, Wawer, Gray, Serwadda & Reynolds (2007), WHO clinical criteria missed half the patients with CD4 cell counts of 200 cells/mm^3 or less. Yet, in another study conducted in Saudi Arabia, Edathodu, Ali & Alrajhi (2009), reported that the WHO clinical staging correlates well with CD4 counts. Therefore, the WHO clinical criteria remain valuable but CD4 cell measurements are crucial for the scale-up of ART provision in resource-limited settings.

2.5.8.4 HIV diagnosis and testing methods

HIV testing is usually indicated in patients seeking medical attention for the various opportunistic infections such as sexually transmitted infections and any of the infections listed in Table 2.7. Although the enzyme-linked immunosorbant assay (ELISA) tests are standard procedure for detecting HIV antibodies, several tests which are cheaper and more rapid have been devised for remote areas where sufficiently equipped laboratories are not available (Tortora, Funke & Case, 2012).

The basic principle for HIV screening tests is based on antigen-antibody binding. The formation of antibodies after infection generally begins with antibodies to p24 and gp120 viral proteins. Therefore, antibodies to p24 and gp120 viral proteins are detectable at early stages of HIV infection (Noah, 2012). Antibodies to the other viral proteins such as the polymerase proteins (p31) generally occur in later stages of HIV infection (Fiebig, 2003).

Central to ELISA tests is a micro-titer plate with 96 wells which are coated with HIV antigens and HIV antibodies. In the event of the serum having the antibodies to HIV viral proteins or HIV antigens, the antibodies bind to the antigens coated on the plate whereas the antigens bind to the antibodies coated on the plates. For detection of the reaction, enzyme-linked antibodies that bind to human antibodies are then added. Addition of a suitable substrate to the enzyme results is a colour change which can be measured by colorimetric detectors.

Generally, the optical density correlates with the concentration of HIV antibodies in the sample of the patient. Several improvements have been incorporated into the original ELISA ideas. For example, in modern test systems, the solid phase consists

of microparticles coupled with the virus antigens and antibodies (Perry, Ramskill, Eglin, Barbara & Parry, 2008). Typical among the modern ELISA systems is the microparticle enzyme immunoassay or MEIA method.

It is important to note that despite the strict demands for screening tests to have high specificities of more than 99% false-reactive results may still occur. False-reactive results may occur in immunological conditions such as pregnancy, viral infections, vaccinations and autoimmune diseases (Noah, 2012). Thus, in certain patient groups an increased proportion of false reactive test results can occur.

In Western Blot analysis, viral proteins are separated by their molecular weight using electrophoresis and transferred to a membrane. Recombinant HIV antigens are then directly sprayed onto a test membrane. The test membrane is incubated with the serum of the patient. If HIV-specific antibodies are present, they bind to the antigens. The resulting antigen-antibody complex is detected using enzyme-linked antibodies and corresponding substrates.

Guidelines on HIV testing still differ between countries (Noah, 2012). For example, guidelines on HIV testing in Germany stipulate that Western Blot test result be considered positive when antibodies against an *env* protein and also against a *gag* protein and/or a *pol* protein are detected. However, the WHO guidelines recommend that Western Blots be considered positive when antibodies against at least two *env* proteins are detectable.

A weak or inconclusive Western blot result may indicate an early phase of an HIV infection and further tests such as polymerase chain reaction (PCR) which can detect HIV RNA should be carried out. The PCR is recommended in case of a highly positive screening and negative confirmatory test result as well as in cases of suspicion of acute infection or vertical transmission. However, HIV PCR cannot replace the HIV antibody tests (Noah, 2012). It is important to note that PCR can only detect the viral RNA circulating in the blood and not in HIV-infected cells. It is also important to note that current methods of HIV testing cannot detect all of the variants of rapidly mutating HIV, but are limited to HIV subtypes that are prevalent in a population (Tortora, Funke & Case, 2012).

2.5.8.5 CD4 cells and the mechanisms of immunological response in HIV infection

HIV infects cells expressing CD4 receptor molecules on their surfaces. To understand the concepts of CD4 counts and the methods of measurement in use, one has to probe further into the concepts of lymphocyte differentiation. All lymphocytes, like all blood cells, are derived from hematopoietic stem cells which have the rare capability of self-renewal and differentiation (Virella, 2001). Whereas self-renewal is the ability to give rise to at least two daughter cells at the same stage of development as the parent, differentiation is the orderly sequence of events that leads to cell maturation and restricted lineage potential.

A given cellular antigen identified by each monoclonal antibody is designated by the prefix CD, which stands for clusters of differentiation followed by a number. The numbers are assigned based on the order of discovery, and the developmental order of appearance. CD4 refers to glycoproteins found on the surfaces of antigen presenting cells such as macrophages, T helper cells, dendritic cells and monocytes (Harrison, 1993; Murphy, 2012). These cells also express another glycoprotein called CD8 on their surfaces which is structurally different from the CD4 molecule. CD4 generally defines the T helper cell population. Although HIV infects a variety of cells, the virus mainly affects T helper cells (Wood, 2006). The CD4 molecule contains four immunoglobulin-like domains, as shown in Figure 2.12.

CD8, another surface molecule important in immunological response, is expressed on T helper cells, some natural killer cells and most thymocytes. CD8 generally defines cytotoxic T cell population because CD8 is found on natural killer cells. CD8 binds to major histocompatibility complex (MHCc) class I antigens during antigen presentation (See Figure 2.12). MHC class I molecules present peptides from pathogens, which are viruses in most cases, to CD8 cytotoxic T cells.

The CD4 molecules function as co-receptors that facilitate interaction of the T cell receptor with antigen-presenting cells. CD4 molecules use their D_1 domains to interact with β_2-domains of MHC class II molecules during antigen presentation as shown in Figure 2.12. CD4 molecules are also important in the sense that they amplify the signal generated by T-cell receptors (Murphy, 2012; Wilson *et al.*, 2008).

CD4 molecules play a central role in the two stages of HIV viral infection. CD4 cells facilitate binding of HIV to the host cell, and also facilitate fusion of the viral particles with the cell membrane to allow the virus to enter the cell (Wood, 2006). CD4 molecules, gp120, gp41, and co-receptors, CCR5 and CXCR4, facilitate entry of HIV-1 viruses into T cells (Murphy, 2012).
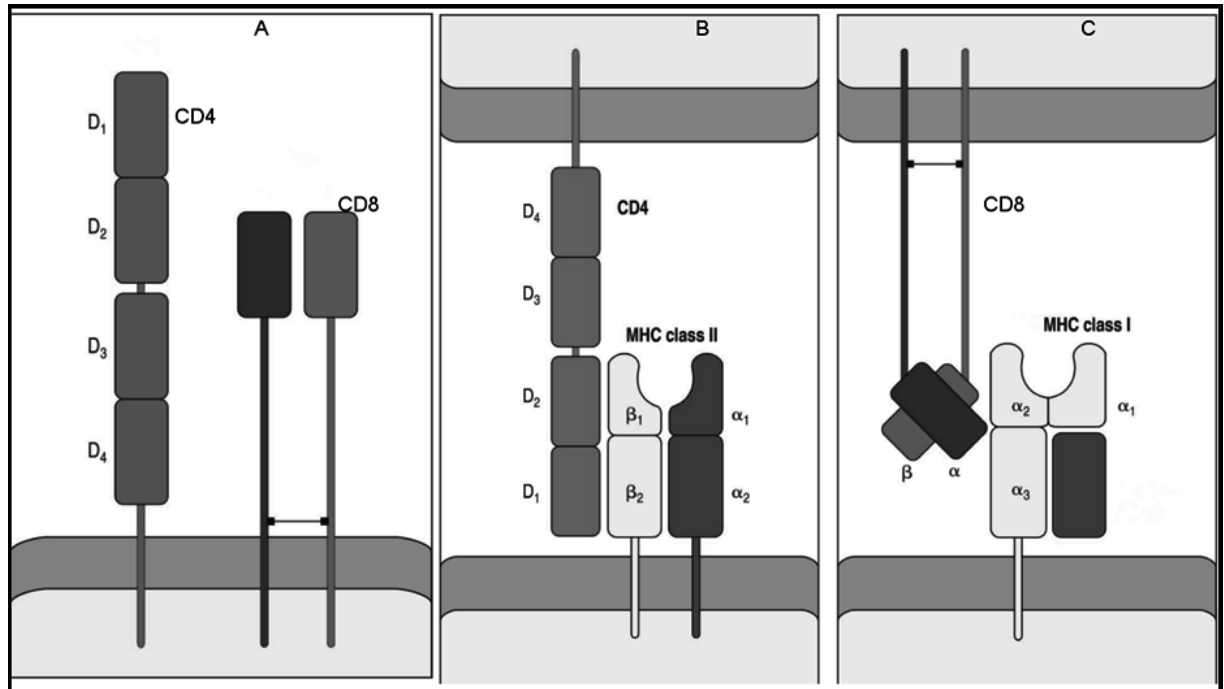
Figure 2.12: The schematic structures and interaction of the CD4, CD8, and the MHC molecules.

(Murphy, 2012).

During viral entry, the first event to occur is the binding of g120 to the CD4 molecule of the host cell (Wood, 2006). The next event to occur depends on the nature of gp41 on the surface of the virus. Gp41 of some HIV variants bind to CCR5 whilst some HIV variants can only bind to CXCR4. This selective binding is one other source of the existence of HIV variants. CCR5 is found on CD4 T cells, monocytes and dendritic cells whilst CXCR4 is only found on CD4 T cells. This means that a virus with an affinity for CCR5 may infect CD4 T cells, monocytes or dendritic cells. Such viral variants are called M-tropic viruses. Other HIV variants bind to CXCR4, which is only found on CD4 T cells but not on monocytes or dendritic cells (Wood, 2006). Such HIV variants can therefore only infect T cells and are called T-tropic viruses.

The binding of the HIV-1 virus results in a conformational change in the viral proteins which eventually leads to viral entry (Murphy, 2012). The genetic expression of CCR5 chemokines on CD4 cells has been given as the reason why some individuals are resistant to HIV infection. A progressive decrease in the number of T cells expressing CD4 molecules is the hallmark of HIV infection because HIV enters the cells carrying the CD4 receptors, usually destroying the cells. The CD4 count is therefore used as a marker of HIV disease progression, including when to begin treatment during HIV infection and the success of ART.

Several possible mechanisms through which CD4 cells decrease in HIV patients have been put forward. The mechanisms include direct destruction of CD4 cells by HIV and increased programmed cell death (Eggena, Barugahare, Okello, Mutyala, Jones, Ma, Kityo, Mugyenyi & Cao, 2005; Wilson *et al.*, 2008). To understand mechanisms of CD4 cell destruction, more concepts have to be put in perspective.

It is important to note that CD4 cells that are infected by the virus express viral antigens on their surface either as antigens presented by MHC class I or as soluble gp120 bound to CD4 molecules. CD4 T cells are therefore killed because of the expression of viral antigen (Wood, 2006). The killing of CD4 cells can occur through complement-mediated, antibody mediated, or CD8 mediated cytotoxic killing. Although there is nothing wrong with the killing of CD4 cells expressing viral antigens, the killing normally goes too far. Bearing in mind that the main type of infected cell is the CD4 T cells, the body ends up killing the same cells that are required to generate immune responses (Wood, 2006).

Other important changes to the immune system occur after HIV infection. In the initial stages of HIV viral infection, the lymph nodes swell due to an influx of CD8 T cells. The influx of CD8 T cells destroys the integrity of the lymph nodes resulting in non-functional lymph nodes (Wood, 2006). Another major change to the immune system is the increase in serum immunoglobulins that are impaired and therefore unable to generate specific antibody responses. Moreover, another change is the increase in production of autoantibodies to red blood cells, spermatozoa or myelin sheath of nerves which possibly explains the appearance of eczematous skin reactions in some untreated HIV patients (Wood, 2006).

Lesotho guidelines recommend that HIV patients be treated with ART drugs when the CD4 counts fall below the threshold of 350 CD4 cells/mm^3. Above 350 CD4 cells/mm^3 threshold, patients are thought to be still immune-competent enough without the aid of ART drugs. CD4 counts that are below the threshold of 200 cells/mm^3 are generally associated with AIDS-defining illnesses (Wilson *et al.,* 2008).

2.5.8.6 Methods of measurement of CD4 cells and factors affecting CD4 counts

CD4 and CD8 cellular markers are most often measured using flow cytometry. Flow cytometry, based on immunofluorescence analysis, is the reference standard for CD4 counts and also the method of choice if large samples are analysed (Zijenah *et al*., 2006). Immunofluorescence analysis involves counting cells that are stained in suspension using fluorescence-labelled monoclonal antibodies (Paraskevas, 2009).

In immunofluorescence analysis, a diluted blood sample is incubated with one or more fluorescent-labeled monoclonal antibodies (Virella, 2001; Paraskevas, 2009). The samples are then diluted further so that they can flow as a unicellular stream for analysis of light scattering and fluorescence properties. Forward light scattering measures sizes of cells and lateral light scattering determines the granularity of the cells. Fluorochromes are molecules that absorb light of one wavelength and emit light of another wavelength, usually a higher wavelength.

The development of monoclonal antibodies against cell-surface markers of blood cells and their conjugation with certain fluorochromes enabled accurate detection of surface molecules such as CD4 cell markers (Paraskevas, 2009). The CD4 count value together with CD3, CD4, and CD8 is obtained by analysing the relative intensity of fluorescence emitted by the cells after staining with the specific monoclonal antibodies. The final computation of the CD4 count is based on cell gating which, in principle, means restricted analysis of cells of interest with similar characteristics.

Efforts to develop affordable CD4 counting methods for resource-limited settings are on-going. To date, single-purpose or dedicated flow cytometers have been designed

for performing CD4 counts. Such machines include Becton Dickinson FACSCount and Partec CyFlow (Rodriguez, Christodoulides, Floriano, Graham, Mohanty, Dixon, Hsiang, Peter, Zavahir, Thior, Romanovicz, Bernard, Goodey, Walker & McDevitt , 2005). Another CD4 measurement technology in the affordable range is the PIMA CD4 machine (Thakar, 2012). The PIMA CD4 machine, believed to eventually bring CD4 counts to the point of care, has been evaluated with success in India (Thakar, 2012).

Absolute and percentage CD4 (CD4%) are the two parameters that CD4 machines can measure. The absolute CD4 counts measure the amount of CD4 cells circulating in the blood. The absolute CD4 count is obtained from the total and the differential white cell count. On the other hand, CD4% is measured directly on a flow cytometer and is less variable with a smaller coefficient of variation compared to the absolute CD4 count (Giorgi, Cheng, Margolick, Bauer, Ferbas, Waxdal, Schmid, Hultin, Jackson, Park & Taylor, 1990). CD4% is more economical because it requires only the flow cytometer results not the white blood cell count and differential count which are required for the absolute CD4 count. In addition, a study conducted by Burcham, Marmor, Dubin, Tindall, Cooper, Berry & Penny (1991), has shown that the CD4% is a better predictor of HIV clinical progression. However, the absolute CD4 count remains popular among clinicians.

CD4 counts are affected by many factors, including biological variations such as diurnal variation, gender, stress and variation in challenges to the immune system due to infections (Malone, Simms, Gray, Wagner, Burge & Burke, 1990; Grinsztejn, Smeaton, Barnett, Klingman, Hakim, Flanigan, Kumarasamy, Campbell & Currier, 2011). Viral, bacterial, parasitic and fungal infections have been shown to have a marked impact on CD4 counts. The other factors listed in the literature include trauma, cancer chemotherapy and malnutrition. Even over-exercising, corticosteroid use, smoking, and pregnancy have been demonstrated to have effects on CD4 counts (Malone *et al.,* 1990).

2.5.8.7 Utility and limitations of CD4 counts in monitoring HIV treatment

Discrepancies between CD4 counts and viral load tests where immunological failure based on CD4 counts did not correlate with viral load results have been found in

some HIV patients. Some of the reasons given to explain the phenomenon include myelo-suppressive effects of ARV drugs such as Zidovudine (Huttner, Kaufmann, Battegay, Weber & Opravil, 2007); thymic involution related to old age (Teixeira, Valdez, McCune, Koup, Badley, Hellerstein, Napolitano, Douek, Mbisa, Deeks, Harris, Barbour, Gross, Francis, Halvorsen, Asaad & Lederman, 2001) and abnormal cell death due to higher immune activation related to frequent challenge by opportunistic infections (Eggena *et al.*, 2005).

Misgena (2011) recently reviewed the risk factors for immunological response or virological failure. The factors mentioned in the review include old age, poor adherence, previous exposure to ART, lower baseline CD4 count, co-morbidities such as Hepatitis B or C and tuberculosis; and low body weight. Prabhakar, Banu, Pavithra, Chandrashekhara & Sasthri (2011) recommended close monitoring to ensure strict adherence to ART in the patients starting ART at very low CD4 counts below 100 cells/mm^3.

Currently the WHO recommends defining immunological failure based on CD4 counts in HIV patients whose CD4 count have either declined to pre-ART values, CD4 count dropped to less than 50% of peak on-treatment value or failure of the CD4 count to achieve a value greater than 100 cells/mm^3 over six month intervals (WHO, 2010; Prabhakar *et al.*, 2011). As a guideline, effective antiretroviral therapy should result in CD4 counts rising by at least 100 cells/mm^3 in the first year and about 50-80 cells/mm^3 in the subsequent years (Wilson *et al.*, 2008).

The debate on the utility of CD4 counts in detecting sub-optimal treatment outcomes is still going on. Some of the issues that have been commonly reported include the low predictive value of CD4 counts in detecting virological failure and the existence of discordant results between CD4 counts and viral load results (Badri, Lawn & Wood, 2008; Kanapathipillai *et al.*, 2011). Therefore, the use of CD4 counts has limited utility and may be more useful if combined with clinical criteria and viral load tests where they are available. Table 2.8 shows a summary of selected studies on the occurrence of sub-optimal immunological outcomes based on CD4 counts and viral load tests and the utility of CD4 counts in detecting sub-optimal treatment outcomes.

Table 2.8: A summary of studies on immunological outcomes based on CD4 counts.

Study reference, Place of Study	Study design; duration	Measure of ART outcome	Main Findings
Amenyah *et al.* (2006), Ghana	Retrospective review of medical records of 3806 patients	CD4 counts	40 (1.1%) had immuno-virological failure. Of the 40, 77% had IMF. 68% of immuno-virological failure occurred in treatment naïve patients. Three patients had documented poor adherence. Half of the patients failed treatment within the first year of treatment.
Chaiwarith *et al.* (2007), Thailand	Retrospective cohort of 327 patients; 6-42 months	VL, CD4 counts, clinical criteria	9.2% had VLF. Using IMF criteria and clinical criteria to detect treatment failure, the sensitivity was 20.0% and the specificity was 85.9%.
Badri *et al.* (2008), South Africa	Observation cohort of 330 patients with initial viral load suppression	CD4 count, VL	The association between a CD4 count decrease and VLF was poor (area under curve = 0.59; sensitivity = 53.0%; specificity = 63.6%; positive predictive value = 10.9%).
Kantor *et al.* (2009), Kenya	Observation cohort of 149 patients; 23 months	CD4 count, VL	IMF as a sole indicator of VLF resulted in a premature switch to second-line regimens for 58% of patients who experienced a 25% decrease in CD4 count and for 43% patients who experience a 50% decrease in CD4 count.
Reynolds *et al.* (2009), Uganda	Observation cohort of 1133 patients; 20.2 months (median)	CD4 and VL	125 (11.0%) had IMF. VLF with VL >400 copies/ml reached by 112 patients (9.9%). Only 26 (2.3%) had both IMF and VLF (2 VL>400 copies/ml) during follow-up.
Bello *et al.* (2011), Brazil	Observation cohort of 139 patients	VL, CD4 count	12.2% had VLF. TB was a significant predictor of VLF.

Charpentier *et al.* (2011), Cameroon	Observation cohort of 819 patients	CD4 count, VL, genotypic resistance testing	36% had VLF. 17% had CD4 counts <200 cells/mm^3 and 37% < 350 cells/mm^3, indicating either immunorestoration or IMF. 20% of patients with VLF showed wild-type viruses susceptible to all ARV, indicating poor adherence.
Kanapathipillai *et al.* (2011), Malawi	Retrospective cohort of 227 patients with IMF	CD4 count, VL	155 (68.2%) had VL testing. The positive predictive value of CD4 count was 28.4%. Repeated CD4 counts showed that 41% of patients initially positive for IMF did not have the IMF.
Prabhakar *et al.* (2011), India	Observation cohort of 251 patients; 3.7±1.14 years	CD4 count, VL	28 (13.59%) had discordant results (low CD4 counts despite viral suppression). IMF without VL testing can result in unnecessary switches to 2nd line therapy.
Zoufaly *et al.* (2011), Germany	Multicenter cohort of 14,433 patients with viral suppression.	VL, CD4 count	Patients with discordant VL and CD4 count results had a higher AIDS event incidence. Discordant group had an incidence rate of 55.06 and the immune responder group had a rate of 24.54.
Eshun-Wilson *et al.* (2012), South Africa	A retrospective cohort of 691 patients	CD4 count, VL and TB incidence.	141 (20.4%) had IMF at six months on ART.
Péré *et al.* (2012), Central African Republic	Observation cohort of 386 patients; 24 months (median)	CD4 counts, VL, genotypic resistance testing	28.5% had VLF (VL >3.7 log (10) copies/ml). 24% of patients with VLF showed wild-type viruses, indicating poor adherence. There is a need for VL tests to monitor therapeutic failure.

IMF = immunological failure; VL = Viral load; and VLF = Virological failure.

2.5.9 Viral load tests and drug resistance tests: Rationale; utility; current technologies; and availability in resource limited settings

By definition, viral load is the quantity of viruses in a given volume of body fluid. Viral load measures the magnitude of a viral infection such as HIV-1 (Puren, Gerlach,

Weigl, Kelso & Domingo, 2010). Viral load is usually given in RNA copies/ml of plasma. Viral load is useful in monitoring patients on ART. Three types of viral load tests are currently in use–namely: commercial nucleic acid amplification based tests, in-house nucleic acid amplification based tests and non-nucleic acid-based tests (Puren *et al.*, 2010).

Nucleic acid amplification-based tests either use the basic polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR) or the nucleic acid sequence based amplification (NASBA) method. RT-PCR is a form of PCR which uses RNA as the starting material and is then converted to double-stranded DNA, using the reverse transcriptase enzyme. The NASBA method is a form of PCR which uses RNA as the target to make a copy of DNA. The DNA copy is then transcribed into RNA and amplified (Greengrass, Turnbull & Hocking, 2005).

Viral load monitoring is not yet widely accessible in most resource limited settings such as in Lesotho although viral load tests are required more in these resource-limited settings. Patients often continue first-line ART until immunologic failure is detected by WHO criteria of diagnosing immunological failure (Wilson *et al.*, 2008).

Currently, three main methods are used for detecting HIV antiretroviral resistance. The first method measures the virus phenotypic susceptibility to drugs directly by culturing virus in the presence of increasing concentrations of the antiretroviral drug of interest (Dunne, Mitchell, Coberly, Hellmann, Hoy, Mijch, Petropoulos, Mills & Crowe, 2001; Mazzotta, Lo Caputo, Torti, Tinelli, Pierotti, Castelli, Lazzarin, Angarano, Maserati, Gianotti, Ladisa, Quiros-Roldan, Rinehart & Carosi, 2003). The concentration of drug required to inhibit viral replication gives a measure of antiretroviral drug resistance.

The second method uses genotyping assays to measure drug resistance and is based on determining the sequence of the HIV gene targeted by the antiretroviral drug and use the information to deduce drug susceptibility (Dunne *et al.*, 2001). A third method of virtual phenotypic assays is a mixture of the first two methods, whereby the viruses of interest are sequenced and large databases of all known gene variants are created. Drug resistance is then tested by comparing the gene

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sequences of viral repertoire in an individual to the stored dataset (Mazzotta *et al.*, 2003).

Acquired and transmitted HIV drug resistances are confirmed phenomena which occur in many HIV patients. HIV drug resistance is transmissible to other individuals who contract the disease from one with drug resistance (Clavel & Hance, 2004). It is claimed that there are minor drug-resistant variants in every patient infected with HIV (Gianella & Richman, 2010). In addition, drug resistance to one drug can cause resistance to other drugs in the same drug class.

According to Chakraborty, Smith, Dunn, Green, Duong, Doerholt, Riordon, Lyall, Tookey, Butler, Sabin, Gibb & Pillay (2008), poor adherence is the major cause of treatment failure in developing countries. Poor adherence implies that sub-inhibitory drug levels persist in the patient's blood resulting in the development of drug resistance.

HIV has a high rate of replication and a high number of errors during replication (Clavel & Hance, 2004). During viral replication, mutations happen resulting in daughter genomes differing from the parent template. Worse still, the mutated daughter genomes which favour survival tend to prevail. Hence, in individuals on ART therapy, there is a natural selection for viral progeny that are resistant to antiretroviral therapy (Clavel & Hance, 2004).

Drug resistance tests allow clinicians to choose or adjust ART drug combinations. The information also enables national treatment programmes to make informed treatment guidelines especially in resource limited settings (Bennett, Myatt, Bertagnolio, Sutherland & Gilks, 2008). However, several questions remain to be answered regarding best use of drug resistance assays in resource limited settings such as the need for training clinical practitioners on how to interpret drug resistance tests.

In developed countries, most patients on ART are monitored regularly by HIV-1 RNA viral load measurements and genotypic resistance testing (Hammer, Eron, Reiss, Schooley, Thompson, Walmsley, Cahn, Fischl, Gatell, Hirsch, Jacobsen, Montaner, Richman, Yeni & Volberding, 2008). For example, guidelines in Europe stipulate that HIV therapy should suppress viral loads to undetectable levels by 24

weeks. If this does not happen and the patient has consistently elevated viral load during treatment, the patient should be tested for HIV drug resistance and the ART regimen modified accordingly (Clumeck, Pozniak & Raffi, 2008).

2.6 Assessment of Nephrotoxicity of Tenofovir and Immunological Outcomes: Chapter Summary

Assessing the extent to which TDF may cause renal toxicity is a huge challenge given the numerous drug combinations commonly administered to patients and other possible risk factors. The inevitable existence of comorbidities associated with HIV and AIDS such as diabetes, hypertension and metabolic syndrome may complicate the ability by researchers to attribute renal insufficiency solely to TDF. Moreover, the kidneys being a major organ for concentrating and excreting toxic metabolites and drugs, is by default at risk of drug toxicity through numerous mechanisms which are difficult to disentangle without suitable study controls. Unraveling the labyrinth of the confounding factors in resource limited areas is likely to be complicated by lack of baseline laboratory tests such as viral hepatitis screening; and limited laboratory capacity to assess the kidney condition routinely.

Although the extent to which TDF may cause renal disease is not clear, the drug needs to be administered with caution. There is a need, meanwhile, to set aside enough resources to meticulously monitor patients on TDF in resource limited areas such as Lesotho. Efforts to find drug intervention means of reducing nephrotoxicity of TDF are likely to continue given the efficacious outcomes of TDF unless a drug with similar or better outcome model and affordability is developed (De Clercq, 2009). It seems effective vaccines for HIV continues to evade researchers.

Besides exposing the limitations facing researchers in resource limited areas with respect to researching on nephrotoxicity of TDF, the chapter indicated that there are some knowledge gaps that future research may cover. For example, there is a knowledge gap in viral hepatitis sero-status of HIV positive patients (Ford, Singh, Cooke, Mills, von Schoen-Angerer, Kamarulzaman & du Cros, 2012). Filling the gap would indicate the extent to which viral hepatitis may be contributing to renal insufficiency.

Assessing for immunological failure early in HIV patients on ART is also a challenge in resource limited settings due to the unavailability of viral load tests. CD4 counts, though helpful have limited utility in detecting sub-optimal immunological response (Kantor, Diero, Delong, Kamle, Muyonga, Mambo, Walumbe, Emonyi, Chan, Carter, Hogan & Buziba, 2009; Reynolds, Nakigozi, Newell, Ndyanabo, Galiwongo, Boaz, Quinn, Gray, Wawer & Serwadda, 2009; Prabhakar *et al.,* 2011), However, detecting early signs of immunological failure based on CD4 counts and clinical signs remains vital to avoid the emergence of drug resistance in HIV patients on ART. Finding ways of improving access to viral load tests, by any means, though it may be elusive at the moment, should remain the prime target if the gains achieved so far are to be maintained.

CHAPTER 3: METHODOLOGY

CHAPTER 3: METHODOLOGY

3.1 Study Design

This study utilised an analytical design in two phases. Phase 1 of the study compared renal function outcomes of patients on TDF-based ART and patients on non-TDF-based ART. Phase 2 of the study, which was based on re-sampling the study population, analysed immunological outcomes based on CD4 counts.

3.1.1 Rationale of the study design

The research study was designed primarily to assess the renal function outcomes following the use of TDF based on a retrospective case-control method, and to evaluate the immunological outcomes following the use of TDF and other ARVs based on CD4 counts at Roma Health Service Area (RHSA) in Lesotho. A retrospective case-control method was chosen because of two reasons: (1) A retrospective case-control method allowed a long period of observation which was required between the exposure to TDF and the possible development of detectable sub-optimal renal function outcomes; (2) The method also allowed the comparison of renal function outcomes for the patients exposed to TDF and individuals not exposed to TDF.

The assessment of immunological outcomes at RHSA was based on CD4 counts since data on viral load tests were very scarce because of limited access to viral load tests in Lesotho. The assessment of immunological outcomes was based on a pooled sample used in phase 1 of the study. Therefore, based on the study design, the main variables of interest were serum creatinine and CD4 counts.

3.2 Study Setting

The Roma Health Service Area (See map in the Addendum on page 185) has about 114 000 inhabitants, a number which was estimated to be about 6% of Lesotho's population according to the 2006 national census. There is one mission hospital known as St Joseph's Mission Hospital and five satellite health centres which cater for areas that are far from the Mission Hospital. The cumulative number of patients on ART in the different Health Centres since 2006 is shown in Table 3.1. Sampling

could not be done at all the health centres at Roma Health Service Area due to resource limitations. Patients at St Joseph's Mission Hospital and at Nazareth Health Centre made up 80% of the total number of HIV patients on ART when combined. Sampling was therefore carried out at St Joseph's Mission Hospital and Nazareth Health Centre as shown in Table 3.1.

Table 3.1: Number of patients on ART at Roma Health Service Area since 2006.

Health Centre	Cumulative number in HIV care	Cumulative number on ART (%)	Sample size (N, %)
St Joseph's Hospital	4 175	2 410 (58%)	351 (15%)
Nazareth	1 782	841 (47%)	188 (22%)
Fatima	726	366 (50%)	
St Benedict	385	156 (41%)	
St Bernard	367	155 (42%)	
Tlali	309	188 (61%)	
Total	7 744	4 116 (53%)	539 (13.1%)

Cumulative number of patients in HIV care refers to the number of patients who tested HIV positive but are not yet on ART.

The HIV clinics followed national guidelines on ART which recommend prescribing TDF-containing ART regimens to all adults above 18 years of age with creatinine clearance equal or above 50 ml/min calculated using the Cockcroft-Gault formula as shown in Section 3.5. Patients on ART at the selected two hospitals were monitored for serum creatinine, urea and electrolytes, CD4 counts and liver function tests at least once every six months. Patients' ART medical records were not computerised.

3.3 Study Population and Sampling

3.3.1 Sampling methods

The research was carried out in two phases. The first phase was a retrospective case-control study to assess the nature of the association between exposure to TDF and the incidence of renal injury. This was conducted on adult HIV patients enrolled on ART at St Joseph's Mission Hospital and at Nazareth Health Centre between

December 2006 and December 2012. Patients on TDF-based regimen with baseline serum creatinine and at least one other serum creatinine value recorded after the first six month interval were assigned to the sample group. Patients on ART regimens not containing TDF were assigned to the control group. The study procedures are outlined in Appendix A.

Data for the two study phases were collected from the medical records of 539 patients. During data cleaning stage, some patients had to be eliminated from the analysis of renal function outcomes because they did not meet the inclusion criteria. The final number of patients who were suitable for the assessment of renal function outcomes was 485 and was distributed in the different ARV regimens as shown in Table 3.2. Out of the 485 patients, 173 (36%) patients met the selection criteria for the control group (Non-TDF group), whilst 312 (64%) met the criteria for the test group (TDF group). For the second phase of the study, some patients also had to be eliminated from the analysis of immunological outcomes because they did not meet the inclusion criteria. A total of 516 patients met the selection criteria for the evaluation of immunological outcomes.

Table 3.2: ART regimens included in the assessment of renal function outcomes.

Group	ART Regimen formula	Number of patients (%)	Sample size (N, %)
Non-TDF group	D4T+3TC+NVP	1 (0.6%)	173 (35.7%)
	D4T+3TC+EFV	2 (1.2%)	
	AZT+3TC+NVP	58 (33.5%)	
	AZT+3TC+EFV	112 (64.7%)	
TDF group	TDF+3TC+NVP	13 (4.2%)	312 (64.3%)
	TDF+3TC+EFV	299 (95.8%)	
Total		485	485

D4T = Stavudine; 3TC = Lamivudine; NVP = Nevirapine; EFV = Efavirenz; AZT = Zidovudine; and TDF = Tenofovir disoproxil fumarate.

Six ART regimen categories were being taken by the patients included in the study, of which two regimens contained TDF. All the patients taking regimens containing TDF were put in the TDF group and the patients taking regimens without TDF were

put in the non-TDF group. Besides Lamivudine (3TC), which was part of all the regimens, Zidovudine (AZT), TDF, Nevirapine (NVP), and Efavirenz (EFV) were the main components (97%) of the ART regimens at the two centres included in the study. Two-thirds of the patients (See Table 3.2) were on TDF-containing regimens. Only a few patients (3%) were on Stavudine (D4T) because the drug was in the process of being phased out countrywide.

3.3.2   Study population

As outlined in section 3.3.1, the analysis of renal function outcomes was based on 485 patients. On the other hand, the analysis of immunological outcomes based on 516 patients. Figure 3.1 clarifies how the sample sizes were distributed in the two study phases and how the study sampling fitted into the research process.

3.3.3   Inclusion and exclusion criteria

To address the objectives of the study, the selection criteria for the patients were based on two main variables; serum creatinine values and CD4 counts. The specific requirements for inclusion and exclusion of patients in the study are outlined in the sections on inclusion and exclusion criteria.

3.3.3.1 Inclusion criteria

i. Patients treated for HIV with TDF or non-TDF based ART regimen from December 2006 up to December 2012;
ii. Patients with at least one baseline creatinine value recorded while still ART-naïve;
iii. Male or female adult HIV patients aged 18 or older at baseline;
iv. Patients who had been on TDF or non-TDF based ART for at least six months;
v. Patients who had the baseline CD4 counts recorded in the medical records and at least one other CD4 count result recorded over six months.
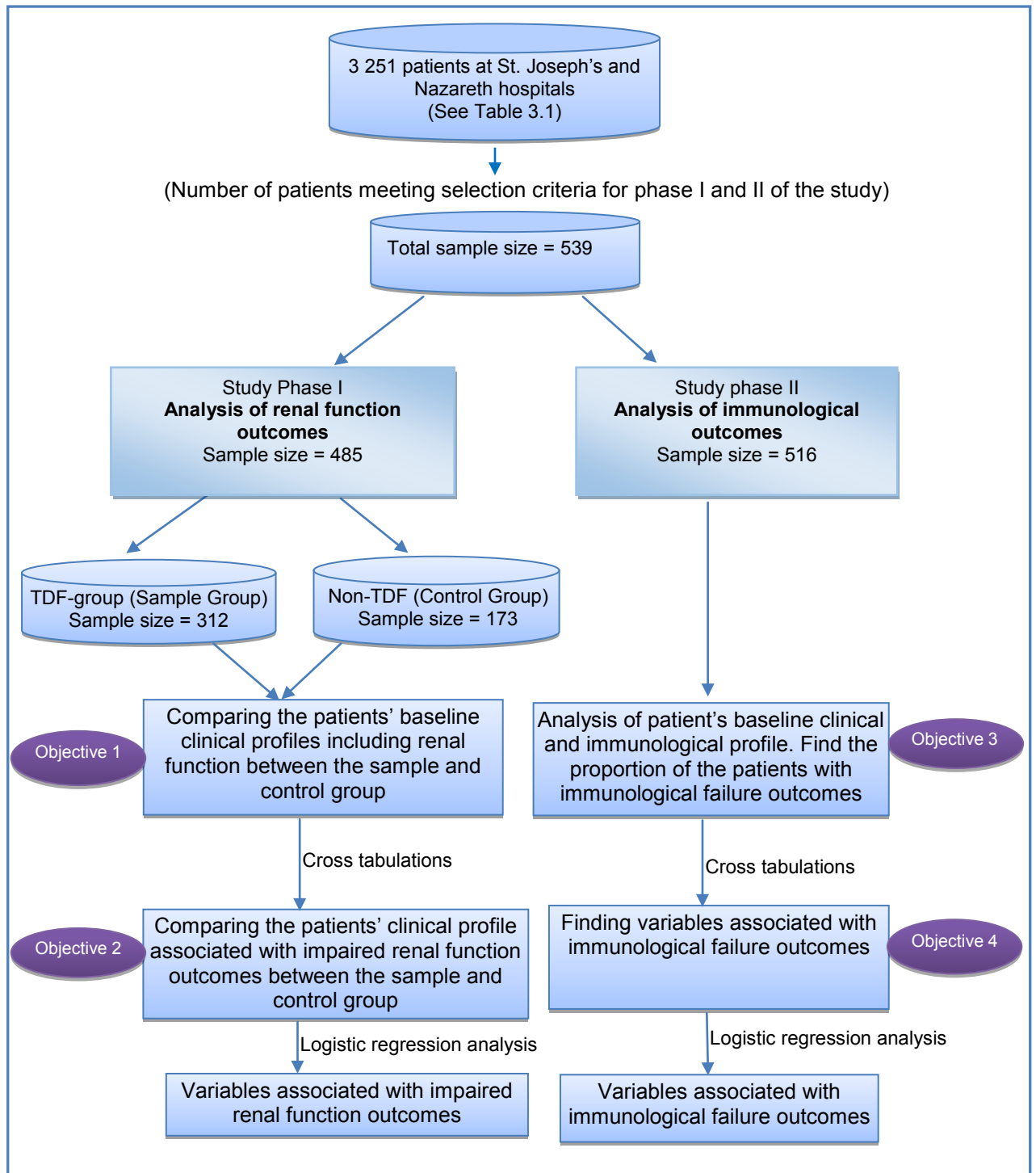
Figure 3.1: Summary of study population and objectives

3.3.3.2 Exclusion criteria

The following patient categories were excluded from the study:

  i. Patients on TDF or non-TDF based ART regimen who were younger than 18 years
 ii. Pregnant women;
iii. Patients on TDF or non-TDF based ART regimen who had no recorded values of serum creatinine at baseline;
 iv. Patients who had been on ART regimen for less than six months;
  v. Patients who had no baseline CD4 counts recorded or patients who had no other CD4 count values recorded after baseline.

3.4 Data Collection

3.4.1   Data collection tool

The data collection tool was designed using Microsoft Access® 2007 (Microsoft Corporation, Redmond, USA). After setting up all the variables required for the assessment of renal function outcomes and the assessment of immunological outcomes based on CD4 counts in database format, the data collection tool was compressed into a single data collection form (See Appendix B). The data collection tool was pilot-tested for suitability and functionality at St Joseph's Mission Hospital before it was used to collect the final data.

3.4.2   Data collection procedure

Demographic data, diagnoses, treatments and laboratory values of HIV-positive patients were extracted from patients' medical records according to the inclusion criteria. Baseline data collected included demographic data such as weight, age and gender; WHO clinical stage, blood pressure, CD4 count, serum creatinine, blood urea nitrogen (BUN), full blood count (FBC), differential count, and liver function test (LFT) profile. The collection also included baseline ART regimen, ART drug switches, date of ART commencement, and TB treatment dates. The data collection procedure was repeated at six month intervals up to seven data sets or up until the entire patient's records were captured, whichever came first.

3.5 Statistical Analysis

3.5.1   Data cleaning

The data collected in Microsoft Access[®] 2007 database (Microsoft Corporation, Redmond, USA) were then exported to Microsoft Excel[®] 2007 (Microsoft Corporation, Redmond, USA) for data cleaning and conversion of non-binary data such as medical conditions to binary data as shown in Appendix C1. The data were then exported to STATA[®] version 11 (StataCorp, Texas, USA) for analysis.

3.5.2   Assessment of renal function outcomes

STATA[®] version 11 was used to analyse the data. Patients' clinical profiles were categorized using the cross tabulation functions available in STATA[®] according to the criteria shown in Appendix C2. Throughout data analysis, sub-optimal renal function outcome was defined as serum creatinine clearance below 50ml/min (Young *et al.,* 2007). Creatinine clearance was calculated using the Cockcroft-Gault equation as follows:

$$\text{Creatinine clearance (ml/min)} = \frac{[(140-\text{age}) \times \text{weight (kg)}]}{[\text{serum creatinine (µmol/l)} \times 1.23 \text{ (men) or } 1.04 \text{ (women)}]}$$

Blood pressure measurements were classified into different stages of hypertension as outlined in section 2.5.4 using the algorithm shown in Appendix C1.2. Diabetes mellitus, Hepatitis B or C, was defined by physician diagnosis or receipt of drugs indicating presence of the conditions.

Due to the unavailability of height data which are required for calculating body mass index (BMI), a cut-off value for defining underweight had to be adopted. Patients weighing less than 50 kg were subjectively classified as underweight.

Summary statistics which included mean, median and inter-quartile ranges for patients' characteristics at baseline and at six-month intervals were tabulated and the variables were assessed for normality graphically. Graphical histograms were plotted to assess for skew in the data and for visual comparisons of the differences in patients' characteristics between the TDF group and non-TDF group at baseline

and during treatment. Differences in patients' clinical profiles at baseline and at six-month intervals were analysed for significance using the t-test and Fisher's exact test (See Appendix C2). Patient's clinical profiles in non-numerical form such as patient medical conditions and prescribed drugs were converted to numerical data as outlined in Appendix C2.1.3

Renal function was further categorised into CrCl>50 ml/min and CrCl<50 ml/min where CrCl<50 ml/min represented patients with baseline renal function contraindicated for TDF (Young *et al*., 2007). For full analysis of baseline renal function and renal function outcomes, CrCl values were categorised as follows: normal (90 ml/min), mild (60–89 ml/min), moderate (30–59 ml/min) and severe renal impairment (CrCl <30 ml/min) (Young *et al*., 2007). However, the classification of CrCl into two categories of CrCl<50 ml/min and CrCl>50 ml/min was used in the determination of the variables associated with sub-optimal renal function outcomes (CrCl<50 ml/min).

Univariate and multivariate logistic regression analyses were done to determine significant variables associated with creatinine clearance less than 50 ml/min versus creatinine clearance greater than 50 ml/min at baseline. *P*-value and odds ratio outputs of the different variables tested were then compared. The variables tested for significance included age, weight, gender, baseline CD4 count, use of concomitant drugs, WHO clinical stage, conditions diagnosed, haematological factors, baseline serum creatinine clearance, the ART regimen, duration of treatment, among other variables. Duration of treatment was calculated as shown in Appendix C2.1.3.

Predictors with *p*-values less than 0.1 in the univariate analysis were included in multivariate logistic regression analysis. Significant variables ($p<0.05$) were selected to remain in the final regression model.

Due to limitations in the data recorded in the medical records, sub-optimal renal function outcomes were defined as follows:

i. Renal function outcome where the latest value of CrCl was less than 50 ml/min
ii. Renal function outcome where baseline CrCl was greater than 50 ml/min and the latest value of CrCl was less than 50 ml/min

iii. Renal function outcome where baseline CrCl was less than 50 ml/min and the latest value of CrCl was less than 50 ml/min

iv. Renal function outcome where baseline CrCl was less than 50 ml/min and the latest value of CrCl was greater than 50 ml/min

v. Renal function outcome where the average of CrCl values after baseline dropped by 25% or more from baseline

vi. Renal function outcome where the latest value of CrCl dropped by 25% or more from baseline

The six definitions above were developed into algorithms for detecting and classifying renal function outcomes. Definition (II) defined the primary outcome which was renal impairment as an outcome from normal baseline renal function. Definition number (I) was used to define the incidence of renal impairment, definition number (III) was used to define failure to improve renal function during treatment. Definition number (IV) was used to define improved renal function outcomes. Definitions (V) and (VI) which were adopted from Nishijima *et al*. (2011), were used to define the overall change in renal function during treatment. The actual algorithms generated from the definitions of impaired renal function are shown in Appendix C2.1.4.1.

Univariate and multivariate logistic regression analyses were done for the different predictors comparing CrCl<50 ml/min and CrCl>50 ml/min outcomes as defined by definition (II) above. Predictors with a *p*-value less than 0.1 in the univariate analysis were included in multivariate logistic regression. Significant variables (*p*<0.05) were selected to remain in the final regression model.

To put into perspective the effect of the variables that emerged as significantly associated with impaired renal function outcomes, case studies of patients with severe or end-stage renal impairment were constructed from the clinical profiles of the patients in those categories. Clinical profiles of patients with severe or end-stage renal impairment outcomes were generated as shown in Appendix C2.1.4.2.3.

3.5.3   Assessment of immunological outcomes

Three criteria were used to define immunological failure. Criterion (I) was adopted from Kantor *et al*. (2009). Criterion (II) and (III) were adopted from the WHO, (2010)

criteria for defining immunological failure. Therefore, immunological failure was defined according to the following criteria:

i. The latest CD4 count values which were less than baseline CD4 count by more than 25%;

ii. Patients with the latest CD4 count values which were less than 50% of the peak CD4 count result.

iii. Patients with the latest CD4 count results which were lower than 100 cells/mm^3.

The three definitions above were developed into algorithms for detecting immunological failure. The actual algorithms are shown in Appendix C2.2.3.

Logistic regression analyses were performed to determine the variables associated with immunological failure. *P-value* and odds ratio outputs of the different variables tested were then compared.

Possible variables tested included age, weight, gender, baseline (BL) CD4 count, WHO clinical stage, conditions diagnosed, the ART regimen, among other variables. Predictors with a *p*-value less than 0.1 in the univariate analysis were included in the multivariate logistic regression. Significant covariates (*p*<0.05) were selected to remain in the final regression model.

To put into perspective the variables associated with immunological failure, patients with latest CD4 count values below 100 cells/mm^3 were further categorised as having critical immunological failure and case studies were constructed from the characteristics of the patients in that category as shown in Appendix C2.2.6.

3.6 Ethical Clearance

The research protocol was submitted for ethical approval to the Ministry of Health and Social Welfare in Lesotho. The study was approved by the ethics review committee of the Ministry of Health and Social Welfare. Letters of approval are shown in Appendices D1–D3.

CHAPTER 4:   RESULTS

CHAPTER 4:   RESULTS

The chapter presents results of the two study phases–namely: renal function outcomes based on serum creatinine clearance and immunological outcomes based on CD4 counts. Phase 1 begins by comparing the baseline clinical profiles of patients in the non-TDF group and the TDF group. Then the following results are presented: renal function outcomes and the variables associated with impaired renal function outcomes. Phase 2 of the study begins by presenting the clinical profiles of the patients followed by variables associated with sub-optimal immunological outcomes.

4.1 Renal Function Outcomes and the Variables Associated with Impaired Renal Function Outcomes

4.1.1   Clinical profile of the study population at baseline

Phase 1 presents results of the 312 patients who met the inclusion criteria for the sample group (TDF group) and 173 patients who met the inclusion criteria for the control group (non-TDF group). The presentations of the results begin by analysing differences in the clinical profiles of the TDF group compared to the non-TDF group.

The ages of the study population ranged from 20 to 78 (See Figure 4.1). Age was normally distributed both in the TDF and the non-TDF groups. However, the 30-39 age group was the most prevalent age group included in the study.
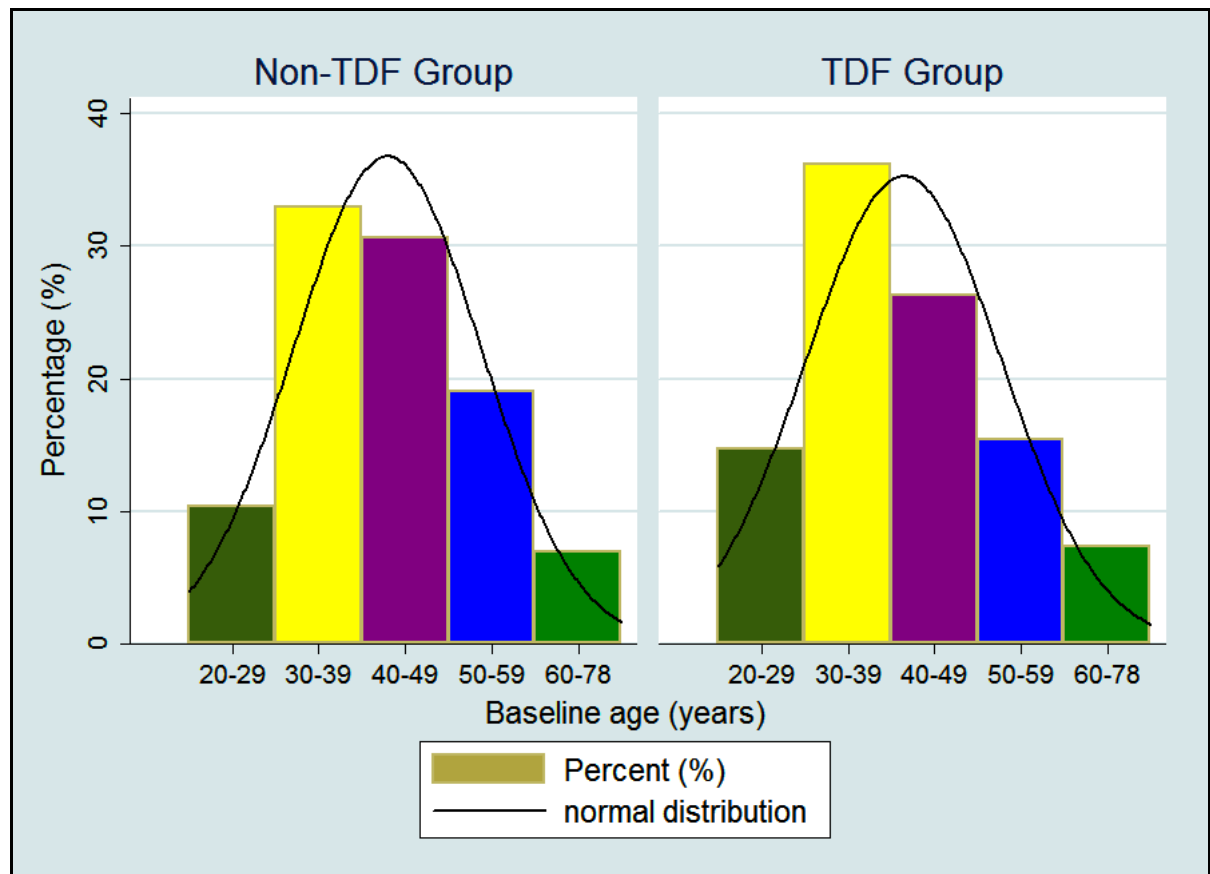
Figure 4.1: The age profile of patients in the study

Table 4.1 presents the clinical profile of the patients. With respect to gender, the number of females was higher than the number of males in both the TDF group and the non-TDF group. The number of females in the non-TDF group was 63.6% compared to the number of males which was 36.4%. However, there were no significant differences in gender distribution between the TDF and the non-TDF group (p=0.258).

Although the clinical profiles of the patients in the TDF group were generally comparable to the non-TDF group, a few variables were significantly different. For example, the mean baseline weight in the TDF group was significantly lower than the mean baseline weight in the non-TDF group (p<0.001). The TDF group also had a higher proportion of patients (29.8%) with baseline weight below 50 kg compared to the non-TDF group (18.5%).

Table 4.1: Clinical profile of the study population.

Baseline (BL) characteristic	Non-TDF Group (n=173)	TDF Group (n=312)	*P-value*
Age, median years (Range)	42 (20-69)	38 (20-78)	0.235
Gender, male	63 (36.4%)	130 (41.7%)	0.258
Gender, female	110 (63.6%)	182 (58.3%)	0.258
Weight (kg), mean (IQR)	58.9 (50.1-65.7)	54.9 (48.0-60.0)	<0.001
Weight (>60)	67 (38.7%)	84 (26.9%)	0.005
Weight (50-60)	74 (42.8%)	135 (43.3%)	-
Weight (<50)	32 (18.5%)	93 (29.8%)	-
WHO stage I—III	165 (95.4%)	299 (95.8%)	0.819
WHO stage IV	8 (4.6%)	13 (4.2%)	-
CD4 count: median(IQR)	142 (76-209)	167 (76.5-257)	0.029
Low CD4 count (<50 cells/mm^3)	23 (13.3%)	41 (13.1%)	1.000
Hypertension stage I or II	24 (17.5%)	53 (20.4%)	0.593
Systolic BP mean (IQR)	119.4 (110-130)	116.5 (100-120)	0.120
Diastolic BP mean (IQR)	75.4 (70-80)	74.6 (70-80)	0.556
Tuberculosis	12 (6.9%)	49 (15.7%)	0.006
Hepatitis B or C	0	0	1.000
Anaemia	94 (54.3%)	162 (52.8%)	0.775
Haemoglobin less than 10.0 g/dl	13 (7.5%)	51 (16.6%)	0.005
Raised ALT (ALT > 60)	12 (7.4%)	16 (5.3)	0.415
Mean CrCl (IQR)	74.6 (55-90%)	72.1 (56-84%)	0.318
CrCl<50 ml/min	32 (18.5%)	56 (17.95%)	0.902
Normal CrCl (>90 ml/min)	44 (25.4%)	61 (19.6%)	-
Mild CrCl (60-90) ml/min	73 (42.2%)	153 (49.0%)	-
Moderate CrCl (30-60) ml/min	55 (31.8%)	98 (31.4%)	-
Severe CrCl (15-30) ml/min	1 (0.6%)	0	-

CrCl = serum creatinine clearance; ALT = alanine amino-transferase; IQR = inter-quartile range; BP = blood pressure in mmHg; CD4 count is in cells/mm^3; Significant *p*-values are highlighted in yellow.

Another baseline variable that was notably different between the non-TDF and the TDF groups was the baseline CD4 count of patients in the TDF group which was significantly higher than the baseline CD4 counts in the non-TDF group (p = 0.029).

Tuberculosis was significantly more common in the TDF group than in the non-TDF group (p=0.006). The number of patients with hypertension stage I or II at baseline was high (about 20%) although there was no statistically significant difference between the TDF and the non TDF group with respect to the number of patients with hypertension (p=0.593). No patients were positive for Hepatitis B or C at baseline, both in the TDF group and the non-TDF group.

There were no significant differences between the non-TDF and the TDF group with respect to the number of patients who were in WHO clinical stage IV; the number of patients with hypertension; Hepatitis B or C; anaemia; baseline laboratory values of ALT; and baseline serum creatinine clearance (CrCl). This means the baseline clinical features of the patients between the two groups were comparable.

Concerning blood pressure levels at baseline, the TDF group and the non-TDF group did not differ significantly with respect to systolic (p=0.120) and diastolic (p=0.556) blood pressure. The numbers of patients with hypertension stage I or II also did not differ significantly between the two groups (p=0.593). However, the number of patients with hypertension was generally high considering that the percentage of patients with hypertension was about 20% across the two groups.

When the baseline CrCl values were categorised according to Chronic Kidney Disease (CKD) stages, there were no significant differences in baseline renal function between the TDF and the non-TDF group. Two of the patients with liver disease at baseline were in the TDF group and the other two were in the non-TDF group.

When the ranges of haemoglobin values were compared, the TDF group had a significantly higher number of patients with haemoglobin values less than 10 g/dl (p=0.005), meaning that there were more severe cases of anaemia in the TDF group than in the non-TDF group. The distributions of anaemia types in the TDF and

the non-TDF group are presented in Figure 4.2. There was a higher proportion of normocytic anaemia in both the TDF and the non-TDF groups.
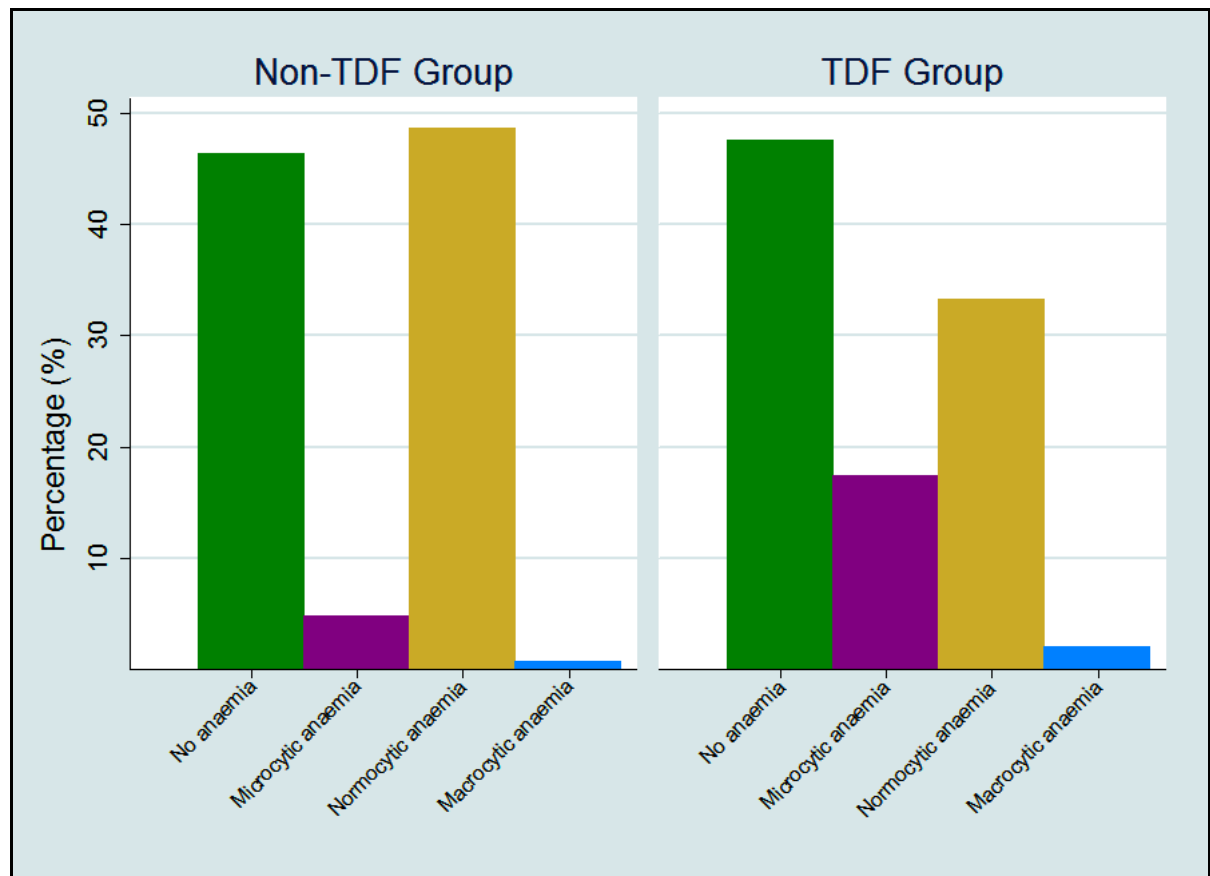
Figure 4.2: Anaemia types at baseline in the non-TDF and TDF groups

4.1.2 Variables associated with impaired renal function at baseline

Underweight, hypertension stage I or II, and older age were significant predictors of baseline CrCl<50 ml/min in univariate logistic regression analysis (See Table 4.2). For instance, considering odds ratios (OR), patients with a baseline body weight less than 50 kg were eight times more likely to have CrCl<50 ml/min outcome (p=0.001); and patients aged 60 or older were 24 times more likely to have baseline CrCl<50 ml/min (p=0.001). Furthermore, patients with hypertension stage I or II were more than three times more likely to have renal insufficiency at baseline.

Table 4.2: Variables of the study population associated with impaired baseline renal function (CrCl<50 ml/min).

Variable	CrCl>50 ml/min (n=397)	CrCl<50 ml/min (n=88)	Unadjusted OR (95% CI)	P- value	Adjusted OR (95% CI)	*P*-value
Age 20-29	61 (15.4%)	3 (3.4%)	1	-	1	-
Age 30-39	156 (39.3%)	14 (15.9%)	1.8 (0.5-6.6)	0.358	2.1 (0.4-10.6)	0.351
Age 40-49	111 (28.0%)	24 (27.3%)	4.4 (1.3-15.2)	0.019	8.6 (1.8-40.9)	0.007
Age 50-59	53 (13.4%)	28 (31.8%)	10.7(3.1-37.4)	<0.001	20.9 (4.3-102)	<0.001
Age 60-78	16 (4.0%)	19 (21.6%)	24.1(6.3-91.9)	<0.001	82.2 (13.8-488)	<0.001
Weight>60 kg	141 (35.5%)	10 (11.4%)	1	-	1	-
Weight 50-60 kg	176 (44.3%)	33 (37.5%)	2.6 (1.3-5.5)	0.010	4.4 (1.8-10.8)	0 .001
Weight<50 kg	80 (20.2%)	45 (51.1%)	7.9 (3.8-16.6)	<0.001	21.0 (8.0-54.9)	<0.001
Male	168 (42.3%)	25 (28.4%)	1	-	1	-
Female	229 (57.7%)	63 (71.6%)	1.8 (1.1-3.1)	0.017	1.8 (0.9-3.6)	0.078
No HTN	274 (85.1%)	46 (61.3%)	1		-	-
HTN stage I or II	48 (14.9%)	29 (38.7%)	3.6 (2.1-6.3)	<0.001	3.0 (1.5-6.0)	0.002

CD4>50 cells/mm^3	343 (86.4%)	78 (88.6%)	1		-	-
CD4<50 cells/mm^3	54 (13.6%)	10 (11.4%)	0.8 (0.4-1.7)	0.575	-	-
WHO stage I—III	380 (95.7%)	84 (95.5%)	1	-	-	-
WHO stage IV	17 (4.3%%)	4 (4.15%)	1.1 (0.3-3.2)	0.913	-	-
Anaemia negative	188 (48.0%)	36 (40.9%)	1	-	-	-
Anaemia positive	204 (52.0%)	52 (59.1%)	1.3 (0.8-2.0)	0.241	-	-

OR = Odds Ratio; CI = confidence interval; HTN = hypertension; WHO stage = WHO clinical stage; Significant *p*-values are highlighted in yellow.

Although females had about twice as much chance as males of having baseline CrCl<50 ml/min (p=0.01), gender had no significant effect on baseline renal function (p=0.078). In addition, baseline CD4 counts below 50 cells/mm^3 and WHO clinical stage had no significant effect on baseline renal function (p=0.575; and p=0.913 respectively). Having anaemia at baseline was also not a significant predictor of having impaired renal function at baseline (p=0.241). Therefore, baseline CD4 count, anaemia and WHO clinical stage were excluded in the multiple logistical regression steps.

When the variables that were significant in univariate analysis were tested concurrently in multiple logistic regression steps, older age (age of 60 or above) emerged as the most decisive predictor of baseline CrCl<50 ml/min (OR = 82.2) followed by underweight (weight <50 kg) which had OR of 21.0, and lastly high blood pressure (OR=3.0). Female gender became insignificant (p=0.078) in multiple regression which means the effect of gender was less decisive compared to age, body weight and high blood pressure.

Figure 4.3 presents the proportions of patients who were taking ARVs containing TDF despite having baseline CrCl<50 ml/min. On investigating the possible reasons why so many patients were taking ARVs containing TDF contrary to the guidelines, it appeared as if the ART centres were only calculating patients' creatinine clearance values if the serum creatinine values were at least double the upper limit of the normal range irrespective of the patient's age, gender or weight.

Having found that underweight, high blood pressure, and older age were significant predictors of baseline CrCl<50 ml/min and that close to a fifth (17.9%) of the patients were erroneously treated with TDF, the focus switched to the renal function outcomes and the variables that were associated with impaired renal function outcome.
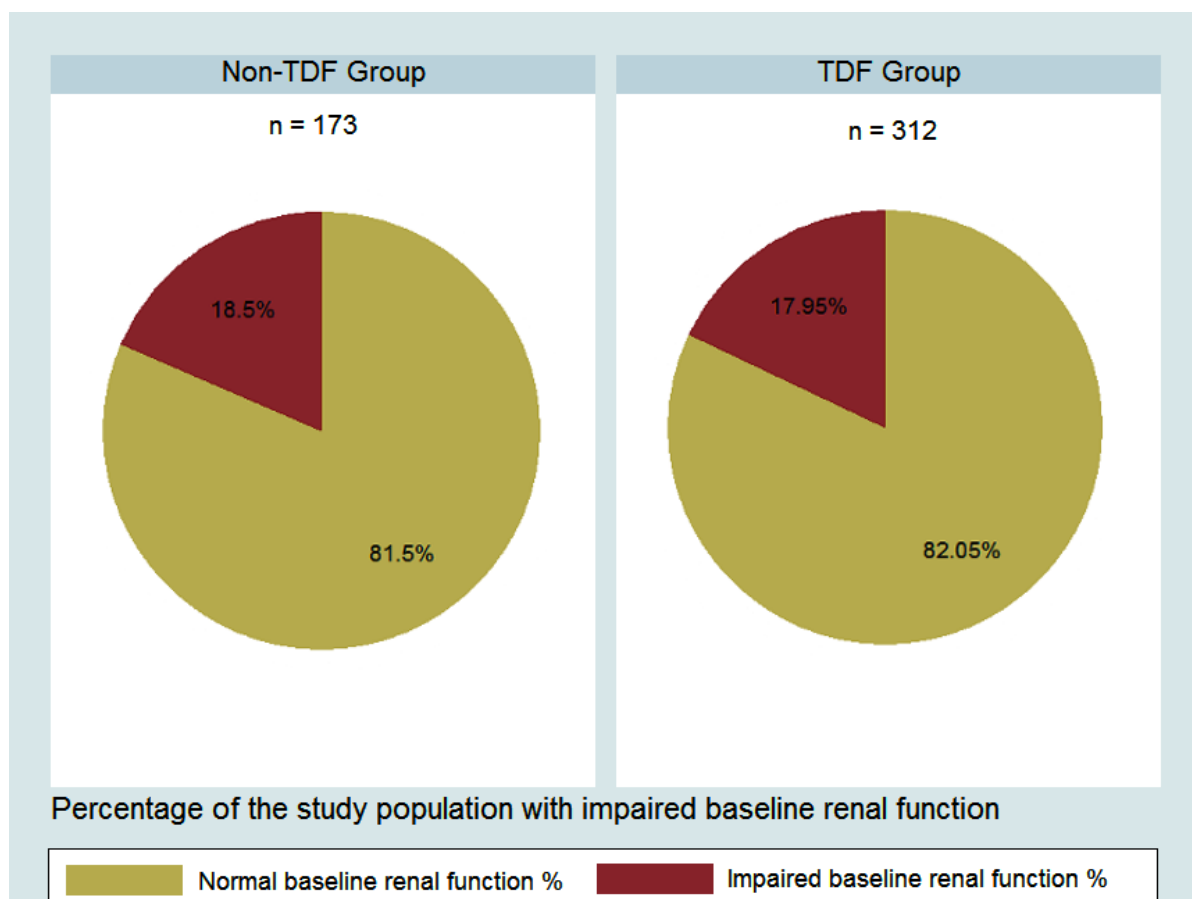
Figure 4.3: Proportion of the study population with impaired baseline renal function

Cut-off value for impaired baseline renal function = CrCl<50 ml/min; CrCl = serum creatinine clearance in ml/min

4.1.3 Renal function outcomes and the variables associated with impaired renal function outcomes

Although the renal function outcomes improved by a median change of +4 ml/min at 12 months from a baseline median of 69 ml/min (IQR 55-84) to a median of 73 ml/min (IQR 57-92) and by another median change of +2 ml/min at 24 months, 10 patients (2.1%) had severe impairment; and three patients had end-stage renal disease. Table 4.3 summarises the renal function categories at baseline and during treatment.

The main outcome of interest between the TDF group and the non-TDF group was an impaired renal function outcome (CrCl<50 ml/min) with a normal baseline renal function because such a result would indicate that the patient developed renal

impairment during treatment. However, there were several possible outcomes depending on whether a patient initiated on ART with either a normal baseline renal function or impaired baseline renal function, had an improvement or deterioration with respect to renal function during treatment.

Table 4.3: Baseline renal function and renal function outcomes of the study population.

Renal function category in ml/min	Baseline renal function (CrCl in ml/min)		Renal function outcome (Latest CrCl in ml/min)	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Normal GFR (>90)	105	21.7	120	24.7
Mild (60-89)	226	46.6	216	44.5
Moderate (30-59)	153	31.5	136	28.0
Severe (15-29)	1	0.2	10	2.1
ESRD (<15)	0	0	3	0.6
Total	485	100.0	485	100

BL = baseline; CrCl = serum creatinine clearance; and ESRD = end-stage renal disease.

Table 4.4 presents the outcome combinations together with the results of the comparisons between the TDF and the non-TDF group. Table 4.4 also summarises how selected variables differed between the TDF and the non-TDF group. The variables include the number of patients whose latest absolute values of CrCl in ml/min dropped by 25% or more from baseline and the number of patients whose mean values of CrCl in ml/min dropped by 25% or more. Other variables include the number of patients who lost more than 5% of body weight during treatment and the number of patients who tested positive for various comorbidities that include tuberculosis and hypertension.

Table 4.4: Renal function (RF) outcomes and variables of the study population.

Variables	Non-TDF Group (n=173)	TDF Group (n=312)	*P-value*
Duration on ART in months, median (Range)	31 (6-48)	18 (6-48)	<0.001
Defaulted for 6 months or more	10 (5.8%)	29 (9.3%)	0.222
Impaired RF outcome with normal or impaired baseline RF	21 (12.4%)	60 (19.3%)	0.043
Impaired RF outcome with normal baseline RF	11 (5.8%)	40 (12.5%)	0.030
Impaired RF outcome with impaired baseline RF	10 (5.8%)	20 (6.4%)	0.846
Normal RF outcome with impaired baseline RF	21 (12.1%)	34 (10.9%)	0.765
Latest CrCl dropped by >25% from BL	28 (16.5%)	72 (23.2%)	0.100
Mean CrCl dropped by >25% from BL	20 (11.8%)	57 (18.3%)	0.069[**]
Lost more than 5% body weight	25 (14.5%)	21 (6.7%)	0.009
History of tuberculosis	58 (33.5%)	111 (35.6%)	0.691
Hypertension stage I or II	36 (25.9%)	68 (25.1%)	0.905
History of diabetes	0	2 (0.6%)	0.540
History of Hepatitis B or C	0	1 (0.3%)	1.000
History of herpes zoster	10 (5.8%)	11 (3.5%)	0.252
ALT > 60 IU/l	11 (6.5%)	15 (4.8%)	0.527
Had 1 or more comorbidity from BL	34 (19.7%)	96 (30.8%)	0.010

CrCl = serum creatinine clearance in ml/min; RF = renal function; LFT = liver function test; IQR = inter-quartile range; [**] = marginally significant *p*-value; Significant *p*-values are highlighted in yellow.

Significant differences between the TDF group and the non-TDF group during treatment were noticed in three variables–namely: duration of treatment, number of patients with impaired renal function outcomes, and the number of comorbidities. Patients in the non-TDF group had longer duration of treatment than the TDF group (p<0.05). There were higher proportions of patients in the TDF group compared to the non-TDF group with respect to the incidence of CrCl<50 ml/min outcomes (p=0.043), and incidence of comorbidities (p=0.010). There was a marginally significant (p=0.069) difference between the number of patients whose mean CrCl dropped by 25% or more from baseline between the TDF and the non-TDF group.

Duration of treatment differed significantly between the TDF and the non-TDF group (p<0.001). For instance, patients in the non-TDF group spent more months on treatment on average (median = 31 months; Range 6-48) than patients in the TDF group (median = 18 months; Range 6-48).

The number of patients in the TDF group (See Table 4.4) who had impaired renal function outcome (19.3%, n=312) was significantly higher (p=0.043) than the number of patients with impaired renal function outcome (12.4%, n=173) in the non-TDF group. When baseline renal function outcome was controlled by excluding patients with impaired baseline renal function (CrCl<50 ml/min), the number of patients in the TDF group who had impaired renal function outcome (12.5%, n=312) remained significantly higher (p=0.030) than the number of patients with impaired renal function outcome (5.8%, n=173) in the non-TDF group.

When positive renal function outcome was considered, 12.1% of the patients in the non-TDF group had a positive renal function outcome compared to 10.9% in the TDF group. There were no significant differences in the proportion of patients with hypertension stage I or II between the TDF and the non-TDF group (p=0.905). However, the proportion of patients with hypertension stage I or II was very high (close to 25%) in both TDF and non TDF groups (See Table 4.4). The number of patients on treatment for hypertension was lower than the number of patients in stage I or II of hypertension in both groups (about 30% in patients with CrCl<50 ml/min and 16% in patients with CrCl>50 ml/min outcome). This could have been

due to the fact that some patients whose data indicated stage I of hypertension were not put on treatment.

When changes in CrCl were compared between the groups, the TDF group had a marginally significant proportion of patients whose mean CrCl values dropped by 25% or more from the baseline (p=0.069). Moreover, when the baseline and the latest values of CrCl were considered, the proportions of patients with the latest absolute values of CrCl that dropped by 25% or more after baseline in the TDF group (23.2%) were not significantly different (p=0.100) from the non-TDF group (16.5%). However, the TDF group still had a comparatively higher proportion of patients with absolute CrCl values that dropped by 25% or more. Therefore, more patients in the TDF group had a negative outcome (40 or 12.5%) compared to the positive outcome (34 or 10.9%). In the non-TDF group, more patients had a positive outcome (21 or 12.1%) compared to the negative outcome (11 or 5.8%). In summary, although positive and negative outcomes occurred in both groups, the TDF group had a higher inclination towards the negative outcome.

Figure 4.4 presents renal function outcomes in another perspective. The figure reflects that the TDF group had a larger proportion of patients with impaired renal function outcomes even when the renal function outcomes were controlled for baseline renal function.

After considering the distribution of the variables of the study population between the TDF and the non-TDF group, the variables were analysed by univariate and multivariate logistic regression for association with impaired renal function outcomes (CrCl<50 ml/min).
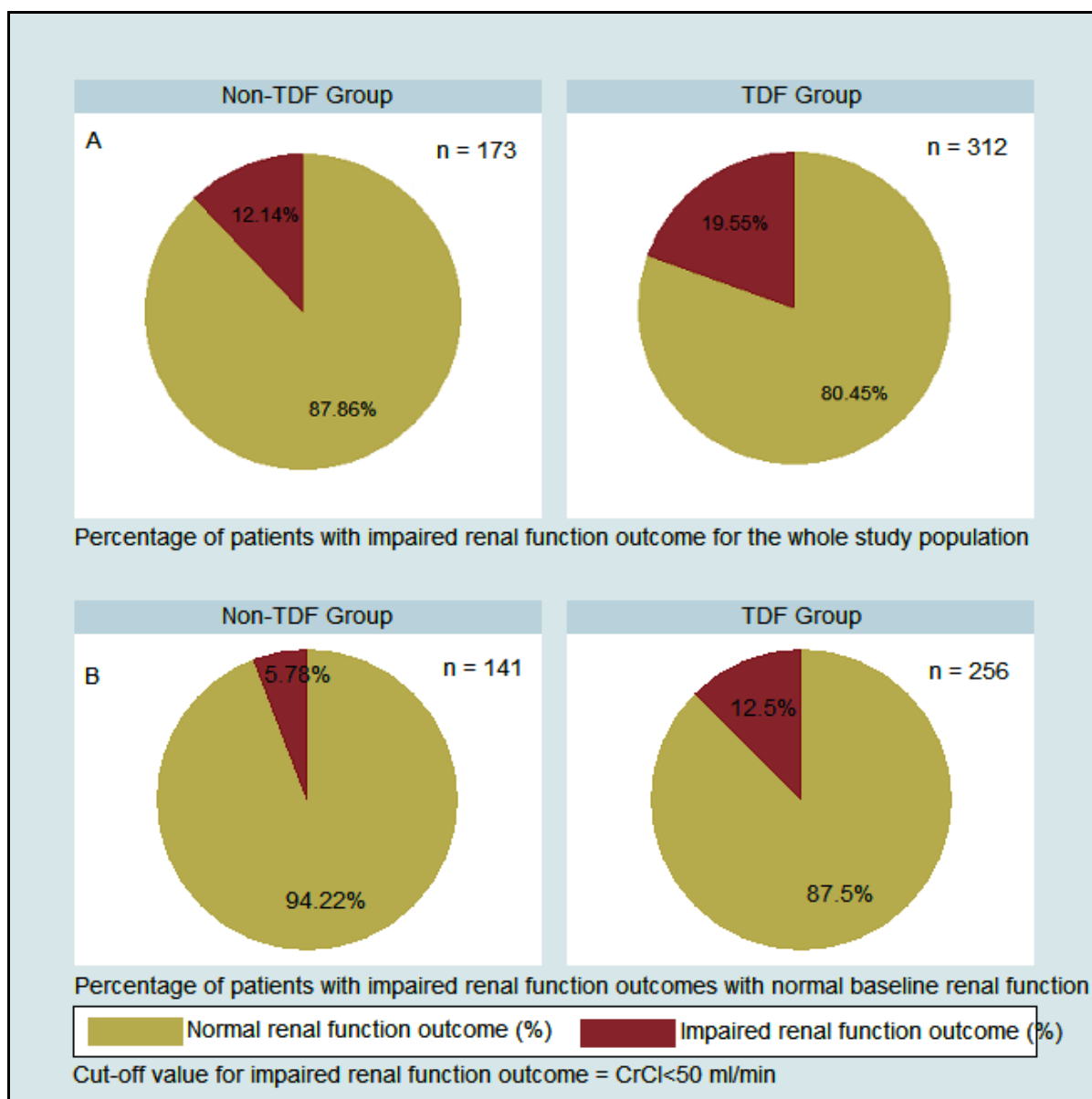
Figure 4.4: Proportion of patients with impaired renal function outcomes versus baseline conditions.

CrCl = serum creatinine clearance in ml/min

Table 4.5 presents results for univariate (Unadjusted Odds Ratio) and multivariate (Adjusted Odds Ratio) logistic regression analysis. In the univariate logistic regression analysis, the variables that had higher odds of predicting impaired renal function outcomes were: (1) use of TDF (OR=1.7); (2) age of 60 or higher (OR=14.2); (3) body weight <50 kg (OR=6.3); (4) female gender (OR=2.1); (5) hypertension (OR=2.8); and (6) baseline renal insufficiency (OR=4.2).

Table 4.5: Logistic regression analysis of variables associated with impaired renal function outcomes.

Variable	CrCl>50 (n=403)	CrCl<50 (n=82)	Unadjusted OR (95% CI)	*P-value*	Adjusted OR (95% CI)	*P-value*
Non-TDF	152 (87.6)	21 (12.4)	1	-	1	-
TDF group	251 (80.7)	61 (19.3)	1.7 (1.0-2.9)	0.054[**]	1.7 (0.9-3.2)	0.122
Age 20-29	61 (14.8)	3 (3.2)	1	-	1	-
Age 30-39	145 (37)	25 (31)	3.5 (1.0-12.0)	0.046	3.2 (0.9-11.6)	0.077
Age 40-49	176 (43.7)	40 (48.8)	4.0 (1.1-13.9)	0.029	3.5 (1.0-12.9)	0.060[*]
Age 50-59	176 (43.7)	40 (48.8)	5.6 (1.6-20.0)	0.008	3.8 (1.0-14.9)	0.053[**]
Age 60-78	21 (5.2)	14 (17.1)	14.2 (3.7-54.6)	<0.001	9.1 (2.0-41.2)	0.004
Weight >60 kg	142 (35.2)	9 (11.0)	1	-	1	-
Weight 50-60 kg	169 (41.9)	40 (48.8)	4.1 (1.9-9.1)	<0.001	5.3 (2.1-13.6)	< 0.001
Weight <50 kg	92 (22.8)	33 (40.2)	6.3 (2.8-14.4)	<0.001	8.6 (3.3-22.8)	< 0.001
Male	232 (57.8)	60 (73.2)	1	-	1	-
Female	171 (42.4)	22 (26.8)	2.1 (1.2-3.6)	0.005	2.2 (1.2-4.1)	0.016

No HTN	267 (79.0)	39 (57.3)	1	-	1	-
HTN I or II	71 (21.0)	29 (42.7)	2.8 (1.6-4.8)	<mark><0.001</mark>	2.2 (1.2-4.0)	<mark>0.009</mark>
CrCl >50 ml/min	347 (86.1)	50 (60.9)	1	-	1	-
CrCl <50 ml/min	56 (13.9)	32 (39.0)	4.2 (2.5-7.1)	<mark><0.001</mark>	1.6 (0.8-3.2)	0.218

HTN = Hypertension; CrCl = baseline serum creatinine clearance in ml/min; OR= odds ratio; ** = marginally significant; Significant *p*-values are highlighted in yellow.

When multivariate regression analysis was performed, a number of significant variables remained in the final model (see Table 4.5). The significant variables included old age especially ages over 60 (p=0.004), body weight less than 50 kg (p<0.001), female gender (p=0.016), and high blood pressure (p=0.009). Although the use of TDF had higher odds ratio of developing impaired CrCl outcome (adjusted OR=1.5) compared to the non-TDF group, the use of TDF became insignificant in the final adjusted model (p=0.122). When odds ratios (OR) were compared, older ages above 60 had the highest odds ratio (adjusted OR=9.1) of having CrCl<50 ml/min followed by having a baseline weight less than 50 kg (adjusted OR=8.6).

Table 4.6 presents the number of patients with different categories of renal function outcomes. Sixty patients (19.6%) in the TDF group had impaired renal function outcome compared to 21 patients (12.1%) in the non-TDF group. Forty patients (12.5%) who had CrCl>50 ml/min at baseline developed impaired renal function outcomes in the TDF group compared to 11 patients (5.8%) in the non-TDF group.

Table 4.6: Categories of renal function outcomes with different baseline renal function conditions.

Renal function category	Number of patients with impaired renal function outcome for the whole study population		Number of patients with impaired renal function outcome with impaired baseline renal function		Number of patients with impaired renal function outcome with normal baseline renal function	
	Non-TDF group	TDF group	Non-TDF group	TDF group	Non-TDF group	TDF group
30-50	19 (90.5%)	49 (81.7%)	10 (100%)	15 (75%)	9 (81.8%)	34 (85.0%)
15-30	1 (4.8%)	9 (15.0%)	-	4 (20%)	1 (9.1%)	5 (12.5%)
≤ 15	1 (4.8%)	2 (3.3%)	-	1 (5%)	1 (9.1%)	1 (2.5%)
Totals	21 (100%)	60 (100%)	10 (100%)	20 (100%)	11 (100%)	40 (100%)

Renal function categories are in ml/min; cut-off value for impaired renal function outcome was CrCl<50 ml/min; CrCl = creatinine clearance in ml/min.

Results for further analysis of clinical profiles of patients who had severe or end-stage renal impairment are presented in Table 4.7. A review of the patients' clinical profiles indicated that the TDF group had five patients with severely impaired renal function outcome (CrCl values between 15 and 30 ml/min) compared to one patient in the non-TDF group; and that the patients in the TDF group either had various conditions at baseline associated with impaired renal function outcome or were diagnosed of the conditions during treatment. The two patients who had end-stage renal disease; one in the TDF group and the other one in the other non-TDF group; also had conditions associated with impaired renal function outcome. For example, the one patient in the TDF group had hypertension, low baseline weight, and tuberculosis whilst the other one patient in the non-TDF group had tuberculosis and raised CrCl at baseline.

The clinical profiles of the patients presented in Table 4.7 further illustrate the importance of baseline renal function screening. For instance, most of the patients on TDF who had severely impaired renal function outcomes had baseline serum creatinine close to the upper limit of the normal range.

While still on the clinical profiles of the patients with severely impaired renal function outcome or worse, the effect of the variables that emerged as significant predictors of impaired renal function outcome became apparent. For example, the four patients who developed severe renal impairment were taking ARVs and anti-TB drugs such as Rifampicin concurrently.

4.1.4   Renal function outcomes: Summary of the findings

Overall, the renal function outcomes improved by a median change of +4 ml/min from a baseline median of 69 ml/min (IQR 55-84) to a median of 73 ml/min (IQR 57-92) at 12 months and by a median change of +2 ml/min (IQR 56-92) at 24 months. Ten patients (2.1%) developed severe renal impairment outcomes and three patients developed end-stage renal disease outcomes.

Table 4.7: Clinical profiles of patients with severely impaired renal function and end-stage renal disease outcomes.

Severely impaired renal function outcome (CrCl: 15-30ml/min) with baseline CrCl>50 ml/min

Non-TDF Group (n=1)	TDF Group (n=5)
Female aged 38; was diagnosed with TB while on ART; was taking anti TB drugs; BL creatinine (69 µmol/l); had normocytic anaemia at BL with neutropaenia; had macrocytic anaemia after six months; Had raised ALT at BL(160 IU/l) and normal ALT& AST at six months; was on AZT+3TC+EFV; with BL CD4 count of (62-381 cells/mm^3)	1. Male aged 41 with a low BL weight (49); was in WHO stage III at BL; BL creatinine (99 µmol/l); CD4 count (164-399 cells/mm^3) 2. Female aged 52 with BL weight (62.1 kg); BL creatinine (80 µmol/l); normal BL ALT and AST; CD4 count (254-311 cells/mm^3) 3. Male aged 48, in stage III at BL who lost weight and had IMF. Normal BL liver profile; BL creatinine (98 µmol/l); had neutropaenia with macrocytosis at BL; CD4 count (28-27 cells/mm^3) 4. Female aged 49 diagnosed with TB while on ART; was taking anti-TB drugs; was anaemic at BL (Hb less than 10.0); BL creatinine (96 µmol/l); CD4 count (63-550 cells/mm^3), had hypertension 5. Male aged 70 with a low BL weight (52 kg); Had TB at BL; lost weight during ART; normal BL ALT and AST. CD4 count (199-408 cells/mm^3). Patient 1 to 5 were all on TDF+3TC+EFV

End-stage renal disease (CrCl <15ml/min) with baseline CrCl>50 ml/min	
Non-TDF Group(n=1)	TDF Group (n=1)
Female aged 46 who had TB at BL; BL weight (56 kg); WHO stage III at BL; was taking anti-TB drugs. Had BL creatinine (100 µmol/l) and urea 3.4 mmol/l; normal BL ALT(45 IU/l)	Female aged 53 diagnosed with TB while on ART; was taking anti-TB drugs. Baseline creatinine (66 µmol/l); Low baseline weight (42 kg); BL ALT(57); ALT at six months (88 IU/l); had hypertension; was on TDF+3TC+EFV; CD4 (274-400 cells/mm^3)

BL= baseline; Values of considered parameters are given in brackets where available; For CD4 count results, the BL CD4 count and the latest CD4 counts are given.

When patients were categorised according to TDF and non-TDF groups, 60 patients (19.5%) in the TDF group and 21 patients (12.1%) in the non-TDF group had

impaired renal function outcomes. Five patients (1.6%) in the TDF group compared to one patient (0.6%) in the non-TDF group (CrCl>50 ml/min) developed severe renal impairment and one patient in the TDF group compared to one patient in the non-TDF group developed end-stage renal disease despite having baseline CrCl>50 ml/min.

The use of ARVs containing TDF emerged as a marginally significant factor associated with impaired renal function outcomes in the univariate logistic regression analysis (p=0.054) but emerged as an insignificant factor (p=0.122) in the multivariate analysis. CrCl<50 ml/min outcomes were significantly associated with underweight (p<0.001), high blood pressure (p=0.009), female gender (p=0.016) and older age above 60 (p=0.004).

4.2 Immunological Outcomes and the Variables Associated with Sub-optimal Immunological Outcomes

4.2.1   Clinical profile of the study population

Phase 2 of the study presents results of the 516 patients who met the selection criteria for the assessment of immunological outcomes based on CD4 counts. The presentations of the results begin by analysing differences in the clinical profiles of the patients who developed immunological failure (IMF) compared to the patients who did not. As a recap, patients were categorised as having IMF if their latest CD4 count values were less than the baseline CD4 count by more than 25%; had latest CD4 counts less than 50% of the peak CD4 count result; or if the latest CD4 counts values were below 100 cells/mm^3, whichever was the case.

Table 4.8 presents the clinical profiles of the patients included in the study. More males had results indicating IMF than females (p=0.002). Regarding age, there was no significant difference in age between the group that developed IMF and the group that did not (p=0.238). However, age was not equally represented in the study. The most prevalent age group was the 30-39 age group. Almost half (48.6%) of the 35 patients with IMF belonged to the 30-39 age group. Only one patient from the 60-78 age group had IMF.

Table 4.8: Clinical profiles of the patients analysed for immunological outcomes.

Variables	IMF negative (n=481)	IMF positive (n=35)	*P-value*
Male	183 (38.1%)	23 (65.7%)	0.002
Female	298 (61.9%)	12 (34.3%)	-
Age, median (Range)	40 (19-71)	36 (21-78)	0.084
Age 19-29)	59 (12.3%)	6 (17.1%)	0.238
Age 30-39)	172 (35.8%)	17 (48.6%)	-
Age 40-49)	133 (27.6%)	9 (25.7%)	-
Age 50-59)	80 (16.6%)	2 (5.7%)	-
Age 60-78)	37 (7.7%)	1 (2.9%)	-
Baseline weight , median (IQR)	55 (49-60)	53.7 (50-60)	0.493
Baseline weight (>60 kg)	141 (29.3%)	9 (25.7%)	0.733
Baseline weight (50-60 kg)	211 (43.9%)	18 (51.4%)	-
Baseline weight (<50 kg)	129 (26.8%)	8 (22.9%)	-
Baseline CD4, median (IQR)	151 (76-241)	89 (32-262)	0.082
Baseline CD4 (>200)	165 (34.3%)	10 (28.6%)	0.014
Baseline CD4 (100-200)	164 (34.1%)	6 (17.1%)	-
Baseline CD4 (50-100)	83 (17.3%)	7 (20.0%)	-
Baseline CD4 (<50)	69 (14.4%)	12 (34.3%)	-
History of TB	171 (35.6%)	13 (37.1%)	0.856
History of hypertension	78 (18.9%)	5 (23.8%)	0.572
History of diabetes	1 (0.2%)	0	1.000
Hepatitis B positive	4 (0.9%)	0	1.000
History of Herpes zoster	21 (4.5%)	0	1.000
History of anaemia	298 (61.9%)	22 (61.4%)	1.000
History of abnormal LFT	8 (17.4%)	0	1.000
WHO clinical stage I—III	461 (95.8%)	34 (97.1%)	1.000
WHO clinical stage IV	20 (4.2%)	1 (2.9%)	-
TDF Group	300 (62.4%)	24 (68.6%)	0.587
Non-TDF Group	181 (37.6%)	11 (31.4%)	-

LFT = liver function test; IQR = Inter-quartile range; Significant *p*-values are highlighted in yellow.

With respect to baseline weight, there was no significant difference in median baseline weight between the group that developed IMF and the group that did not (p=0.493). However, a number of patients who developed IMF had baseline weight below 50 kg. For example, in the group that did not develop IMF, just over one quarter (26.8%, n=481) weighed below 50 kg at baseline.

Baseline CD4 count values for the group that developed IMF were significantly lower than the group that did not develop IMF (p=0.014). Although the median value for the CD4 counts for the IMF group were not significantly lower than the group that did not develop IMF (p=0.082), the IMF group generally, had a lower median baseline CD4 count value (89 cells/mm^3) compared to the other group (151 cells/mm^3). Moreover, about one-third (34.3%, n=35) of the patients who developed IMF outcomes, had baseline CD4 count below 50 cells/mm^3.

The other variables such as the proportion of patients having tuberculosis, hepatitis, diabetes and hypertension were not significantly different between the group that developed IMF outcomes and the group that did not. In addition, the proportion of patients having Herpes zoster, anaemia or liver disease were also not significantly different between the group that developed IMF outcomes and the group that did not develop IMF outcome. Similarly, the proportions of patients who were categorised under WHO clinical stage IV as well as the proportion of patients on TDF-based ART were also not significantly different between the group that developed IMF outcomes and the group that did not develop IMF outcome.

4.2.2 Immunological outcomes and the variables associated with immunological failure

Having presented the results on the clinical profiles of the patients, the focus switched to presentation of detailed results of the immunological outcomes and the variables associated with IMF outcomes obtained after carrying out logistic regression analysis. The presentation is carried out in this order: (1) summaries of the immunological outcomes of the study population; (2) analysis of immunological outcomes relative to ART regimens; (3) analysis of the variables associated with IMF.

Although on average 6.8% of the patients developed IMF, the benefits of ART were still noticeable based on the CD4 counts. Generally, CD4 counts increased significantly by more than double (233%) from a baseline average of 163 cells/mm^3 to an average of 380 cells/mm^3 after treatment (p<0.001). The histogram of CD4 counts after treatment was more normally distributed compared to the histogram of CD4 counts at baseline (See Figure 4.5).
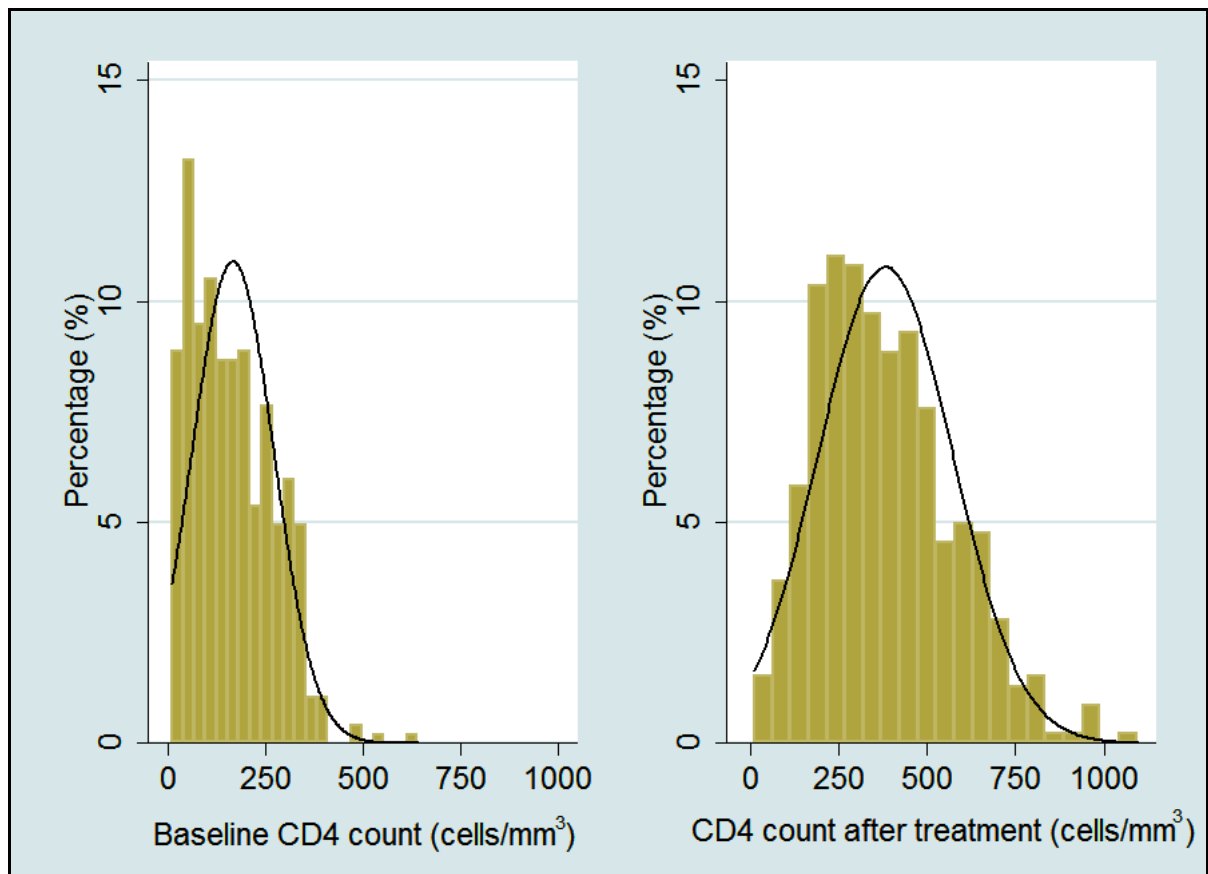
Figure 4.5: Histogram of baseline CD4 counts and CD4 count outcomes

The three different criteria used to define IMF yielded different results. Table 4.9 highlights the distribution of IMF results by criteria used to detect IMF outcomes. Out of the 516 patients included in the study, 35 patients (6.8%) had sub-optimal treatment outcome based on CD4 counts. Fourteen patients had the latest CD4 count values below 100 cells/mm^3 and 15 patients had the latest CD4 count values which were below the baseline CD4 count values by 25% or more. Only six patients had CD4 counts that indicated a 50% drop from the peak value. Therefore, the most critical indicators of IMF were: (1) having the latest CD4 count below the baseline

CD4 count by 25% or more; and (2) having the latest CD4 count value lower than 100 cells/mm^3.

Table 4.9: Distribution of immunological failure results by criteria of detecting immunological failure

Criteria for determining IMF	IMF (%) (n=35)	% of study population (n=516)
Latest CD4 count < 75% of BL CD4 count	15 (42.9)	2.9
Latest CD4 count <50% peak CD4 count	6 (17.1)	1.2
Latest CD4 count lower than 100 cells/mm^3	14 (40.0)	2.7
Total	35 (100)	6.8

IMF = immunological failure; % = percentage

When baseline CD4 count cut-off values and ART regimens were tabulated against IMF results (See Table 4.10), three important points were noted: (1) D4T-based ART regimens had the least number of patients (11 out 516) in the study meaning that TDF and AZT-based ART regimens were the two main regimens evaluated in the study; (2) highest rates of IMF outcomes for AZT-based and TDF-based regimens occurred in the patient category with baseline CD4 counts below 50 cells/mm^3; (3) The proportion with Immunological failure outcome for TDF-based regimens (7.4%, n=309) was not significantly different (p=0.458) from that of AZT-based regimens (5.7%, n=192). This indicates that immunological outcomes of TDF-based ART regimens were not significantly different from AZT-based regimens.

Table 4.10: Comparison of immunological failure (IMF) outcomes versus ART regimens and baseline CD4 cut-off values.

BL CD4 cut-off values	D4T+3TC+ EFV/NVP	AZT+3TC+ EFV/NVP	TDF+3TC+ EFV/NVP	Total
<50 cells/mm^3 [IMF (n)]	0 (5)	4 (32)	6 (44)	10 (81)
IMF%	0	12.5	13.6	12.3
50-100 cells/mm^3 [IMF (n)]	0 (4)	3 (35)	3 (51)	6 (90)
IMF%	0	8.6	5.9	6.7
100-200 cells/mm^3 [IMF (n)]	0 (5)	3 (70)	4 (95)	7 (170)
IMF%	0	4.3	4.2	4.1
>200 cells/mm^3 [IMF (n)]	1 (1)	1 (55)	10 (119)	12 (175)
IMF%	100	1.8	8.4	6.9
Total [IMF (n)]	1 (11)	11 (192)	23 (309)	35 (516)
IMF%	9.1	5.7	7.4	6.8

BL = baseline; IMF = immunological failure outcomes; %IMF = Percentage of patients with IMF in each category; CD4 counts are in cells/mm^3; AZT = Zidovudine; EFV = Efavirenz; D4T = Stavudine; and 3TC = Lamivudine; EFV/NVP means regimen contained either EFV or NVP.

As a recap, the analysis of the variables associated with IMF was based on univariate and multivariate logistic regression analysis. Several variables were analysed for possibility of association with IMF outcome, including gender, age, body weight, baseline CD4 count, use of TDF-based ART regimens, and patients' adherence to treatment.

Table 4.11 presents variables that were significantly associated with IMF in the univariate analysis.

Table 4.11: Variables and baseline CD4 cut-off points associated with immunological failure outcomes.

Variables	IMF Negative (n=481)	IMF Positive (n=35)	Unadjusted OR (95% CI)	*P-value*	Adjusted OR (95% CI)	*P-value*
Female	298 (61.0%)	12 (34.3%)	1			
Men	183 (38.0%)	23 (65.7%)	3.1 (1.5-6.4)	0.002	2.8 (1.3-5.8)	0.005
Age 19-29	59 (12.3%)	6 (17.1%)	1			
Age 30-39	172 (35.8%)	17 (48.6%)	1.0 (0.3-2.6)	0.954	-	-
Age 40-49	133 (27.6%)	9 (25.7%)	0.6 (0.2-2.0)	0.459	-	-
Age 50-59	80 (16.6%)	2 (5.7%)	0.2 (0.04-1.3)	0.093		
Age 60-78	37 (7.7%)	1 (2.9%)	0.3 (0.03-2.3)	0.228	-	-
BL Weight>60	141 (29.3%)	9 (25.7%)	1			
BL Weight 50-60	211 (43.9%)	18 (51.4%)	1.3 (0.6-3.1)	0.492	-	-
BL Weight <50	129 (26.8%)	8 (22.9%)	1.0 (0.4-2.5)	0.954	-	-
Weight loss <5%	440 (91.5%)	29 (82.9%)	1		1	

Weight loss ≥5%	41 (8.5%)	6 (17.1%)	2.2 (0.8-5.6)	0.095[**]	2.3 (0.9-6.0)	0.095
No treatment default	440 (91.5%)	33 (94.3%)	1		-	-
Defaulted treatment	41 (8.5%)	2 (5.7%)	0.7 (0.2-2.8)	0.564	-	-
Non-TDF Group	181 (37.6%)	11 (31.4%)	1			
TDF Group	300 (62.4%)	24 (68.6%)	1.3 (0.6-2.8)	0.465	-	-
BL CD4>200 cells/mm^3	165 (34.3%)	10 (28.6%)	1	-	1	
BL CD4 100-200 cells/mm^3	164 (34.1%)	6 (17.1%)	0.6 (0.2-1.7)	0.339	0.6 (0.2-1.7)	0.308
BL CD4 50-100 cells/mm^3	83 (17.3%)	7 (20.0%)	1.4 (0.5-3.8)	0.518	1.3 (0.5-3.7)	0.584
BL CD4<50 cells/mm^3	69 (14.4%)	12 (34.3%)	2.8 (1.2-7.0)	0.020	2.5 (1.0-6.1)	0.049

BL = baseline; [**] Marginally significant; OR = Odds ratio; IMF = immunological failure; Significant *p*-values are highlighted in yellow.

The variables that were significantly associated with IMF in the univariate analysis included male gender (p=0.002); baseline CD4 counts below 50 cells/mm^3 (p=0.020); and weight loss of more than 5% from baseline (only significant at 90% confidence level).

In multivariate analysis, male gender (p=0.005) and baseline CD4 count below 50 cells/mm^3 (p=0.049) remained significant predictors of IMF outcome. Although weight loss of 5% or more was an important variable determining the development of sub-optimal immunological outcomes (p=0.097), the variable was not statistically significant at 95% confidence level (p=0.095).

With respect to the use of TDF-containing ART, there were no significant differences in immunological responses between the patients using TDF and the other patients using non-TDF based ARVs (p=0.587). Therefore, the use of TDF-based ARVs had immunological outcomes that were essentially similar to non-TDF-based ARVs.

To put into perspective the variables associated with IMF, patients with the latest CD4 counts below 100 cells/mm^3 were categorised as having *critical IMF* and their clinical profiles were further analysed. This was done to highlight the significance of the variables associated with IMF outcomes.

More than half (57%) of the patients with IMF had critical IMF. Most of the patients with critical IMF were males. Only two patients out of the 20 patients with IMF were aged 50 or higher which means critical IMF was not associated with old age. One fifth of the patients with critical IMF lost at least 5% body weight. Interestingly, all the five patients who had weight loss during treatment had baseline CD4 counts below 100 cells/mm^3. Apparently, advanced baseline WHO clinical stages such as stage III and IV did not make a decisive trend. Only one patient was in WHO clinical stage IV at baseline. Therefore, the clinical stage of the disease was not a critical factor determining critical IMF outcome. Although the disease TB was not a significant predictor of critical IMF outcome, just above one-third (35%) had TB at baseline or had the disease TB diagnosed during treatment. Only two patients had records confirming failure to collect ARVs on at least one occasion. Therefore, the occurrence of immunological failure could not be explained by treatment defaulting.

4.2.3. Immunological outcomes: Summary of the findings

Generally, CD4 counts increased significantly by more than double (233%) from a baseline average of 163 cells/mm^3 to an average of 380 cells/mm^3 after treatment (p<0.001). However, out of the 516 patients included in the study, 35 patients (6.8%) had sub-optimal immunological outcomes based on CD4 counts. Twenty of the 35 patients with IMF had critical IMF defined in the study as CD4 outcome below 100 cells/mm^3. Further analysis of the 20 patients with critical IMF showed that most of the patients with critical IMF had multiple variables associated with IMF.

With respect to gender, the male gender was a significant predictor of IMF (p=0.005). One of the reasons for males having a higher likelihood of developing IMF could be poor adherence to ART. Although weight loss of 5% or more was an important variable determining the development of sub-optimal immunological outcomes, the variable was not statistically significant at 95% confidence level (p=0.095).

The other important variable determining the likelihood of a patient developing IMF was baseline CD4 count below 50 cells/mm^3 (p=0.049). However, baseline CD4 count was a confounding variable in the study because baseline CD4 count values for the group that developed IMF were significantly lower than that for the group that did not develop IMF (p=0.014).

There were no significant differences in immunological responses between the patients using TDF and the other patients using non-TDF based ARVs (p=0.587). The two main regimens in use were TDF and AZT-based regimens. When TDF and AZT-based regimens were compared, there was no significant difference between the two with respect to the proportion with immunological outcomes (p=0.458).

CHAPTER 5: DISCUSSIONS

CHAPTER 5: DISCUSSIONS

The chapter presents discussions of the results presented in chapter 4 in the same order in which the results were presented. The chapter begins with discussions on renal function outcomes based on serum creatinine clearance and then moves on to discussions on immunological outcomes based on CD4 counts.

5.1 Renal Function Outcomes

5.1.1 Clinical profile of the study population and renal function at baseline

5.1.1.1 Clinical profile of the study population at baseline

Although there was no significant difference between the TDF and the non-TDF group with respect to age (p=0.235), the 30-39 age group constituted more than one-third of the study population. The high frequency of this age group might mean that there is a higher risk of HIV infection in this age group. The results were similar to the results of another study conducted in Lesotho by Nagesh (2008), who reported that the 30-39 age group constituted up to 43.7% (n=255) of the study population.

The most common infection at baseline was tuberculosis. This was in line with the national TB statistics. According to the WHO (2008), there was a phenomenal increase in the number of TB cases between 2006 and 2008 in Lesotho possibly because of the increase in the number of HIV and AIDS cases. Moreover, according to a report by the Government of Lesotho, about three-quarters (76%) of TB patients in Lesotho are co-infected with HIV (GoL, 2012).

Patients in the TDF group had higher baseline CD4 counts than patients in the non-TDF group. This could be due to the change in the national guideline on CD4 count threshold below which patients could begin ART in Lesotho. Beginning in 2007, HIV patients were put on ART when the CD4 count fell below 350 CD4 cells/mm^3 (GoL, 2010). Before then, HIV patients were only put on ART if the CD4 count fell below 200 cells/mm^3. Interestingly though, there was no significant difference in the proportions of patients with very low CD4 counts (CD4 count <50 cells/mm^3)

between the TDF and the non-TDF group (p=1.00) as reflected in Table 4.1 on page 104.

The distribution of anaemia types in the TDF and the non-TDF group, which had a higher proportion of normocytic anaemia in both the TDF and the non-TDF group, was consistent with a typical anaemia profile in HIV and AIDS disease. Normocytic anaemia is common in HIV and AIDS patients (Wilson *et al*., 2008). However, anaemia was not a significant predictor of having impaired renal function at baseline.

Although the current guidelines do not indicate an upper age limit at which patients may not be given TDF, some patients older than 70 were put on TDF-containing regimens. Older patients were at a higher risk of having impaired renal function outcome than younger patients because according to Thomson (1995) & Naughton (2008), GFR decreases with age.

Although the percentages of patients who had baseline renal impairment in the two groups were not significantly different, when the levels of serum creatinine clearance values at baseline were looked at closely, two interesting points became clear: 56 (17.9%) were put on TDF-based regimens despite having compromised baseline renal function; and some patients with creatinine clearance values as low as 32 were put on TDF contrary to the guideline which states that patients with baseline CrCl below 50 ml/min should not be put on TDF (GoL, 2010). This implies that some clinicians might not have been informed enough about the screening criteria.

5.1.1.2 Variables associated with impaired renal function at baseline

Having TB at baseline and WHO clinical stage IV were not significant predictors of having impaired renal function at baseline. The study findings concurred with the findings by Bygrave *et al*. (2011a) who also did not find WHO clinical stage and having TB at baseline to be significant predictors of having impaired renal function at baseline.

Having anaemia at baseline was not a significant predictor of having impaired renal function at baseline (p=0.241). The results of the study differed from the findings in Zambia by Mulenga, Kruse, Lakhi, Cantrell & Reid (2008), who reported that having anaemia was predictive of impaired renal function at baseline. However, the results concurred with the findings by Bygrave *et al*. (2011a) who also did not find anaemia to be a significant predictor of having impaired renal function at baseline.

The results of the study concurred with the findings in the UK by Nelson *et al*. (2007) and in the USA by Crum-Cianflone *et al*. (2010) with respect to the variables that emerged as significant predictors of having impaired renal function at baseline. The two studies reported that underweight, female gender and older age were significant predictors of impaired renal function at baseline.

The results of this study were also similar to the findings at another site in Lesotho by Bygrave *et al*. (2011a) with respect to gender and age but differed with respect to CD4 counts. Whereas findings by Bygrave *et al*. (2011a) showed that having baseline low CD4 counts was predictive for the development of renal impairment, this study did not show that low baseline CD4 was a significant predictor for development of renal impairment. The results might have differed from the results reported by Bygrave *et al*. (2011a) due to differences in the study populations.

5.1.2 Renal function outcomes and variables associated with impaired renal function outcomes

Use of low baseline CD4 as a criterion for determining who should be screened, as suggested by Bygrave *et al*. (2011a), might pose a challenge. The baseline CD4 counts for the same patients were inconsistent, with some patients having baseline CD4 counts equal to or greater than 200 cells/mm^3 despite having severe or end-stage renal impairment. Bygrave *et al*. (2011a) investigated the feasibility of screening for renal function only for the patients of ages above 40 and CD4 count less than 200 cells/mm^3.

Five (1.6%) of the nine patients who had baseline CrCl>50 ml/min in the study developed severe renal impairment. A study in Spain by Padilla, Gutiérrez, Masiá, Cánovas & Orozco (2005), reported that five patients (4%, n=122) in the TDF group

versus 1 out of 194 patients in the control group developed moderate renal impairment. Another study by Young *et al*. (2007) in the USA reported that renal disease was diagnosed in seven patients (1%, n=593) who were exposed to TDF and three patients (0.5%, n=521) who were not exposed to TDF. Nelson *et al*. (2007) in the UK reported that 51 (0.5%, n=10 343) developed severe renal disorder whilst 227 patients (2.2%, n=10 343) developed moderate renal impairment. Another study by Patel *et al*. (2010) in India reported that 79 patients (6.2%, n=1 271) developed moderate renal impairment and five patients (0.4%, n=1 271) developed Fanconi's syndrome.

In this study, the four patients who developed severe renal impairment were taking ARVs and anti-TB drugs such as Rifampicin concurrently. Rifampicin is associated with interstitial nephritis (Schetz *et al*., 2005; Loh & Cohen, 2009). Attributing impaired renal function outcomes to TDF alone, therefore, becomes a challenge. However, one reason why the use of nephrotoxic medications was not a significant factor in the study could be because one of the drugs defined as nephrotoxic in the study, Rifampicin, was used in almost equal proportions between the TDF (35.6%, n=312) and non-TDF group (33.5%, n=173).

In this study, six out of 312 patients in the TDF group had end-stage renal disease compared to two out of 172 patients in the non-TDF group. In terms of renal function outcomes, these results are more or less comparable to findings by Young *et al*. (2007) who reported that renal disease occurred in seven patients in the TDF group (n=593) and three patients in the non-TDF group (n=521).

The overall prevalence of moderate renal insufficiency (30-60 ml/min) at baseline which was 31.5% in this study almost concurred with the findings by Mulenga *et al*. (2008) who reported a prevalence rate of 23.4% for moderate renal insufficiency in Zambia. However, the prevalence of mild renal insufficiency at baseline in this study was higher than the one reported by Franey, Knott, Barnighausen, Dedicoat & Cooke (2009), in South Africa which was 13.1% (n=2 189). There are differences in age groups assessed for renal function in the studies. For instance, Franey *et al.* (2009) considered patients whose median age was 36 (IQR 30-43). The median

age in this study was 40 (IQR 32-49). This study therefore considered older patients, which may explain the higher prevalence of mild renal insufficiency.

The results are also similar to findings by Padilla *et al.* (2005) who reported that five patients in the TDF group (n=122) versus one patient in the non-TDF group (n=194) developed serum creatinine elevations associated with WHO stage I or higher kidney disease. Other studies with findings that concur with the findings of this study to some extent, were by: Post, Moyle, Stellbrink, Domingo, Podzamczer, Fisher, Norden, Cavassini, Rieger, Khuong-Josses, Branco, Pearce, Givens, Vavro & Lim (2010), in the UK; De Beaudrap *et al.* (2010) in Senegal; Tordato, Cozzi, Lepri, Cicconi, De Luca, Antinori, Colangeli, Castagna, Nasta, Ladisa, Giacometti, d'Arminio & Gori (2011), and Calza *et al.* (2011) in Italy, who reported that there were increases in markers of tubular dysfunction in the TDF groups but did not observe significant differences between the TDF and the non-TDF groups and that the observed differences between the TDF and the non-TDF groups were only mild changes with little clinical significance.

The results from the study by Brennan *et al.* (2011) in South Africa which showed that TDF may only worsen a pre-existing renal disorder, also concurred with the results of this study. Screening for pre-existing renal disorder therefore may need to be emphasised in Lesotho. The results of the study showed that there were many leakages (20 patients or 19.5%) in the screening of baseline renal function before patients were put on ARVs containing TDF. Therefore, selective screening of baseline serum creatinine may be controversial because of the high likelihood of missing patients with impaired renal function at baseline. According to the study carried out in the area of Morija in Lesotho by Bygrave *et al.* (2011a), selective screening of baseline renal function missed 14 patients (or 8%) with CrCl<50 ml/min at baseline, two of whom had CrCl<40 ml/min at baseline.

The results of this study differed from a number of studies that did not find any association between the use of TDF and the development of renal toxicity. Notable among the studies that found no evidence linking TDF and renal toxicity was a study by Viganò *et al.* (2011) in Italy. The study however only included patients with age groups up to the young adult category. Another study whose results did not agree

with the results was by O'Donnell *et al.* (2011) which did not find any correlation between impaired renal function outcomes and exposure to TDF.

According to Bygrave *et al*. (2011a) in Lesotho, TDF-associated renal toxicity was rare and the authors concluded that there may be no need to switch patients who develop renal toxicity while on TDF because the renal toxicity that may occur while taking TDF is only transient. Although the number of patients with impaired (CrCl<50 ml/min) baseline renal function (176 or 18.9%) was similar to the number of patients with baseline CrCl<50 ml/min in the study (56 or 18.0%), more patients developed impaired renal function outcomes in this study than in the study by Bygrave *et al*. (2011a). While only 31 (5.5%) of the patients with baseline CrCl>50 ml/min developed renal toxicity while on TDF during follow up and only 3 developed severe impairment, 40 patients (12.8%) with baseline CrCl>50 ml/min in the study developed renal toxicity while on TDF.

Although there were differences in underlying conditions, age, gender and body weight between the patients in the study and the study by Bygrave *et al*. (2011a) which may make comparisons less effective, the results of this study still showed that use of TDF might be a contributing factor towards impaired renal function outcomes.

Moreover, the debate on what to do with patients who develop renal impairment while taking TDF is another issue which may be difficult to resolve given the limited number of alternative ART regimens available in Lesotho. The patients who developed renal toxicity while taking TDF in the study by Bygrave *et al*. (2011) were not switched and three patients developed severe renal impairment. In this study, despite the guidelines recommending an ART switch in patients who develop impaired renal function outcomes, only three of the six patients who developed severe renal impairment, were switched to Abacavir-based ART regimens.

The results of this study did not show that having comorbidities such as Hepatitis B or C are significant variables that contribute towards the development of impaired renal function outcomes as found by Young *et al*. (2007). Why these results might have deviated from findings by Young *et al*. (2007) and Nelson *et al*. (2007) may be

because of a smaller sample size compared to other studies and probably the limited number of patients who were tested for Hepatitis B or C. For instance, in the study by Young *et al.* (2007), 70 patients were positive for Hepatitis C in the non-TDF group and 70 patients were positive for the same condition in the TDF group from a sample size of 521. From a sample size of 485 used in this study, only two patients were positive for Hepatitis B. However, according to Ford *et al.* (2012), many cases of Hepatitis B or C are undiagnosed in Lesotho.

High blood pressure, which was high (about 20%) among the patients included in this study, poses a huge challenge as a critical contributing factor to renal impairment. Therefore, in addition to the need for screening for hypertension at baseline, patients diagnosed with hypertension may need frequent screening for renal function while taking ART drugs that may cause or exacerbate renal impairment.

TDF was not a significant predictor for the development of impaired renal function outcomes in the multivariate analysis, despite being marginally significant in the univariate analysis. The results might indicate that TDF may be a contributing factor towards renal impairment but probably with less power of influence when compared to other variables such as female gender, high blood pressure, older age, and underweight.

5.1.3 Limitations of the study

The clinical profiles of the patients in the TDF group and the non-TDF group differed in a number of variables. The variables that were were significantly different between the two groups were namely: (1) the mean baseline weight (p<0.001); (2) baseline CD4 count (p=0.029); (3) number of patients with tuberculosis at baseline (p=0.006); (4) the number of patients with anaemia at baseline (p=0.005). The disproportionate distribution of these variables between the TDF and the non-TDF groups means that these variables were confounders in the study.

Several other limitations might have had a negative impact on the analysis of the effect of TDF on renal function. The sample size of the data collected (539) was

66% of the calculated target sample size (816). There was a significant discrepancy in number of patients belonging to cases group (312 or 64%) compared to the number of patients belonging to the control group (173 or 36%) as intended in the study design.

Data on patients' height which is required for the calculation of body mass index (BMI) was not available. Lesotho does not measure patients' heights as part of the ART program. Therefore, the effect of BMI on kidney function was not assessed in the study.

According to the guidelines for case control studies by Lwanga (1991), the total sample size required to address the objectives of the study was 816 assuming an estimated odds ratio of 2 at 95% confidence level, and relative precision of 25%. However, due to resource limitations, poor accessibility, the selection criteria and the data cleaning procedure, only 539 patients who met the set inclusion criteria from the two centres were included in the study.

The disproportionate numbers of patients between the TDF and the non-TDF group was a major limitation of the study. Close to two-thirds (64%) of the patients included in the study were in the TDF group mainly due to the scarcity of serum creatinine data for patients in the non-TDF group. Therefore, further studies are necessary in African settings where the number of patients in the non-TDF group is more proportional to the number of patients in the TDF group.

The duration of treatment for the patients varied from 6 to 48 months. Moreover, the data on other adverse drug reactions which was important for the evaluation of ART outcomes was scarcely indicated in the medical records which again made comparisons disproportionate. The data for diagnosis of diabetes which depended solely on diagnostic reports of diabetes in the medical records or reported use of anti-diabetic drugs had limited reliability. Actual data on blood glucose levels for the patients were largely unavailable.

5.2 Immunological Outcomes

5.2.1 Clinical profile of the study population at baseline

With regard to clinical profiles of the patients at baseline, more males had results indicating IMF than females (p=0.002) although there was no indication that males defaulted treatment more than females. Defaulting rates between males and females did not differ significantly (p=0.564).

With respect to baseline CD4 count, the baseline CD4 count values for the group that developed IMF were significantly lower than the group that did not develop IMF (p=0.014). This means that baseline CD4 count was a confounding variable in the study. Therefore, the effect of low baseline CD4 count on immunological outcome needed to be interpreted in the context of other variables.

5.2.2 Immunological outcomes and the variables associated with immunological failure

The proportion of patients with IMF (6.8%) was lower than the one found by Eshun-Wilson, Taljaard & Nachega (2012), who reported a failure rate of 20.4% in a study they carried out in South Africa. However, the results of this study were closer to findings by Reynolds *et al*. (2009) in Uganda who reported that 11.0% of the patients had IMF.

The low predictive value of CD4 counts in detecting treatment failure emphasises the need for viral load tests. Without viral load tests, some of the patients in this study may be switched to second line probably inappropriately. According to Kantor *et al*. (2009), using CD4 count results unconfirmed by viral load tests resulted in inappropriate switching of 43% of the patients who had latest CD4 counts which were lower than 50% of the baseline CD4 cell count.

The low predictive value of CD4 counts in detecting virological failure is a major challenge in limited settings. For example, the number of patients who had virological failure according to Reynolds *et al.* (2009) in Uganda was 20.8% out of 125 patients who had immunological failure. In another study by Amenyah *et al.*

(2006) in Ghana, 31 patients (77%) had virological failure out of 40 patients who had immuno-virological failure.

The reasons why the rates of immunological failure differ by wide margins from virological failure rates may be because of low specificity of the CD4 count results in detecting treatment failure and the differences in the definitions of immunological failure. While most settings use the criteria recommended by WHO (2010), some settings define immunological failure as a decrease in CD4 counts by more than 25% from baseline (Kantor *et al*., 2009).

Another reason why reports of immunological failure differ widely may be because of the different thresholds at which patients start ART. For example, in this study, the median baseline CD4 count value was 154 cells/mm^3 (IQR 11-641) which means that some patients started ART despite having CD4 counts above 500 cells/mm^3 (patients sometimes start ART with higher baseline CD4 counts if they happen to have a life-threatening condition such as TB). Unfortunately, using the criteria for defining immunological failure without modifications in such patients may lead to errors because patients with higher baseline CD4 counts would probably need a higher percentage decrease in CD4 counts before the CD4 counts can indicate immunological failure.

A closer look at the clinical profiles of the patients with critical IMF further highlights the challenge of measuring poor adherence to treatment in Lesotho. According to Tiam (2008), poor adherence to treatment is the most common cause of ART treatment failure in Lesotho. Male patients on ART especially migrant workers who work in South Africa sometime default treatment due to constant relocations (Bygrave, 2010). A study in Central African Republic by Péré, Charpentier, Mbelesso, Dandy, Matta, Moussa, De Dieu Longo, Grésenguet, Abraham & Bélec (2012), reported that 24% of patients with virological failure showed wild-type viruses, which indicated poor adherence. The existence of poor adherence in African settings therefore poses a major setback to the success of ART programmes.

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According to Prabhakar *et al*. (2011), low baseline CD4 count is one of the risk variables for immunological failure. Prabhakar *et al*. (2011) recommended close monitoring of patients with baseline CD4 counts below 100 cells/mm^3 to ensure strict adherence to ART. The results of this study therefore further emphasize the need to closely monitor patients with low baseline CD4 counts.

Adherence to ART drugs could be one possible reason why the male gender was more likely to have immunological failure than females. However, there were no significant differences in the defaulting records of males and females (p=0.556). This study did not find any significant association between the development of IMF and poor adherence and co-morbidities such as Hepatitis B or C as reported by Prabhakar *et al*. (2011) possibly because of the small sample size of patients who were positive for Hepatitis B or C.

The results of this study did not confirm that having TB condition was a significant predictor of IMF. According to a study in Brazil by Bello, Correia, Marins, Merchan-Hamann & Kanzaki (2011), TB was a significant predictor of virological failure. In this study, the number of patients who had TB and IMF was 13 (37.1%) of the total number of patients with IMF and TB was not a significant predictor of IMF. However, the proportion of patients in the group with IMF was slightly higher than the prevalence rate (35.6% or 184 patients) in the group of patients considered in the study. Moreover, 8 of the 20 patients (40%) who were classified as having critical IMF based on CD4 count results, had TB.

5.2.3 Limitations of the study

The major limitation of this study was that the number of patients who had immunological failure was not compared to the number of patients who had virological failure. This was due to the fact that Lesotho does not test for viral load in many of the patients whose CD4 count results indicate immunological failure due to resource limitations. However, the results may indicate the need for viral load tests in Lesotho to reduce the number of patients switched to second line therapy inappropriately.

Another major limitation of the study was baseline CD4 counts. Baseline CD4 count below 50 cells/mm^3 was a significant predictor of developing IMF (p=0.049). However, baseline CD4 count was a confounding variable because baseline CD4 count values for the group that developed IMF were significantly lower than those for the group that did not develop IMF (p=0.014). Therefore, more studies with comparable baseline CD4 counts are warranted in this regard.

CHAPTER 6:   CONCLUSIONS

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Following the results presented in chapter 4 and discussed in chapter 5, conclusions on the following are drawn: renal function outcomes following the use of TDF; the variables associated with sub-optimal renal function; the occurrence of sub-optimal immunological outcomes based on CD4 counts; and the variables associated with sub-optimal immunological outcomes. The chapter ends with recommendations on how the occurrence of sub-optimal renal function outcomes and sub-optimal immunological outcomes may be minimised in light of the results obtained.

6.1 Renal function outcomes

Although the overall renal function outcomes were positive, more patients in the TDF group had a negative outcome (40 or 12.5%) compared to the positive outcome (34 or 10.9%). In the non-TDF group, more patients had a positive outcome (21 or 12.1%) compared to the negative outcome (11 or 5.8%). Although positive and negative outcomes occurred in both groups, the TDF group had a higher inclination towards negative outcomes.

The proportion of patients with impaired (CrCl<50 ml/min) baseline renal function (176 or 18.9%) in the study was similar to the proportion reported by Bygrave *et al*. (2011a) in Lesotho which was (56 or 18.0%). However, about twice as many patients (40 or 12.8%) developed impaired renal function outcomes in this study compared to the study by Bygrave *et al*. (2011a) where 31 or 5.5% developed renal function impairment with baseline CrCl>50 ml/min. Furthermore, in the study by Bygrave *et al*. (2011a), only one patient (0.2%) developed severe impairment and none developed end-stage renal disease. In this study, five patients (1.6%) in the TDF group developed severe renal impairment and one patient in the TDF group developed end-stage renal disease.

The use of ARVs containing TDF emerged as a marginally significant factor associated with impaired renal function outcomes in the univariate logistic regression analysis (p=0.054) but was an insignificant factor (p=0.122) when adjusted for age (p<0.05), gender (p=0.005), high blood pressure (p=0.009), and body weight (p<0.05). The results indicated that TDF may be a weak contributing

factor towards the development of renal impairment when compared to other variables such as female gender, high blood pressure, older age, and underweight or TDF may only worsen a pre-existing renal disorder as reported by Brennan *et al.* (2011) in South Africa.

The reported incidence of severe renal impairment following the use of TDF ranges from below 0.5% to just above 2%. The results of this study showed that 1.6% (n=312) of the patients developed severe renal impairment. The proportion with severe renal impairment was comparable to proportions reported in other studies. For example, a proportion of 1% was reported in the USA by Young *et al*. (2007), 0.5% in the UK by Nelson *et al*. (2007); and 0.4% in India by Patel *et al*. (2010).

With reference to the effect of baseline variables, the results of the study concurred with the findings by Bygrave *et al*. (2011a) at another site in Lesotho with respect to the effect of gender and age but differed with respect to CD4 counts. The results did not show that low baseline CD4 count was a significant predictor for development of impaired renal function outcome. The reason could be due to the wider range of baseline CD4 counts in the study than the patients in the study by Bygrave *et al*. (2011a).

The results of this study did not show that having comorbidities such as Hepatitis B or C is a significant factor that may contribute towards the development of renal impairment as found by Young *et al*. (2007). A number of reasons may explain why these results differed from other studies–namely: (1) differences in clinical profiles of the study populations; (2) a smaller sample size in this study compared to other studies; (3) the limited number of patients who were tested for Hepatitis B or C. As reported by Ford *et al*. (2012), many patients remain untested for Hepatitis B or C in Lesotho.

Differences in methodologies used in the studies may explain why the results in some studies were different. For example, the study by Viganò *et al.* (2011) in Italy included patients with age groups up to the young adult category. The impasse over TDF nephrotoxicity is likely to continue unless more research studies that include

large sample sizes selected from as many patient groups as possible are undertaken.

6.2 Immunological outcomes

Out of the 516 patients included in the study, 35 patients (6.8%) had sub-optimal immunological outcomes based on CD4 counts. The proportion of patients with sub-optimal immunological outcomes or IMF was therefore low. The benefits of ART were noticeable based on the CD4 counts as indicated by the increase in CD4 counts from a baseline average of 163 to 380 cells/mm^3 during treatment. There were no significant differences in immunological responses between TDF and non-TDF based ARVs (p=0.442). This means that TDF-containing regimens were more or less equally effective with respect to immunological response.

In multivariate analysis, male gender (p=0.005) and baseline CD4 count below 50 cells/mm^3 (p=0.049) remained significant predictors of IMF outcome. Although weight loss of 5% or more was an important variable determining the development of sub-optimal immunological outcomes (p=0.097), the variable was not statistically significant at 95% confidence level (p=0.095).

The proportion of patients with IMF (6.8%, n=516) was lower than the one from a study by Eshun-Wilson *et al*. (2012) who reported an immunological failure rate of 20.4% in South Africa. However, the results of the study were closer to findings by Reynolds *et al*. (2009) in Uganda who reported that 11.0% of the patients had IMF. The differences in the definitions of IMF might contribute to the differences in the reported rates of IMF.

There is also a knowledge gap in Lesotho with respect to the reasons why more men have higher rates of immunological failure than women. Interestingly, this study did not find any significant association (p=0.556) between the development of IMF and poor adherence and co-morbidities such as Hepatitis B or C as reported by Prabhakar *et al*. (2011). However, the small number of patients with documented evidence of poor adherence and patients who were positive for Hepatitis B or C in this study may explain why the results of this study differed from those of Prabhakar *et al*. (2011).

The results of this study did not indicate that having TB was a significant predictor of IMF. According to a study by Bello *et al.* (2011) in Brazil, having TB was a significant predictor of virological failure. However, in this study, 8 (40%) of the 20 patients who had critical IMF defined as having an endpoint CD4 count below 100 cells/mm^3, had TB. Therefore, further studies are necessary to establish the interaction between the TB and sub-optimal immunological outcomes.

6.3 Recommendations

6.3.1 Renal function outcomes

i. The screening for pre-existing renal disorder therefore may need to be emphasised in Lesotho.

ii. Female gender, high blood pressure, older age and underweight are the variables that should be closely monitored for changes in renal function while on ART.

iii. Patients who develop impaired renal function outcomes while on TDF-based regimens need to be switched to other regimens such as Abacavir-based regimens.

iv. Testing of Hepatitis B or C in HIV patients need to be up-scaled to the maximum level that the available resources may allow.

v. The numerous exit points for the patients in the study made the assessment of the effect of duration of treatment on renal function outcomes less effective. More studies with controlled exit points may be necessary to study the effect of duration of treatment on renal function outcomes.

6.3.2 Immunological outcomes

i. Male patients, patients with baseline CD4 count less than 100 cells/mm^3, and patients who fail to gain weight should be monitored more closely especially for adherence to drugs.

ii. Although the extent to which patients may be monitored for adherence to drugs may be limited, giving extra counselling to patients at risk of developing IMF may probably reduce the development of IMF.

iii. The recommendation for close monitoring of males may face some challenges in Lesotho due to push factors among males which tend to force males to relocate to South Africa in search of employment. The challenge may be addressed by moving towards more integrated HIV treatment programmes between South Africa and Lesotho.

iv. There is need to up-scale viral load tests. Although it may not be feasible to test all patients on ART, there is need to ensure that the patients whose CD4 counts indicate IMF are tested for viral load.

In conclusion, the results of this study indicated that there is a weak association between use of TDF and the development of renal impairment (p=0.054) before controlling for other variables and no association when the results were controlled for other variables (p=0.122). Therefore, TDF is a weak contributing factor associated with renal impairment outcomes compared to other variables such as hypertension, older age, underweight and female gender. More research on long term effects of TDF is recommended. Baseline renal function screening should be improved to minimise leakages of patients contraindicated of TDF.

The proportion of patients who developed immunological failure in this study was low (6.8%, n=516). The mean CD4 counts of the study population increased significantly after treatment (p<0.001). Therefore, generally immunological outcomes were favourable. There were no significant differences in immunological responses between TDF and non-TDF based ART (p=0.465) which means TDF-based ART has comparable effect on immunological outcomes. However, males and patients with low baseline CD4 counts should be monitored closely while on ART.

CHAPTER 7:   REFERENCES

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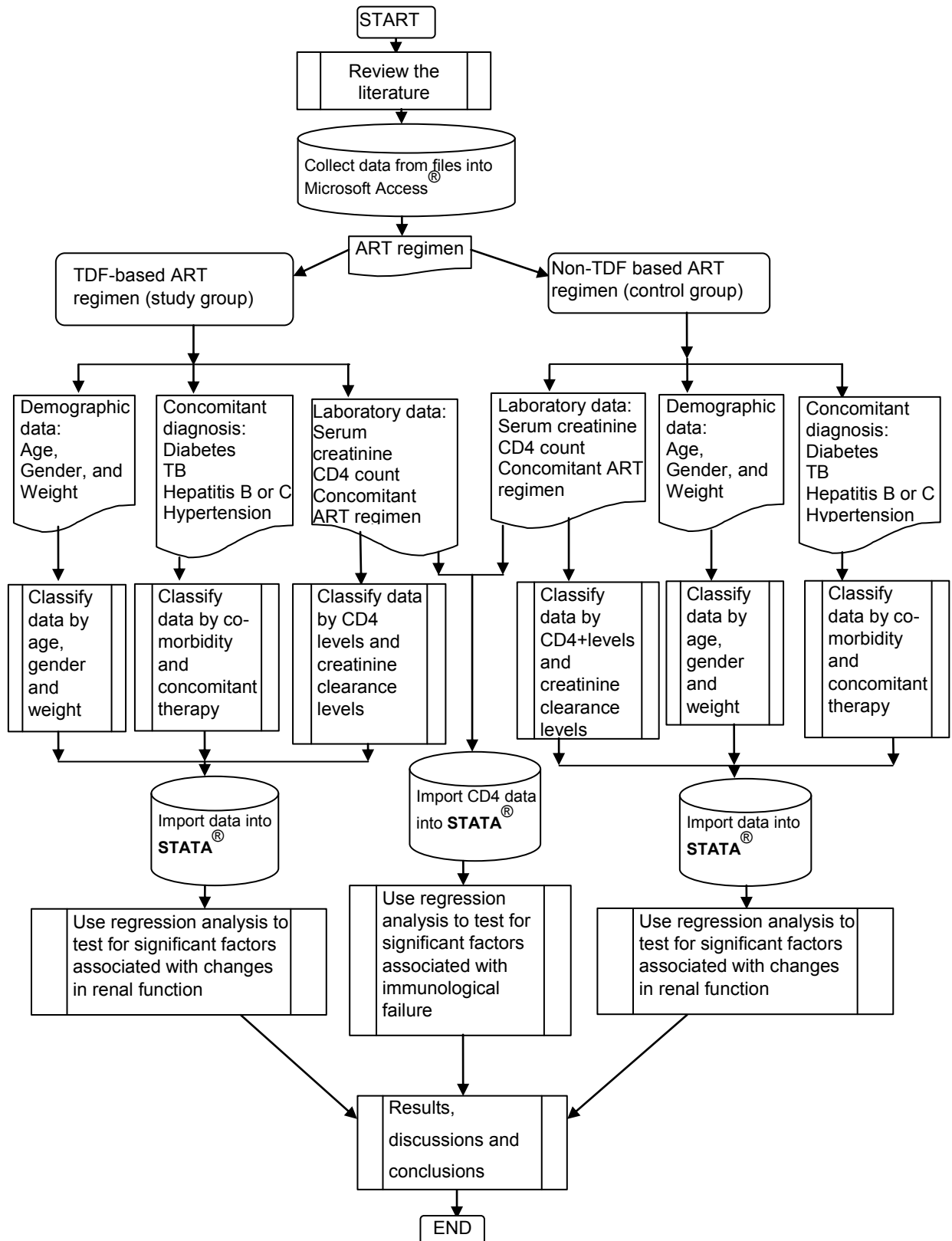
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CHAPTER 8: APPENDICES

Appendix A: Study Scheme

Appendix B: Microsoft Access® data collection tool

Group data-Microsoft Access

The effect of Tenofovir on renal function and immunological response in HIV-positive patients in Lesotho

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DEMOGRAPHIC INFORMATION

- RECORD NUMBER
- ID-NUMBER
- AGE at Baseline
- YEAR OF BIRTH
- SEX(M/F)
- MARRITAL STATUS
- HEALTH CENTRE:
- CARE ENTRY POINT:

ART REGIMEN

- HIV DATE OF DIAGNOSIS:
- HIV TYPE:
- ART START (0/12) DATE
- ART REGIMEN-BASELINE
- ART NAIVE/EXP
- SUB within 1st-LINE DRUGS-1
- SUB-1 DATE
- REASON FOR SUB-1 SWITCH:
- SUB within 1st-LINE DRUGS-2
- SUB-2 DATE
- REASON FOR SUB-2 SWITCH:
- TB Rx INITIAL DATE
- TB Rx END DATE
- ART LAST RECORD DATE
- PATIENT STATUS
- ART ADHERENCE RECORD:

BASELINE LABORATORY DATA

- DATE AT 0/12M:
- WHO CLINICAL STAGE @ 0/12:
- CONDITIONS @ 0/12:
- DRUGS @ 0/12:
- W(kg) at 0/12M
- CREAT(umol/L) at 0/12M
- UREA @ 0/12:
- CD4+(cells/uL) at 0/12

BASELINE FBC: WBC@0/1, RBC@0/12, HGB@0/1, HCT@0/12, MCV@0/1, MCH@0/1, MCHC@0, PLT@0/12

BASELINE DIFF COUNT: LYM%@0/, MXD%@0, NEUT%@0, NEUT#@0, LYM#@0/, MXD#@0

BASELINE LFTs: ALK Phos, ALT@0/12, AST@0/12, GGT@0/1, Tot prot@0, Albumin@, Total bili@, Direct bili, Indirect b

6 MONTHS LABORATORY DATA

- DATE AT 6/12M VISIT
- WHO CLINICAL STAGE @6/12:
- Conditions at 6/12:
- Drugs at 6/12:
- W(kg) at 6/12M
- CREAT at 6/12M (umol/L)
- UREA @ 6/12:
- CD4+ at 6/12M(cells/ul)

FBC AT 6 MONTHS: WBC@6/1, RBC@6/12, HGB@6/1, HCT@6/12, MCV@6/1, PLT@6/12, MCHC@6, MCH@6/1

DIFF AT 6 MONTHS: LYM%@ 0/, MXD%@ 0, NEUT%@ 0, NEUT#@ 0, LYM#@ 0/, MXD#@ 0

LFTS AT 6 MONTHS: ALK Phos, ALT@6/12, AST@6/12, GGT@6/1, Tot prot@6, Indirect b, Direct bili, Total bili@, Albumin@

12 MONTHS DATA

- DATE at 12/12M
- WHO CLINICAL STAGE @12/12
- Conditions at 12/12:
- Drugs at 12/12:
- W(kg) at 12/12M
- CREAT at 12/12M(umol/L)
- UREA @ 12/12:
- CD4+ at 12/12M(cells/ul)

FBC AT 12 MONTHS: WBC@12/, RBC@12/1, HGB@12/, HCT@12/1, MCV@12/, MCH@12/, MCHC@1, PLT@12/12

DIFF AT 12 MONTHS: LYM%@ 0/, MXD%@ 0, NEUT%@ 0, NEUT#@ 0, LYM#@ 0/, MXD#@ 0

LFTs AT 12 MONTHS: ALK Phos, ALT@12/1, AST@12/1, GGT@12/, Tot prot@1, Albumin@, Total bili@, Direct bili, Indirect b

DATA AT 18 MONTHS

- DATE at 18/12M
- WHO CLINICAL STAGE @18/12
- Conditions at 18/12:
- Drugs at 18/12:
- W(kg) at 18/12M
- CREAT at 18/12M(umol/L)
- UREA @ 18/12:
- CD4+ at 18/12M(cells/ul)

FBC AT 18 MONTHS: WBC@18/, RBC@18/1, HGB@18/, HCT@18/1, MCV@18/, MCH@18/, MCHC@1, PLT@18/12

DIFF AT 18 MONTHS: LYM%@ 0/, MXD%@ 0, NEUT%@ 0, NEUT#@ 0, LYM#@ 0/, MXD#@ 0

LFTs AT 18 MONTHS: ALK Phos, ALT@18/1, AST@18/1, GGT@18/, Tot prot@1, Albumin@, Total bili@, Direct bili, Indirect b

DATA AT 24 MONTHS

- DATE at 24/12M
- WHO CLINICAL STAGE @24/12
- Conditions at 24/12:
- Drugs at 24/12:
- W(kg) at 24/12M
- CREAT at 24/12M(umol/L)
- UREA @ 24/12:
- CD4+ at 24/12M(cells/ul)

FBC AT 24 MONTHS: WBC@24/, RBC@24/1, HGB@24/, HCT@24/1, MCV@24/, MCH@24/, MCHC@2, PLT@24/12

DIFF AT 24 MONTHS: LYM%@ 0/, MXD%@ 0, NEUT%@ 0, NEUT#@ 0, LYM#@ 0/, MXD#@ 0

LFTs AT 24 MONTHS: ALK Phos, ALT@24/1, AST@24/1, GGT@24/, Tot prot@2, Indirect b, Albumin@, Total bili@, Direct bili

DATA AT 30 MONTHS

- DATE at 30/12M
- WHO CLINICAL STAGE @30/12:
- Conditions at 30/12:
- Drugs at 30/12:
- W(kg) at 30/12M
- CREAT at 30/12M (umol/L)
- UREA @ 30/12:
- CD4+AFTER 30/12M(cells/ul)

FBC AT 30 MONTHS: WBC@30/, RBC@30/1, HGB@30/, HCT@30/1, MCV@30/, MCH@30/, MCHC@3, PLT@30/12

DIFF AT 30 MONTHS: LYM%@ 0/, MXD%@ 0, NEUT%@ 0, NEUT#@ 0, LYM#@ 0/, MXD#@ 0

LFTs AT 30 MONTHS: ALK Phos, ALT@30/1, AST@30/1, GGT@30/, Tot prot@3, Albumin@, Total bili@, Direct bili, Indirect b

DATA AT 36 MONTHS

- DATE at 36/12M
- WHO CLINICAL STAGE @36/12:
- Conditions at 36/12:
- W(kg) at 36/12M
- Drugs at 36/12:
- CREAT at 36/12M (umol/L)
- UREA @ 36/12:
- CD4+ at 36/12 (cells/uL)
- CRITICAL NOTES

FBC AT 36 MONTHS: WBC@36/, RBC@36/1, HGB@36/, HCT@36/1, MCV@36/, PLT@36/12, MCHC@3, MCH@36/

DIFF AT 36 MONTHS: LYM%@ 0/, MXD%@ 0, NEUT%@ 0, NEUT#@ 0, LYM#@ 0/, MXD#@ 0

LFTs AT 36 MONTHS: ALK Phos, ALT@36/1, AST@36/1, Indirect b, Direct bili, Total bili@, Albumin@, Tot prot@3, GGT@36/

Appendix C: Data Analysis Procedures

Appendix C1:   Summary of data cleaning, and preparation in Microsoft Excel®

START EXCEL PROGRAM

C1.1: Data cleaning

All numerical data such as age and weight, serum creatinine, CD4 counts, LFTs were checked for validity using the following formulae:

*=IF(*data is outside normal range*) OR (*cell contains string data*), check data validity*

C1.2: Medical conditions and use of concurrent Non-ART nephrotoxic drugs

The presence of a specific medical condition such as Hepatitis C was detected by combining *IF; OR; and ISNUMBER(SEARCH)* formula functions available in Microsoft Excel® 2007 using the formula:

*=IF(OR(ISNUMBER(SEARCH("*Condition name*", Cell# for conditions))), "1","0")* where Cell# = Cell address

Hypertension was defined and graded into the stages of hypertension by assessing the maximum values for the systolic and diastolic blood pressure readings for each patient by combining MAX and IF formula functions. The syntax for the MAX IF formula is:

=MAX(IF (logical test, value if true, value if false))

Maximum BP level for each patient was given by:

=MAX(IF(AND(Systolic+Diastolic BP = BLANK),NA(),MAX(Systolic BP + Diastolic BP))) where BP = Blood pressure in mmHg, and NA() was used for censorship of missing data.

Systolic and Diastolic blood pressure was given by:

Systolic BP =IF(ISBLANK(BP),"",IF(AND(120<=BP,BP<140),1, IF(AND (140<=BP, BP<160), 2,IF(BP=160,3,0))))

Diastolic BP =IF(ISBLANK(BP),"",IF(AND(80<=BP,BP<90),1,IF(AND(90<= BP,BP<100),2, IF(BP>=100,3,0))))

Similarly, history of treatment for hypertension (BP) was assessed by:

=MAX(IF(AND(BP = BLANK),NA(),MAX(BP))) where again, *NA() was included to detect missing data.*

Patients were screened for use of nephrotoxic drugs against a database of non-ART nephrotoxic medications using the following formula:

=*IF(OR(ISNUMBER(SEARCH("Drug name", Cell#)))),"1","0")* where Cell# = Cell address

C1.3: Presence of anaemia and classification based on MCV

The presence of anaemia was detected based on haematological reference ranges of the people of Basotho lineage outlined in Table 2.6 using the formula:

=*IF(AND(Gender="Male",Hb<13.7),1,IF(AND(Gender="Female",Hb<12.7),1, 0)*

Anaemia was classified as microcytic, normocytic, and macrocytic using the results from the formula above as follows:

=IF(AND(anaemia=1,MCV < 80),"microcytic anaemia", IF(AND(anaemia =1,MCV>80, MCV<100),"normocytic anaemia", IF(AND(anaemia =1,MCV > 100),"macrocytic anaemia",0)))

C1.4: Liver condition

Liver condition was classified based on ALT, AST and GGT as follows:

=IF(OR(ALT>60)+(GGT>50)+(AST>60),1,0)

Export data to STATA® Version 11

END EXCEL PROGRAM

Appendix C2:   Summary of data analysis steps in STATA® Version 11

START STATA PROGRAM

C2.1: Stage 1: analysis of renal function outcomes

C2.1.1: Tests for normality of continuous data

Tests for normality of continuous data were performed as:

Plot histograms AND RUN Sktest for continuous variables

C2.1.2: Demographic and baseline data summary

Demographic and baseline data were summarised as:

Tabulate demographic characteristics versus Non-TDF and TDF groups

Re-group continuous variables AND generate new group variables

Tabulate variables versus baseline CrCl<50 ml/min and baseline CrCl >50ml/min: Where CrCl = creatinine clearance in ml/min

Re-group baseline CrCl according to CKD classification AND generate new group variables

C2.1.3: Conditions post-baseline: Tabulation summary

Re-group continuous variables, Generate new group variables

IF variable is non-numeric, convert to binary using regular expressions:

gen binary variable = *regexm(non-numeric variable 1, "seach term 1") OR regexm(non-numeric variable 2, "seach term")*

ELSE

Tabulate variables versus TDF group and Non-TDF group

Duration of treatment was calculated as:

Treatment duration in months = (year (*latest record date*) – year (*ART start date*)) ^{X} 12 + month (*latest record date*) – month *(ART start date)*

Siginificance of the differences between the TDF and Non-TDF group was calculated as:

Tabulate TDF group vs variables, AND do Fishers exact test (if variable is categorical) or Ttest (if the variable is continuous)

Summary row data for blood pressure measurements (For example, Systolic BP) were tabulated as:

bysort BP Treatment Status*: summ* Row Mean of Systolic BP

where Row Mean of Systolic BP was calculated as*:*

egen Row Mean of Systolic BP = *rowmean* (baseline Systolic BP– latest Systolic BP)

C2.1.4: Analysis of renal function outcomes

Renal function outcomes were analysed as:

Generate variable latest CrCl value = rowlast (baseline CrCL up to latest CrCl value)

Re-group variable latest CrCl value according to CKD classification, Generate new group variable

C2.1.4.1: Definitions of sub-optimal renal function outcomes:

Sub-optimal renal function outcomes were detected and defined by:

Generate outcome variable 1 IF CrCl outcome<50 ml/min AND baseline CrCl < 50 ml/min OR baseline CrCl>50

Generate outcome variable 2 IF CrCl outcome<50 ml/min AND baseline CrCl>50

Generate outcome variable 3 IF CrCl outcome<50 ml/min AND baseline CrCl<50

Generate outcome variable 4 IF CrCl outcome>50 ml/min AND baseline CrCl<50

C2.1.4.2: Factors associated with renal insufficiency at baseline:

C2.1.4.2.1: **Analysis of variables associated with impaired baseline CrCl (*baseline CrCl<50)*

RUN Logistic Regression for baseline CrCl<50 ml/min versus categorical variables, Show Odds Ratio results

RUN Logistic Regression for baseline CrCl<50 ml/min versus all categorical variables significant in univariate analysis, Show Odds Ratio results

C2.1.4.2.2: Analysis of variables associated with sub-optimal renal function outcomes

RUN Logistic Regression for CrCl <50 ml/min outcome versus test categorical variables, Show Odds Ratio results

 RUN Logistic Regression for CrCl <50 ml/min outcome versus all test categorical variables significant in univariate analysis, Show Odds Ratio results

Repeat Multivariate analysis step until significant variables remain in the multivariate model, Show Odds Ratio results

C2.1.4.2.3: Clinical profile of patients with sub-optimal renal function outcomes

Clinical profiles of patients with sub-optimal renal function outcomes were generated as:

> *List* demographic data, laboratory data, conditions, concomitant treatment, baseline CrCl and Latest values of CrCl *IF* the patient has normal baseline renal function *AND* severe or end-stage renal impairment outcome

C2.2.1: Stage 2: analysis of immunological outcomes based on CD4 counts

Test for normality of continuous data were performed as:

> *Plot histograms* for CD4 counts and other continuous variables

> *RUN Sktest* for continuous variables

C2.2.2: Definitions of CD4 count variables

Summary statistics for CD4 count values were generated as:

> *Re-group* baseline CD4 count AND Generate group variable

> *Generate* (latest CD4 value) = rowlast (baseline CD4 count up to latest CD4 value)

> *Generate* (CD4 row mean) = rowmean (baseline CD4 count up to latest CD4 value)

> *Generate* (CD4 row max) = rowmax (baseline CD4 count up to latest CD4 value)

C2.2.3: Definitions of immunological failure (IMF)

Sub-optimal immunological response was defined as:

> *Generate* outcome variable 1 if latest CD4 value $<$(baseline CD4$^\times$0.75)

> *Generate* outcome variable 2 if latest CD4 value $<$(CD4 row max$^\times$0.5)

Generate outcome variable 3 if latest CD4 value <100

Generate IMF result if variable 1 OR variable 2 OR variable 3 = 1

C2.2.4: Testing significance of the differences in characteristics between patients with and without immunological failure outcomes

Testing significance of the differences in characteristics between patients with and without immunological failure (IMF) outcomes was performed as:

Tabulate IMF versus test variables, AND do Fishers exact test (if variable is categorical) or Ttest (if variable is continuous)

C2.2.5: Univariate and multivariate analysis of the factors associated with immunological failure outcome

Univariate and multivariate analysis of the factors associated with immunological failure (IMF) outcome was performed as:

RUN logistic regression for IMF result versus categorical variables, Show Odds Ratio results

RUN logistic regression for IMF result versus all categorical variables significant in univariate analysis, Show Odds Ratio results

C2.2.6: Clinical profiles of patients with critical sub-optimal immunological outcomes

Clinical profiles of patients with critical sub-optimal immunological outcomes were defined and listed as:

Define critical IMF outcome as latest value of value of CD4 count is <100 cells/mm^3

List patient's demographic data, BL CD4 counts and Cd4 count outcomes if the patient has a critical IMF outcome

END STATA PROGRAM

Appendix D: **Study Ethical Approval Letters**

Appendix D1:   **Study ethical approval letter from the Ministry of Health and Social Welfare Research and Ethics Committee**

LESOTHO

Ministry of Health and
Social Welfare
PO Box 514
Maseru 100

Date: 13 January 2012

Eltony Mugomeri
Department of Pharmacy
NUL

Dear Mr. E. Mugòmeri,

**Re: The Link between use of Tenofovir in HIV treatment and changes in GFR:
Findings from Roma Hospital, Lesotho**

Thank you for re-submitting the above mentioned protocol. The Ministry of Health and Social Welfare Research and Ethics Committee having reviewed your protocol hereby authorizes you to conduct this study among the specified population. The study is authorized with the understanding that the protocol will be followed as stated. Departure from the stipulated protocol will constitute a breach of the permission.

We are looking forward to have a progress report and final report at the end of your study.

Best regards,

Dr. M. M. Moteetee
Director General of Health Services and
Chairperson Research and Ethics Committee

Appendix D2: Study approval letter from St Joseph's Hospital

Received 06/09/2012
Med. Sup.

LESOTHO

Ministry of Health and
Social Welfare
PO Box 514
Maseru 100

Date: 13 January 2012

Eltony Mugomeri
Department of Pharmacy
NUL

Dear Mr. E. Mugomeri,

**Re: The Link between use of Tenofovir in HIV treatment and changes in GFR:
Findings from Roma Hospital, Lesotho**

Thank you for re-submitting the above mentioned protocol. The Ministry of Health and Social
Welfare Research and Ethics Committee having reviewed your protocol hereby authorizes
you to conduct this study among the specified population. The study is authorized with the
understanding that the protocol will be followed as stated. Departure from the stipulated
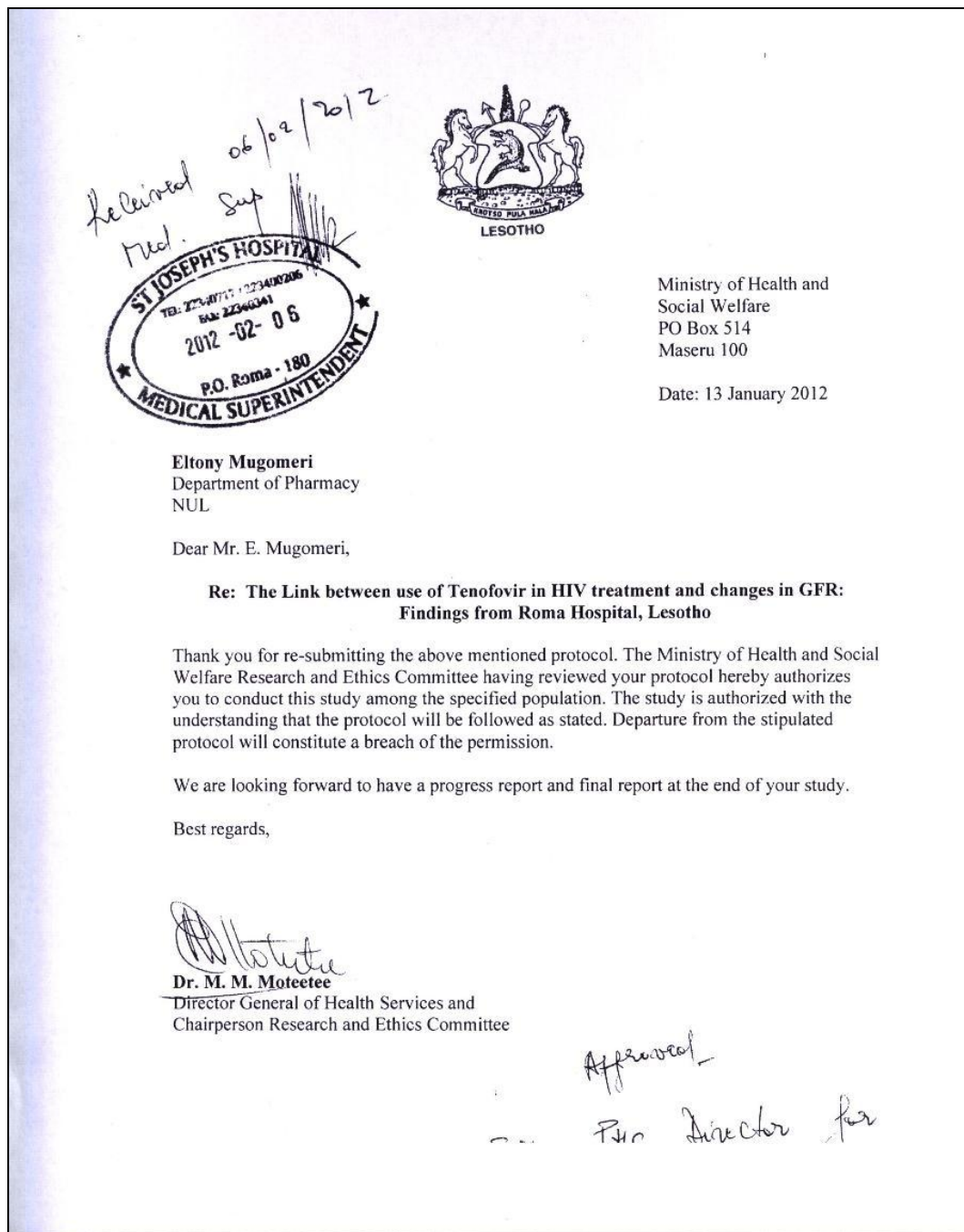protocol will constitute a breach of the permission.

We are looking forward to have a progress report and final report at the end of your study.

Best regards,

Dr. M. M. Moteetee
Director General of Health Services and
Chairperson Research and Ethics Committee

Approved
PHC Director for

Appendix D3:   **Study approval letter authorizing data collection from Nazareth Health Centre**

05/03/2012

To whom it may concern

Re: Eltony Mugomeri

Dear Sir/Madam;

The holder of this letter is conducting a study of the use of TDF (ARV), would you accept him in Nazareth Clinic where he would like to collect other data in order to further his study.

Your usual cooperation is highly appreciated.

Dr Kayembe

Medical Superintendent

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Addendum: Map of Lesotho showing Health Centres in Roma
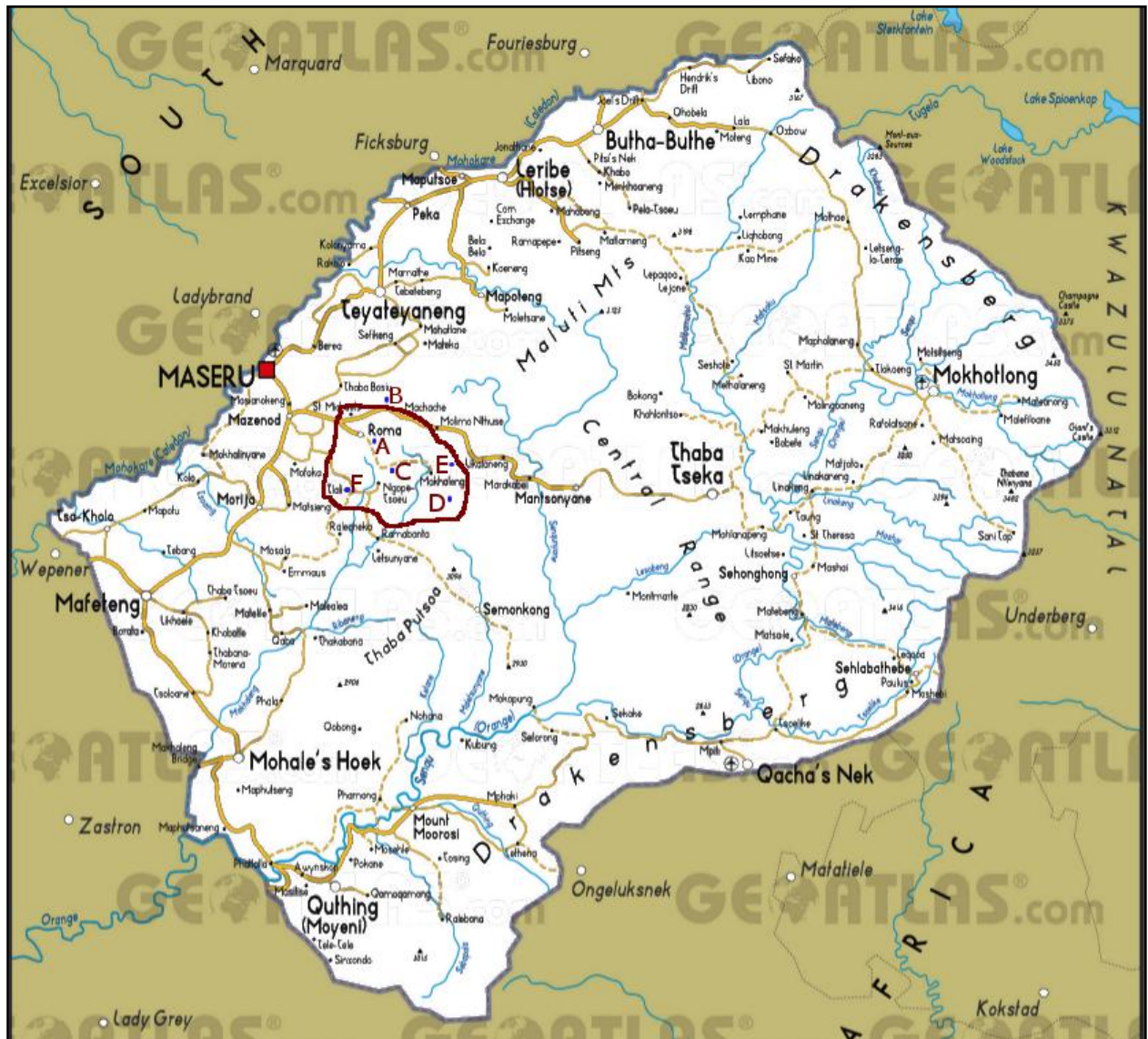
Map of Lesotho showing Roma Health Service Area (RHSA). RHSA (highlighted in red colour) is comprised of St Joseph's Hospital (A) and 5 Health Centres (B—F): B = Nazareth; C = Fatima; D = St. Benedict; E = St. Bernard and F = Tlali. Adapted from Geoatlas (2010).

Reference:

Geoatlas. 2010. [Online] Available at www.geoatlas.com/africa. Accessed: 04/08/2013