PHARMACOGENETIC AND PROTEIN BIOMARKERS ASSOCIATED WITH TENOFOVIR-INDUCED RENAL TOXICITY IN HIV-POSITIVE PATIENTS IN ZAMBIA

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy (PhD) in Pharmacology

by

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21st July 2021

Declaration

I can confirm that the work presented in this thesis represents my original work, and where information has been derived from other sources, this has been appropriately referenced and credited.

I conceived the general research concept to investigate the pharmacogenetics and biomarkers of TDF-induced nephrotoxicity in the Zambian population. This concept was approved, structured, and narrowed down to the specific research objectives as outlined in the thesis. I applied for a Commonwealth Scholarship Commission with support from my supervisors, Prof Sir Munir Pirmohamed and Dr Sudeep Pushpakom, to undertake this PhD in the University of Liverpool.

Through the institutional and intellectual support of my collaborating Supervisor in Zambia, Prof Lloyd Mulenga, the National HIV Program Coordinator, Ministry of Health, Zambia, and Director of Infectious Diseases, University of Zambia, School of Medicine. I carried out the recruitment of all research participants from the University Teaching Hospital, Adult Infectious Diseases Centre (AIDC) at the University Teaching Hospital, in Lusaka, Zambia. I primarily recruited the cohort for the pharmacogenetic study. The prospective cohort for the biomarker study was completed with the support of staff at AIDC.

I primarily performed extraction of DNA from collected blood samples, quality control and TaqMan real-time PCR genotyping. All the preliminary work for iPLEX MassArray genotyping (iPLEX PCR, Shrimp Alkaline Phosphatase treatment, CleanResin and iPLEX product extension) were done in the molecular lab of the Wolfson Centre for Personalised Medicine. I performed SpectroChip spotting and genotyping analyses for some samples with the help of Dr Eunice Zhang. Due to technical problems with the equipment, SpectroChip spotting and analysis for the rest of the samples was conducted at Agena Bioscience, Hamburg, Germany through the support of Dagmar Kasper and Hicham EL Mahmoudi.

The GWAS analysis of TDF-FS cohort was conducted by Dr Pushpakom and Prof Pirmohamed in collaboration with researchers from the Kings College, London; statistical analysis of GWAS data was conducted by Dr Ben Francis. Validation of GWAS results was conducted by me with guidance from my supervisors; in addition, I conducted the clinical data analysis, genotyping analysis and biomarker analysis with statistical guidance from Prof Andrea Jorgensen and my supervisors.

Audrey Hamachila

Abstract

Investigation of Pharmacogenetic and Protein Markers of Tenofovir induced Renal Toxicity in HIV positive patients in Zambia

Tenofovir Disoproxil Fumarate-induced renal toxicity (TDF-RT) in HIV positive patients has been associated with genetic variants in genes encoding drug transporters. I set out to investigate the association of TDF-RT with previously reported single nucleotide polymorphisms (SNPs) in drug transporter genes (*SLC22A11, ABCC2, ABCC4 and ABCC10),* genes involved in Fanconi syndrome (FS) (*OCRL1*), and transmembrane (*TMEM*) and peroxisomal (*PEX14* and *ITSN)* genes. I also performed validation of genetic markers identified from a GWAS in a cohort of UK HIV patients with TDF-FS. Finally, I explored the potential for novel kidney injury biomarkers for monitoring RT outcomes in treatment naïve patients initiated on TDF therapy.

We recruited HIV positive patients who were receiving antiretroviral therapy containing TDF from the University Teaching Hospital (UTH) in Lusaka, Zambia, between 2007 and 2017. RT was defined by a creatinine clearance of <60ml/min. 24 selected SNPs were genotyped by iplex MassARRAY® System and validated by TaqMan Real-Time PCR. 887 participants of whom 17.8% had RT were recruited. In the multivariate logistic regression, females (Odds ratio (OR),13.22; baseline age (OR,1.12,; BMI (OR, 0.50 and SCr (OR, 1.2) were independently associated with TDF-RT. 22 SNPs were analysed for an association with TDF-RT. None of the SNPs analysed was associated with TDF-RT. *PEX14*, rs284301 G>A was significantly associated with RT ($p<0.012$) but did not remain significant after adjusting for multiple testing. We identified novel haplotypes in the *ABCC2* and *PEX14* variants although they were not associated with TDF-RT.

We also confirmed an association between TDF-FS and GWAS identified SNPs *atTMEM120A*, *PEX14*, and *ITSN* genes in a case-control study. The role of *TMEM120A* in adipocyte differentiation, *ITSN* as a key regulator in several signalling pathways and *PEX14* in tubular β-oxidation of fatty acids and detoxification of ROS may represent alternative pathways and targets for the most serious TDF-RT phenotype, FS, this area needs further investigation.

52 treatment naïve HIV positive patients (aged ≥ 18 years) who were commenced on TDF ART were prospectively recruited to the biomarker study. Urine samples were collected at baseline, and at 2 and 4 weeks after TDF treatment to analyse KIM-1 and RBP4 corrected for urine creatinine. Participants were followed and monitored for TDF-RT events for up to 6 months. 12 (23.1%) presented with RT after a median of 3.5 months (IQR, 0.75–5.5 months) of TDF exposure. Both KIM-1/Cr ($p=0.021$) and RBP4/Cr ($p=0.01$) were significantly different after 4 weeks. KIM-1/Cr was significantly associated with RT outcomes (difference of means, 1.271ng/mg, 95% CI (0.26-2.52), $p=0.046$). The AUC for KIM-1/Cr (0.713) showed a relatively good sensitivity in predicting RT compared to RBP4/Cr (0.462) and CrCl (0.64).

Our findings indicate that baseline factors are important predictors of TDF-RT, but SNPs were not associated with TDF-RT in our cohort. However, a GWAS together with larger sample size is needed to investigate the contribution of unknown gene variants in the African population. We have identified some novel SNPs in patients with FS, but this will need further mechanistic and replication studies. We have also demonstrated that cumulative TDF exposure leads to tubular abnormalities and KIM-1 could be utilized to monitor and predict TDF-RT outcomes in HIV patients, but this will need further validation. The thesis highlights that it is possible to undertake mechanistic and genetic investigations in African patients with HIV, where the greatest burden of disease lies, to ensure that any findings and future interventions are relevant and appropriate.

Acknowledgements

I would like to express my profound gratitude to my supervisors, Prof Sir Munir Pirmohamed and Dr Sudeep Pushpakom for their time, guidance and intellectual resources throughout my PhD programme. Their unwavering patience and support has been a great source of inspiration through which I have developed my professional ambitions with a wider perspective.

To Prof. Andrea Jorgensen for the intellectual guidance on conducting the biostatistics analyses.

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To the Almighty God, for making this possible. Ebenezer!!

Abbreviations

Abbreviation	Meaning
ACR	Albumin creatinine ratio
AIDCOE	Adult Infectious Diseases Centre of Excellence
AIDS	Acquired immune deficiency syndrome
AIN	Acute interstitial nephritis
AKI	Acute Kidney injury
APOL1	Apolipoprotein L1
APR	Protein creatinine ratio
ART	Antiretroviral therapy
ARVs	Antiretroviral drugs
ATN	Acute tubular necrosis
ATP-ABC	Adenosine triphosphate-binding cassette
AUC	Area under the curve
BAF	Bioanalytical Facility
ΔCrCl	CrCl difference from baseline
cART	Combined Antiretroviral therapy
CKD	Chronic Kidney Injury
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CrCl	Creatinine clearance
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DTG	Dolutegravir
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
FAO	Fatty acid oxidation
FIs	Fusion Inhibitors
FSGS	focal segmental glomerulosclerosis
HAART	Highly Active Antiretroviral Therapy

HIV	Human immunodeficiency virus
HIVAN	HIV-associated nephropathy
HIVICK	HIV associated immune complex kidneys disease
hMATE1	multidrug and toxin extrusion proteins 1
hMATE2-k	multidrug and toxin extrusion proteins 2-k
hOAT	Human organic anion/cation transporters
hOCT	Human organic anion/cation transporters
INSTIs	Integrase Strand inhibitor
KDIGO	Kidney disease: Improving Global Outcomes
KIM-1	Kidney Injury Molecule-1,
KTD	Kidney Tubular disease
LMWP	Low molecular weight proteins
LMWP	Low molecular weight proteins
MALDI-TOF	Matrix assisted laser desorption/ionisation - Time of flight
MDRD	Modification of Diet in Renal Disease
MnSOD	Manganese superoxide dismutase
MRPs	Multidrug resistance-associated proteins
MSD	Meso Scale Discovery
mt	Mitochondria
mtDNA	Mitochondrial DNA
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NHRA	National Health Research Authority
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NSAIDs,	Non-steroidal ant-inflammatory drugs
NtRTI	Nucleoside/Nucleotide reverse transcriptase inhibitors
OCRL1	Oculo-Cerebro-Renal lowe protein-1
PCR	Polymerase chain reaction
PIs	Protease inhibitor
PK	Pharmacokinetics

PT	Proximal Tubules
RBP4	Retinol-binding protein 4
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SCLC22A6	Solute Carrier Family 22 Member 6)
SCr	Serum Creatinine
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase (SOD)
TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
TDF-FS	Tenofovir induced Fanconi syndrome
TDF-RT	Tenofovir disoproxil fumarate-induced renal toxicity
UNAIDs	United Nations Programme on HIV/AIDS
UTH	University Teaching Hospital
VL	Viral Load
WHO	World Health Organisation
β2MG	Beta-2-microglobulins

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview of HIV infection and antiretroviral therapy.

The human immunodeficiency virus (HIV) is a complex lentivirus of the retroviridae family that targets vital cells in the human immune system such as the $CD4^+$ T-cells, macrophages, and dendritic cells. This results in a progressive depletion of the human $CD4^+$ T-cells by several mechanisms that include a direct viral attack of $CD4^+$ T-cells, chronic immune activation and inflammation and pyroptosis and apoptosis[1, 2]. $CD4^+$ T-cells are central in mediating immune responses in humans, crucially coordinating cellular and humoral immune responses against infections. A progressive decline in $CD4^+$ T-cells to critical levels results in severe immune suppression leading to the development of an acquired immune deficiency syndrome (AIDS), a complex of life-threatening opportunistic infections[3, 4]. HIV-AIDS is a global pandemic that has been around for nearly four decades and is undoubtedly the most defining global public-health crisis over the years. In 2019, an estimated 38 (31.6–44.5) million people globally were living with HIV, of which, nearly 70% were from Sub Saharan Africa. Furthermore, approximately 690,000 people have died from HIV/AIDS-related conditions representing a one-third decline in morality since 2010 (Figure 1.1)[5].

Before the advent of antiretroviral therapy, infected people rapidly progressed to HIV-AIDS status due to progressive multiplication of the virus leading to severe immune suppression. However, advances in the knowledge of HIV biology and pathogenesis introduced combined anti-retroviral therapy (cART), a combination of potent antiretroviral drugs (ARVs) that aim to suppress the virus and restore the immune system[6]. Moreover, improvement in antiretroviral therapy efficacy, safety and accessibility has demonstrated positive results on the quality of life and survival of people living with HIV and AIDS (PLWHA)[7].

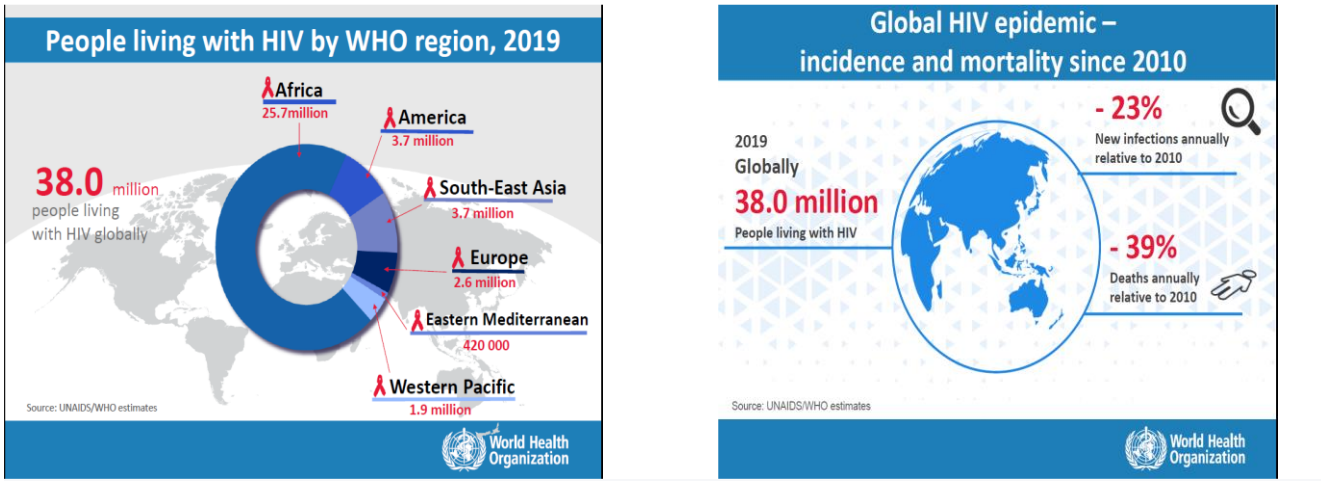


Figure 1.1. The global status of the HIV pandemic
Data Source, WHO 2019 Report[8].

Indeed since the introduction of cART, the disease course of HIV has changed significantly from a chronic fatal illness to a manageable condition with a nearly 40% decline in the mortality rate from AIDS-related causes[9, 10]. In Zambia, the current HIV status follows the trend in the global epidemiology data as shown in figure 1.2. According to the 2019 Joint United Nations Programme on HIV/AIDS (UNAIDS) data, about 1.2 (1.2–1.3) million adults and children in Zambia were living with HIV and AIDS-related deaths declined by nearly 28% since 2010[11]. The breakthrough in antiretroviraltherapy (ART) outcomes of HIV disease has been attributed to the availability of new classes of antiretroviral drugs that have allowed for the expansion of access to cART to most people living with HIV and AIDS[7]. This triggered the UNAIDS and partners to set the ambitious '90-90-90 targets'; aiming to diagnose 90% of all HIV positive people, provide ART for 90% of those diagnosed and achieve viral suppression for 90% of those treated, by 2030[11].

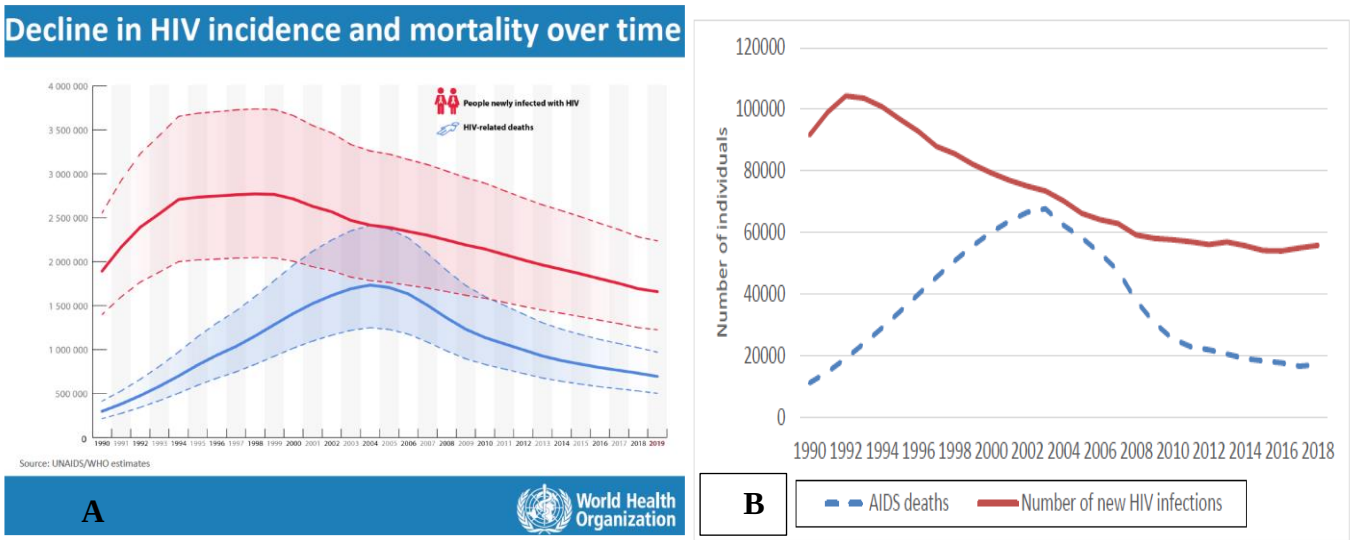


Figure 1.2. Trends in incidence and HIV related deaths following widespread use of combination anti-retroviral therapy; global (A) and Zambian (B) trends

To date, more than 30 antiretroviral agents have been approved for the management of HIV infection. However, despite the undisputed success of modern cART, many challenges remain. These include, for reasons still under research, that once the HIV infects the host, it cannot be completely eradicated by the available antiretroviral drugs (ARVs)[12, 13]. Besides, drug adverse effects associated with old and novel antiretroviral drugs remain a challenge in clinical care, both in the developed and low-income resource countries, raising the incidences of non-HIV associated morbidity and mortality. While some adverse effects may be predictable and controlled, others are often idiosyncratic and unexpected leading to a negative effect on the course of treatment and the quality of life of a patient. It would seem desirable that similar efforts and resources would be disbursed towards monitoring and studying the short and long-term impact of adverse effects recorded with the most used antiretroviral drugs. A brief outline of the ARVs and their effects is given below.

1.2 HIV Replication cycle and Antiretroviral Therapy

Under this section, I have reviewed the pathological mechanism of the HIV infection, briefly outlining the HIV replication cycle and the specific therapeutic targets and the basis for ART.

1.2.1 The HIV life Cycle – mechanism of infection

HIV life cycle demonstrates a multi-step phased replication cycle comprising of successive steps that are essential for viral maturation, infection and survival. These steps have been exploited in drug discovery, design, and development of different classes of ARVs and are the basis on which different therapeutic targets were identified and several ARVs were developed[3, 14, 15](Figure 1.3). HIV is a single-stranded enveloped virus that consists of two copies of the viral genome RNA in its core (capsid). Its viral envelope is encircled in spikes like glycoproteins, gp120 and gp41 while the core contains important enzymes such as reverse transcriptase, integrase, and protease, which are essential for viral replication[14, 16].

The first step in the HIV infection of the host cell and replication involves an integrated two-step process involving HIV attachment and entry into the $CD4^+$ cell. This starts with the interaction of the viral gp120 protein with specific host CD4 cell receptors on the membrane[14, 16]. This interaction leads to the virus binding to either the C-X-C chemokine receptor type 4 (CXCR4) or C-C chemokine co-receptors type 4 (CCR5) or both, which triggers conformational changes culminating in the fusion of the viral membrane and the CD4 cell membrane[16]. Fusion precedes entry and release of viral genetic material (single-stranded RNA) into the host CD4 cell cytoplasm which is the second step in the life cycle. These two stages have been identified as important targets to prevent viral binding and entry into the host cells. Antiviral agents such as fusion inhibitors (eg. Enfuvirtide) and CCR5 (coreceptor) antagonists

(eg. Maraviroc) have been designed to inhibit attachment plus fusion and entry of the virus [14]. Once the viral RNA is released into the host cell cytoplasm, it proceeds to reverse transcription (step 3), and conversion of single-stranded viral RNA into viral DNA copies, a process catalysed by the viral RNA reverse transcriptase enzyme using the host deoxyribonucleoside triphosphates [dNTPs] as substrates[3, 17]. Interference with viral reverse transcription activity is another important therapeutic target. However, if not inhibited, the formed viral pre-integration complex (PIC) comprising of viral DNA copies and host proteins migrates to the nucleus where viral integrase enzyme integrates (inserts) the viral DNA into the host genome to form a provirus DNA (step 4). Viral mRNAs are then transcribed and translocated to the protein assembly complex in the host cell cytosol[3]. A class of agents, integrase strand transfer inhibitors (INSTIs) have been designed to block integration of the viral DNA into the host DNA.

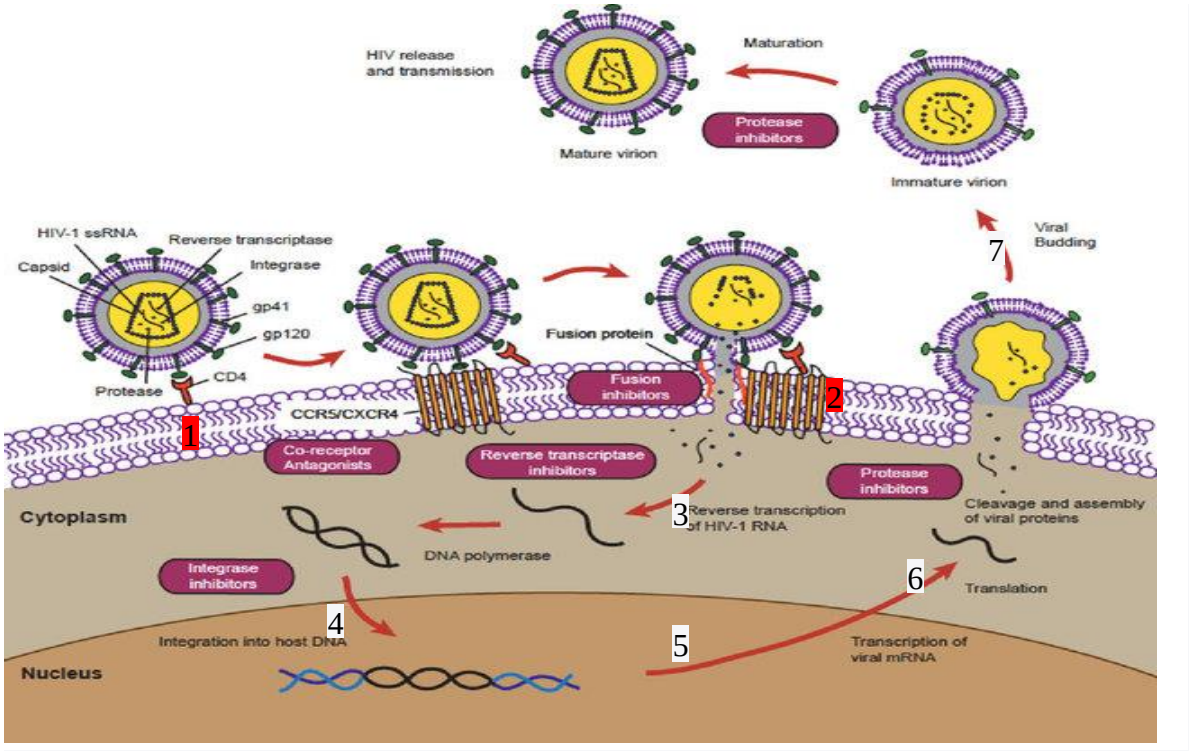


Figure 1.3. Diagrammatic illustration of the key molecular events in the HIV replication cycle 1.Attachment (Binding) of gp120 to CD4 receptor 2. Fusion (Entry) of genomic RNA into CD4 cell, 3. Reverse transcription, 4. Genomic integration 5. Viral

Replication, 6. Cleavage and assembly of new HIV protein and HIV RNA and protease catalysed viral maturation 7. Budding and Reinfection of more CD4. Figure adapted from[18].

At this stage, the HIV may remain dormant for prolonged periods in the host CD4 cell DNA, a stage called latency, where the virus may be undetectable, and the host remains asymptomatic for a prolonged period (latency infection)[19-21]. Alternatively, an immune response may trigger activation of immune-competent cells infected with HIV and initiate synthesis of messenger RNAs, the precursor of HIV viral structural polyproteins (long-chain viral proteins) (Step 5) catalysed by the host RNA polymerase enzyme. Thus, generated HIV viral proteins are assembled at the cell surface into immature products by the viral protease enzyme (step 6)[4, 22]. These exit from the host $CD4^{+}$cell by a process called budding; long viral protein chains are cleaved into individual functional viral proteins by the viral protease. The virions acquire their outer envelope forms new infective mature HIV virions capable of initiating a new life cycle and continue multiplying by continuous infection of other immune-competent host cells (step 7). Inhibition of viral protease activity is another mechanism for viral suppression; this results in the synthesis of immature virions that lack the glycoprotein envelope structure and therefore, incapable of affecting the host CD4 cells[15, 18, 23].

The final stage in viral maturation involves protein precursor Gag, which induces major structural and morphological changes in the HIV particle. Maturation inhibitors are an important novel and unique class of ARV drugs that inhibit processing of the Gag protein to prevent viral protein assembly into mature infectious viral particles. However, maturation inhibitors are still undergoing clinical trials and yet to be available for use[24, 25].

1.2.1 Principles of Combination ART (cART) in HIV Management

The availability of a diverse range of classes of ARVs with varied pharmacokinetics, resistance, safety and tolerability profiles provide a wider option for the selection of drugs[26]. Previously, ART treatment guidelines were formulated to achieve pre-determined therapeutic goals that include: to provide maximal and durable suppression of HIV-viral load (VL); restore and preserve immune function, reduce HIV-related opportunistic diseases (infectious and non-infectious morbidity), prolong life expectancy and improve quality of life, prevent onward transmission of HIV[26].

These therapeutic goals are achieved by a combination of different antiretroviral agents from different pharmacological classes targeting different steps of the HIV life cycle[27]. cART is recommended on the principle of offering greater efficacy secondary to additive or synergistic interactions among components of the combination which prevents or reduces the emergence of drug resistance. This approach also has the potential to reduce individual drug toxicity by reducing doses of drugs that show synergistic antiviral effects while increasing penetration in cells and tissue that may be difficult to reach. cART may also target different cell activation states, different tissues or different cell reservoirs while taking advantage of varied pharmacokinetics among individual drugs and drug combinations. Cumulative evidence has shown that cART has succeeded in achieving improved clinical outcomes resulting in prolonged life expectancy and improved quality that has redefined the spectrum of HIV infection from a fatal infection to a chronic manageable condition[7, 28, 29]. Moreover, significant developments in simplifying the regimen of cART with fixed-dose combinations to reduce the pill burden have improved the experience of lifelong ART. The durability of ART outcomes is sustained using drugs that combine excellent potency with convenience and a good safety

profile. As a result, treatment should further aim to minimise the long-term adverse effects of cART[26].

Based on positive reports from randomised clinical studies[30, 31], the WHO recently issued ART guidelines that recommend initiating therapy, with rare exceptions, to all newly diagnosed HIV positive patients regardless of their $CD4^+$ cell count or WHO clinical stage to prevent disease progression and maximise clinical outcomes. In line with these guidelines, the preferred first-line ART regimen for adults and adolescents in Zambia has changed since this study was conducted. In Zambia and other international ART guidelines[8, 32, 33], all cART regimens must include a backbone of two Nucleoside/Nucleotide reverse transcriptase inhibitors (NtRTIs) plus either a ritonavir-boosted protease inhibitor (PI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), and CCR5 receptor antagonist or integrase inhibitors[32, 34, 35]. Until recently, tenofovir disoproxil fumarate (TDF) has been the only nucleotide prescribed as part of the first-line cART. A novel NtRTI, tenofovir alafenamide(TAF) which has a better renal profile compared to TDF has been introduced in the latest guidelines for special population groups only. The classification of approved ARVs agents, their mechanism of action and available fixed-dose combinations (FDC) is summarised in Table 1.1.

1.2.1.1 **Therapeutic Benefits and Challenges of Combined Antiretroviral Therapy**

According to the 2020 UNAIDS global report, improved access to ART by many populations has led to a decline in HIV/AIDS-related deaths by 38% (690 000) in 2019[36]. Provided a choice of ARV drugs are available, accessible, effective, safe and well-tolerated, long term exposure to cART has reported very positive epidemiological outcomes[37-40]. When cART is initiated before severe immune suppression (CD4 count <200 cells/ml), it has achieved immune resuscitation, increased CD4 cell count of ≥550 cells/μL and reduced viral replication to viral

load (VL) below detectable levels and improved quality of life[41-44].

In South Africa, a theoretical model projected a reduction in annual HIV incidences and mortality to 1 in 1000 persons per 10 years and a decline in the prevalence of 1% in 50 years[45]. Recent reports have continued to report astounding reductions in new infections in Africa [40], Western populations[46-49] and special populations[42, 50-52]. Several clinical trials and observational studies reported a reduction in HIV related mortality and morbidity and a narrowing gap in life expectancy between HIV positive patients compared to the general population[53, 54]. Despite the reported benefits of cART in HIV infection, significant challenges remain[25, 36, 55-58].

At the heart of the challenges facing HIV as a chronic condition are determinants of persistent immune suppression, systemic inflammation, coagulation abnormalities and long-term non-HIV morbidities and mortality[19, 59]. These challenges border on critical issues relating to specific characteristics of the virus (HIV), available ARV agents and host characteristics.

With regards to HIV infection, the main challenges include the presence of drug-resistant variants of the virus in seminal fluids persists[13, 60, 61], the existence of virus reservoirs that become latent triggers of infection despite apparent immune resuscitation[12, 62] and impossibilities of eradicating the persistent form of the virus[60, 63, 64]. The challenges with the current ARVs are specific to safety and efficacy[65]. These include prolonged exposure to cART and survival that lead to subsequent morphologic, metabolic disorders and age-related non-HIV adverse effects. [66-68]. Additionally, adverse drug effects of specific ARVs and drug interaction involving the cytochrome P450 (CYP450) metabolic isoenzymes[69-71] and interaction with drug transporters[72-74] potentially complicates ART and management of toxicity. Furthermore, there exists a varying degree of *in vivo* viral resistance to almost all

ARVs that may lead to virologic failure which further challenges the selection and design of effective ART regimens[75, 76] and, the influence of genetic factors in response to ART[76].

Finally, specific host characteristics can impact the success of ART. Evidence shows that obese patients with organ impairment and a higher risk of adverse drug effects and the existence of comorbidities[77] has an impact on patient compliance. In addition, the increasing ageing HIV population requires special treatment adjustments to meet their need relative to organ function has increased[25, 77]. However, implementing these adjustments may be a challenge in optimising ART outcomes.

The focus of this thesis is the pathogenesis of nephrotoxicity associated with tenofovir disoproxil fumarate (TDF), a NtRTI widely used in HIV patients in several African countries including Zambia. Therefore, I will give a general overview of kidney disease in HIV and discuss specific aspects relating to kidney disease in general and ART

Table 1.1. Approved ARVs according to classification and their common FDCs

DRUG CLASS	APPROVED AGENTS	MECHANISM OF MECHANISM	COMMON FIXED DOSE COMBINATION
NtRTI	Zidovudine (ZDV)-Retrovir Lamivudine (3TC)-Epivir Abacavir (ABC)-Ziagen Didanosine (ddl)-Videx Stavudine (d4T)- (Zerit) Emtricitabine (FTC)-Emtriva TDF-Viread TAF (Vemlidy)	Inhibit reverse transcriptase via termination of chain elongation	ABC/3TC (Epzicom) ABC/3TC/ZDV (Trizivir) TDF/FTC (Truvada) TAF/FTC (Descovy) ZDV/3TC (Combivir)
NNRTIs	Efavirenz (EFV)- (Sustiva) Nevirapine (NVP)-Viramune Etravirine (ETR)-Entelence Rilpivirine (RPV)-Edurant	Inhibit reverse transcriptase enzyme via direct binding to enzyme catalytic site and inactivation	DOR/TDF/3TC (Delstrigo) EFV/TDF/FTC (Atripla) RPV/TAF/FTC (Odefsey) RPV/TDF/FTC (Complera)
PIs	Atazanavir (ATV)-Reyataz Darunavir (DRV)- Prezista Fosamprenavir (FPV): Lexiva Indinavir (IDV)-Crixivan	Inhibit the HIV protease enzyme and prevents catalytic cleavage of proteins needed for viral replication	LPV + RTVr (LPV/r) (Kaletra

	Nelfinavir (NFV)-Viracept Ritonavir (RTV)-Norvir Tipranavir (TPV)-Aptivus		
FIs	Enfurvitide (ENF or T-20)-(Fuzeon)	Prevent entry of HIV into CD4 via co-receptor blockage	EVG/c/TAF/FTC (Genvoya)
INSTIs	Dolutegravir (DTG)-Tivicay Elvitegravir (EVG)-Vitekta Raltegravir (RAL)-(Isentress)	Inhibit integrase, the enzyme necessary for the integration of viral DNA into host cell	DTG/RPV (Juluca) DTG/3TC (Dovato) EVG/c/TDF/FTC (Stribild) EVG/c/TAF/FTC (Genvoya)

1.3 General Overview of Kidney Disease

This section will describe the anatomical-functional features of the kidneys as the basis for the pathophysiology of different spectrums of kidney diseases which will be highlighted in subsequent sections.

The kidney is a paired anatomical organ responsible for the regulation of fluids, electrolytes and acid-base balances of the body to create a stable environment for physiological functions that are vital for tissue and cell metabolism[78]. This life-sustaining function is accomplished by regulating arterial pressure, excretion of metabolic waste products (hydrophilic xenobiotics, nephrotoxic drugs, and endogenous compounds). Other kidney functions include the synthesis of endocrine substances of physiological importance[79]. The normal human kidney is bean-shaped measuring approximately 12cm long, 6 cm wide, 4cm thick and weighs 150 grams. It is situated in the retroperitoneal space and the posterior abdominal wall precisely between the 12th thoracic (T12) and third lumbar vertebrae (L3). The left kidney is slightly longer than the right and lies closer to the midline while the right kidney lies slightly lower due to displacement by the liver[78, 80](Figure 1.4.). Collectively, the two kidneys constitute a structurally complex but essential organ for maintaining its functions. Moreover, the two ureters, the urinary bladder and the urethra together form the renal-urologic system. The ureters extend from the kidney carrying urine to the urinary bladder which serves as a reservoir for urine before it is expelled from the body through the urethra[78](Figure 1.4A). The kidney consists of the outer cortex and the inner medulla which are highly specialized cells of the renal parenchyma with distinct structural functions. The medulla is divided into renal pyramids; a series of cone-like wedges and the renal calyx chambers through which urine passes from the collecting ducts and water flows onto the ureter (Figure 1.4B)[81].

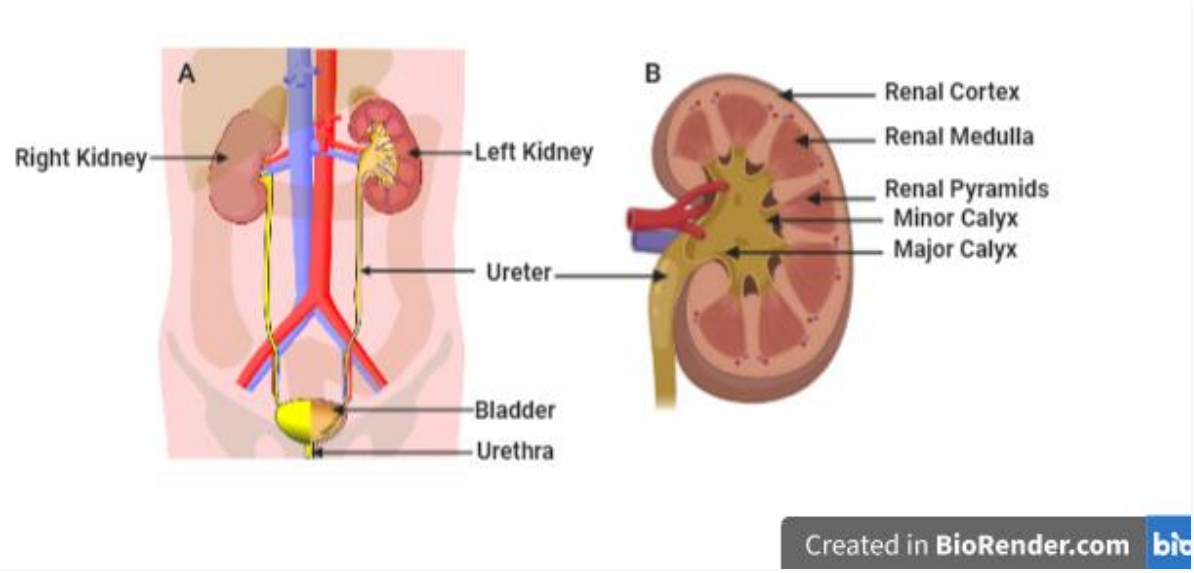


Figure 1.4. Features of the urinary system: A-anatomical structure and B-cross section of the kidney.

1.3.1 The Functional Unit of the Kidney: The Nephron

Anatomically the renal medulla in each kidney contains nearly 1.2 million nephrons which are the functional unit of the kidneys. Thus, functional subunits are integrated with the renal blood circulation to carry all the physiological functions[78]. These subunits of the nephron include the renal corpuscle (Glomerulus and Bowman's capsule) and renal proximal tubules (proximal convoluted tubule (PCT) located in the cortex and the loop of Henle that penetrates the medulla through the cortex and connects to the distal convoluted tubule (DCT) before draining in the collecting duct (Figure 1.5). Functionally, the glomerulus consists of a specialised porous capillary network that has an afferent and efferent arteriole at either end that regulates the intraglomerular pressure necessary for passive filtration of all solutes (except proteins) from approximately 160 to 180 litres of plasma[82, 83]. The formed ultrafiltrate then flows through the Bowman's capsular space into the first segment of the renal tubule, the proximal tubules (PT). The PT cells are made of the cuboidal epithelium with acidophilic cytoplasm rich in microvilli

brush border designed to increase the surface area necessary for the secretion and reabsorption of electrolytes and water lost in the filtrate. These cells, express a variety of charge selective (anionic or cationic) renal transporter proteins at the basolateral and apical membranes that are vital in tubular secretion and reabsorption of solutes and drug molecules in the kidney[84, 85]. The PT are also abundant in mitochondria involved in generating ATP necessary for facilitated intracellular transport of nearly 80% of the solute[86]. As a sole site for solute transport and metabolite recovery, almost all of the filtered glucose and amino acids are reabsorbed through the Sodium/Glucose transporter 1 and 2 (SGLT1 and 2), SGLT1 (*SLC5A1*) and neutral amino acid transporter B(0)AT1 (*SLC6A19*) or Na^+-H^+-dependent cotransporters respectively. About 70% of the filtered sodium chloride (NaCl) and water (H_2O) are reabsorbed facilitated by the Na^+K^+–ATPase pump[87, 88] while urea, minerals (calcium, magnesium and phosphates) and exogenous anions are also reabsorbed and secreted through various mechanisms. Although most reabsorption and secretion occurs in the PT, varying extents of these processes also occur throughout the different segments of the renal tubules[86]. The PT filtrate continues its transit through the cortex and then flows down onto the second tubular segment, the thin descending limb (DL) of the loop of Henle which has minimal mitochondrial and microvilli for reabsorption allowing only passive water diffusion from the filtrate. Compared to the DL, the ascending limb has functional microvilli and mitochondrial cells to facilitate Na^+ transport to dilute urine[86] before finally dipping into the renal medulla. As the filtrate transits through the collecting duct, the antidiuretic hormone (ADH) regulates H_2O permeability, reabsorption of NaCl, HCO_3^- and urea as well as secretion of K^+, H^+ and NH_3^+ resulting in concentrated urine. Figure 1.5 illustrates the solute reabsorption/secretion in the different segments of the nephron.

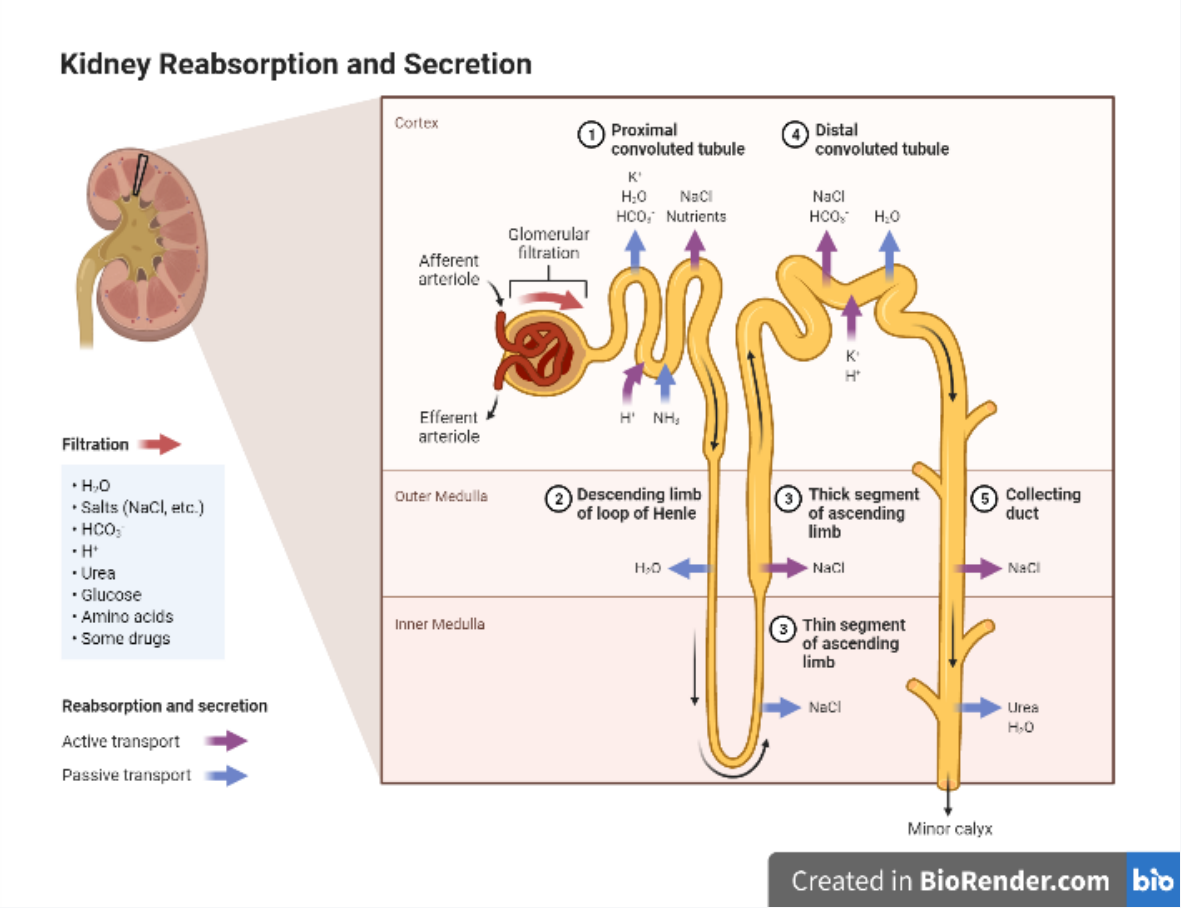


Figure 1.5. The figure shows the different segments of the nephron and the respective metabolic activities of the different solutes.

In addition, the nephron is integrated with the juxtaglomerular apparatus (JA), a specialized structure that is responsible for the regulation of glomerular pressure and filtration rate in response to Na^+ levels in collaboration with the renin-angiotensin-aldosterone system[89, 90]. This regulation is multifaceted involving the interaction of the renal, cardiovascular and autonomic nervous systems. For this reason, the kidney is an important organ in the long-term regulation of blood pressure, and it is considered as an independent risk factor for various cardiovascular conditions and vice-versa[91].

1.3.2 Proximal Renal Tubules and Disposition of drugs

In recent years, transporter proteins have been recognised as important determinants of drug disposition, transport for endogenous compounds (bile acids, electrolytes) and xenobiotics (toxins) across biological membranes[92]. The greatest proportion of prescribed drugs are excreted through the kidneys, either as a metabolite or as an unchanged molecule. A report from the USA showed that nearly 32% of the top 200 prescribed drugs are excreted in the urine while more than 25% of the absorbed dose is excreted unchanged [93]. Transporter proteins expressed in the intestinal membrane impact the absorption of drugs, while transporters in the liver and kidney impact the excretion by regulating their influx and efflux from cells where they are metabolised (hepatocytes)[84, 85]or secreted (renal tubule cells)[92]. Transporter proteins found in the PT basolateral and apical membranes have an important role in facilitating renal transport of drugs and are also increasingly recognised as a target for clinically significant drug-drug interactions that modify the pharmacokinetics and pharmacodynamics of drugs[85, 93, 94]. Most transporters are encoded by the human genome and generally fall under two super-families: i) adenosine triphosphate (ATP)-binding cassette (ABC) family that are energy-dependent efflux transporters which transport against their electrochemical gradients, and ii) solute carrier (SLC) transporters or Organic anion/cation transporters *(OAT/OCT)* that are influx transporters and utilise varied mechanisms[85, 95, 96]. In humans, basolaterally expressed organic cationic transporter 2 (hOCT2) and the multidrug and toxin extrusion proteins 1 and 2-K (*hMATE1* and *hMATE2-K*) on the apical membranes are mediators of tubular transport of cationic drugs[85, 95]. Both hMATE1/2-K share a broad spectrum of substrates and inhibitors with the hOCT2 and coordinates with hOCT2 in the secretion of cationic compounds [93, 94, 96]. In addition, P-glycoprotein (P-gp) located in the apical membrane facilitates the excretion of larger and more hydrophobic cations. On the other hand, organic anion transporters 1 and 3

(hOAT1 and *hOAT3*) and to a smaller extent *hOAT4* expressed on the basolateral membrane and several ABC- superfamily of multidrug resistance-associated proteins (MRPs) expressed apically in proximal tubule are major transporters of anionic drug molecules[85, 95, 97]. Clinically relevant cationic and anionic drugs that are substrates or inhibitors of *hOCT2/MATE1/2-K* and *hOAT1/2* and MRPs are illustrated in Figure 1.6.

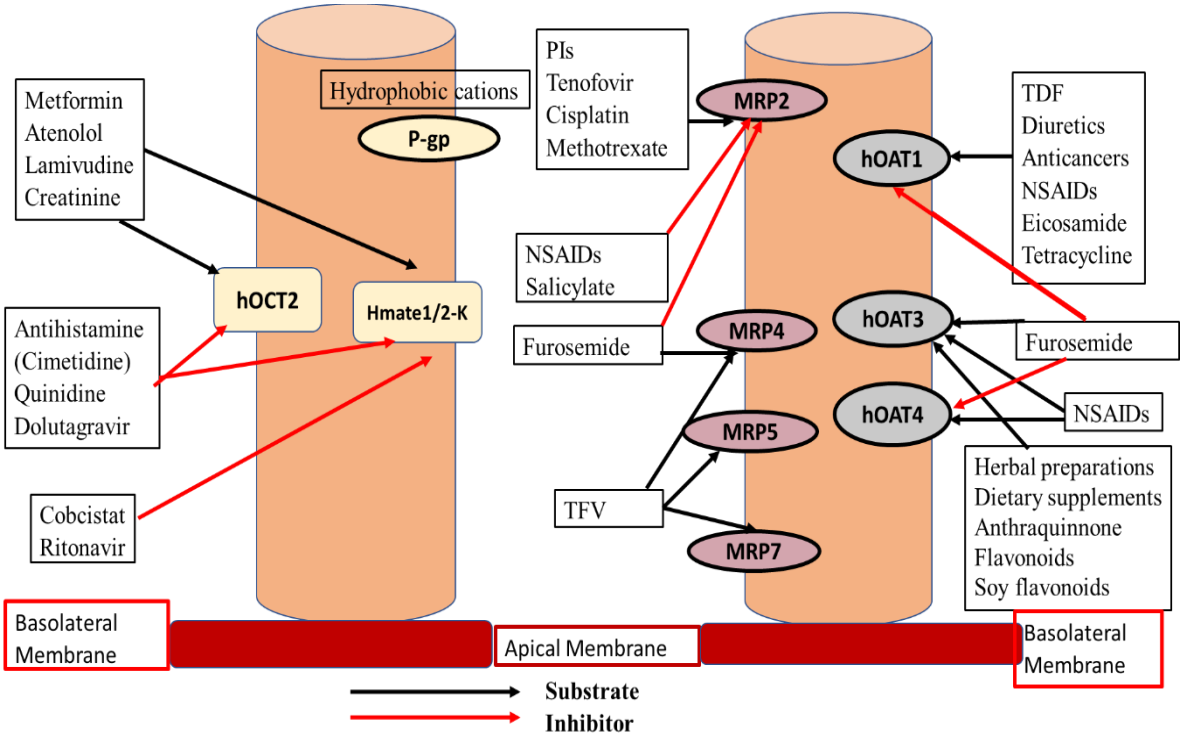


Figure 1.6. Illustration of the Proximal Tubular Cell transport system of common cationic and anionic drugs

The transport pathway is mediated by influx transporters the organic cationic transporter and efflux through the multidrug and toxin extrusion proteins 1 and 2-K. Anionic drugs are transported into tubular cells by the organic anionic transporter 1.3 and 4 and efflux through the multidrug resistance proteins MRP2, 4,7 and 8.

1.4 Kidney Disease: Definition and Classification

In this section, we briefly review types of kidney abnormalities that are likely to develop in the event of an insult to the functional structure of the kidney.

Nephropathies are quite complex and different mechanisms of damage are responsible for affecting each segment of the nephron. It is noteworthy that one injury may affect multiple structures due to the interdependence of different segments of the functional unit. Whereas glomerular injuries are often mediated by immunological reactions, tissue hypoxia and ischaemia, tubulopathies and interstitial injuries are mainly caused by nephrotoxic exogenous agents (active drugs and metabolites), endogenous substances (glucose and proteins), infections and genetic defects[86]. Unlike other organ disorders; kidney disease is often insidious, asymptomatic and presents with non-specific symptoms long after significant structural and functional changes have occurred[98] and can affect the function of other organs.

According to the kidney disease: Improving Global Outcomes (KDIGO) guidelines, kidney disease is defined as morphological and functional abnormalities of the kidney that has health implications. This injury can be classified based on the duration, cause, and extent of structural and functional abnormalities. Kidney disease may present as acute which is injury evolving over a few days and lasting less than three months of duration or as chronic kidney dysfunction which lasts more than three months[99]. Either type of injury is further classified by severity measurements of tissue damage (albuminuria, abnormalities in the urine sediment, imaging, or biopsy) and function (glomerular filtration rate (GFR) or creatinine clearance (CrCl) or serum creatinine (SCr) or decreased urine output). Thus, understanding the pathophysiology of kidney injury entails a collective consideration of the renal structural and physiological characteristics.

1.4.1 Acute Kidney disease

Acute kidney disease also referred to as acute kidney injury (AKI) is a complex heterogeneous group of kidney disorders of multifactorial origin and is characterised by a rapid decline in glomerular function evidenced by 1.5 times rise in baseline serum creatinine and/or oliguria (< 0.5ml/kg/h for 6 hours)[100, 101]. AKI mainly presents in more than 20% of critically hospitalised patients and regardless of patient characteristics and the context of the injury. It is often associated with a high mortality rate and short and long-term adverse events[102-107]. AKI is initiated by various clinical insults that are classified by three main aetiologies of injury such as pre-renal, renal and post-renal aetiologies[108, 109]. The mean features of each aetiology and the common causes are summarised in Table 1.2.

Table 1.2. Aetiologies and common causes of AKI

AETIOLOGY	MAIN FEATURE/ CHARACTERISTICS	MAJOR CAUSES
Prerenal	The rapid decline in GFR due to a decrease in renal perfusion and Insufficient intraglomerular pressure	Intrarenal: Vasoconstriction: Medication: NSAIDs, ACE Inhibitors, ARB, Calcineurin, Cardiorenal/Hepatorenal syndrome Volume depletion: Vomiting, diarrhoea, burn, bleeding Systemic Vasodilation: sepsis, shock
Postrenal	Acute obstruction of the urinary flow resulting in impaired renal perfusion and inflammation,	Intrarenal causes: crystal precipitation of insoluble drugs, kidney stones, Extrarenal causes malignancies
Intrarenal (interstitial renal damage)	Damage of different segments of the nephron (Glomerular and renal	Glomerular damage: Glomerulonephritis, Lupus erythematosus. Infection, Hypertension, Diabetes mellitus

	tubules)110, 114, 120	**Tubular damage:** Pre-renal and post-renal causes aetiologies. Exogenous toxins: Drugs aminoglycosides, methotrexate, cyclosporin, TDF.NSAIDs, Endogenous toxins: haemolysis, rhabdomyolysis, tumour lysis
		Vascular damage: Decrease in renal perfusion and GFR, Malignant hypertension, renal vein thrombosis. Interstitial Nephritis: Drugs(NSAIDs, penicillin analogues, cephalosporin, antiviral Viruses: HIV, Cytomegalovirus, Epstein-Barr virus

1.4.2 Drug-induced Acute Tubular Necrosis

The role of renal tubular cells in modifying the glomerular filtrate through the secretion and reabsorption processes potentially makes them vulnerable to a high level of circulating nephrotoxins[110, 111]. Almost all drugs may exhibit and retain varying degrees of nephrotoxicity depending on their structure, dose/concentration, metabolism, excretory pathway through the kidney. Patient characteristics like comorbidities and genetic predisposition may also play a role in developing nephrotoxicity[110, 112, 113]. Drug-induced tubular toxicity results from a combination of mechanisms that include interfering with tubular transport, impairing mitochondrial function, formation of free radicals and oxidative stress[112]. Furthermore, nephrotoxic drugs may accumulate and exhibit toxicity by either direct tubular injury or crystal-induced nephropathy that present as sublethal changes in tubular cells and cause a major impact

on GFR which further causes tubular cell damage and death[114, 115]. In addition, some nephrotoxic drugs may precipitate and cause a direct acute tubular injury or alter intraglomerular haemodynamics and interfere with the kidneys' ability to regulate its glomerular pressure and glomerular filtration capacity[115, 116]. Therefore, knowing the mechanism of toxicity is vital in identifying and preventing renal impairment. Commonly prescribed drugs can also induce ATN by direct tissue injury, triggering inflammatory responses or inducing rhabdomyolysis[117-122].

Once toxicity occurs, ATN follows a well-defined four-part sequence of clinical phases that include initiation, extension, maintenance, and recovery that are characterised by different pathological features as illustrated in figure 1.7. The phases are further characterised by different biomarkers that are useful in monitoring and treating the injury.

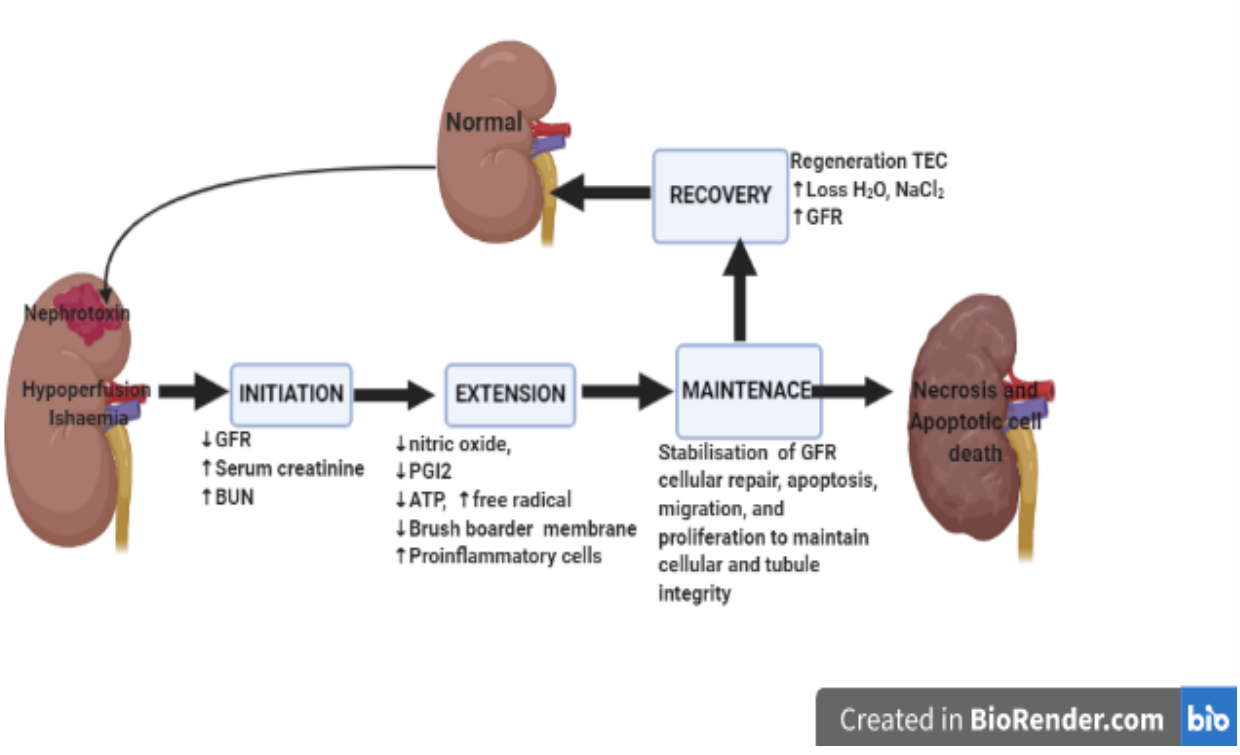


Figure 1.7. Pathophysiology phases of acute tubular necrosis following hemodynamic changes and or a nephrotoxic event.

1.4.3 Chronic Kidney Disease

Chronic kidney disease (CKD) is a major global health burden that affects millions of people from diverse ethnic backgrounds[123]. Recently, a report showed an increase in the global prevalence of CKD between 1990 and 2017 by 29·3% (95% CI, 26·4-32·6) while the global all-age mortality rate increased by 41·5% (95% CI, 35·2 - 46·5)[124]. The kidney has limited capacity to respond to an injury and therefore, repeated and sustained injuries resulting in the nephron adapting to the functional deficit due to poor regenerative capacity. CKD is characterised by an irreversible loss in renal function lasting for at least 3 months duration that may be congenital or acquired[125, 126]. There are several events occurring in isolation or simultaneously that may propagate a chronic kidney injury due to partial recovery of drug-induced nephrotoxicity[120], inadequately treated infections[127] and urolithiasis[128].

The nephron's microvasculature and the position of renal tubules in relation to the glomeruli (glomerular - peritubular circulation) facilitate the spread of abnormal glomerular ultrafiltrates and some inflammatory mediators which lead to glomerular disease[129]. Furthermore, the dependence of glomerular filtration on intra-trans-glomerular pressure renders the glomerular vasculature at a high risk of injury resulting from glomerular hypertension and hyperfiltration[125, 129].

The early stage of CKD is characterised by an increase in intraglomerular pressure resulting in hyperfiltration as a compensatory mechanism. This apparent increase in GFR from the remnant nephrons, despite a progressive kidney tissue injury, maintains the observed normal renal function. Effectively compensated nephrons will allow for normal creatinine clearance and other solutes even in the presence of injury[130, 131]. Furthermore, an increase in glomerular capillary pressure alters the glomerular membrane making it permeable to anionic

macromolecules leading to proteinuria; a hallmark of progressive kidney injury[129]. As a consequence of progressive kidney damage, proteinuria may also worsen the progression of CKD because of lost tubular cell capacity to reabsorb proteins[125, 129]. Moreover, reduced glomerular perfusion leads to peritubular hypoxia which explains the often-observed tubule interstitial injury and tissue remodelling[125, 129]. Progression of kidney injury by this mechanism has been reported with uncontrolled hypertension and untreated kidney injury is known to induce the production of inflammatory cells and pro-fibrotic cytokines which unleashes a cascade of reactions leading to oxidative stress and tissue hypoxia; the major players of CKD[130]. Further, when persistent inflammation and tissue damage reach irreversible levels, thus, when a 50% decline in renal function manifests clinically, there is already nearly 50% to 75% irreversible loss in functional nephrons[130, 131].

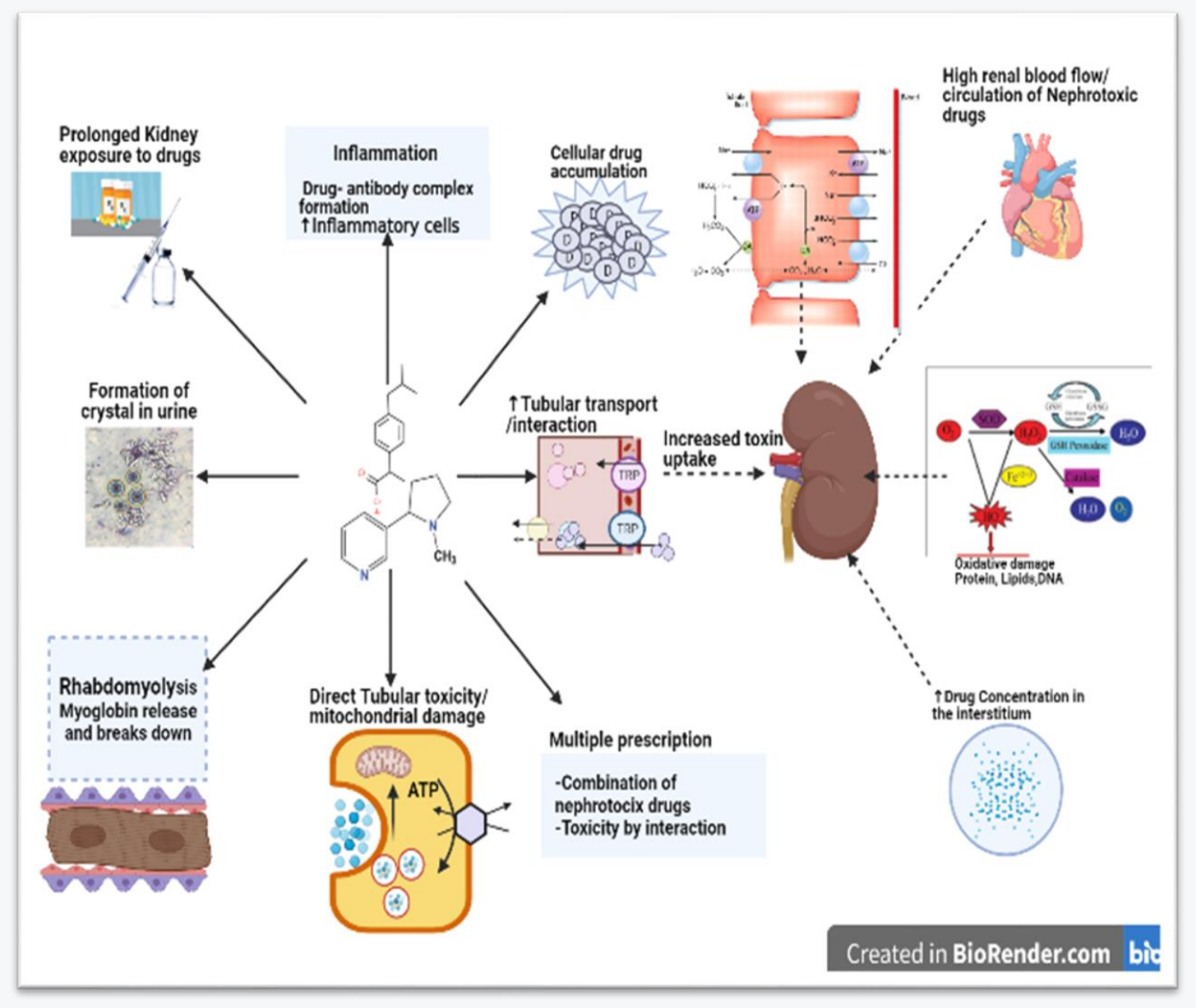


Figure 1.8. Drug-induced nephrotoxicity: drug and kidney factors associated with an increased risk of different types of nephrotoxicity. Figure adapted from Perazella[111].

1.5 Kidney Disease in HIV Infected Patients.

After nearly four decades since the first report of HIV infection, cART has dramatically overturned the spectrum of the HIV disease from an acute and fatal disease to a chronic manageable condition with improved survival[38, 41, 132]. The cART has further reduced AIDS-related mortality and morbidities[133]. However, an increase in life expectancy of HIV

patients has consequently increased the risk of non-HIV age-related comorbidities like kidney disease, diabetes mellitus and cardiovascular disease[5, 57, 132, 134, 135]. As a result, one-third of HIV patients are affected by kidney disease[136, 137] and they remain at higher risk of morbidity and mortality associated with the burden of both AKI and CKD compared to the general population[138-141]. The spectrum of kidney diseases in HIV range from HIV associated nephropathies[142-144], proteinuria[145, 146`], electrolyte disorders[133, 145], AKI[147-149] and a wide-ranging severity of CKD[101, 139]. Consequently, kidney disease further reflects a shift from HIV associated nephropathies before the ART era[142, 143] to HIV nephropathies post ART that include immune complex kidney diseases[150-152], ART-induced nephropathies[107, 153, 154] as well as nephropathies associated with age-related comorbidities (hypertension, diabetes and nephroangiosclerosis)[136, 146, 155, 156]

In the following sections, we will present a brief review of renal pathologies in the HIV setting in the context of HIV associated nephropathies in the pre and post-ART eras. We will then detail the role of ART in kidney disease with a focus on TDF.

1.5.1 Pre-ART era: HIV Associated Nephropathy (HIVAN)

Before the introduction of Highly Active Antiretroviral Therapy (HAART), now called cART, severe immunosuppression due to untreated HIV infection was synonymous with opportunistic infection and CKD being the highest cause of end-stage renal disease (ESRD) in HIV-infected patients[157-160]. Direct toxic effects of the virus on the kidneys was responsible for a characteristic kidney disease known as HIV-associated nephropathy (HIVAN) which typically presented with proteinuria and a rapid loss of renal function[158-162]. Definitive diagnosis of HIVAN is based on biopsy features characterised by a pattern of collapsing focal segmental glomerulosclerosis (FSGS) with tubular interstitial injury and fibrosis[163] (Figure 1.9).

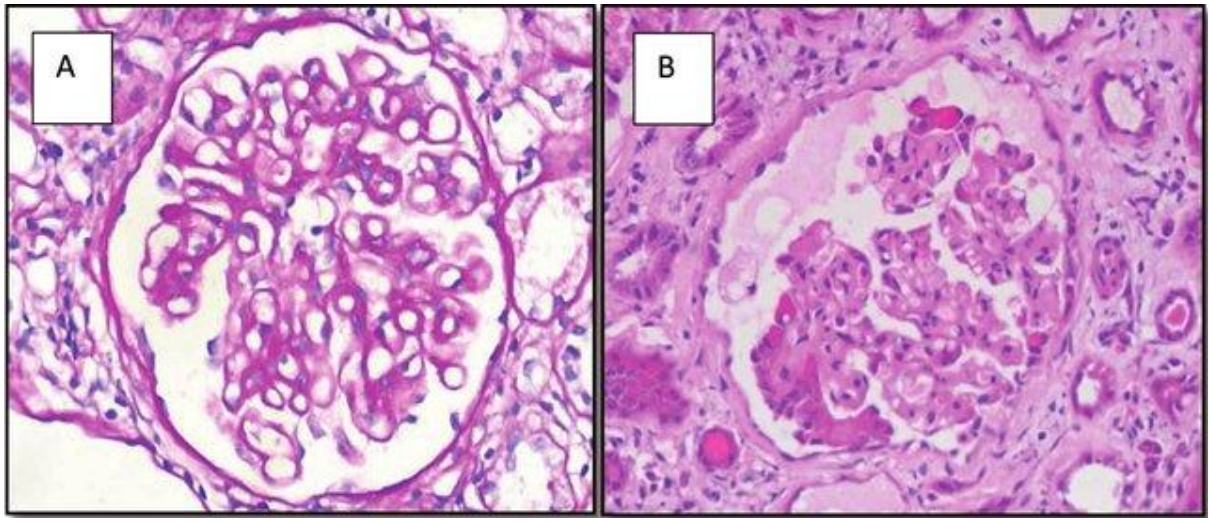


Figure 1.9. Normal glomerulus with patent capillary loops A vs. B: The collapsing form of focal segmental glomerulonephritis.
(Typical glomerular lesion observed in HIV-associated nephropathy) adopted from Wearne et al[144].

Between 1999 and 2003, the US renal data reported 90% of ESRD attributed to HIV infection in the black population[164]. In a study of a group of HIV-positive patients with CKD, 60% of renal biopsies had consistent features of FSGS[158]. A systematic review of nephropathies in South African HIV patients identified a 57.2% prevalence of HIVAN[165]. A combination of multiple mechanisms that include a direct viral cell injury and host susceptibility factors are linked to the development of HIVAN[166]. It is understood that the expression of viral DNA in parietal and tubular epithelial cells and its infestation of CD4 cells and macrophages have a role in the primary pathogenesis of HIVAN[167, 168] while cell apoptosis occurs secondary to dedifferentiation and proliferation of infected podocytes and tubular epithelial cells. In addition, the prognostic markers of progressive tissue injury have been linked to lymphocytic and macrophage infiltrations that cause interstitial inflammation[166] and interstitial tubular microcysts[143].

Host characteristics that favour HIVAN includes a low plasma $CD4^+$ cell count and high VL[169].

Individuals with these characteristics reportedly have a 4-fold increased risk of albuminuria[170]. These findings have remained consistent in a series of South African studies where HIVAN has widely been investigated HIVAN albeit with varying incidences[143, 159, 171, 172]. Although HIVAN has been common in black patients independent of their genetic predisposition[173, 174], evidence shows a strong predilection for a genetic predisposition of recessive variants of the Apolipoprotein L1 (*APOL1*) gene in patients of African descent[175-177]. A polymorphism G1 and G2 in *APOL1* chromosome 22 particularly in the black population is linked to HIV1-RNA expression on the renal epithelial cells resulting in dysregulation n of various cellular pathways involved in cell differentiation such as deactivation of redox-stress responses leading to the observed histopathology[157, 178-180].

Following the introduction of cART, many nephropathies are reported to improve[181]. while incidences of HIVAN have significantly declined[154]. However, some studies have observed that despite the clinical treatment of HIVAN, histological lesions do not retain the same response to treatment[182] and FSGS persist requiring renal replacement therapy[154].

1.5.2 Post-ART era: HIV Associated immune complex kidney disease.

While HIVAN remains common, observations show that it has been outnumbered by immune complex glomerulonephritis, diabetic nephropathy, and drug-induced toxicity in HIV patients in the cART era[142]. A study of features of kidney biopsies in cART experienced patients between 1997 and 2004 reported a 60% decline in features of FSGS in parallel with ART while a significant increase in non-collapsing FSGS renal pathologies by 22%, acute interstitial nephritis (AIN) by 8% and diabetic nephropathies by 5% was noted[158]. Other studies have also reported similar observations[142, 146]. These findings suggest a shift of the landscape of pre-ART HIVAN towards other forms of kidney diseases but without completely eradicating HIV

associated nephropathies[142]. The post-ART nephropathies are a group of disorders characterised by HIV associated immune complex kidneys disease (HIVICK) describing different glomerulopathies identified by glomerular immune complex deposition on immunostaining or electron microscopy of renal biopsies. These include HIV glomerulonephritis that includes post-infectious glomerulonephritis (PIGN), lupus-like glomerulonephritis, Immunoglobulin A nephropathy and renal disease associated with thrombotic microangiopathy (TMA)[159, 183, 184].

HIVICK is predominantly observed in patients with prolonged exposure to ART[143, 144]. Unlike HIVAN, patients with HIVICK are immunologically stable with a high CD4 count and higher eGFR[183, 185, 186], hepatitis C co-infection and a positive lupus serologic test[143, 187]. Generally, HIVICK is likely to progress to ESRD compared to HIVAN[151, 183]. On renal biopsy, distinct lesions show a histological pattern of "ball in cup", which is a large subepithelial deposit that is a direct consequence of HIV[143, 159] as well as non-collapsing forms of FSGS[143, 152, 188]. A multicentre study characterised renal pathologies in HIV patients who underwent biopsy; 92 (34.7%) had features characteristic of HIVICK (65 HIVICK and 27 IgA nephropathy) while 70 (26.4%) had HIVAN and 103 nephropathies of different pathologies[185]. Another study describes 58.8% of renal biopsies as HIVICK lesions in patients on ART[189].

The genetic predilection for HIVICK remains controversial. Earlier studies reported higher incidences in Caucasians[190, 191], however, a higher expression of the *APOL1* risk allele was later observed in African American patients with a combination of host characteristics[174]. Subsequent reports continue to show a high incidence of HIVICK in patients of African ethnicity[143, 159, 183, 192] although the genetic heterogeneity has been demonstrated by varying estimates of kidney disease among Africans[181, 193-197]. HIVICK is becoming an important renal

condition in the post-ART era that may result from a combination of mechanistic factors including continuous viral RNA multiplication characterised by increased T cell turnover and disposition of the HIV antigen-antibody complexes within the glomerulus plays a significant role in the pathogenesis of HIVICK[136, 139, 185, 198, 199]. It is not yet known why HIVICK does not benefit from ART exposure as observed with HIVAN; understanding the extent to which response to therapy differs between patients with HIVICK and those previously with HIVAN may help to further understand these outcomes.

1.6 Antiretroviral therapy-induced Nephropathies

Renal insufficiency appears to remain prevalent in HIV patients and changes in renal function occur throughout ART although these changes may not appear to be significant in the short term[200]. Antiretroviral therapy has been associated with 14% late-onset episodes of AKI and a CKD prevalence ranging from 2-30%[201]. Almost all antiviral agents can cause some degree of nephrotoxicity either directly by inducing acute tubular necrosis, acute interstitial nephritis, crystal nephropathy, and renal tubular disorders or indirectly via drug-drug interactions[202]. While it may be difficult to distinguish between ART-induced nephrotoxicity from complications of HIV infections or other non-HIV related renal diseases, several mechanisms have been described for the causal link of individual nephrotoxic agents not limited to ARVs[111, 196]. These include direct tubular toxicity, hypersensitive reactions and deposition of insoluble drug crystalline[111, 203, 204] and all the other drug factors as previously illustrated in figure 1.8.

In this section, I will give a brief review of common antiretroviral drugs (other than TDF which will be detailed later) that contribute to kidney injuries in patients with HIV infection.

1.6.3 Integrase Strand Transfer Inhibitors

Dolutegravir (DTG) is among the novel ARV agents whose renal effect has been assessed in clinical trials. There were no significant renal events to warrant drug withdrawal observed in a comparative study of dolutegravir and raltegravir, although the DTG arm recorded a small rise in SCr[204]. A similar increase in SCr with DTG arm was observed when compared against Atripla (TDF+FTC+EFV) without significantly affecting CrCl[205]. The mechanism behind these results is not fully understood and does not seem to be explained by the metabolic pathway of DTG or its excretion (< 1% of the drug is renally excreted). There are suggestions that this is an apparent benign, non-progressive, effect of DTG on SCr that causes a slight change in mean SCr (0.1-0.15 mg/dl) without affecting structural functions of the glomerulus or renal tubules in HIV patients on ART[206, 207]. It is also hypothesised that interference with creatinine uptake from the blood to the proximal renal tubules by inhibition of creatinine transporter, OCT2 may also play a role[205, 206]. Another concern has been the rise of SCr in regimens containing integrase inhibitors in combination with cobicistat (COBI). COBI is a pharmaco-enhancer with no antiviral activity but inhibits the metabolic cytochrome enzyme CYP3A to increase the concentration of ISTIs that are metabolised by CYP3A. COBI is also a potent inhibitor of MATE1 transporter on the apical membrane of the proximal tubule cells responsible for the efflux of creatinine into the urine[204]. In a comparative study of regimens containing cobicistat +elvitegravir + TDF+FTC with Atripla® and atazanavir/r +TDF+FTC, a significant increase in area under the curve (AUC) and peak serum concentration (Cmax) of TDF along with a stable rise in SCr was observed. However, this interaction was not linked to the involvement of MATE1 which is unlikely to affect TDF concentration[208], but the involvement of COBI on inhibiting the gut P-glycoprotein transporter on the absorption of TDF[204, 209]. P-glycoprotein is also an apical efflux transporter of TDF and its inhibition

increases the intestinal absorption and systemic exposure of TDF, which may trigger significant and progressive nephrotoxicity in patients with comorbid conditions or pre-existing renal impairment[204, 210].

1.6.4 Protease inhibitors (PIs)

PIs were initially associated with nephrotoxic effects characterised by nephrolithiasis secondary to deposits of insoluble crystalline drug in poorly hydrated kidneys[211]. Toxicity presenting as crystalluria has been reported in 20% of patients and 3% of those on indinavir progressed to nephrolithiasis while other manifestations of deposition of crystalline, dysuria, haematuria, renal atrophy, acute interstitial nephritis, and acute and chronic renal failure have also been reported[212-214]. The potential for atazanavir (ATV) to form deposits of insoluble crystal precipitates in the kidney tissue can progress to chronic renal impairment[215]. The risk is reported to be higher in patients with a history of urolithiasis, higher plasma drug concentrations, dehydration and alkaline urinary pH (pH>7)[216-219]. An incidence of 7.3-23.7 per 1000 person-years, particularly associated with dehydration, has been significantly associated with at least two years of ATV suggesting a cumulative dose-related effect[218].

In general, most PIs cause nephrotoxicity due to their interaction with several drugs that share or compete for their CYP450 metabolic or excretory pathway. Most PIs are substrates for *OCT*s because of their positively charged moieties, and, therefore competes for and inhibit the transport of cationic compounds causing toxicity[210]. On the other hand, ritonavir, lopinavir and atazanavir have been reported to inhibit efflux transport of TFV via the MRP*2* and MRP4 transporters. This interaction potentially increases tubular exposure to TFV leading to TDF-RT[220-222]. PIs have also been shown to be potent inhibiotrs of MDR1 Pgp, MRP and BCRP although they are poor substrates of other MRPs that may contribute to specific drug advese

effects and dru-dru interactions[223].

1.6.5 Non-Nucleoside Reverse Transcriptase Inhibitors

Very limited data is available associating NNRTIs with nephrotoxicity. Neither of these agents demonstrated adverse renal effects during preclinical trials. However, a few cases of obstructive crystalluria and urolithiasis have been described in patients on efavirenz (EFV) regimens which have also been linked to interstitial nephritis secondary to hypersensitive reactions[64, 224, 225]. A new generation NNRTI, rilpivirine (RPV), reported a stable increase in SCr and a decline in eGFR during clinical trials. This effect is thought to be secondary to inhibition of creatinine secretion via the *OCT2* in the proximal renal tubular cells[226] which is observed as a rise in SCr. In the ECHO study[227], an increase of baseline SCr by 0.064-0.10 mg/dl and a subsequent stable decline in eGFR of 8-11 ml/min/1.73m^2 were observed, similar results were also reported in the THRIVE study[44]. Both studies ruled out nephrotoxicity by RPV but attributed the clinically insignificant decline in eGFR to the transient rise in SCr due to inhibition of the *OCT*2 transporter[227].

1.6.6 Nucleoside and Nucleotide reverse transcriptase inhibitors

During clinical trials, both nucleoside and nucleotide reverse transcriptase inhibitors (NtRTIs) were rarely associated with a significant decline in renal function. However, in preclinical studies, NtRTIs reported induction of metabolic abnormalities that included hypokalaemia and hypomagnesemia as potential indicators of renal toxicity[203, 228, 229]. Intracellular NtRTIs have been implicated in clinical toxicities most of which are attributed to their non-specificity inhibition of mitochondrial (mt) DNA polymerase gamma (γ) in parallel with inhibiting reverse transcriptase enzyme. This leads to subsequent reduction in mtDNA replication resulting in

depletion of mtDNA cells and decline in various mitochondrial functions[111, 230]. The subsequent anaerobic respiration, lactic acid production and oxidative damage further leads to cell apoptosis and subsequent clinical symptoms[228, 230, 231]. With the widespread use of these agents in HIV positive patients, varying forms of kidney injury resulting from one or a combination of these mechanisms have been reported[111]. The above theory is backed by the evidence of hyperlactatemia induced lactic acidosis and AKI observed in 20-30% of HIV patients administered with nucleoside analogues particularly stavudine, didanosine and zidovudine[232, 233].

Although it is uncommon to observe NtRTI induced kidney toxicity outside the involvement of mitochondrial depletion, other forms of kidney injury may be influenced by the patient characteristics and their response to individual antiretroviral agents. Examples of such cases include carriers of the genetic variants such as HLA-B∗5701 that develop acute interstitial nephritis and renal failure secondary to abacavir induced hypersensitivity syndrome[234]. Other studies have reported abacavir-induced Fanconi syndrome of unknown cause[235].

Among all the NtRTIs, clinical reports have focussed mainly on TDF-induced renal toxicity (TDF-RT). Despite the reports of insignificant nephrotoxicity during early randomised controlled trials, considerable safety concerns regarding the cumulative nephrotoxic potential of TDF emerged given its structural similarity to the acyclic nucleotide phosphonates, adefovir and cidofovir (Figure 1.10) whose nephrotoxicity had been demonstrated[236]. Because of similarities in the pharmacokinetic profile and renal disposition, understanding their molecular mechanism and differences in observed tubular toxicity has been an area of research interest for a considerable period. Inconsistent reports regarding TDF-nephrotoxicity has characterised published studies; from an initial safety profile with lack of significant renal toxicity in

randomised controlled clinical trials[203, 237] to reports of wide-ranging forms of nephrotoxicity in various populations over the years[238-240].

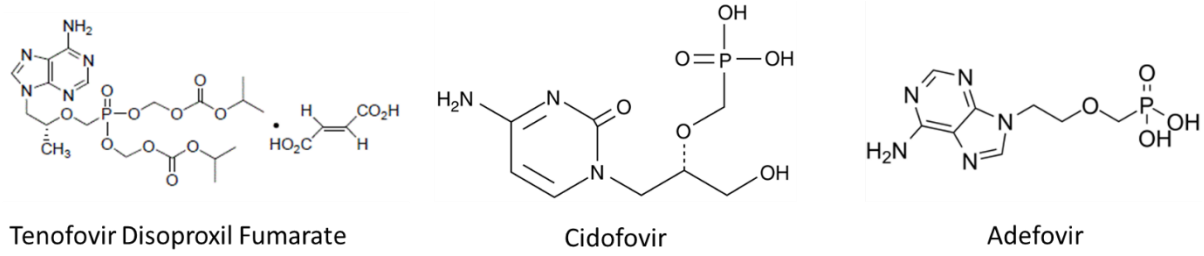


Figure 1.10. Structural similarities of tenofovir with acyclic nucleotide phosphonates adefovir and cidofovir

TDF is a known nephrotoxic antiretroviral drug that can cause direct tubular damage due to reduced tubular secretion or by mechanisms that can induce mitochondrial depletion[115, 239, 241, 242]. It has also been associated with nephrotoxicity secondary to genetic defects in tubular transporter proteins[210, 243]. In this present research, we focused on investigating TDF-induced renal toxicity (TDF-RT) in a cohort of HIV positive patients that were recruited from the University Teaching Hospital in Lusaka, Zambia. We will therefore present a more detailed review on the background of TDF-RT in the context of its pharmacology and the mechanisms involved. Furthermore, we will highlight the pharmacogenetic basis of TDF-RT and its role in urinary biomarkers that could potentially be useful as early predictors of TDF-RT.

1.7 Tenofovir Disoproxil Fumarate: Pharmacology and its association with TDF-RT

Tenofovir disoproxil fumarate (TDF) is a prodrug of tenofovir (TFV); an acyclic nucleotide diester analogue of adenosine monophosphate that inhibits the HIV DNA polymerase reverse transcriptase and the hepatitis B viruses[244, 245]. TDF requires dephosphorylation to its active antiviral metabolite. Following oral administration, TDF is rapidly converted to tenofovir by intestinal carboxylesterase. Intracellular phosphorylation by the adenyl kinase converts it to its monophosphate intermediate and rapidly dephosphorylated by the nucleoside diphosphate kinase to its active form TFV diphosphate. Dephosphorylation of TFV occurs in both active and resting cells[230]. The activated antiviral metabolite then acts as an HIV viral transcriptase inhibitor of HIV-1 replication in macrophages and other non-dividing cells by incorporating itself into the viral DNA to terminate the DNA chain[246]. Unlike other NRTIs, TFV weakly inhibits cellular DNA polymerase γ, a principal polymerase for mitochondrial synthesis[239, 247, 248]. Non-utilised tenofovir-diphosphate may be dephosphorylated to its inactive form by cellular phosphatase or transported out of the cell and excreted as unchanged tenofovir [246].

1.7.1 Pharmacokinetics of tenofovir

The bioavailability of TFV following a once-daily recommended adult oral dose of 300mg/day is 25%[246]. However, TFV is not subject to intracellular deamination or deglycosylation, this stability, therefore, results in prolonged systemic circulation that confers TFV a long intracellular half-life of 17 hours in activated lymphocytes and ≥60 hours in resting intracellular lymphocytes[230, 249, 250]. Furthermore, TFV has a low potential for drug-drug interactions because it is poorly protein-bound (<0.7%), and it is not metabolised by the hepatic cytochrome P450 isoenzymes via this metabolic pathway[246]. Tenofovir has shown excellent antiviral efficacy following persistent viral suppression observed in clinical studies long after drug

discontinuation[244, 251].

TFV is excreted unchanged via a combination of glomerular filtration and active tubular secretion mediated by several different drug transporters such as the hOAT1 and 3 uptake transporters and efflux transporters, MRP4[252], MRP7[253] and MRP8[254]. About 70-80% of the drug is excreted unchanged[246, 255] and therefore may require dose adjustments in patients with compromised renal function. A pharmacokinetic (PK) study of TDF in healthy subjects with varying degrees of renal impairment recommended 300mg every 48 hours for patients with CrCl 30–49 mL/minute[251] or 150 mg of TDF every 24 hours[256]. Further assessment is proposed when TDF is co-administered with drugs like lopinavir/ritonavir (LPV/r) that potentially interact with TDF and increase TDF levels by 32%[220, 245, 257].

The excellent pharmacokinetic and pharmacodynamic profile exhibited by TDF led to its U.S. Food and Drug Administration (FDA) approval as the first nucleotide reverse transcriptase inhibitor (NtRTI) in 2001. Since its approval, TDF has enjoyed worldwide acceptance and has been the most preferred first-line component of most antiretroviral therapy regimens.

1.7.2 Tenofovir-induced renal toxicity in HIV infected patients.

TDF demonstrated a favourable renal safety profile in preclinical[246, 258] and early observational studies[203, 259-262] and these findings remained consistent with post-market consumer-based data[263]. The initial preliminary safety reports were part of Study 902 from Gilead Sciences, Inc. trials in which HIV patients were randomly assigned to different TDF dosages (75mg, 150mg and 300mg/day) and placebo for 48 weeks and found insignificant elevations of SCr across the different treatment groups during the study period[260]. It is noteworthy that insignificant elevation of tubular injury marker, phosphorus, was also observed but was not clinically

significant to warrant any concerns[260, 264]. However, after TDF approval, isolated cases of potentially serious and dose-limiting nephrotoxicity were reported[265-269]. Following the nephrotoxicity associated with other antiviral acyclic nucleotide phosphonates, concerns about the potential for tenofovir-induced renal toxicity have been expressed and widely investigated[239, 270]. Like tenofovir, these antivirals share the same elimination pathway[246, 270] and their potential for nephrotoxicity due to tubular accumulation has been demonstrated[236]. However, during experimental studies, tenofovir exhibited low intracellular accumulation and cytotoxicity in isolated proximal tubular cells[271] and these findings correlated with earlier observed mild and insignificant renal toxicity in early clinical trials. Following the growing clinical experience with TDF in a real clinical setting of patients with various comorbidities and different characteristics, an increase in case reports of nephrotoxicity manifested as ATN, Fanconi syndrome (FS), and rare cases of nephrogenic diabetes insipidus[272] consistent with observations in adefovir and cidofovir studies[273] have been observed. With these reports, the earlier held views on TDF renal safety profile came under scrutiny and concerns surrounding TDF nephrotoxicity following chronic exposure in ART has been a research interest in the past two decades. Figure 1.11 illustrates the TDF pathway from administration, distribution, metabolism and excretion (ADME)

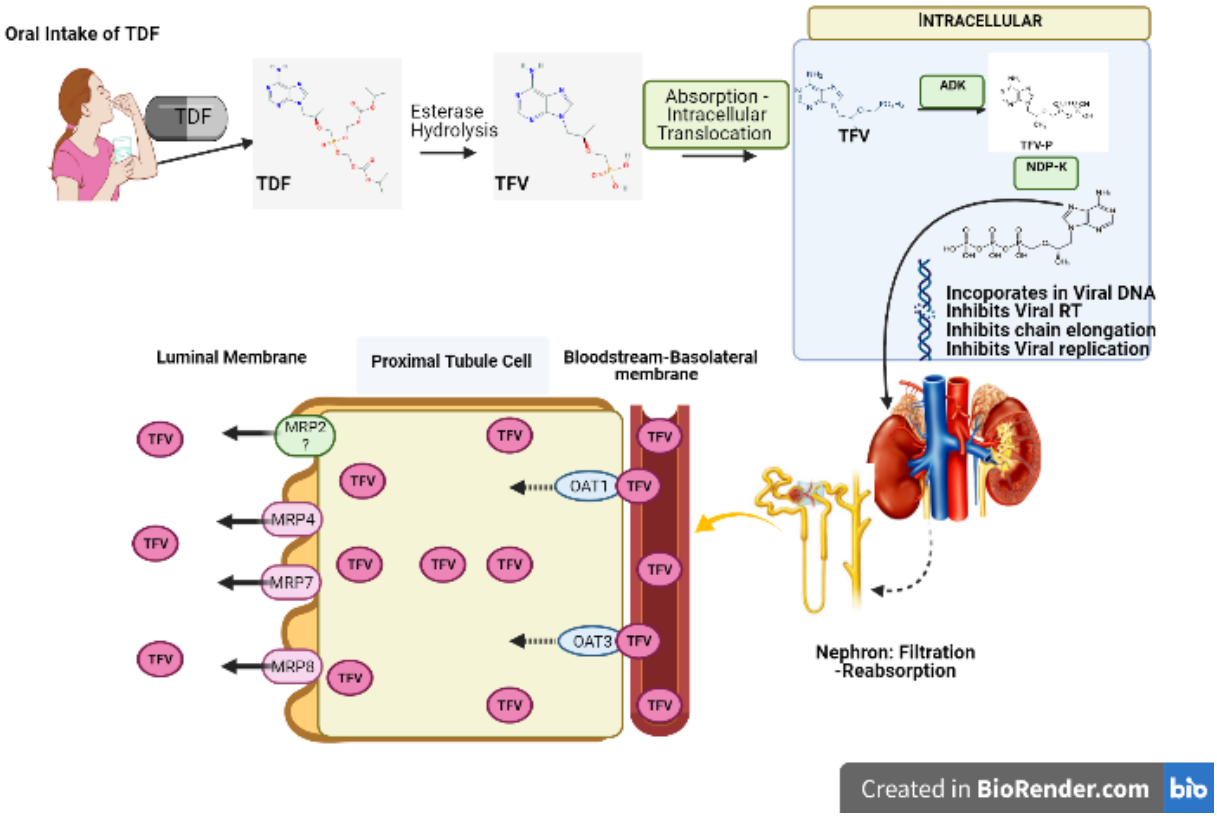


Figure 1.11. The activation and excretion pathway of TFV:
TDF esterase hydrolysis and a two-step adenyl kinase phosphorylation converting it to the active antiviral TFV-DP which inhibits the viral reverse transcriptase enzyme. The excretory pathway of TFV via the proximal tubular epithelial cell involves tubular uptake by the organic anionic transporters 1 and 3 and tubular efflux by the MRP4, MRP7 and MRP unconfirmed mechanism by MRP2.

In the following section, we briefly review the published evidence of TDF-RT as reported by various researchers.

1.7.3 TDF-induced renal toxicity: Review of the evidence.

TDF-RT in patients with HIV remains one of the most challenging issues in clinical practice with studies reporting 15% incidences of renal abnormalities during a 2-9 years study period[274]. A systematic review and meta-analysis of 17 studies reported that HIV patients exposed to TDF were associated with a modest but significant decline in glomerular function which

correlated with a higher risk of acute kidney injury[238]. Another systematic review constituting cohorts from African studies of patients exposed to TDF for a period ranging from baseline up to a maximum of nine years reported a significant association between TDF and a decline in renal function[240]. Based on these reports, TDF-RT may manifest at any time point during tenofovir therapy. Researcher Andrew Hall[275], a nephrologist from the Centre of Nephrology, University College London, reported that an internal audit of nephrologist specialist consultations showed that TDF-RT was the common reason for specialist consultations accounting for at least 20% of all consultation[275].

The exact pattern of TDF nephrotoxicity (clinical phenotype) and the mechanism of kidney involvement remains poorly understood. Proximal tubulopathy and FS have been widely reported[237, 239, 242, 261, 273]. In addition, reports of impaired glomerular function presenting as AKI, CKD or different descriptions of a decline in CrCl or estimated glomerular filtration rate (eGFR) from baseline have also been documented[239, 240, 276]. Literature suggests that all forms of TDF-RT share a common pathogenesis and pathology, but the varied range of phenotype descriptions associated with TDF use confirms the inconsistency in the understanding of TDF-RT. Moreover, the lack of a standardised and specific diagnostic method to accurately measure TDF-RT may be another challenge that further makes it impossible to reliably compare case by case and study by study reports of TDF-RT.

1.7.3.1 **Effect of TDF on Serum creatinine and Glomerular function**

Several studies have investigated the relationship between TDF exposure with CrCl and eGFR estimated by the Cockcroft Gault (CG) and Modification of Diet in Renal Disease (MDRD) methods respectively. The formulas for the two estimations are thus given below.

The Cockcroft Gault formula for the estimation of CrCl

$$CrCl = \left\{\frac{(140 - age) \times weight(kg)}{72 \times SCr}\right\} \times 0.85(if\ female)$$

Note: CrCl = ml/min, Age =years, SCr = mg/dL .

MDRD formula for the estimation of GFR

$$GFR = 175\ X\ SCr^{-1.154} \times Age^{-0.203} \times 1.212\ (patients\ if\ black) \times 0.742\ (if\ female)$$

Note: GFR=ml/min per 1.73 m^2

These formulas have acknowledged weaknesses because they rely on SCr which is not a specific marker for renal injury[277-280]. In addition to glomerular filtration, tubular secretion is responsible for 10-40 % of creatinine renal clearance. Clinical studies in patients receiving TDF based ART have reported a decline in CrCl or eGFR associated with TDF use in the first three months of therapy without further decline with continuing TDF use[281-283]. These temporal changes and variations have been attributed to impaired tubular secretion of creatinine rather than glomerular function[284]. However, in a 17-study systematic review, a small but significant reduction in kidney function compared with controls (eGFRs, 3.9 mL/min; 95%CI, 2.1-5.7) was reported; however, a large statistical heterogeneity attributed to a wide variation in study design and demographics of patients was acknowledged by the authors[238].

Characterisation of the safety profile of TDF in a cohort of over 10,000 patients observed that only 2.2% and 0.6% of patients reported an increase in SCr levels of ≥0.5 or ≥2 mg/dL respectively[261]. It has been argued these low incidences of TDF-RT in early studies were attributed to a selection of thoroughly screened participants in clinical trials with strict

exclusion criteria and also the use of SCr, a poor surrogate estimate of renal function[285]. However, the multinational D.A.D study of almost 50,000 HIV infected patients reported that in 22,603 patients treated with TDF with normal baseline serum creatinine levels, 2.1% experienced ≤ a 70ml/min decline in eGFR while 0.6% progressed to CKD during a median follow-up of 4.5 years[189]. A similar study reported a significant but modest reduction in CrCl (5.7 vs 2.6mg/dL) in TDF-treated versus TDF-sparing regimens[286]. Elsewhere, TDF treated patients experienced an 18.1% increase in SCr and a 4.8% decline in eGFR compared to a 1.8% increase in SCr and 5.1% gain in eGFR observed in TDF-sparing controls[287]. Studies reporting TDF-RT using CrCl or eGFR need a cautious interpretation that takes into account the physiology of SCr and the possible inter-individual variations.

Progressive TDF-RT is widely observed while severe cases may be uncommon. Nartey and colleagues reported a 21.0% (95% CI 6.5–26.1) incidence in renal impairment (CrCl<50mL/min) of varying degrees with 18.3% showing moderate and 2.3% with severe impairment[288]. The DART trial; an observational study of a sub-Saharan African cohort (Zimbabwe and Uganda) of treatment naïve HIV infected patients reported a severe and progressive decline in renal function (<30–60ml/min) after 96 weeks of TDF treatment compared to none-TDF regimens. In this cohort, mild (≥60ml/min , <90 mL/min/1.73 m2) and moderate (≥30 but <60 mL/min/1.73 m2) incidences of renal dysfunction was observed in 45% and 7% of patients respectively[196]. Several conceptually similar studies in African populations from Senegal[289], Ghana[290] and South Africa [291-293] have reported varying severities of glomerular dysfunction in HIV patients treated with TDF-based ART.

1.7.3.2 **Effect of TDF on Renal Tubular cells**

Based on existing evidence, TDF-RT may manifest as proximal tubular dysfunction with or without evidence of glomerular dysfunction[275, 294, 295]. Moreover, it is noteworthy that estimating GFR that rely on measurements of serum creatinine levels, a marker filtered by the glomerulus and secreted across the proximal tubule may vary (appear normal or low) in the presence of proximal tubular dysfunction. In a multinational randomised trial of HIV treatment-naïve patients initiated on d4T/3TC/EFV and TDF/3TC/EFV, both arms reported an increase in CrCl by 18.1 mL/min and 14.2 mL/min respectively. However, a greater loss of hip bone density was observed with TDF containing regimen suggestive of an insidious but progressive phosphate wasting, a sensitive marker of tubular dysfunction[296]. Moreover, Labarga et al[294] reported an association between TDF and tubular impairment in the absence of evident glomerular dysfunction[294]. Tubulopathy has also been reported in case studies of patients with normal baseline renal function, before commencing TDF based ART[297].

TFV-induced tubulopathy is typically observed as increased secretion of tubular proteins, hyperphosphaturia/hypophosphataemia, glycosuria and metabolic acidosis[273]. While TDF-induced tubulopathy may manifest as AKI (0.7–10% of patients), studies have reported a persistent subclinical and undiagnostic proximal tubular impairment in 22-81% of patients receiving TDF[295, 298]. In a comparative, longitudinal prospective study of treatment naïve HIV-positive patients, PTD was reported in 6% and 9% at 12 and 24 weeks respectively[299]. In a similar prospective cohort, the incidence of hypophosphatemia (2.0–2.4 mg/dL) was reported in 12.7% vs 6.7% outpatients on TDF based treatment compared to other regimens respectively[300]. Many studies have shown a correlation between TDF use with markers of progressive kidney injury leading to CKD. Heavy proteinuria (a marker of CKD) of

≥130mg/day of tubular origin was reported with the use of TDF based cART[301]. Elsewhere, measuring urine protein ratios (protein/creatinine (uPCR), albumin creatinine (uACR) and albumin/protein (uAPR)) has been suggested to detect and manage early CKD in HIV-infected patients presenting with normal or sub-normal eGFR[302]. In a cross-sectional study of ambulatory HIV positive adults in Montpellier in France, measurements of spot uACR ≥30mg/g or uPCR ≥ 200mg/g with uAPR ≥ 0.4 defined glomerular dysfunction while ≥ 200mg/g uPCR or with uAPR < 0.4 confirmed tubulopathy. In this cohort, 18.2% patients with eGFR ≥ 60ml/min per 1.73m2 recorded proteinuria and 50.7% of these consisted tubular impairment. Current use of tenofovir [OR 3.52 (95%CI: 1.86–6.65)] in the presence of other risk factors was also associated with proteinuria[302].

1.7.3.3 **TDF-induced Fanconi syndrome**

TDF has been implicated in the development of FS[273, 295, 303], a very rare, but the most severe form of proximal tubulopathy characterised by a global breakdown in the proximal tubular solute transport system rather than an isolated problem due to a specific transporter defect[118, 304]. In FS, tubular reabsorption of substances is impaired and may manifest as hypophosphataemia, low molecular weight proteins (LMWPs) wasting, mild metabolic acidosis, normoglycaemic glycosuria, amino acids, hypouricemia and/or amino-aciduria[118, 263] and some degree of electrolyte imbalance[118]. While some patients may only present with a milder tubulopathy, phosphate wasting is the most significant clinical feature which if not managed, may lead to complications of bone mineral disorders such as osteomalacia and rickets after several months or years of undetected hypophosphataemia[118, 305].

The actual incidence of FS is unknown and often underestimated, largely due to a lack of standardized definitions; however, features of FS have been reported in 22 - 53% of clinically

asymptomatic HIV patients treated with TDF based ART[294, 306]. Although many patients present with milder tubular defects[118, 295], severe TDF-induced FS (TDF-FS) is estimated to occur in <1% of patients[275, 295]. Diagnostically, sequential development of generalised tubulopathy following exposure to TDF may be suggestive of FS while complications of TDF-FS may occur after several months or years of exposure[307, 308]. Therefore, a combination of the biochemical data and clinical manifestations may lead to a diagnosis of TDF-FS[309]. While glomerular function may be preserved in some patients[310], mitochondrial cytopathy is the main presentation on biopsy following TDF-FS toxicity[311] evidenced by acute tubular necrosis with eosinophilic intracytoplasmic inclusions in proximal tubular cells and large-irregular mitochondrial structures[275, 310, 312]. Figure 1.12 shows a biopsy image with distinct features of tubular necrosis.

TDF-FS has been described in many single case studies and a small cohort of HIV infected patients[269, 305, 307, 313]. In the FDA Adverse Event Reporting System, 164 cases met the FS definitions criteria; the largest TDF-FS cohort ever reported[263]. In some settings, patients receiving TDF based ART only present with bone disorders associated with hypophosphataemia and FS is determined incidentally[308, 309]. The majority of FS cases are characterised by the use of ritonavir-boosted protease inhibitors (83%), particularly lopinavir/ritonavir (22%) alone or combined with Didanosine in 55 (34%) and 24% of concomitant users of trimethoprim/sulfamethoxazole for opportunistic infection prophylaxis[263]. The interaction of PIs with TFV renal transporters leading to increased plasma and intracellular tenofovir concentration with consequent toxicity has been described in the previous section. Although tubular function recovers within weeks to months of TDF withdrawal, partial recovery with residue toxicity and proteinuria may persist[314] while complications of bone mineral disorders may continue[263]. of nucleotides with a potential of

mediating their accumulation that may lead to nephrotoxicity. However, TDF-RT may result from an interaction of several factors that may include dose-dependent tissue accumulation, functional alteration in renal transporter proteins[210, 315-317], drug-drug interactions[107, 153, 245] as well as patient-specific factors[107, 239, 274, 288].

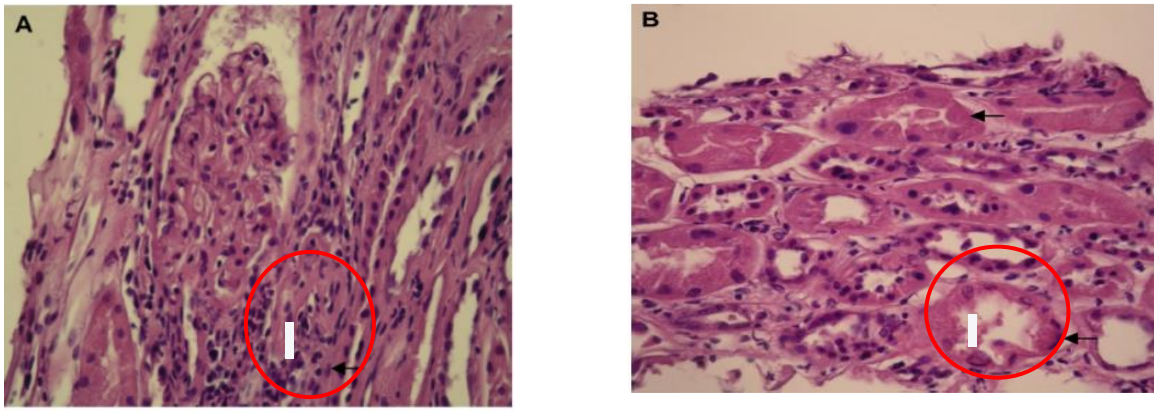


Figure 1.12. Distinguishing features of acute tubular necrosis.
A renal biopsy specimen showing normal glomeruli (A). The arrow shows acute tubular necrosis and interstitial inflammation. (B): Eosinophilic intracytoplasmic inclusions (arrow) are seen within the proximal tubular epithelial cells. Image adapted from Tsai et al.[310].

1.7.4 Factors associated with tenofovir-induced renal toxicity

1.7.4.1 The influence of Dose, Concentration and Distribution in RT

Cumulative tenofovir plasma concentration has been associated with incidences of TDF-RT[318]. Moreover, the influence of drug Pk on alterations of renal function and toxicity has been described in TDF-RT[319]. TFV-RT is dependent on successive and cumulative plasma drug concentration which is also dependent on other Pk factors like the dose, the volume of distribution (body mass), and the rate of renal clearance. Clinical studies have demonstrated that TFV exposure is independently associated with a high risk of TDF-RT[320-322] and patients may benefit from dose adjustment[251, 256]. A study showed that after multivariable adjustment,

cumulative exposure to TFV increased the risk of developing proteinuria[320]. Furthermore, the distribution of TFV across the tissue is dependent on body mass, vascular permeability, cardiac output and regional blood flow (perfusion rate). Thus, low body weight [261, 298, 323] and cardiovascular disorders like hypertensive and dyslipidaemia [321, 322, 324] have been identified as independent risk factors for TDF-RT. In addition, obesity, pre-existing renal and diabetes mellitus impairment may indirectly affect drug distribution by negatively impacting renal blood flow and glomerular filtration[261, 293, 325, 326].

1.7.4.2 **The role of Transporter Proteins in tenofovir elimination and TDF-RT.**

The uptake of TFV via basolateral brush membrane into PT has been demonstrated to be predominantly via hOAT1 and to a lesser extent hOAT3[72, 210, 271, 327-330]. The affinity of TFV for anionic transporter proteins is given by the negatively charged phosphate group in its structure at physiologic pH[246]. Although hOAT3 encoded by the *SCL22A8* gene is highly expressed in the kidneys than hOAT1 encoded by the *SCLC22A6* gene, tenofovir has > 20-fold higher affinity for *SLC22A6* transporter[271]. The high expression-lesser affinity of hOAT3 in the influx of TFV has been linked to a parallel low affinity-high capacity influx pathway for TFV[252, 329, 331]. Overexpression of the hOAT1 has been associated with tubular impairment and decreased GFR and while kidney tissue with hOAT1 knocked cells retained proximal tubular mtDNA abundance suggesting that dysfunctional hOAT1could prevent TDF accumulation and toxicity[257]. In addition, transporter-mediated drug-drug interactions may dramatically influence the tenofovir elimination rate and affect intracellular concentration and toxicity[210].

Conversely, TFV exits the PT into the lumen via the substrate-specific multidrug-resistant protein (MRP) transporter family expressed on the apical membrane of renal proximal tubule cells[210, 332]. Until recently, MRP isoforms MRP2, MRP4 and MRP7 encoded by *ABCC2*,

ABCC4 and *ABCC10* genes respectively were the only known mediators of TFV efflux, however, a recent study reported that TFV was a novel substrate of MRP8 encoded by *ABCC11*[254].

There are conflicting findings on whether TFV is a substrate of MRP2 (*ABCC2*) transporter because earlier independent studies failed to demonstrate this. Imaoka et al [315], multiple *in vitro* studies by Ray's group[252] and a recent *in vitro* transport assay[333] did not observe any ATP-dependent uptake of tenofovir in cell membranes expressing MRP2 in tubular tissue. Nevertheless, clinical pharmacogenetic studies have identified genetic variants in the *ABCC2* associated with TDF-RT[334, 335]. However, these pharmacogenetic studies suggest an apparent hypothesis of MRP2's role in TFV efflux albeit through an indirect yet to be identified MRP2 linked pathway that could in parallel, mediate TFV efflux from the tubular cells[336, 337].

The role of MRP4 (*ABCC4*) in regulating TFV concentration has been well characterised in renal proximal tubular cells expressing MRP4 in the apical membrane[257, 338]. Moreover, experimental studies have demonstrated a low accumulation of tenofovir in MRP4 expressing cells, but when its activity is inhibited, TFV levels begin to rise to confirm MRP4-mediated efflux of TFV into the urine[252]. Similar studies have confirmed the substrate specificity of TFV for MRP4[220, 315]. On the other hand, single nucleotide polymorphisms in the *ABCC4* gene was associated with high TFV concentration and renal toxicity[257]. These findings have consistently been backed by evidence in pharmacogenetic studies where polymorphisms in the *ABCC4* gene may reduce tenofovir elimination from tubular cells and is therefore postulated to result in tubular impairment observed in patients on TDF based ART[334, 339, 340].

The MRP7 encoded by the gene, *ABCC10* has functional similarities with other MRPs and it was reported as a TFV efflux transporter from an experimental study[253]. The *ABCC10* gene is

universally expressed in more than 40 human tissues but highly expressed in the kidney, colon and brain and has nucleotide phosphates binding sites[210]. Pushpakom et al.[253] demonstrated a significant low accumulation of TFV in *ABCC10* transfected HEK293 cell lines and, substrate specificity was demonstrated in the presence of cepharanthine, an inhibitor of MRP7. Parental HEK293 cell lines demonstrated an increase in TFV accumulation similar to what was observed in *ABCC10* knockdown cells. The study further demonstrated that patients with variants in the *ABCC10* may exhibit poor renal tubular transport of TFV and contribute to tubular toxicity[253]. The presence of allelic variants in *ABCC10* was reported in two case reports of HIV-infected patients presenting with tubular dysfunction following TDF administration further confirming the role of *ABCC10* in TDF transport and toxicity[297]. Recently, MRP8 transporter protein (*ABCC11*), localised in the proximal tubular cells became the latest novel tubular efflux mechanism of TFV which was demonstrated in MRP8 overexpressing cells where a 50% cytotoxic concentration of TDF was nearly 5 times higher than that of parental cells. However, a high intracellular TFV level in MRP8-overexpressing cells was correlated with 55 times lower accumulation than in parental cells and this was partly reversed by an inhibitor, MK-571[254].

Other transporters responsible for TFV disposition include the P-Glycoprotein (Pgp), encoded by *ABCB1* and also *ABCB2*, both of which are found in humans[341]. Both genes are widely expressed in many luminal cell membranes[210, 307]. *ABCB1* has a broad substrate specificity of particularly hydrophobic neutral or cationic compounds[342] and plays a vital role in regulating the tissue absorption of potentially toxic compounds. Unlike, *ABCB1*, *ABCB2* substrates include hydrophilic organic anions and contributes to renal excretion of some drugs[341, 343]. It is hypothesised that their role in transporting a wide range of xenobiotic compounds may potentially represent an alternative pathway for TFV elimination[243, 252, 344]. The prodrug, TDF

and not TFV-diphosphate is a substrate to both *ABCB1* and *ABCB2*, and they facilitate intestinal TDF absorption and disposition[333], but not in the kidneys[252] because the parent drug is not available at the blood-kidney basolateral membrane due to its rapid (<1 min) esterase hydrolysis. Moreover *in vitro* and *in vivo* studies have failed to show a significant role of *ABCB1* and *ABCG2* in the tubular transport of TFV[252, 333].

Other studies have suggested that efflux transporters of cationic substrates such as *SLC47A1* and 2, also known as multidrug and toxin extrusion (MATE) transporters may play a role in the efflux of TDF[210]. Both *SLC47A1* and *SLC47A2* are highly expressed and localised to the apical membrane of the renal proximal tubular cells. Many of the substrates and inhibitors of SLC47 transporters overlap with those of *SLC22A1*, *SLC22A2*, and *SLC22A3* and they cooperate to control the concentration of several substrates within the proximal tubule cells, such as creatinine[210].

1.7.4.3 **The role for drug-drug interactions in TDF-induced renal toxicity**

It has been observed that of all the published cases relating to TDF-RT, nearly 70% involve co-administration of low-dose ritonavir in some regimens[225, 345]. This significant interaction is explained by the co-administration of ritonavir which is often given as a PI enhancer to inhibit the cytochrome P450 3A4 (CYP3A4) metabolising enzyme[245, 346]. Studies have shown an increase of TFV plasma concentration by 37% with atazanavir, 32% with lopinavir, 22% with darunavir and 14% with saquinavir[246, 347, 348]. Others have shown an increase in plasma exposure of TFV by 25–35% with ATV and LPV/r[245]. In other studies, a reduction in tenofovir clearance by 17.5% in patients receiving TDF+ PI/r compared to those on non-PI combinations has been reported[345]. This interaction is explained by *in vitro* inhibitors of transporters *SLC22A8* (hOAT3) and *ABCC4* (MRP4) by ritonavir and lopinavir by a magnitude of 62% and

37% respectively[220]. An interaction of PIs with efflux transporters, Pgp and MRP2[349, 350] equally alters the TFV efflux system and may contribute to renal toxicity[153, 336, 351]. Based on these findings, it is plausible to suggest a poor TFV disposition in patients with concomitant use of PI/r. In the case of MRP2, its exact role remains to be confirmed. However, an alternative pathway suggests a yet to be identified cofactor that is excreted by MRP2 may be responsible for TFV excretion and toxicity or competes for TDF excretion at MRP4[275]. In other studies, findings show that although PIs are poor substrates of ABC transporters, they are potent inhibitors of MDR1, P-gp, and MRP1 and may contribute to drug-drug interactions leading to adverse events, particularly ritonavir and lopinavir[341].

Apart from antiviral drug interactions involving transporter mechanisms, commonly prescribed non-antiviral drugs for comorbidity management may enhance TDF-RT through different mechanisms. This has been well illustrated under mechanisms of drug-induced nephrotoxicity in Figure 1.8 under subsection 1.5, subheading "Kidney Disease: Definition and Classification". Some drugs may interact with TFV by affecting transporter protein function, compete for elimination or induce toxicity through their known pharmacological actions[222, 242]. At transporter level, substrates of the hOAT 1 and 3 (diuretics, NSAIDs and some β-lactam antibiotics and other antivirals) may compete for uptake and increase TFV systemic exposure while efflux transporter inhibitors (NSAIDs, salicylates and furosemide) or substrates (methotrexate, cisplatin) will cause TFV tubular accumulation leading to TDF-RT[222, 352]. In addition, NSAIDs have the potential to increase TDF renal toxic effects by inhibiting prostaglandins and renal flow which may reduce the GFR and therefore clearance of TDF[353]. Concomitantly administered drugs may also potentiate TFV tubular cytotoxicity by direct mitochondrial damage or oxidative stress[111, 114, 242, 354-356] while others may cause nephrotoxicity through multiple mechanisms[353, 357, 358]. Other nephrotoxic drugs can trigger

inflammation in the glomerular, tubular and surrounding cellular matrix that can alter normal kidney functions and induce toxic glomerulonephritis, acute and chronic interstitial nephritis[120, 122, 128]. Drugs like NSAIDs, rifampicin, calcineurin inhibitors and some anticancer drugs cause a varying degree of interstitial nephritis that may precipitate TFV disposition and toxicity[111, 359]. Formation of dose- and urine-pH dependent precipitates that form insoluble crystals by some drugs can significantly contribute to crystal nephropathy with features of nephrolithiasis and crystalluria[112, 117]. In other settings, statins can break down skeletal muscle and release muscle fibre in circulation through a condition known as rhabdomyolysis; the released muscular myoglobin and creatine kinase into the blood can potentially damage or reduce glomerular function resulting in acute tubular necrosis or renal failure[114, 121, 122]. In addition, thrombotic microangiopathy is a condition that may occur following kidney damage secondary to inflammation or direct renal tissue toxicity[114, 122, 360, 361]. Concomitant prescription of these drugs should be assessed in patients at high risk particularly those with multiple mechanisms of nephrotoxicity. More drug-drug interactions that can potentiate renal toxicity can be checked on the University of Liverpool HIV drug interaction checker website (https://www.hiv-druginteractions.org).

1.7.4.4 **The role of Clinical and host factors**

An association between immunologic factors (CD4 count and viral Load), comorbidities and wide-ranging patient characteristics with TDF-RT have been reported[322]. Severely suppressed patients with CD4 count <200 cells and high HIV RNA are at higher risk of TDF-RT[261, 293, 325].

Comorbidities such as hepatitis C coinfection, through hepato-renal pathophysiology and hypertension, pre-existing renal impairment can affect cardiac out and renal blood flow. In addition, metabolic disorders diabetes and obesity are independent risk factors through diabetes

nephropathy and vascular resistance, respectively. Renal function declines with age, which may explain why increased age is also a risk factor for TFV[261, 322, 362, 363]. In patients established on cART with stable renal function, TFV toxicity can subsequently occur if renal function rapidly deteriorates due to other unrelated reasons like septicaemia, hypotension shock, or toxicity from another drug[364]. Many studies have reported at least one or a combination of advanced age, duration of TDF treatment, a low body weight, obesity, elevated baseline creatinine levels, pre-existing renal impairment[293, 322, 325, 365]. Besides, traditional risk factors for kidney disease in the general population also impact greatly on TDF potential for renal toxicity in HIV^+ patients[261, 293, 325, 326]. Most of these risk factors may be present in isolation or combination. In a single-centre cohort of Japanese HIV positive patients, comorbidities, advanced age, immunosuppression, high baseline eGFR, low serum creatinine and concurrent use of nephrotoxic agents were identified as determinants of TDF-RT[323]. Elsewhere, pre-existing renal disease (eGFR <60ml/min), hepatitis C coinfection, and metabolic disorders[365-367] and low body weight, advanced age, diabetes and a lower CD4+ cell count[282] were significantly associated with TDF-and a decline in eGFR respectively.

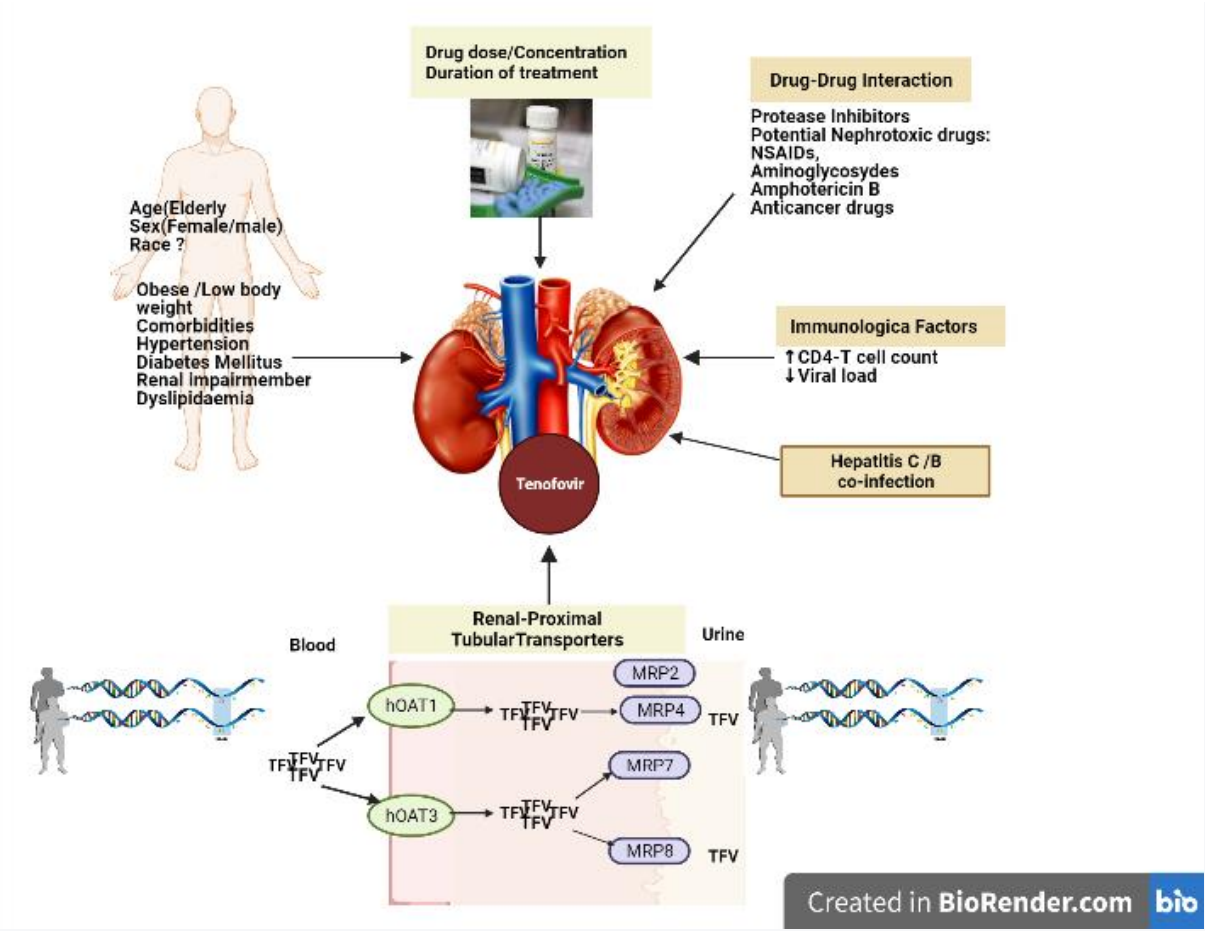


Figure 1.13. Summary illustration of determinants of tenofovir induced nephrotoxicity: Patients factors, drug interactions, comorbidities.

1.7.4.5 **Mitochondrial Toxicity.**

Mitochondrial DNA (mtDNA) variations including single nucleotide polymorphisms (SNPs) have been proposed to be involved in idiosyncratic drug reactions. Mitochondria are vital players in the development of drug-induced toxicity owing to their indispensable role in cellular bioenergy synthesis[368]. This is because abnormalities in mitochondrial DNA (mtDNA) may cause clinically significant effects that may seem silent in the beginning but may predispose individuals to idiosyncratic drug responses when exposed to potentially nephrotoxic drugs[368].

Several drug-induced injuries have been associated with dysfunctional mtDNA which results in rapid depletion of mitochondria and loss of function[368]. mtDNA encodes thirteen proteins by initially encoding two important RNAs and 22 tRNAs which are necessary for the synthesis of the proteins[369]. The proteins are subunits of the complexes involved in oxidative phosphorylation, a very important process during ATP production. Therefore, a genetic mutation in the mtDNA encodes dysfunctional genes leading to the development of serious disorders such as mitochondrial encephalomyopathy with lactic acidosis[370]. While polymorphism in mtDNA may be present without causing significant effect, However, its persistency may in the long-term lead to a decline in cell respiratory capacity[371]. In addition, If SNPs in mtDNA accumulates, this may be worsened by drugs whose effect is likely to affect mtDNA function and subsequently lead to a clinically significant mitochondria function impairment. This is the case of TDF which, on one hand, depends on mt ATP production for its transporter-mediated excretion, but on the other hand, inhibit mt polymerase responsible for mt synthesis[372].

The kidney, particularly PT are enriched with mitochondrial as their primary source of energy to accomplish their function of the acid-base regulation, reabsorption and excretion of metabolites and nutrients. Whereas mitochondrial ATP is energy-dependent derived from fatty acid oxidation (FAO), systolic ATP is anaerobic and dependent on glycolysis (carbohydrate oxidation). Thus, the primary source of energy from mitochondrial abundant PTs and distal tubules is from β-oxidation of fatty acids in the mt cell. Unlike PT, other segments of the tubules like the loop of Henle rely on anaerobic glycolysis for energy production.

Mitochondria are also generators of reactive oxygen species (ROS) which may be toxic to the cells including mitochondrial cells at high concentrations. Thus, mitochondrial toxicity may

result from multiple mechanisms, mutations in mtDNA or drug inhibition of mtDNA that lead to the production of defective mitochondrial that becomes permeable to low molecular weight proteins leading to deficiency in *ATP* synthesis, the unopposed effect of ROS, mtDNA depletion, and inhibition of cell respiration (apoptotic cell death)[373]. On the other hand, the abundance of drug transporters on tubular membrane also exposes mitochondrial to toxic drug concentration and, high mitochondrial demand, oxidative stress and tubular cell death.

1.8 Mechanisms of TDF-RT

1.8.1 Tenofovir effect on Mitochondria

Published *in-vitro* studies, case reports and observational studies support the that PT cells are the main target of TDF-RT because of the abundance of membrane transporters in PT that mediate the uptake and efflux of TDF[269, 374-376]. While the exact mechanism of TDF tubular injury remains unclear, two mechanisms have been suggested. The first one is associated with mitochondrial toxicity resulting from additive or synergistic effects of multiple factors[229, 239, 257, 377, 378]. The second proposed mechanism of TDF-RT involves intracellular accumulation of TFV and factors contributing to increased intracellular concentration, slower renal clearance and tubular accumulation have been detailed under sections describing the PK factors and transporters' role in TDF-RT.

Experimental studies have shown that the proximal renal tubule is susceptible to mitochondrial damage resulting from limited anaerobic energy-generating capacity[377]. Moreover, both clinical and animal studies have demonstrated mitochondrial depletion and damage associated with TFV treatment[242, 377, 379]. One way for mtDNA depletion is through the cumulative inhibition of mtDNA polymerase γ, the sole polymerase for mtDNA replication[246], leading to

mitochondrial depletion[239, 246, 247]. In an HIV transgenic model, a decrease in mtDNA abundance in renal proximal tubules was observed following TDF treatment while histological analysis showed an increase in irregular mitochondrial morphology with sparse fragmented cristae[377]. Another study confirmed the TDF effect on renal mtDNA depletion and respiratory chain dysfunction[374] which, therefore, due to the high metabolic activity and limited anaerobic ATP-generating capacity, PT mitochondria became dysfunctional, resulting in toxicity[380, 381].

In clinical studies, a review of biopsies for HIV infected patients treated with TDF confirmed abnormal features such as acute tubular necrosis with cytoplasmic inclusions indicating enlarged mitochondria while electron microscopy showed depleted mitochondria with structural changes[307]. A comparison of measured mtDNA to nuclear DNA ratios of patients stratified to TDF plus ddl, ddl alone and HIV uninfected controls revealed lower mean mtDNA ratios in TDF treated group> ddl group > HIV negative controls (7.5, 95%CI, 2.0-12.1; P=0.046, 6.4, 95%CI, 2.8-11.9; p=0,82; and 14.3, 95%CI, 6.0-16.5, P=0.014) respectively. These findings suggest TDF-induced mitochondrial damage and subsequent renal toxicity could also be a consequence of multiple drugs or patient factors[275, 379] although other studies suggest otherwise[380]. However, it was findings by Herlitz et al[312] that made a strong assertion for TFV as a toxin for PT mitochondria in humans. Pathological findings in HIV infected patients with acute kidney injury or proteinuria attributed to TDF were examined to correlate with TDF-RT in patients exposed to TDF for a duration ranging from 3 weeks to 8 years, with SCr, 5.7 ± 4.0 mg/dl, proteinuria,1.6 ± 0.3 g/d with normoglycemic glycosuria in some patients. Biopsy findings showed a wide range of chronic tubulointerstitial scarring of proximal tubules with features consistent with toxic ATN[312]. Biopsies visualised on light microscopy showed evidence of toxic ATN with eosinophilic inclusions in proximal tubular cytoplasm characteristic of large and irregular mitochondria. On electron microscopy, overall, evidence

of a depleted number of mitochondria was observed. Different mitochondrial sizes and irregular shapes and disoriented cristae were observed in giant cells[312]. These findings were consistent with the proposed mechanism of TDF induced tubular injury that can occur with varied severity[374, 377].

Despite the above evidence scholars argue about the involvement of this mechanism because TDF only has a weak effect on DNA polymerase γ compared with other NRTIs, and therefore may unlikely affect mtDNA levels via this pathway[382, 383]. Additional theories support the role of lipid peroxidation and oxidative stress secondary to tubular mitochondrial damage as the main drivers of TDF-RT. Following TFV altered mitochondrial function, inevitable oxidative stress also occurs because mitochondria are both generators and targets of reactive oxygen species (ROS)[384]. Oxidative stress occurs secondary to a decrease in the glutathione antioxidant system and the superoxide dismutase catalyst, manganese superoxide dismutase (MnSOD)[384, 385]. Superoxide Dismutase (SOD) is the first-line antioxidant and neutraliser of superoxides which is the main ROS produced by the mitochondria. Continuous depletion of the antioxidant (AO) defence and unopposed ROS (superoxide) leads to excessive accumulation of free radicals (superoxide radicals) targeting the macromolecule structures of tubular cells, lipids, proteins, and DNA[386]. Thus, oxidative stress plays a vital role in the development and progression of acute tubular necrosis by further triggering proinflammatory responses in the renal parenchyma[387].

1.8.2 Gene polymorphisms in the development of TDF-RT

Genetic variants in genes encoding for specific drug targets, receptors, metabolizing enzymes, and drug transporters can contribute to variations in antiviral drug disposition, efficacy and toxicity[388-391]. Understanding interindividual pharmacogenetic variabilities have the potential

to assist clinicians in individualizing ART to maximize therapeutic benefits. Studies have demonstrated that TDF may mediate a concentration-dependent inhibition of some MRP transporters[392] and the role of transporter proteins on TDF disposition and nephrotoxicity in HIV patients receiving TDF based ART has been studied[210]. Other studies suggest confirming an indirect pathway similar to acyclovir which causes tubular impairment without glomerular dysfunction by inducing the downregulation of genes that encode for the efflux transporter proteins like sodium-phosphate cotransporter IIa, potassium/hydrogen exchanger 2 (NKCC2) and aquaporin 2 (AQP2) involved in the excretion of some nephrotoxic compounds[393, 394]. It has been hypothesised that single nucleotide polymorphisms (SNPs) in tenofovir drug transporters could play a role in the pathogenesis of TDF-RT by increasing systemic concentration, accumulation and toxicity.

This section presents a review of the most relevant findings from previous investigations on the role of SNPs in tenofovir renal transporter proteins in the pathogenesis of TDF-RT in HIV infected patients.

1.8.2.1 **Association between single nucleotide polymorphisms in MRP2 (ABCC2)**

The controversy surrounding the role of MRP2 as a functional renal efflux transporter of TFV remains unresolved with studies showing inconsistencies in pharmacogenetic findings in the populations studied. Several researchers have investigated the association of *ABCC2* SNPs with the risk of TDF-RT in cohorts that are predominantly Caucasian or Asians. Investigated *ABCC2* SNPs include: -24T>C (rs717620), 1249G>A (rs2273697), 3563A>T (rs8187694), 4544A>G (rs8187710) and 3972C>T (rs3740066). Regardless of the role that MRP2 plays in the disposition of TDF, what is consistent in most pharmacogenetic studies is the predictive role of variants in position -24, particularly genotype CC of the SNP, rs717620). Carriers of

genotype -24CC in *ABCC2* were independently associated with a higher risk of TDF-RT, phosphate wasting and beta 2 microglobulins (β_2MG) excretion[298]. Also, an association between -24CC genotype with high plasma TFV levels and a low eGFR was reported[335] following 48 weeks of TDF exposure implying a poor disposition of TFV by patients with this genotype[335]. In other studies, the interaction between patient factors (sex, age, weight, comorbidities) and pre-existing renal impairment with -24CC genotype significantly influence renal outcomes[395]. In case studies of HIV patients with tubular dysfunctions, the risk C allele was present[297] while a study in a recent African population, this polymorphism was not associated with TDF use[396].

Other *ABCC2* variants seem to influence isolated tubular markers: SNP 1249G>A (rs2273697) was independently associated with a higher risk of KTD[395] and high expression of the allele A in the TDF-RT case group (42.3%) than controls (17.6%). Elsewhere, the variant genotypes, GA and AA, were linked to amino acid excretion[340] and functional studies have confirmed that the variant may affect plasma TFV concentrations[345]. Conversely genotype CC of the 3972C>T; (rs3740066) affects β_2MG excretion[298] although these findings may not agree with other studies[397] with different cohort characteristics and phenotype definitions. On the other hand, carriage of the mutant allele for both 3563A>T; (rs8187694) and 4544A>G; (rs8187710) have been suggested to confer protection against the development of TDF-RT[397].

In a pharmacogenetic candidate gene study of TDF-FS, Dahlin and colleagues[398] reported novel *ABCC2* rare alleles not previously observed reported. [398]. *ABCC2* SNPs, rs17222519, rs7899457, rs17216177 were associated with TDF-FS although they did not remain significant after correcting for multiple testing[398]. Furthermore, although rs79174032 and rs8187707 variants were associated with high serum creatinine, rs8187707 encoding synonymous amino

acid change (His = His) in *ABCC2* remained significant with eGFR after adjusting for multiple testing[398].

Izzedine[397] et al found an association between the haplotype 'CATC' for a combination of SNPs at positions -24, 1249, 3563 and 3972 in the *ABCC2* gene with a higher risk of TDF-induced tubulopathy suggesting that the combination could impair the effective secretion of TFV in renal proximal tubules[397]. Furthermore; a higher tubular secretion of TFV was proposed with a protective 'CGAC' haplotype[397]. The risk predisposition for variants -24C and 1249A (CA haplotype) and a protective predisposition described with −24T and 249G was confirmed in subsequent studies[395]. Despite these findings, experimental studies have failed to demonstrate ATP dependent uptake of TFV by cells expressing MRP2[252, 315, 339, 348].

1.8.2.2 **Single Nucleotide Polymorphisms in MRP4 (ABCC4) in TDF-RT**

The role of the MRP4 (*ABCC*4) in the excretion of TFV has been established, and therefore alterations in gene expression for this protein could significantly change the TFV secretion process. However, no statistically significant associations directly implicate SNPs in the *ABCC4* gene with any phenotype of TDF-RT. Nevertheless, studies have demonstrated a wide-ranging association with abnormalities in the secretion of tubular markers. The *ABCC4* 3348T>C (rs1751034) was reported to independently affect the urinary output of TFV in HIV patients suggesting an underlying effect on tubular function and toxicity[399]. In another study, polymorphism in *ABCC4,* 3463A>G SNP, with a missense gene consequence was significantly associated with a 35% higher intracellular TFV diphosphate concentration compared to the common genotype in a multivariate analysis adjusted for race, treatment duration and eGFR (p=0.04), this remained higher after adjusting for the area under the curve although it did not attain statistical significance[339]. These findings were consistent with trends of higher TFV

concentrations in 3463 AG/GG genotype carriers in another Thai cohort following one year of TDF exposure (p=0.07)[400] where a significant association with tubular impairment was reported (OR 4.67, 95% CI 1.25–17)[400]. Moreover, another study found that GG genotype was frequently expressed in patients with tubular dysfunction (14.3%) than controls (3.5%) p = (0.01)[401]. A predilection of 3463GG genotype in the black population than other ethnic groups was reported[339].

Investigation of a non-synonymous polymorphism in *ABCC4* 669C>T in a cohort comprising 73% Caucasians showed a higher frequency in the group of patients with the tubular disease (26.9%) vs controls (8.8%) p=0.04[397], but these findings were not replicated in another study, although genotype CC was associated with phosphaturia in patients with TDF-RT[340]. The *ABCC4* 669C>T SNP is thought to increase the probability of altering messenger RNA (mRNA) splicing and potentially affect MRP4 expression[393].

ABCC4 4131T>G has been investigated in the Thai[402] and Caucasian[340] populations. Carriers of the mutant alleles TG or GG genotypes were independently associated with high tenofovir concentration with 30% higher mean TFV levels than those with the common genotype[402]. The same variant was more likely to cause altered excretion of uric acid in another cohort[340] confirming the hypothesis of impaired TFV elimination in MRP dysfunction. The influence of this polymorphism on TFV handling is thought to be similar to the effect on lamivudine intracellular concentrations of which were 20% higher in the 4131T >G variant carriers (P = 0.004)[388].

Another *ABCC4* 4976T>C (rs1059751) investigated in a Thai cohort showed that of the 20% (54/273) patients presenting with β2MG, the CC genotype (allele frequency=0.602) was significantly associated with β2MG[403]. In a similar study, a large proportion of this variant C

allele was highly expressed in Italian HIV positive patients (85% white patients) with reduced urinary excretion of tenofovir although the observation did not reach statistical significance[404] while a marginal association (p=0.09) with TFV- induced proximal tubulopathy (PT) was reported elsewhere[395]. These findings suggest that predisposition of the *ABCC4* 4976C allele could potentially impair tenofovir excretion resulting in its accumulation to tubular toxic levels and impair proximal tubular function. In a Fanconi syndrome cohort, a novel *ABCC4* rs2274409 C>T variant was highly expressed in the case group but did not remain significantly associated after adjusting for multiple testing[398].

1.8.2.3 **Single Nucleotide Polymorphisms in MRP7 (*ABCC10*) in TDF-RT**

MRP7 encoded by the *ABCC10* gene is highly expressed in intestinal, hepatic, brain and kidney cells[210, 341]. The potential for MRP7 to transport TFV was first demonstrated in *in-vitro* models of *ABCC10* transfected human embryo kidney 293 (HEK293) cells and ex-vivo cells of *ABCC10* small interfering RNA (siRNA) knocked down cells[253]. In subjects treated with TDF, a synonymous intronic variant rs9349256 was associated with tubulopathy (72.2%) while the mutant C allele of rs2125739 was only present in 39% of the cases. *ABCC10* 526G>A (rs9349256) (p=0.02), rs2125739 (2759T>C) (p=0.05) and their haplotype (p=0.05) were significantly associated with tubular disease. However, studies by Nishijima [395] and Salvaggio[401] failed to show any association between these SNPs with TDF-induced tubular disease in Japanese and Caucasians respectively with significant exposure to TDF. The probable evidence for the role of MRP7 as a TDF tubular transporter has already been discussed in previous sections of this thesis. Independent studies have also reported an association between ABCC10 526G>A (rs9349256) with a varying disposition of TFV and poor secretion of injury biomarkers in patients with TDF; urine excretion of phosphorus and β2MG[253],

abnormal urine retinol-binding protein to creatinine ratios (uRBP/Cr)[405] and high urinary-plasma tenofovir exposure ratios[404]. These findings are supported by other findings of a heterozygous genotype for *ABCC10* rs2125739 T>C in an HIV patient with generalised tubulopathy in FS[297] and a significant association with indicators of poor renal function elsewhere[396].

1.8.2.4 **Other suggested SNPs associated with TDF-RT**

Novel rare variants have been reported in a cohort of HIV patients infected with TDF induced FS. These include variants in the Oculo-Cerebro-Renal lowe protein 1 (OCRL1,) and chloride channel 5 (CLCN5) genes that are associated with mendelian FS. In this study, two OCRL SNPs (128701401T>C and 128699579T>C) were associated with TDF-FS; four intronic SNPs of OCRL (128718244C>T, 128718318A>G, rs113165732C>T, rs7057639 C>T) were also independently associated with increased serum creatinine[398]. Interestingly, none of these SNPs identified in cases was present in the control group representing novel candidate loci suggesting that TDF-FS may be affected by other unknown rare genetic variants.

1.9 Biomarkers of TDF-RT in HIV positive patients – the current scenario

There are currently no validated biomarkers that are specific to measure TDF-RT[406-408]. The wide-ranging phenotypes of TDF induced nephrotoxicity reported have demonstrated the lack of uniformity due to differences in markers used to define TDF-RT. A review of yellow cards of HIV patients demonstrated the inconsistency in reporting phenotypes of TDF-RT. Among the patients that satisfied the definition criteria of TDF-RT, 50% had tubular impairment, 33% glomerular dysfunction while 17% had FS[409]. The lack of consensus and standard in the accepted surrogate biomarkers of kidney disease may have significantly contributed to the

current uncertainty regarding the exact prevalence and pattern of TDF related nephrotoxicity[238].

1.9.1 Traditional markers: Use of Serum Creatinine to estimate TDF-RT

Creatinine is a 113 Dalton molecule product of creatinine metabolism from the muscle that is freely filtered by the glomerulus and excreted in the urine with minimal reabsorption. Approximately 10% of creatinine is secreted by the proximal renal tubules adding to the final creatinine concentration excreted through the urine[407, 410, 411]. Therefore, rising serum creatinine is indicative of several other factors and kidney injury of glomerular origin. Although widely used in practice, serum creatinine has a few limitations. In the first place serum creatinine has inter and intra-personal variations that are affected by factors that alter its production (age, gender, muscle mass, high protein diet, general nutritional status of an individual), elimination (previous kidney disease), secretion (drugs) and concentration according to haemodynamics[101, 412]. Secondly, because only 10% of the filtered creatinine is secreted by proximal renal tubules, most abnormalities in creatinine concentration are due to glomerular defects than tubular[406, 410, 411]. Current measurements of renal function use serum creatinine-based formulas/equations. These equations eGFR by the MDRD formula and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) and CrCl by Cockcroft-Gault (CG)[413]. However, none of these equations accounts for all factors that influence serum creatinine concentration to accurately estimate renal function. Moreover, evident abnormalities in eGFR or CrCl reflected through serum creatinine manifest after the extensive loss of about 75% renal mass tissue following failure in the compensation and adaptative mechanism of the nephron tissue[414, 415]. This may preclude an early diagnosis of renal dysfunction and consequently the provision of early therapeutic interventions.

Against this background, the rationale for using traditional markers of SCr-based estimation of CrCl, eGFR and ACR or PCR to describe TDF-RT in some settings has been questioned, principally because of the inability to detect sub-clinically or predict early kidney injury associated with TDF[268, 416]. On the other hand, albuminuria and proteinuria are generally indicators of established and progressive renal dysfunction which rarely presents in the initial stages of tubular dysfunction.

The apparent normal eGFR observed in patients with a 5-fold increase in albumin-creatinine ratio (ACR) in PLWH further interrogates the plausibility of using any measurements that rely on SCr in reporting cases of TDF-RT[417]. While SCr may be an important surrogate marker to estimate glomerular function, myriad problems affecting its production exist, and lacks specificity and sensitivity to detect subclinical glomerular and tubular injury. A serum creatinine-based estimate of renal function may be inappropriate in TDF-RT where cases of subclinical tubular injury[295] and tubular impairment without evident glomerular dysfunction[294] have been reported. Thus, coupled with the measures of glomerular function, proteinuria is certainly a valuable tool to detect and monitor the progression of chronic glomerular dysfunction.

1.9.2 Low Molecular Weight Proteins TDF-induced Renal Toxicity

Several candidate low molecular weight proteins (LMWPs) excreted in urine have been proposed as potential surrogate markers of tubular injury[407]. Varying degrees of TDF-induced tubular impairment has previously been reported by measuring tubular markers that include urinary glucose, amino acids, phosphates and urinary β2MG with or without glomerular impairment. Hypophosphatemia is an important and sensitive indicator of tubular impairment because 80-85% filtered phosphate is reabsorbed by the proximal tubules. However,

hypophosphataemia in HIV infection may be multifactorial and could be affected by HIV infection, ART, nutritional state, alcoholism and hyperparathyroidism are known to independently affect serum phosphate levels[418-421]. On the other hand, normal glycaemic glycosuria may be an alternative tubular marker that presents as a defect in the proximal tubular disposition of glucose. Nearly 180 grams of glucose per day is filtered from the glomeruli, all of which is reabsorbed through glucose transporter proteins that are present in cell membranes within the proximal tubules[422, 423]. Therefore, urinary glucose may be indicative of proximal tubular dysfunction. However, glycosuria manifests late and may represent a symptom of other conditions like insulin resistance, metabolic syndrome and therefore, may be short for being ideal as a timely indicator of tubular injury in isolation[406, 407]. Therefore, a biomarker that can report sub-clinical kidney injury early in TDF exposed to ART will be important in risk stratification of these patients before initiating ART and plan for routine renal monitoring. High β2MG urinary concentrations (≥ 500µg/l) have been reported in patients treated with TDF[303, 306, 424, 425]. β2MG is an excellent marker of tubular dysfunction but high enzymatic activities and urinary pH <5.5 causes protein degradation which interferes with the urinary stability of the protein[426, 427].

1.9.3 Novel Biomarkers for TDF-RT in HIV Patients

HIV-infected patients with different risk factors have been associated with TDF-RT of varying degrees of proximal tubular toxicity, from persistent subclinical renal dysfunction to the most severe FS. However, the clinical significance of isolated tubular dysfunction observed while on TDF treatment remains unclear. Also, some minor tubular abnormalities and in some cases severe ones may be missed until they affect the glomerular function[308, 428]. While currently used markers of tubulopathy remain clinically useful, they are neither specific nor sensitive for

subclinical and early detection of kidney injury[406, 407]. Recent investigations have identified novel biomarkers for AKI. Unlike conventional SCr, ideally, these novel biomarkers are can identify patients at risk, determine the origin of the injury and, provide an early diagnosis and predict the prognosis of the injury[101]. Several clinical and experimental studies have demonstrated that in the pathogenesis of proximal tubular injury, markers such as kidney injury molecule-1(KIM-1), Neutrophil Gelatinase-Associated Lipocalin (NGAL) and retinol-binding protein (RBP4) rise within hours of injury compared to days of creatinine (Figure1.15).

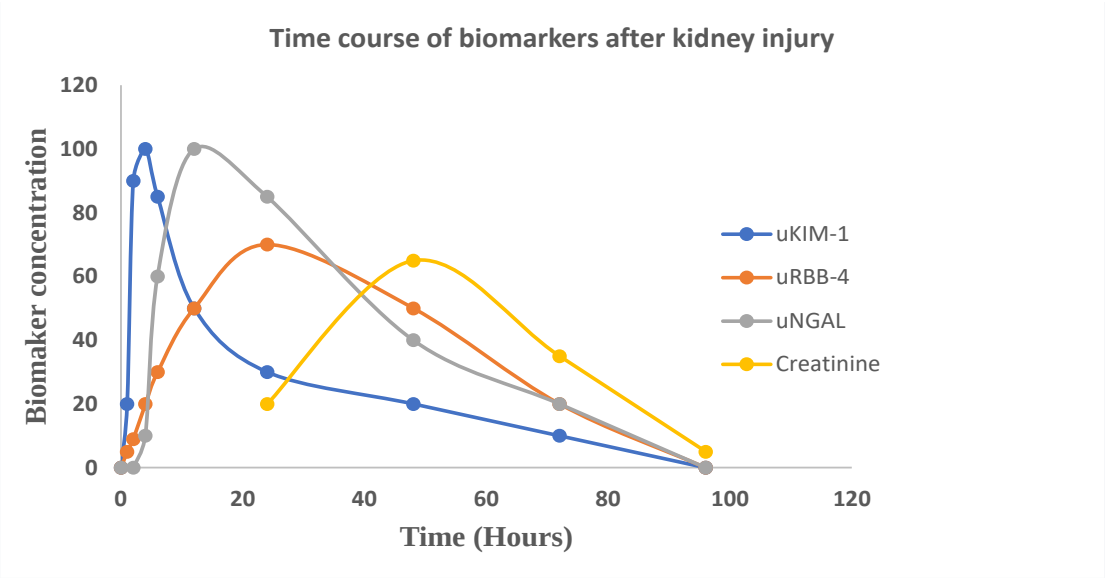


Figure 1.14. Time course of the rise in urine levels of KIM1, NGAL and RBP4 compared to serum creatinine in detecting kidney injury.

It has further been demonstrated that the contribution of these biomarkers in different clinical phases of AKI and progression to CKD may be useful in monitoring kidney injury (Figure 1: 13.)[429]. Since, TDF targets proximal tubular tissue, measuring the transportation capacity of LMWP appears to be more specific to detect proximal tubule alteration[430, 431]. It has been postulated that delayed detection of AKI is one of the reasons intervention trials aimed at treating AKI have postulated that delayed detection of AKI is one of the reasons intervention

trials aimed at treating AKI have failed. Therefore, efforts have been directed towards finding biomarkers of early kidney injury expressed on the surface of kidney tubular cells[432]. Its trans-membrane domain undergoes proximal membrane cleavage, releasing the ectodermal KIM-1 that is excreted and quantifiable in urine[432]. It is often found in very low concentrations in normal kidney tissue but highly expressed following proximal tubular earlier. Some novel biomarkers proposed for this role include KIM1, NGAL and RBP4 and a review of each is detailed below.

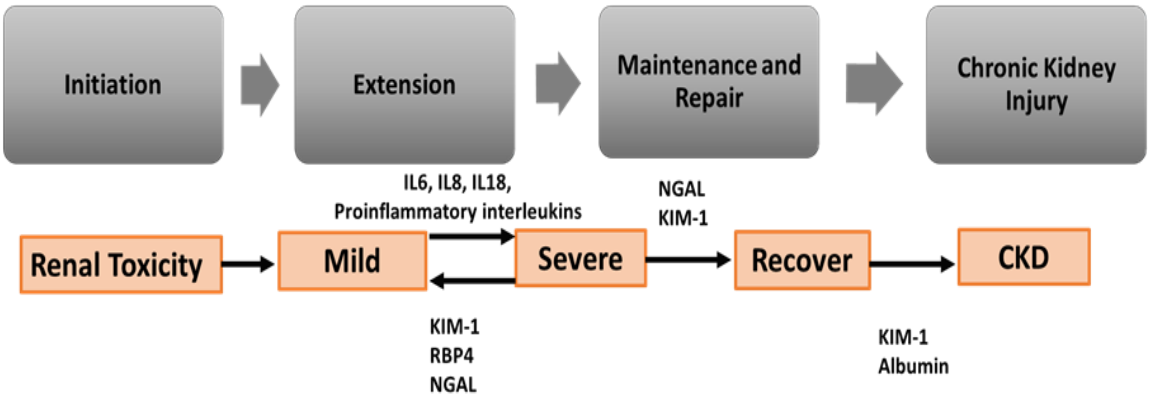


Figure 1.15. Illustration of the course of kidney injury and associated biomarker with each phase.
Figure adopted from[429] https://cjasn.asnjournals.org/content/10/1/147

1.9.3.1 **Kidney Injury Molecule -1 (KIM-1)**

KIM-1 (kidney injury molecule 1) is a putative type-I transmembrane tubular adhesion glycoprotein upregulated in cells undergoing injury, ischemic reperfusion injury and metabolic/infective stress[433]. KIM-1 was defined and proposed as a potential AKI biomarker in 2002 following observation of high levels in the urine of patients with acute renal tubular injury compared to lower levels in AKI of other origins. This was a cohort of patients undergoing cardiac surgical procedures; urinary KIM-1 (uKIM-1) levels at 3 hours post-

surgery were highly predictive of AKI compared to raised SCr levels 3 days post-surgery[434]. Subsequent experimental studies demonstrated the upregulation of KIM-1 levels following a nephrotoxic insult on the epithelial cells of the proximal renal tubules with or without a rise in creatinine or blood urea nitrogen[435-438]. without a rise in creatinine or blood urea nitrogen[435-438].

In multi-site preclinical toxicology studies, KIM-1 outperformed the traditional "gold standard" biomarkers as predictors of tubular injury[439]. Thus, its potential as an early predictor of renal injury has been analysed and validated in assays involving a healthy population with a proposed normal range that falls between 59-2146 pg/mL[440]. Several clinical studies have explored the relationship between KIM-1 and the development of AKI in different populations. In one observational study, preoperative levels of uKIM-1 could predict the development and severity of AKI[441] while in another, uKIM-1 was associated with high morbidity and mortality[442]. Its potential to predict early tubular injury in renal transplant recipients[443] and progressive renal failure[444] has been demonstrated. There is very limited information about the experience with KIM-1 in patients on cART. A cross-sectional study reported ratios of KIM-1/creatinine values that were associated with a high risk of renal tubular disease in HIV–Hepatitis C co-infected patients who were on TDF based regimens[445]. In a few studies involving HIV infected women, raised KIM-1 was one of the defining markers for tubulopathy and glomerular dysfunction[446] as well as mortality[447].

1.9.3.2 **Neutrophil Gelatinase-Associated Lipocalin (NGAL)**

NGAL is a 25 kDa protein comprising 180 amino acids which is a member of the lipocalin superfamily. It is produced by neutrophils and renal epithelial cells following inflammatory or malignant disorders[430, 448]. The exact function of NGAL is still unclear although it is believed

to be involved in kidney tubular regeneration[449]. Upregulation and an increase in the expression of NGAL in tubular cells have been observed following exposure to metabolic, inflammatory, and infective stress[450]. The interest in NGAL's potential as an early kidney disease diagnostic biomarker developed following an increase in urinary levels in *in-vivo* models within three hours of cisplatin 20mg/kg exposure[451-453]. A meta-analysis of NGAL confirmed that plasma and urinary NGAL (uNGAL) levels were excellent diagnostic and prognostic predictors of kidney injury[454].

As a prospective kidney injury marker, NGAL has been extensively studied in varied clinical settings in both HIV cohorts and the general population[450, 454]. In paediatric patients undergoing cardiopulmonary bypass, serum and uNGAL levels were diagnostic of AKI within a couple of hours compared to more than 24 hrs of creatinine levels post-surgery. A receiver-operating characteristic (ROC) curve with uNGAL levels demonstrated high sensitivity and specificity in predicting the severity, morbidity and mortality of AKI[455, 456]. Similar results have been observed in **a**dult patients with AKI; NGAL levels were at least four times higher within an hour of surgery compared to serum creatinine indicating its reliable predictive value for AKI[450, 454, 457, 458]. Studies conducted on patients in intensive care units, serum and uNGAL levels were independently associated with the development of AKI two days earlier than serum creatinine levels in both paediatric and adult patients[459, 460126–130]. Similar findings were also reported in patients with heart failure[461] and patients with suspected AKI[462-464]. In other studies, uNGAL levels were reported to distinguish AKI of tubular and glomerular[465]. Very few studies have investigated the utility of serum and urinary NGAL in HIV patients. An investigation comparing uNGAL with serum creatinine in TDF-RT showed that NGAL levels were nineteen times higher following initiation of tenofovir compared to baseline and were associated with proteinuria[466]. A cohort of patients with a confirmed biopsy-based HIVAN diagnosis expressed

higher uNGAL and these levels were reduced with ART introduction a possible indication of improvement of HIVAN and or HIV-infection[467].

1.9.3.3 **Retinol Binding Protein (RBP)**

Retinol binding protein (RBP) is another LMWP freely filtered through the glomerulus and completely reabsorbed through an ATP-dependent endocytic mechanism in the proximal tubules[431]. Mitochondrial tissue damage induced by TDF may cause a possible inhibition of the ATP-dependent endocytic transport mechanism and deplete cellular energy[430, 431] resulting in high urinary RBP (uRBP) levels, as well as RPB/Cr ratio, would be specific predictors of the presence of tubular injury in TDF treated patients[430, 431]. Early studies have confirmed the important role of uRBP in predicting proximal tubulopathy[426, 430]. A cross-sectional study involving patients on TDF and protease inhibitors demonstrated that RBP levels correlated with increased risk of tubulopathy compared to creatinine levels[468] while in another study, uRBP levels were sensitive predictors of AKI that gradually declined with renal improvement demonstrating its role as a monitoring tool of disease progression[469].

A study of treatment naïve HIV infected patients without overt renal impairment suggests that there is 3–10-fold higher urinary concentrations of LMWP (B2M and RBP, but not Cystatin-C) and NAG compared to the general population[470]. In a cohort of HIV infected patients on ART without clinically diagnostic symptoms of tubular injury, participants on TDF treatment had a higher median RBP/Cr ratio (214µg/g; Normal range < 159 µg/g) than those on TDF-free regimen (111.6 µg/g) or naive patients (92.5 µg/g) indicative of TDF's potential of affecting tubular cells and inhibiting RBP reabsorption[295]. However, the values seemed much lower than the ones reported in severe cases of TDF induced tubulopathy(50,000µg/g)[295] an indication of the high sensitivity in predicting the presence of TDF-RT. This could suggest an

important role in predicting and detecting subclinical TDF-tubulopathy in HIV patients[468]. HIV patients with TDF-FS have reported elevated levels of urinary LMWP (RBP and CysC) as a marker of tubular impairment in other forms of TDF-RT[307, 471]. Several studies have quantified LMWP in patients receiving TDF ART; higher levels of urinary RBP or β2MG were noted in participants exposed to tenofovir compared to those on other regimens or no cART[263]. In cross-sectional studies, Increased levels of uRBP and β2MG were also observed in a randomized clinical trial, especially in patients initiated on tenofovir/emtricitabine compared to those on abacavir/lamivudine, with efavirenz [468]. In a similar observational cohort study, TFV based ART was associated with phosphaturia and a five-fold increase in urinary β2-microalbuminuria detectable at 12 weeks of TDF treatment[306]. Co-administration of TFV and PI/r has also been associated with levels of higher RBP defining tubular proteinuria (OR =2 for RBP/ Cr >17mg/mmol and OR=3 of RBP/Cr >38.8mg/mmol)[303, 472].

Reduced mortality and improved longevity associated with cART has improved the quality of life of HIV-positive. However, incidences of renal disease regardless of the aetiology and magnitude of injury remains a challenge in clinical care. Therefore, optimising timely diagnosis by utilising tools or panels that can detect and or predicting early injury will prove efficient in providing care to avoid the associated kidney disease complications in HIV patients.

1.10 Relevance of this study and TDF-RT for the Zambian ART management.

TDF was introduced in the Zambian ART guidelines in 2007, nearly six years after the FDA approval. Its introduction was limited by its then high cost, particularly for an ART programme that was heavily dependent on donor funding. Besides the cost, most settings with inadequate laboratory facilities made it practically impossible to effectively offer simple renal assessments (SCr) required due to its potential for renal toxicity. The introduction of TDF came at a time

when an assessment of patients who had started ART between 2004 and 2007 concluded that renal impairment was prevalent at the point of ART initiation and contributed to a high risk of mortality in adults[195]. Despite the controversy surrounding the cost, funding and anticipated challenges, Zambia became the first African country to introduce TDF as a component of first-line antiretroviral therapy[473].

While some may argue that clinically significant TDF-RT is uncommon, published literature continues to show varying degrees of renal impairment in different populations. In the Zambian context, a higher risk of kidney dysfunction in treatment-naïve patients initiated on TDF based regimens was reported during the initial stages of TDF introduction in cART[474]. Mulenga et al. also observed that TDF treated patients are more likely to develop a mild to severe decrease in eGFR[475] Also, Banda and colleagues reported a 38% prevalence of TDF associated kidney dysfunction in hospitalised HIV patients diagnosed with kidney dysfunction[476]. Efforts to address TDF-RT have resulted in the development of Tenofovir alafenamide (TAF), a novel analogue of tenofovir with a better renal safety profile, which was recently approved by the FDA in 2015 for the treatment of HIV and Hepatitis B infections[477-479]. In Zambia, TAF has been rolled out in a limited and specific population group. However, the long-term safety and benefits of TAF are still unknown. To-date, TDF remains the preferred mainstay of first-line antiretroviral therapy regimens prescribed to almost all HIV-infected patients in commencing cART[32, 480]and elsewhere[107, 481] and will remain in use for an undetermined period. Therefore, it is logical to presume that as more HIV-infected patients are enrolled in the TDF based ART regimen, the incidence of TDF-RT will potentially increase.

Patients with HIV infection on TDF treatment will potentially develop TDF-RT at one point and at any time during treatment. Besides, it is fairly common for HIV infected patients to have

pre-existing renal impairment and host risk factors that favour any-induced nephrotoxicity, and of concern, is that TDF-RT exists even in those with normal baseline renal function[475]. The impact of long-term effects of TDF associated toxicity is of great concern because the ageing population of HIV infected patients also develop non-HIV age-related comorbidities that are risk factors of TDF-RT while multiple drugs administered for these conditions may be a source of interactions that can enhance TDF-RT. Besides, noteworthy is the background tubular proteinuria and subclinical tubular injury that has been established in clinically stable, comorbidity free patients receiving TDF[295, 470] could suggest a subtle long-term TDF-RT which is a matter of when this becomes clinically significant in the presence of an additional trigger.

While discontinuation of TDF has shown improvement and reversibility of kidney injury[482-484], abnormal glomerular dysfunction persist for significant periods (23 months) in some patients[485] while others progress to chronic kidney disease[483, 486]. Elsewhere, cases of chronic tubulointerstitial nephritis due to partial reversibility of kidney injury have been reported[312]. A cohort of patients with a baseline CKD stage 0 or 1 stratified to either TDF or ABC reported a 48.8% vs. 23.7% progression to CKD stage 2 respectively; while 5.8% of those on TDF progressed to stage 3 CKD[487]. Therefore, the clinical environment needs adequate tools and preparation to manage a chronic HIV infection in parallel with chronic kidney comorbidity which could independently trigger cardiovascular and metabolic disorders. Currently, the capacity to effectively manage these remains uncertain.

Currently, the precise mechanism by which transporter proteins play a role in TDF renal secretion and cause kidney toxicity is still unclear. We, therefore, acknowledged that the continual increase in the use of TDF in HIV patients, especially in Zambia, necessitates more research into the pathogenesis of TDF-RT to make an informed decision on the future of TDF

in antiretroviral therapy guidelines. This research proposes to investigate some of the questions raised regarding the mechanisms of TDF-RT. The study will investigate the association between SNPs in genes encoding for reported transporters and other genes indirectly involved in the excretory pathway of TFV in the Zambian population. The study will also serve to establish a clinically well-phenotyped DNA cohort from an African population that will serve as a valuable resource for future genetic reference for future studies to identify toxicity susceptibility markers.

On the other hand, detection and monitoring of kidney function in Zambia and other African countries largely depend on the traditional measurement of eGFR and CrCl which have known limitations. This study also proposes to investigate the utility of novel urinary biomarkers KIM-1, NGAL and RBP4 and how these correlate with TDF administration comparison with SCr to predict TDF-RT.

1.11 Research Hypothesis

The following research hypothesises were formulated following the demonstrated extensive literature review.

1. Genetic variants in the transporter proteins responsible for TFV transport across the renal proximal tubular epithelial cell membranes could potentially modify the influx and efflux transport mechanisms of TFV and may affect the cellular accumulation of the drug leading to renal tubular cell injury.
2. There is potential dysregulation in the expression of renal injury marker proteins, KIM-1, NGAL and RBP during the clinical manifestation of TFV-induced kidney toxicity induced. Understanding how the levels of these biomarkers change with TDF exposure may provide

the basis for their use as clinical markers for prediction, detection and prognosis of TDF-RT in patients actively presenting with renal dysfunction.

To investigate the above-stated hypothesis, we formulated the following objectives that were investigated in the subsequent research experimental chapters 2, 3, 4 and 5.:

1.12 Specific Study objectives:

1. To investigate the clinical determinants of TDF-RT in HIV-positive patients on TDF- based ART recruited from Zambia.
2. To investigate the association of single nucleotide polymorphisms in drug transporters (*ABCC2, ABCC4, ABCC10, SLC22A6 and SLC22A8*) with tenofovir induced renal toxicity in HIV-positive patients on tenofovir recruited in Zambia.
3. To validate genetic variants found associated by a GWAS in a UK Caucasian cohort of TDF-induced Fanconi syndrome
4. To prospectively profile the change of KIM-1 and RBP4 with TDF treatment and renal outcomes in the treatment naïve HIV positive patients

CHAPTER 2

CLINICAL DETERMINANTS OF TENOFOVIR INDUCED RENAL TOXICITY IN HIV POSITIVE PATIENTS IN ZAMBIA

2.2 BACKGROUND

TDF was the first nucleotide reverse transcriptase to be approved by the U.S. Food and Drug Administration (FDA) in 2001 for the management of the HIV and hepatitis B virus infections[244, 246, 488-490]. In Zambia, TDF was introduced in 2007 as part of the components of cART regimens[473]. Before the recent introduction of TAF in some settings, TDF had been the only NtRTI and a preferred first-line component of cART. It is also a preferred backbone of most ART regimens in high and low to middle-income countries[473, 480, 491]. The preference for TDF has been attributed to its excellent antiviral efficacy, a favourable viral resistance and pharmacokinetic profile as demonstrated by low drug interaction and adverse drug effects and, good patient tolerance due to the convenience of a once-daily oral administration [246, 281]. Furthermore, TDF has proved to be superior compared to non-TDF ART regimens in terms of safety, tolerance[492-494] and durability[495].

Although prospective randomised clinical studies[203, 260, 261, 264, 282] and post-marketing-consumer data[203, 496] demonstrated a favourable TDF renal safety profile, various cases and clinical studies have reported an association between TDF with different forms of renal dysfunction in different populations[238, 269, 274, 275, 294]. It has been argued that clinical trials usually comprise cohorts of well-selected sociodemographic and clinically stable patients that do not represent an ideal ART setting of patients with comorbidities, concomitant drugs and other risk factors. Moreover, thorough screening also excludes patients with risk factors. Furthermore, the use of SCr instead of CrCl or eGFR to measure renal toxicity in clinical trials may underestimate renal toxicity[285]. In fact, concerns regarding the potential nephrotoxic effects of TDF were initially raised due to structural similarities to adefovir and cidofovir whose nephrotoxicity has been established[236, 271, 497, 498] although pharmacokinetic studies

suggested that TDF did not share the same transport and elimination pathway[380, 499]. With the widespread use of TDF, reports of a wide-ranging prevalence and severity of renal toxicity have continued to emerge nearly two decades since its approval[276, 292]. Evidence of background or subclinical proteinuria without impaired glomerular function has also been observed with TDF suggesting longstanding toxicity before it is clinically evident and measurable through SCr[294, 295, 424].

TDF-RT is thought to be of multifactorial causes[268, 500] and remains a very challenging issue in clinical settings associated with the highest causes of referrals to nephrologists[275]. TDF induced nephrotoxicity is known to depend on its renal excretion mechanism where it is excreted unchanged by glomerular filtration and tubular secretion processes[246, 397]. Its entry from the blood into proximal tubules is through the efflux transporters *hOAT1* and *hOAT3* expressed on the basolateral membranes [271, 329, 330, 498]while the efflux into the urine through the apical membranes is by MRP2, MRP4 and MRP7 transporters[335],[253, 403, 501]. Recently, MRP8 encoded by the *ABCC11* gene; has been identified as a TFV efflux transporter. Thus, functional changes in these transporter proteins along with any drug-drug interactions may lead to the excessive influx and or reduced efflux of tenofovir leading to intracellular accumulation and toxicity[111, 120, 153, 502]. Furthermore, patients' specific factors that include but not limited to immunologic status, comorbidities and concomitant drugs may influence TDF-RT.

Zambia became the first African country to introduce TDF in the ART regimens in 2007. Although initial assessment of clinical outcomes associated with TDF uses showed comparable effects with other regimens[474], a larger study of 62,230 HIV patients on ART associated TDF with moderate to a severe decrease in eGFR during the course of treatment [475]. TDF remains an important backbone of cART in Zambia despite rising evidence of TDF-RT[32,33]. Besides

more HIV positive patients are likely to be initiated on TDF ART following the recent WHO guidelines recommending lifelong ART for all newly diagnosed HIV^+ patients, regardless of CD4 cell count. Recently, TAF, a novel analogue of tenofovir with a better renal safety profile was approved in 2015[477-479]. Preliminary findings have continued to favour TAF over TDF on renal safety. However, the long-term safety and benefits of TAF are unknown[478, 503-505]. Although TAF unquestionably great advancement and alternative for TDF, TDF is likely to remain the most prescribed over TAF at least for the near future in Africa, South America and most parts of Asia due to the cost advantage. Furthermore, the prevalence of renal disease no matter how small, remains a challenge in long term ART, as such, there is still increased interest in understanding TDF pharmacokinetics and determinants of TDF-RT to determine better strategies for patient care. In this chapter, we investigated determinants of tenofovir-induced renal toxicity in a Zambian population. This is the first Zambian cohort to be investigated for factors associated with TDF-RT considering a longer duration of TDF exposure.

2.2.1 Research Hypothesis and Objectives

We hypothesised that there is an association of various patient-related clinical factors with TDF-induced renal toxicity at the time of commencing TDF based regimen and throughout their treatment course.

2.2.2 Research Objectives.

1. To recruit a cohort of HIV- positive patients on TDF-based antiretroviral therapy from HIV specialist hospitals in Zambia.
2. To collect demographic, clinical, and disease-related data from the clinical records.
3. To investigate the clinical determinants of TDF induced renal toxicity in HIV positive

patients from Zambia.

4. To determine an ideal clinical model of factors independently associated with TDF induced renal toxicity

2.3 METHODS

2.3.1 Study Design

This was a retrospective case-control study with a collection of demographic, clinical and laboratory data in HIV positive subjects who accessed ART services between 2007 and 2017 at the Adult Infectious Diseases Centre of Excellence (AIDCOE) at the University Teaching Hospital (UTH), Lusaka. The study commencement period of 2007 was selected because it coincided with the period TDF was introduced into the Zambian ART treatment program. When this study was conducted, the 2016 Zambia consolidated ART guidelines were in effect[506]. According to these guidelines, when a patient tested positive for HIV, they were assessed for readiness and immediately initiated on ART regardless of the CD4 cell count[506]. Before these guidelines, cART was only initiated in patients with CD4 cell count <350 cells/ml[3]. The preferred first-line ART regimens for adults consisted of two NRTIs plus a NNRTI (Table 2.1). Once patients commenced ART therapy, they were closely monitored for early adverse drug effects at 2 weeks, 4 weeks and 3 months. Thereafter, clinically and immunologically stable patients were routinely monitored every 3 months or 6 months.

Table 2.1. Antiretroviral therapy regimens for adults in Zambia (2017).

ART Regimen	Preferred ART Combinations	Alternative combinations
1st Line ART	TDF + 3TC 300 OD + EFV400 OR TDF + FTC + EFV	AZT + XTC + NVP TDF + 3TC + EFV400 TDF + 3TC (or FTC) + NVP
2nd Line ART	If AZT was used in first-line ART	TDF + XTC + ATV-r or LPV-r
	Or if TDF was used in first-line ART	AZT+XTC + ATV-r or LPV-r
3rd Line ART**	TDF + XTC or AZT + 3TC + RAL-r or DRV-r OR ETR – In the presence of persistent NNRTI mutations genotyping results MVC*** – Prior tropism test before initiation	

XTC-either 3TC or FTC, 3TC-lamivudine, FTC-emtricitabine, ABC-abacavir, AZT-Zidovudine, DRV-Darunavir, DTG -Dolutegravir, EFV-Efavirenz, LPV-lopinavir, NVP-Nevirapine, r-ritonavir, TDF-tenofovir, RAL-Raltegravir, ETR-Etravirine, Maraviroc (MVC), only effective against CCR5-tropic HIV-1 or CCR5 tropism (R5-tropic), ** Used in persistent detectable viral load >1,000 copies/ml and Genotype (resistance) testing. *** Maraviroc is only effective against CCR5-tropic HIV-1 CCR5 tropism (R5-tropic)

2.3.2 Study Setting

The study was conducted at the AIDCOE at UTH in Lusaka, Zambia. The UTH is the biggest public tertiary hospital in Zambia; it serves as the national referral hospital for specialised care and management of many medical conditions not limited to HIV complications. Since early 2002, UTH under the Ministry of Health has been offering free ART services to the general public with the support of the US President's Emergency Plan for AIDS Relief (PEPFAR), the Global Fund and other non-governmental organisations. The AIDCOE was developed to specifically offer specialised ART management to ambulatory and hospitalised HIV infected patients. The AIDCOE provides ambulatory HIV care, treatment and support to nearly 9,000

patients in Lusaka and serves as a referral centre for specialised management of HIV related complications from other care centres in the country. The Centre benefits from the systematic and prospective routine collection of patient sociodemographic and clinical data. Routine immunological and virologic assessments are conducted at regular intervals mainly within 3-6 months. All collected data are manually recorded in patients' files (notes) and later transcribed into a SMARTCARE Tool; this is an electronic clinical care tool and database that aggregates successive clinical and routine monitoring data for patients. As an established and experienced, ART centre, UTH-AIDCOE was chosen as a study site because of the availability and easily accessible patients' clinical data through the SMARTCARE database.

2.3.3 Ethical approval of research protocol

The research protocol approval and clearance were obtained from the University of Zambia's Biomedical Ethics Committee under Ref. No 013-05-17 (Appendix A). Permission to access patients and data facilities at the University Teaching Hospital, Lusaka, was granted through the National Health Research Authority (Appendix B), a research directorate at the Ministry of Health, Zambia. Potential research participants were identified by screening the SMARTCARE database and medical notes. All the potential participants were given a participant information leaflet (PIL) that clearly explained the purpose of the study and a certificate of consent (Appendix C) before enrolment in the study. Only participants that consented and signed the consent form were enrolled on the study. Clinical data and biological samples were collected. All patient information (samples and medical) obtained during this research was treated with maximum confidentiality. All data and sample labels were pseudo-anonymised with a unique study code as shown in the designed data collection tools (Appendix D) and were only known to the research team. The information and samples that were collected were used only for

research purposes as outlined in the patient information leaflet and consent forms.

2.3.4 Study Population

The target population for our research study was HIV-infected adult patients (≥18 years old) receiving anti-retroviral therapy containing TDF combinations. In general, participants were drawn from a diverse socio-economic background, although many of the patients were from low to middle-income classes (record and classification by the electronic database). This observation, although it was not an objective of the study, reflected previous findings that HIV infection distribution in populations is often driven by demographic and socio-economic elements, showing a higher association with poverty and low to middle-income households.

2.3.4.1 **Inclusion and exclusion criteria for participants**

We included consenting clinically stable HIV-infected adult patients (≥18 years) who were initiated on a first-line ART regimen containing TDF 300 mg once daily for at least 3 months. We included participants that had up-to-date clinical data and had a recorded baseline serum creatinine result together with another record within the last 3-6 months by the time of recruitment for estimation of eGFR and or creatinine clearance. We then categorised our participants as cases and controls as follows:

- **Inclusion criteria for cases:**

  - No documented renal disease or risk factors in the last 3 months before TDF initiation.
  - History or clinical diagnosis of the presence of renal disease attributed to TDF treatment.
  - CrCl of less than 60 ml/min/1.73 m2 calculated by the Cockcroft-Gault equation indicative of TDF-RT

- Absence of diarrhoea in the two weeks before the TDR-RT event
- Ability to consent

➢ **Criteria for Controls**

- Patients on TDF treatment for at least 3 months without a record or history of renal disease attributed to TDF based ART.

➢ **Exclusion Criteria:**

- For cases, history or presentation of kidney disease suspected to be due to causes other than TDF treatment
- Individuals with known causes of renal impairment were excluded.
- A history of renal impairment 3 months prior to commencing TDF treatment.
- For controls, patients on TDF for less than 3 months.

2.3.4.2 **Case-Control definitions**

➢ **Research Case definition**

Systematic reviews have reported no uniformity in the case definition of TDF induced renal toxicity. This may be because there are no validated standard criteria to define TDF induced renal toxicity. We have observed reports of various forms of TDF-RT measured by either glomerular or tubular markers. Despite consistent evidence showing tubular mitochondrial deformity induced by TDF toxicity[312, 377, 384], the laboratory at our study site did not routinely measure proximal tubular markers, but only measured serum creatinine. For this reason, we used the reported SCr measured within the last 3 - 6months prior to recruitment to determine CrCl by the Cockcroft-Gault (CrCl-CG) formula. CrCl-CG was estimated based on the

recorded SCr level, weight and sex of the patient. We therefore defined TDF-RT as CrCl less than 60ml/min (CrCl <60ml/min). This is the case definition for our research.

- **Physician case definition of TDF-RT - for sub-analyses**

Based on medical notes and database records, physicians clinically defined TDF-RT using a CrCl-CG <50ml/min. It is understood that this is the threshold recommended according to guidelines for medical intervention to either discontinue or switch TDF to other ARVs with less risk of renal injury like abacavir. We used this definition to categorise participants for a secondary analysis.

2.3.4.3 **Assessment of renal function**

Renal function was assessed using SCr level measurement to estimate the GFR based on the CrCl-CG formula shown below. Note: urine protein, albumin and urea were not routinely measured in the clinic.

$$\frac{(140-Age)\times Body\ weight\ (kg)}{Serum\ creatinine\left(\frac{mg}{dl}\right)\times 72} \times 0.85\ if\ female$$

Based on the calculated CrCl, we calculated the absolute and percentage change in CrCl from baseline to recruitment and compared the difference between cases and controls. To determine the extent of reduction in CrCl was presented as percentage change and categorised in quartiles; < 25%, ≥ 25%, > 50% and >75%.

2.3.4.4 **Sample size determination**

We aimed to recruit a sufficient number of participants to provide us with an acceptable statistical power to investigate both the pharmacogenetic and clinical determinants of TDF-RT

in HIV positive patients. For this reason, we determined the sample size on the basis that detection of an association between single nucleotide polymorphisms and TDF-RT is highly affected by disease prevalence, SNP allele frequency, linkage disequilibrium, inheritance model (homozygous or heterozygous) and effect size[507]. The effect sizes reported for some of the major SNPs associated with TDF-RT range between 2.0 and 3.0 (*ABCC2* 24CT[395], effect size is 3.0; *ABCC10* rs9349256[253], effect size is 2.3; and *ABCC10* rs2125739, effect size is 2.0[253]). Assuming the lower end of the effect size (odds ratio 2.0), to obtain 80% statistical power to identify a SNP with a minor allele frequency of 10%, we determined the sample size of 200 for cases (HIV positive patients with TDF-associated renal toxicity). We used a case-control ratio of 1:4. We used PASS15 software (https://www.ncss.com/software/pass/) to calculate the sample size and aimed to recruit 200 cases and 800 controls (HIV positive patients on TDF-containing regimen but with no evidence of renal toxicity). In this sample size determination, we considered a projected prevalence of 16.7% for TDF-RT in the Zambian population on the basis of a study by Mulenga et al[475].

2.3.5 Recruitment procedure

The recruitment of research participants was achieved with the financial support of the Commonwealth Scholarship Commission, which is the sponsor of my PhD studies. Recruitment of participants was conducted in Zambia for a period of seven months from June 2017 to November 2017. To identify eligible potential research participants, we screened the updated SMARTCARE - electronic database to identify patients that were active on the ART register. Where necessary, we further reconciled the electronic record with the manually entered medical notes. We then screened and identified participants that met the eligibility pre-determined by the inclusion and exclusion criteria.

2.3.5.1 **Patient Visit and participation invitation:**

Based on the local institutional practice, patients that accessed ART services at the AIDCOE were reviewed and monitored routinely at 3 to 6-month intervals. Potential participants identified from the ART database and medical notes were randomly invited to participate through the study patient information leaflet during their routine clinical visits/reviews. Data were then collected from consented patients and were recruited in the study as cases or controls according to our criteria. This study only needed one visit to collect a blood sample for DNA extraction for the pharmacogenetic study, which will be discussed in detail in Chapter 3.

2.3.5.2 **Data collection**

We collected the corresponding demographic and clinical data from two sources: the SMARTCARE database (contains medical, laboratory and pharmacy dispensing records) and medical notes for reconciliation. We used a pre-designed data collection form (Appendix D) which was later transcribed to an Excel spreadsheet. The collected data was categorised as baseline and recruitment data respectively. The data collected included:

- Demographic: gender; baseline/recruitment age and weight.
- Clinical Data: body weight, height, comorbidities, co-infections like hepatitis B or C viruses or pulmonary TB, co-administration of non-ARVs, other ARVs in the regimen and TDF treatment duration,
- Laboratory data: serum creatinine, CD4 count and viral loads.
- Viral load measurements were not routinely done during the time of TDF introduction, and thus most of our participants had no baseline viral load data. For the most recent

viral load, we considered the last record entered 3-6 months prior to recruitment.

2.3.6 Operational Definition

- **Baseline Characteristics or Measurements:**

As some patients were migrated from other regimens containing stavudine prior to starting TDF containing ART, we defined baseline measurements as any recent measurements that were taken prior to migration to TDF-containing regimen. For treatment-naïve patients, the baseline was assigned as measurements taken before TDF initiation.

- **Characteristics at Recruitment:**

These were described by data collected at the time of TDF-RT or the time of recruitment for cases and controls respectively.

- **TDF treatment duration:**

This was the duration from when they a patient started TDF based ART to the time of recruitment / TDF-RT event.

- **Body Mass Index (BMI):**

We adopted the BMI categories as per WHO BMI classification (https://www.euro.who.int/en/health-topics/disease-prevention/nutrition/a-healthy-lifestyle/body-mass-index-bmi): < 18.5 = underweight; 18.5 to <25= normal weight; 25.0 to <30= overweight range and ≥ 30.0 obese weight range.

2.3.7 Statistical Analyses

Demographic, clinical and laboratory parameters were compared between cases and controls.

We summarised and described normally distributed continuous variables using means and standard deviations. The median and interquartile range were used for continuous variables that did not show normal distribution. Normality of distribution of data was assessed using histograms and the Shapiro-Wilk test; p-value > 0.05 was considered as not normally distributed. Categorical variables were summarised using percentages and proportions. Summary statistics for patient's characteristics were provided separately as cases and controls according to the group case definition; CrCl<60ml/min or CrCl <50ml/min groups.

For inferential statistics; we carried out a univariate binary logistic regression to assess the association between individual variables and TDF-RT to filter for variables to be included in fitting a binary multivariate logistic regression model. We applied purposeful selection of variables with a p-value cut off point of less than 0.2 (P-value <0.2) at univariate analysis to include in multivariable logistic regression models. Predictor variables meeting these criteria were selected for inclusion in the binary multivariable logistic regression model to test the hypotheses regarding the association between TDF-RT with baseline patient characteristics. The hypothesis was tested on two models with data for TDF-RT defined by CrCl<60ml/min and CrCl <50ml/min. We performed the analysis using the forward stepwise likelihood ratio method to assess for independent determinants of TDF-RT. The probability for evaluating variable entry and removal from the model was set at P=0.05 and P=0.10 respectively. An individual predictor was considered significantly associated with TDF-RT when the statistical test for the regression coefficients was less than 0.05 (p-value < .05). Adjusted odds ratios and their 95% confidence interval (95%CI) were reported for all variables retained in the final multivariable regression models.

The soundness of the logistic regression models against the observed outcomes was evaluated

by three model parameters; an overall model evaluation to compare the model fit with the null model was done using the likelihood ratio, the goodness of fit statistics against the actual outcomes was based on the Hosmer and Lemeshow (H-L) test and as a supplementary the Cox Snell R^2 indices. The classification tables were obtained to assess each model's sensitivity and specificity in predicting the development of TDF-RT. All statistical analyses were performed with SPSS software IBM SPSS STATISTICS Version 25.0 (IBM Corp, New York, USA.)

2.4 RESULTS

We conducted a cohort analysis of retrospectively collected clinical and laboratory data of patients who accessed ART services during the period June 2017 – November 2017 at the AIDCOE at the UTH in Lusaka Zambia. From the 8600 HIV infected patients recorded on the SMARTCARE database, 8228 (95.7%) were initiated on TDF containing regimens. A total of 915 consenting HIV positive patients were successfully recruited to our pharmacogenetic study. Of these, 887 participants were included in our analysis for clinical determinants of TDF-RT in this cohort after excluding some due to various reasons **(Figure 2.1).**

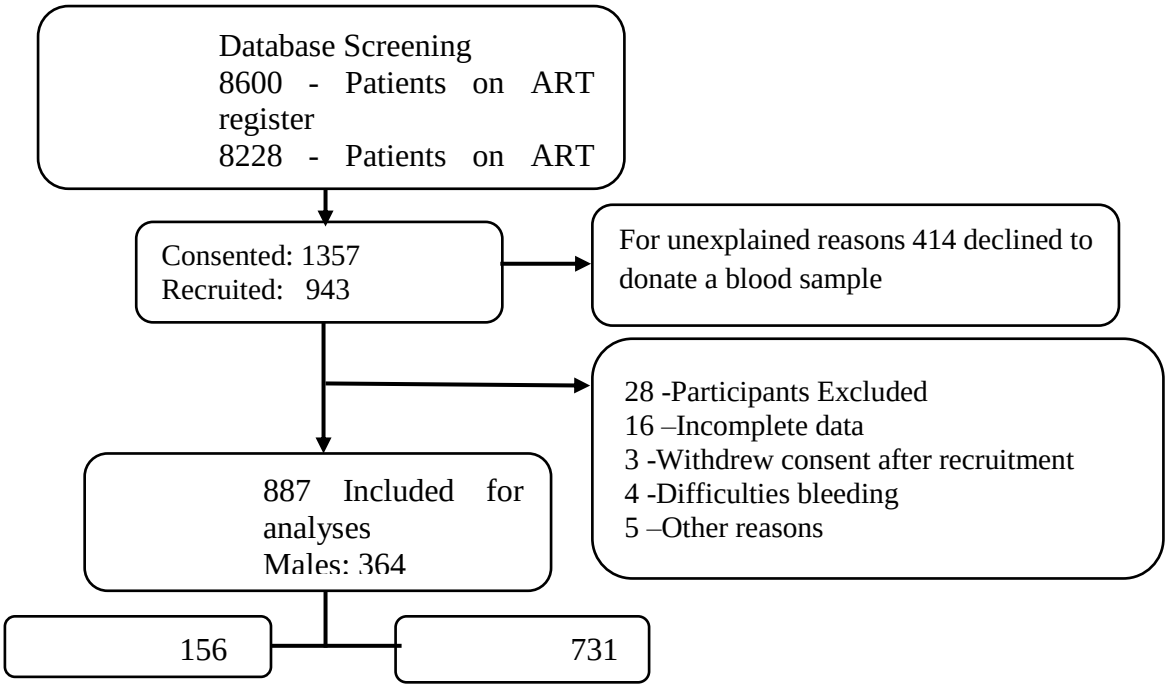


Figure 2.1. Flowchart for the Recruitment of participants

2.4.1 Demographic and Clinical characteristics of patients

We recruited 887 HIV patients from urban, peri-urban and rural areas of Lusaka. Being the capital city, Lusaka is highly heterogeneous; residents are of different cultural backgrounds, beliefs and tribes drawn from the 10 provinces of Zambia. Overall, participants were predominantly female (59%). The mean baseline age was 39 (± 11) years. The median duration of TDF treatment in all participants was 41 (IQR 16-72) months median baseline CD4 cell count was 230 (IQR 110–400) cells/mm^3 and serum creatinine was 69.2 (IQR 58 – 85) µmol/L while the creatinine clearance was (IQR 74.24-118.24)94.54ml/min.

Table 2.2 shows the baseline characteristics of participants stratified according to case definition; CrCl >60ml/min and CrCl <50ml/min. Out of the total 887 participants that were analysed, 156 (18%) were defined as cases (with TDF-RT) and 731(82.4%) controls under the case definition by CrCl < 60ml/min threshold compared to 103 (11.6%) cases and 784(88.2%)

controls under the CrCl <50ml/min case definition in the respective groups. In the case group defined by CrCr <60 there was a similar number of females and males in the case group (78 (50%)) compared to 445 (59%) females in controls. In the case group defined by CrCl <60ml/min, baseline median age was 44 (IQR:36-52) years, median baseline BMI was in the normal range: 20.6 (17.9-23.4) kg/m^2 and 21.2(18.7-24.8) kg/m^2 for cases and controls, respectively. Noteworthy was a median (IQR) baseline SCr of 77.4 (IQR; (61.6 - 95.6) μmol/l in the CrCl<60ml/min defined cases compared to 74 (IQR;60.5-88) μmol/L for the CrCl<50ml/min group, and a corresponding CrCl of 77.7 (59.5-102.7) ml/min vs 88.0 (71.5-108.6) ml/min respectively. For the CrCl<50ml/min case group, the median (IQR) baseline CrCl was higher than in controls 88 (71.5-108.6) ml/min vs 74 (95.6-118.9) ml/min. On the other hand, in the CrCl <60ml/min group, median baseline CrCl was lower in cases 77.4 (59.5 -102.7) ml/min compared to controls 97.5 (78.8 - 120.3) ml/min. Overall, baseline CD4 cell counts were similar in both categories regardless of the TDF-RT status while the sample median CD4 cell count was 200 (IQR:110-499) cells/mm^3.

Table 2.2. Baseline demographics of study participants (N-887)

Variables	**Case Definition-CrCl <60ml/min**		**Case Definition-CrCl <50ml/min**	
	TDF-RT (n=156)	**No TDF-RT (n=731)**	**TDF-RT (n=103)**	**No TDF-RT (n=784)**
	n(%), Median(IQR)	**n(%), Median(IQR)**	**n(%). Median(IQR)**	**n(%), Median(IQ)**
Male	78 (50)	286 (41)	54 (52.4)	310 (39.5)
Female	78 (50)	445 (59)	49 (47.6)	474 (60.5)
Age (years)	44 (36 - 52)	38 (32 - 45)	42.25 (35-46)	38.5 (32-46)
Age range				
≤25	8 (5.1)	76 (9.5)	6 (5.8)	84 (9.5)

26-35	27 (17.3)	201 (25.7)	20 (19.4)	228 (25.7)
36 - 45	57 (36.5)	280 (38)	45 (43.7)	337 (38)
46 - 55	38 (24.4)	128 (18.7)	22 (21.4)	166 (18.7)
≥56	26 (16.7)	46 (8.1)	10 (9.7)	72 (8.1)
weight (Kg)	56 (49-64)	58.6 (49-64)	58 (49.25-65.0)	58 (50-69)
BMI (kg/m^2)	20.6 (17.9 - 23.4)	21.2 (18.7-24.8)	21.1 (18-24.22)	21.0 (19-25)
BMI category				
<18.5	49 (31.4)	167 (24.4)	27 (26.2)	189 (24.1)
18.5-24.9	85 (54.5)	390 (53.6)	59 (57.3)	416 (53.1)
25-29.9	19 (12.2)	125 (16.1)	15 (14.6)	129 (16.5)
≥ 30	3 (1.9)	49 (5.9)	2 (1.9)	50 (6.4)
CD4 ($cells/mm^3$)	230 (109 - 359)	231 (111-416)	230(128.5-351.5)	230.5 (108-416)
CD4 range				
<200	62 (16.7)	319 (17.2)	39 (37.9)	342 (43.6)
200-499	78 (66)	287 (50.2)	58 (56.3)	307 (39.2)
≥ 500	16 (17.3)	125 (32.6)	6 (5.8)	135 (17.2)
SCr (μmol/L)	77.4(61.6- 95.6)	68 (57-83.1)	74 (60.5-88.0)	69 (58-84.3)
CrCl-CG (ml/min)*	77.4(59.5102.7)	97.5(78.8-120.3)	88.0(71.5-108.6)	74(95.6-118.9)

Note: BMI =Body mass index, SCr=Serum creatinine; CrCl-CG=Creatinine Clearance calculated Cockcroft Gault Formula. Summary statistics are mean/ median (SD/IQR) = Standard deviation/ Interquartile range; n (%) = count (percentage). Count % is a column percentage standard deviation/ Interquartile range; n (%) = count (percentage).

2.4.2 Characteristics of patients at recruitment according to TDF-RT

Patient characteristics at recruitment are summarised in Table 2.3. Across the two groups categories, most parameters were similar. For the CrCl<60ml/min vs CrCl<50ml/min groups;

median (IQR) SCr for cases was of 236.5 (131-478.75) μmol/L vs 368.7(220.9-665.8) μmol/L compared to 64.9 (54- 76.35) μmol/L vs 65.8(54.5-79.3) μmol/L for controls respectively. At the time of TDF-RT presentation, the majority of the cases were at least 36 years: 36-45 years (32%); 46–55years (25%) and ≥ 56 years (26%) while controls were between 26–55 years old for both groups. The BMI at recruitment remained similar to baseline values across the two groups with a median (IQR) BMI lying between 21.1 (18.62-23.95) kg/m2 in cases and 24 (20.7-27.6) kg/m2 in controls. As expected, the median (IQR) CrCl values for cases were 3-fold lower (28.5ml/min (12.70-466.28) than in controls (111.1 ml/min; 90.46 -134.83). The median CD4 count was similar with the majority having a CD4 cell count between 200 - 499 cells/mm^3.

Table 2.3. Characteristics of study Participants at RT event or Recruitment (N-887)

	CrCl <60ml/min	No TDF-RT (n= 731)	CrCl <50ml/min TDF-RT(n=103)	No TDF-RT (n=784)
Variables	n(%); Median(IQR)	n(%); Median(IQR)	n(%); Median(IQR)	n(%); Median(IQR)
Age (years)	46 (39 - 56)	42 (35 - 49)	44 (38.0-51.0)	43 (36-50)
Age Range				
≤ 25	6 (3.8)	50 (6.3)	5 (4.9)	51 (6.5)
26 to 35	20 (12.8)	137 (17.7)	15 (14.6)	142 (18.1)
36 to 45	50 (32.1)	271 (36.2)	37 (35.6)	284 (36.2)
46 to 55	39 (25)	189 (25.7)	30 (29.1)	198 (25.3)
≥ 56	41 (26.3)	84 (14.1)	16 (15.5)	109 (13.9)
Weight (Kg)	58 (49.7-55)	66(65.6-77)	58 (50.5-66.0)	65 (56.0-76.0)
BMI kg/m2	21.1(18.6-24)	24.0(20.7-27.6)	21.8(19.1-24.5)	23.7 (20.3-7.5)
BMI category				
<18.5	36 (23.1)	79 (10.8)	22 (21.4)	93 (11.9)
18.5-24.9	88 (56.4)	344 (47.1)	58 (56.3)	374 (47.7)
25-29.9	25 (16)	200 (27.4)	18 (17.5)	207 (26.4)
≥ 30	7 (4.5)	108 (14.8)	5 (4.9)	110 (14)
TDF Tx Duration (Months)	24.5 (8 -60)	48 (20-72)	13 (6.0-60.0)	48 (20.0-72.0)
Recent CD4 (cells/mm3)	355 (236-453)	412 (250-561)	357 (243.0-553)	357 (247.0-556)
Recent CD4 Range				
<200	26 (16.7)	126 (17.2)	39 (37.9)	342 (43.6)
200-499	103 (66)	367 (50.2)	58 (56.3)	307 (39.2)
≥ 500	27 (17.3)	238 (32.6)	6 (5.8)	135 (17.2)

Recent SCr (μmol/L)	236.5 (131-479)	64.9 (54-76.35	369(220.9-665.8)	65.8 (54.5-79.3)
CrCl at RT event/Recruitment	28.5(12.7-466.3)	111.1(90.46-34.83)	17.3 (9.2-31.6)	108.4(86.3-132.9)
% Change in CrCl-CG from baseline	78 (24.5-85.4)	12.2(5.6-38.5)	81 (62.6-90.0)	10.3 (7.6-35.6)

Note: BMI =Body mass index, SCr=Serum creatinine; CrCl-CG=Creatinine Clearance calculated Cockcroft Gault Formula. Summary statistics are mean/ median (SD/IQR) = Standard deviation/ Interquartile range; n (%) = count (percentage). Count % is a column percentage standard deviation/ Interquartile range; n (%) = count (percentage).

2.4.3 Comorbidities and Concomitant Drugs

Table 2.4. summarises comorbidities and concomitant drugs. We noted 8 (5%) participants in the case group had a record of cardiovascular disease (hypertension, heart failure, cardiovascular, accidents and stroke), 4 (2.6%) had diabetes while 4 (2.6%) had hepatitis B. In comparison, the prevalence of CVD, diabetes and Hepatitis B in the control group were 19 (2.6%), 0 and 18 (2.5%), respectively. Noteworthy is a large number of participants who did not have a record of comorbidities; whether they do not have it is highly uncertain.

In our cohort, approximately over 90% were on NRTIs/NNRTIs based ART regimens. Only about ~10. % of cases and ~8% of controls received regimens containing ritonavir-boosted PIs (atazanavir or lopinavir). In general, participants recorded concomitant use of other non-ART drugs. Most patients were receiving cotrimoxazole for prophylaxis for opportunistic infections (~34%, cases and ~43 %, controls). Noteworthy was a small number of participants who were on mycobacterium tuberculosis (TB) treatment. Interestingly, a relatively high number of participants recorded the use of haematinics (cases, 17, 10.9% and controls, 38, 6.2%) and herbal supplements. A very small number of cases (7, 5%) reported the use of non-steroidal

inflammatory drugs compared to controls (34; 4.6%).

Table 2.4. Comorbidities and concomitant drugs of Study Participants.

Variable	Case:CrCl <60ml/min		Case :CrCl <50ml/min	
	TDF-RT (n=156)	No TDF-RT (n= 731)	TDF-RT(n=103)	No TDF-RT(n=784)
	n(%)	n(%)	n(%)	n(%)
Other ARVs				
NRTIs/NNRTIs	140 (89.7)	679 (92.3)	94 (91.3)	725 (92.5)
ritonavir boosted PIs	16 (10.3)	52 (7.7)	9 (8.7)	59 (7.5)
None ARV drugs				
CTX	57 (36.5)	317 (42.2)	31 (31.1)	343 (43.8)
Supplements +Chemotherapy	4 (2.6)	28 (3.6)	3 (2.91)	29 (3.7)
Chemotherapy	4 (2.6)	11 (1.7)	2 (1.94)	13 (1.7)
Anti-diabetics /CVDs drugs	10 (6.4)	29 (4.4)	8 (7.777)	31 (4)
Haematinics	17 (10.9)	38 (6.2)	12 (11.65)	43 (5.5)
Herbal supplements	12 (7.7)	38 (5.6)	11 (10.68)	39 (5)
NSAIDs/Paracetamol	7 (4.5)	34 (4.6)	7 (6.8)	34 (4.3)
Not recorded /Not disclosed	45 (28.8)	235 (31.6)	29 (28.16)	251 (32.1)
Other medical conditions				
CVDs (HTN, HF, HHD, stroke)	8 (5.1)	19 (2.6)	6 (5.8)	21 (2.7)
TB/Meningitis	4 (2.6)	7 (1)	4 (3.9)	7 (0.9)
DM/CVDs+RD	4 (2.6)	0 (0)	4 (3.9)	0 (0)
Anaemia	7 (4.5)	20 (2.7)	3 (2.9)	24 (3.1)

TB/Meningitis	3 (1.9)	10 (1.4)	2 (1.9)	11 (1.4)
Hepatitis B	4 (2.6)	18 (2.5)	3 (2.9)	19 (2.4)
Other OIs	8 (2.6)	15 (2.1)	6 (5.8)	17 (2.2)
GIT Conditions/Not diarrhoea	1 (0.6)	16 (2.2)	1 (1)	16 (2)
None/Not recorded	117 (75)	626 (85.6)	74 (71.8)	669 (85.3)

HTN: Hypertension, HF: Heart failure, HHD CTX: Cotrimoxazole, ATT: Anti-tuberculosis treatment, CVDs: Cardiovascular conditions (hypertension, hypertensive heart disease, heart failure), DM: diabetes mellitus, NSAIDs: Non-steroidal anti-inflammatory drugs, GIT: Gastrointestinal; Chemotherapy (Antibiotics/Antifungal/Antimalarial)

2.4.4 Univariate analyses

Table 2.5 presents results for the univariate analysis of risk factors for TDF-RT impairment at baseline and recruitment. The analyses showed that for TDF-RT defined by CrCl<60ml/min, nearly all baseline and recruitment characteristics; patients' gender, age weight, and CrCl) contributed to TDF-RT and met the purposeful selection (p-value<0.2) for inclusion in the multivariable model analyse. In addition, CD4 cell count at recruitment also met the inclusion threshold. On the other hand, for TDF-RT defined by CrCl <50ml/min, all characteristics except for baseline BMI, SCr and age at recruitment met the inclusion criteria for multivariable model-fitting analyses.

Table 2.5. Univariate analyses of risk factors associated with TDF-RT

Variable	Case Definition-CrCl <60ml/min			Case Definition-CrCl <50ml/min		
	OR	95% CI	p	OR	95% CI	p
Baseline Parameters						
Sex (Male)	1.56	(1.1-2.20)	0.013	1.58	(1.10-2.28)	0.013
Age	1.05	(1.03-1.06)	<0.001	1.02	(1.00-1.04)	0.024
Baseline Weight (Kg)	0.977	(0.96-0.99)	0.001	0.986	(0.971-1.002)	0.007
BMI	0.95	(0.91 - 0.99)	0.007	0.98	(0.94-1.02)	0.333
CD4	1.00	(1.00-1.02)	0.449	0.99	(1.00-1.05)	0.155
SCr (µmol/L)	1.04	(1.0-1.07)	0.021	1.00	(0.995-1.00)	0.874
CrCl-CG	0.98	(0.97-0.99)	<0.001	0.92	(1.90-1.93)	<0.001
Parameters at recruitment						
Age (years)	1.04	(1.02–1.07)	<0.001	1.01	(0.99-1.03)	0.232
Baseline Weight (Kg)	0.95	(0.941-0.97)	<0.001	0.963	(0.947-0.98)	<0.001
BMI (kg/m^2)	0.88	(0.85-0.92)	<0.001	0.90	(0.87-0.95)	<0.001
CD4 ($cells/mm^3$)	0.99	(0.998-1.00)	0.013	0.97	(0.99-1.00)	0.016
SCr(µmol/L)	1.10	(1.08-1.13)	<0.001	1.03	(1.02-1.02)	<0.001
% Reduction in CrCl	0.95	(0.94 - 0.95)	<0.001	0.92	(0.93-0.96)	<0.001

p-value<0.2 cuts off point for inclusion in the multivariable model analyses; OR-odds ratio; 95%CI- 95% confidence interval.

2.4.5 Multivariable Logistic regression analysis of risk factors associated with TDF-RT

We conducted a multivariable logistic regression analysis to investigate the patient clinical and demographic characteristics that are independently associated with TDF-RT (defined by either CrCl <60ml/min or CrCl <50ml/min) . After including the variables that showed a p-value <0.2 in the univariate analysis, we fitted the respective data into a multiple regression to

compare the two models. Table 2.6 shows the covariates that were included in the two multivariable models and Table 2.7 is a summary of statistical results of individual predictors.

Table 2.6. Variables included in the multivariable model analyses

Case Definition <50ml/min)	Case Definition <60ml/min)
Sex	Sex
Baseline Age	Baseline Age
Baseline BMI	BMI at recruitment
BMI at recruitment (kg/m2)	Baseline CD4
Recent CD4 (cells/mm3)	RECENT CD4 arbitrary
Baseline SCr (μmol/L)	Baseline CrCl-CG
Baseline CrCl-CG	% Change in CrCl - CG
Recent SCr(μmol/L)	
% Reduction in CrCl - CG	

When our outcome of TDF-RT was defined as CrCl<60ml/min, baseline characteristics, gender, age, BMI, SCr and the % decline in CrCl and BMI at recruitment were independently associated with TDF-RT. The risk of developing TDF-RT was higher in females (OR: 13.22, 95%CI, 4.05–43.12) and the risk also increased with baseline age (OR, 1.186; 95% CI, 1.12-1.26). Patients with higher baseline serum creatinine levels were more likely to develop TDF-RT (OR:1.198; 95% CI. 1.14-1.26, <0.001) than those with lower levels.

Further, those with a higher baseline BMI were at a lower risk of developing TDF-RT (OR 0.495; 95% CI.0.379 - 0.647, p<0.001), whilst a higher BMI at recruitment was associated with a higher risk of developing TDF-RT (OR: 1.23; 95%CI. 1.028-1.479) which highlight the

influence of HIV infection and metabolic diseases in renal dysfunction.

On the other hand, when our outcome of TDF-RT was defined as CrCl <50ml/min; only baseline SCr, CrCl and the % decline in CrCl were independently associated with TDF-RT at statistical significant of p<0.05. Those with higher baseline SCr were at a higher risk of developing TDF-RT (OR: 1.037; 95% CI, 1.02-1.06). In addition, those with higher baseline CrCl (OR: 0.973; 95% CI. 0.957-0.989,) and greater % decline in CrCl (OR: 0.891; 95% CI,0.870-0.913) were at a lower risk of developing TDF-RT.

Table 2.7. Multivariable logistic regression models for TDF-RT

Variable	CrCl <60ml/min)			CrCl <50ml/min)		
	OR	95%CI	p-value	OR	95%CI	p-value
Sex* (Female)	13.2	(4.06-43)	<0.001			
Age:	1.12	(1.12-1.26)	0.01			
Baseline BMI	0.50	(0.38-0.65)	<0.001			
BMI at recruitment	1.23	(1.03-1.48)	0.024			
SCr*(µmol/L)	1.20	(1.14-1.26)	<0.001	1.04	(1.02-1.06)	<0.001
CrCl-CG* (ml/min)	–	–	–	0.97	(0.96-0.99)	0.001
% Decrease in CrCl CG	0.83	(0.79-0.87)	<0.001	0.89	(0.87-0.91	<0.001

*Are baseline values. Statistical significance is p-value < 0.05; 95% CI - 95% confidence interval

The logistic regression to describe the data fit for both models was obtained by statistical tests of model coefficients, goodness-of-fit tests and the Cox Snell R2 as summarised in Figure 2.2

and The table below demonstrates the classification of cases and control by the two models.

TDF-RT Model	Model Coefficients	H-L goodness of fit	Cox Snell R^2
CrCl<60ml/min	X^2, p-value (df:5,N=887) =711.89, p<0.001	X^2;P-value = 5.14; p=0.647)	55.3%
CrCl<50ml/min	X^2, p-value (df:5,N=887) = 520,67, p<0.001	X2;P-value = 4.68; p=0.791)	44 %

Figure 2.2. Parameters for Model fit for TDF-RT by CrCl<60ml/min and CrCl<50ml/min. The figure shows the parameters used to evaluate the logistic model: tests of model coefficients; X^2 =Chi squared, df=degree of freedom, N= Sample size; hosmer and lemeshow test (H-L) and the the Cox Snell R^2 .

Table 2.8. Percentage accuracy in classification of cases and controls for TDF-RT: CrCl<60ml/min and TDF-RT: CrCl<50ml/min models

Observed	**Predicted**		
	Cases	Controls	% Accuracy
Case (TDF-RT: CrCl<60ml/min	140	16	89.7%
Controls	11	717	98.5%
Overall % Accuracy			96.9%
Case (TDF-RT: CrCl<50ml/min	89	14	89.3%
Controls	12	769	98.5%
Overall % Accuracy			97.1%

2.5 DISCUSSION

2.5.1 Demographics of Research participants

We investigated the clinical determinants associated with tenofovir-induced nephrotoxicity. in a cohort of HIV infected patients that were receiving TDF based regimens in Zambia. We report the results from the analyses of 887 stable, HIV-infected patients who were initiated on TDF based ART from the AIDCOE-UTH, Lusaka, Zambia. Our participants were predominantly female, similar to other studies[321, 508-510]. This is also in line with global figures indicating that over 50% (19.2 million) of HIV infected patients are females (≥15 years) and 59% are in sub-Saharan Africa[36]. Female patients are more vulnerable to acquiring the HIV infection compared to men but are more likely to consent to seek medical help than men [511]. Generally, participants had a normal median baseline CrCl and CD4 cell counts. Moreover, some patients were ART experienced because they were migrated from other ART regimens prior to TDF, hence their normal baseline parameters.

2.5.2 Definition of TDF-RT and incidence of renal impairment

2.5.3 Definition of patients with TDF-RT

Our study determined that 18% HIV infected patients receiving TDF based ART developed nephrotoxicity defined as CrCl < 60ml/min by the Cockcroft Gault (CG) formula. In the same cohort, based on medical notes, we noted that physicians defined toxicity when CrCl was <50ml/min, leading to an incidence of 11.6%. The CG formula remains the gold standard for measuring renal function and a CrCl<60 ml/min is classified as mild to moderate (45-59

ml/min) reduction in renal function according to kidney disease improving global outcomes (KDIGO) classification[512]. The physician definition of TDF-RT (CrCl<50ml/min) is consistent with the ART management guidelines which suggest switching to another NRTI or NNRTI when CrCl is below 50ml/min[32]. This threshold, however, may lead to misdiagnosis and late management of the toxicity given that subclinical TDF-RT and a gradual decline in renal function has been reported even in the absence of overt clinical evidence[289, 295]. Generally, it would take a loss of at least two-thirds of renal mass or at least several days for substantial loss of GFR to be reflected through an elevated serum creatinine[513, 514] which may also preclude early presentation and diagnosis of renal dysfunction. In comparison to the case definition of CrCl <60ml/min, an estimation of GFR using the MDRD equation defined 14% as to cases (eGFR<60ml/min), 4% less than the CG formula in the same cohort.

Our CG case definition appears to contrast the findings of a South African Cohort where a small but significant reduction in eGFR over time with the MDRD formula was accompanied by a small increase in eGFR using the CG equation[293]. Indeed, a similar study observed that renal function based eGFR (CG) increased over time [509]. However, an analysis of 19 patients receiving tenofovir found similar results to the present study, in that eGFR by the Cockcroft-Gault formula declined over time[284]. While these studies may be different in design to ours, it has been argued that the decline in eGFR in TDF induced toxicity is largely influenced by tubular creatinine excretion rather than glomerular dysfunction[284]. This may suggest that the incidence of nephrotoxicity could be higher if more sensitive methods and markers were used. The disparity in case definitions with these formulas may explain, why to date, the incidence of TDF toxicity is not well known and varies across published studies[240, 288, 508, 515] with some being lower than that observed in this study.

Whereas both formulas remain useful and widely used, scientists have acknowledged weaknesses in accurately estimating kidney function. None of the formulas was validated in HIV infected populations although, in a small cohort of HIV patients, it was found that the CG best reflected the real GFR compared to measured glomerular filtrations rate[516]. Furthermore, the MDRD was limited by age while CG does not correct for body surface area which is influenced by weight and BMI leading to overestimation of GFR[517]. On the other hand, the MDRD tends to underestimate GFR at higher SCr levels in HIV infection[278]. Contrary to these assertions, our findings show that the CG formula had lower eGFR values leading to defining a higher incidence of TDF-RT compared to the MDRD suggesting that the incidence of nephrotoxicity could in fact be higher if more sensitive methods are used to measure renal function.

2.5.4 Incidence of TDF-induced renal impairment

Our work shows that 18% of patients on TDF had renal toxicity during the course of their treatment. Following a mean TDF exposure of 41months (IQR:16-72), in the case group, the median baseline serum creatinine increased 3-fold at the time of RT event compared to the baseline value. Our results are similar to those of Nartey and colleagues, who retrospectively analysed a cohort of 300 TDF treated Ghanaian HIV patients and reported a 21% incidence of renal impairment (CrCl<50 mL/min) after 2.9 years (IQR 2.3–3.4) follow-up[288]. Similar findings were reported in Nigeria in an observational study evaluating 186 participants which reported an increase in serum creatinine by 18.1% and a decline in eGFR of 4.8% in the TDF treated group[287]. On the other hand, an incidence rate of 29.2 cases per 1000 person-years (95 % CI 22.1–36.3) of moderate renal impairment (eGFR <60ml/min) was reported in a Spanish cohort[322].

Glomerular dysfunction has been observed with long term use of ART with TDF being independently associated[518]. Moreover, an association of the cumulative effect of longer duration of TDF exposure on renal function has also been investigated[508]. In a cohort of nearly 5000 Nigerian patients, the prevalence in TDF associated nephrotoxicity increased from 10% at 24 weeks to 45% at 144 weeks[508]. In a larger cohort of over 15,000 South African patients with normal baseline GFR (≥90 mL/min), there was a small but significant reduction in renal function after nearly 2 years of treatment[293]. In Zambia, Mulenga et al. observed that TDF treated patients with mild baseline renal injury had a higher risk of progressing to moderate or severe renal impairment than their non-TDF exposed counterparts in the first year of antiretroviral therapy[475]. In addition, another Zambian urban cohort reported that the proportion of TDF treated patients developing renal impairment (CrCl <50ml/min) ranged from 2.6% to 62% at 6 and 12 months, respectively[474]. All these studies along with our findings confirm the evidence that renal toxicity is an important adverse effect experienced by HIV positive patients receiving long term tenofovir based cART in Africa.

Some studies have reported a lower incidence of TDF associated RT compared to this present study. A Ghanaian study reported a 7% incidence during a 20-month study period[290], while 7.8% of patients had >50% decline in baseline GFR after 48-weeks follow-up in another study[519]. In addition, Nishijima and colleagues[520] reported a 9.84% incidence compared to 4.55% in those not treated with TDF. Furthermore, a decline in eGFR of -7ml/min was reported in Caucasian and Indian patients at 24 months[521] similar (7–10mL/min/) to that reported by Rodriguez-Novoa et al[522]. No matter the magnitude and its origin, renal dysfunction in HIV patients is of clinical importance. In the particular case of TDF-RT, subclinical renal impairment progressing to CKD has been observed to raise HIV related referrals to nephrologists by over 20%[275].

Our findings contrast with the early TDF clinical trials and post-marketing studies that reported a safe TDF renal profile in well-selected cohorts of subjects[261, 523]. We note that recent studies in African and Western populations have been reporting a rare but significant small decline in renal function associated with tenofovir use in HIV positive patients[320, 518, 523-525]. Among 59,479 HIV-infected persons, TDF exposure was associated with a higher risk of developing various forms of renal impairment including increased risk of proteinuria by 34%, acute decline impairment by 11% and CKD by 33%[320]. Other studies included Nigerian cohorts where the cumulative incidences of TDF-RT ranged from 4.6 to 4.8% compared to 2.3 to 5.1% in the TDF free arm following 48 weeks of TDF exposure[321, 508]. Small cohorts of treatment naïve HIV positive patients also reported a low prevalence of TDF-RT[362, 486, 526]. In another cohort analysis from Lesotho, the use of TDF was a borderline significant factor for renal toxicity (p-0.054)[527]. These findings suggest that the cumulative toxic effect of tenofovir may not be apparent during a short duration of follow-up. The difference between these studies compared to the present study may be a combination of factors that include study design, larger sample size and a longer period of follow-up. TDF has also been associated with a subclinical, small but persistent decline in renal function in the presence of normal glomerular parameters which may clinically lead to misdiagnosis and underestimation of the accurate incidence of nephrotoxicity in this population[295].

2.5.5 Factors associated TDF-RT- Univariate assessment

Univariate analysis of our data showed that multiple clinical determinants may be associated with TDF-RT. In both analyses of the two sets of data; CrCl <60ml/min and CrCl <50ml/min, baseline and factors at recruitment (sex, age, BMI, Serum creatinine, CD4 cell count at recruitment, CrCl, % and changes in CrCl) were statistically associated with tenofovir induced

renal toxicity ($p<0.05$), showing that TDF induced renal toxicity may be multifactorial in nature. In a very large (>60 000 HIV positive subjects) comparative study, Mulenga and colleagues observed that patients who initiated TDF ART with normal baseline renal function or mild to moderate kidney disease were more likely to have lower eGFR during prolonged exposure to TDF. These patients were also at higher risk (2-3 times) of developing worsening renal impairment[475]. Renal toxicity in HIV patients receiving TDF based cART has been associated with older age, lower BMI, and concurrent use of ritonavir-boosted PIs[238, 320, 528]. Moreover, older patients and low body weight have been associated with progressive decline in renal function (eGFR <60ml/min)[240, 293]. While factors like age and BMI were also observed in our analyses, the use of PI/r was not associated with TDF-RT in our cohort presumably because of a smaller sample size. These findings are important because they are practical pointers of where clinicians may be required to exercise caution when prescribing TDF combined ART and schedule monitoring. Moreover, a review of factors associated with renal toxicity in TDF treated patients in Africans found a high prevalence of multiple patient-related factors[240].

HIV infected patients are known to have comorbidities and therefore likely to be receiving other drugs. As this was the case in our cohort, multiple drugs were reported to be concomitantly used. Among these was the common use of cotrimoxazole (CTX), a combination of trimethoprim/sulfamethoxazole and haematinics (iron boosting drugs). Cotrimoxazole is commonly used in prophylaxis of opportunistic infections but it is pharmacologically known to raise serum creatinine and cases of interstitial nephritis and acute tubular necrosis have been reported[529]. The use of haematincs to treat anaemia, a common finding in HIV infection that may also be due to renal impairment resulting from reduced erythropoiesis. Incidences of anaemia in TDF nephrotoxicity have been reported elsewhere[288].

HIV infection and impaired kidney function have a combined impact on lowering haemoglobin levels, resulting in a higher risk of anaemia[530]. In addition, cardiovascular conditions and not diabetes was relatively common y in the TDF-RT groups for both case definitions. It is noteworthy that the effectiveness of ART has led to an increase in age-related chronic comorbidities such as cardiovascular diseases, a decline in renal function and diabetes. It is therefore expected that tenofovir induced nephrotoxicity may be impacted by cardiovascular risk factors. Our cohort endeavoured to exclude participants where traditional risk factors for renal impairment were present during or before TDF-RT. it was therefore not surprising that our study recorded a small number of these co-morbidities due to a strict pre-determined exclusion criterion. Indeed hypertension, pre-existing renal dysfunction and diabetes mellitus have been reported as risk factors for TDF-RT as well as renal impairment in HIV infected patients[486, 531].

A higher risk of tenofovir-induced renal toxicity has been reported in HIV patients with immunosuppression of CD4 cell count to <200cell/ml[3] [532]. However, our study found no difference in CD4 T cell count between cases and controls similar to a study by Fux et al[420]. Neither did we find an association between TDF-RT with different regimens (with PI/r and with NNRTIs) similar to Nartey et al[288, 533]. Nevertheless, other studies have reported a higher decline in eGFR with concomitant use of PI/s[283, 322] a finding that is supported by the potential role of ritonavir boosted PIs in inhibiting the tenofovir efflux transporter protein MRP2 thereby increasing its accumulation and toxicity to the proximal tubules[397, 534, 535]. However, the role of MRP2 in the cellular transport of TDF is still inconclusive[536].

It should be stressed that our study population comprised almost exclusively ART experienced patients with normal baseline renal function (median SCr=77.4ml/min) before receiving TDF

based ART. We, therefore, hypothesise that the associated nephrotoxicity in our cohort may be due to a combination of immunological, patient and treatment-related factors. In addition, only a very small number of patients were receiving PIs/r and their contribution to TDF-RT in this cohort may be insignificant. We however note that large randomised trials have not reported renal insufficiency with concomitant administration of NNRTIs, a class of drugs not known to have any inhibitory effect on renal transporter proteins [203, 537]

2.5.6 Factors independently associated renal impairment

In a model fitting data for TDF-RT (CrCl <60ml/min). gender (male), baseline age, SCr, BMI at baseline and recruitment as well as the percentage reduction in CrCl were determined as predictors of TDF-RT. According to our findings, females (OR=13) were more likely to develop TDF-RT compared to male patients. In comparison to the TDF-RT (CrCl <50ml/min) model, only baseline SCr, CrCl and the percentage reduction in CrCl were determined as predictors of TDF-RT. We attribute the difference in the predictors to the difference in the number of cases CrCl <60ml/min (156) vs CrCl <50ml/min (103). The CrCl <60ml/min case group may have patients in whom TDF nephrotoxicity may be a result of additional factors like sex and BMI. The predictors of TDF-RT determined by both case definitions have been independently associated with TDF-RT in many African studies[321, 538-540]. Our results support earlier findings in a large Zambian cohort by Mulenga and colleagues where a higher risk of progressive decline in renal functions was observed in patients with normal baseline eGFR[475]. A similar study reported that cumulative and current exposure to TDF in patients with normal baseline eGFR increased the annual incidence of developing chronic kidney disease for up to 6 years[318]. In our study, we showed that for the two case definitions, a higher percentage decline in creatinine clearance (OR= 0.830 and OR= 0.891) was associated with a 17% and 11%

increase in the risk of renal toxicity respectively. Generally, irrespective of case definition, patients with TDF-RT had higher baseline serum creatinine levels compared to controls and therefore, slight deviations from the normal values would be indicative of abnormalities in renal function.

This study observed an independent association of patients with low baseline BMI (body weight) (OR= 0.59) and advanced age (OR= 1.2) with developing TDF-RT. Our results are consistent with previous findings where TDF nephrotoxicity was associated with lower weight, with low BMI and advanced age[288, 293, 323, 324, 509, 541]. A recent retrospective study observed that among other factors, lower BMI (underweight) and older age were positive predictors of TDF impaired renal function[321]. Moreover, a large cohort study (15,156 patients) showed findings similar to our findings: low body weight and older age were independently associated with a greater risk of experiencing a decline in eGFR below 30 mL/min and a successive loss of 1 ml/min in eGFR per year[293]. The association of age and decline in organ function is well known. In the general public, advanced age is a risk factor for chronic kidney disease with an average decline of 0.4ml/min per year decline in renal function in older patients than young ones[542]. This explains a significant association between low body weight and chronic kidney disease in patients living with HIV infection administered nephrotoxic drugs[543] as well as our observation of TDF-RT particularly in patients aged at least 46-years-old. While this association was previously observed along with low CD4 cell count and concomitant use of protease inhibitors in South African studies [293, 509, 544], our cohort did not observe these factors. The difference in our findings could be explained by the fact that most of our participants were ART experienced patients whose CD4 cell count may have improved at the time of commencing TDF based ART.

In our study, people with a higher BMI at the time of recruitment had a greater risk of developing renal toxicity. Since the determination of CrCl is based on age, body weight, serum creatinine and gender, it is highly likely that CrCl may fluctuate with serum creatinine levels that are dependent on muscle mass. Patients on cART are likely to develop a higher muscle mass proportionate to their response to treatment, and this may be more significant in those with low baseline weight reflecting the reversal of wasting in those patients who were most malnourished. Such an increase in muscle mass could then result in higher serum creatinine levels and eGFR despite no significant changes in the actual renal function[323]. This could be why both a low baseline BMI and high BMI at recruitment were both independently associated with TDF-RT.

Based on the variance in the incidence rate between our study and others, and the variance in severity and trajectory of renal injury observed from other studies; we postulate that TDF-RT is due to an interaction of multiple factors that impact renal function in different ways. These may include patient-specific characteristics, HIV infection itself and genetic factors. For example, the genetic predisposition of Africans conferred by a missense mutation in the *APOL1* increases the risk of developing ESRD[177, 545] and genetic variants in renal transporter proteins affecting the handling of tenofovir elimination cannot be ruled out from our cohort.

Based on the logistic regression models fit with data for the two case definitions (CrCl<60mls vs CrCl<50mls), model parameters (Model coefficients, H-L test and the Cox Snell R^2) consistently demonstrated that the respective models provided a better fit to the data compared to a constant model also known as a null model in predicting the development of TDF-RT. Both models fit the data well based on their H-L tests consistent with the model coefficients indicating that predictor variables in both models best explain the observed association with

TDF-RT based on the respective CrCl threshold that defines the cases. These findings were supported by the Cox Snell R^2 indices showing a proportion of the variation in TDF-RT that can be explained by the respective model predictor variables; 55.3% for CrCl<60mls and 44% for CrCl<50mls. It must be declared that while the Cox Snell R^2 is more useful in linear regression, there is no exact equivalent interpretation for logistic models and therefore, we used this parameter as a supplement to other model statistical indices. In our study, these parameters were supported by the observed and predicted classification of TDF-RT. There were no differences in the sensitivity (correctly classified cases) and specificity (correctly classified controls). In both models, misclassification as false cases and controls were lower than 15%. We can conclude that both models can effectively predict the risk of developing TDF-RT despite the different CrCl thresholds used to define the cases. However, based on the multiple aetiologies of TDF-RT, we would recommend the use of the CrCl <60ml/min model because it captures a diverse range of predictors that are common in HIV infected patients with a possibility for early identification of TDF-RT.

2.5.6.1 **Limitations**

We acknowledge that this analysis has limitations that could have impacted our findings, and therefore, interpretation of our findings should take account of the following. Firstly, the study was a retrospective case-control study and not a randomized controlled trial, and we cannot exclude potential confounders. Secondly, our study participants included patients who were initiated on TDF as either treatment-naïve or experienced who were migrated from stavudine to TDF. Treatment-naïve patients could be different from treatment-experienced patients who could have stabilised baseline risk factors for TDF-RT and therefore we could not measure the true baseline effect in this study. In the third place, our case definition was based on the

estimation of renal function (CrCl) using SCr, a surrogate marker that is neither specific nor sensitive to tubular injury induced by TDF. We, therefore, did not account for TDF-related proximal tubular injury which may be present insidiously leading to concealed kidney injury. In addition, the difference between cases and controls was based on renal function estimated by two measurements, at baseline and time of recruitment which could not allow us to determine the cumulative effect of TDF on renal function at different time points. Due to limitations in the hospital laboratory, albumin and proteinuria were not routinely measured to determine the extent of renal failure.

2.5.6.2 **Conclusions**

We have presented findings investigating the determinants of TDF-RT in a cohort of HIV infected patients from Zambia. Our findings show that TDF induced renal toxicity is prevalent in HIV-infected patients receiving TDF based antiretroviral therapy in Zambia and it may be attributed to an interaction of multiple factors, particularly baseline patient characteristics. Furthermore, the use of CrCl <60ml/min in place of CrCl <50ml/min may adequately identify TDF-RT in patients with different ranging risk factors. The significance of these findings places an emphasis on identifying and judiciously monitoring renal function in high-risk patients. More sensitive markers of tubular function would likely identify a greater number of patients, and facilitate the determination of the true prevalence of TDF-RT which currently remains unknown. Our findings add further concern regarding the future of TDF and its place in long term ART, particularly now that ART access has been scaled up to "test and treat" irrespective of the CD4 count and laboratory monitoring of baseline renal performance[33, 480, 546].

CHAPTER 3

PHARMACOGENETICS OF TDF-RT IN HIV PATIENTS RECEIVING TENOFOVIR DISOPROXIL FUMARATE BASED ANTIRETROVIRAL THERAPY IN ZAMBIA - PHARMACOGENETIC STUDY OF TENOFOVIR

3.1 INTRODUCTION

TDF has continued to be part of cART guidelines in the management of HIV infection in many countries despite the associated nephrotoxicity reported as renal proximal tubular injury, nephrogenic diabetes insipidus (NDI), Fanconi syndrome and acute renal failure (ARF). The magnitude of TDF-RT has been demonstrated in observed partial reversibility of kidney injury and progression to CKD long after TDF treatment discontinuation[312, 547]. The continued use of TDF in the management of HIV infection demonstrates that its efficacy, tolerance and durability outweigh the associated renal toxicity.

Given that the intracellular concentration of NRTIs is responsible for many of their adverse effects including lactic acidosis, pancreatitis, peripheral neuropathy, and lipoatrophy[339], evidence suggests that membrane transporter-dependency of TDF in human proximal tubular cell lines is vital in eliciting accumulation and toxicity in the tubular cell[270]. The influence of proximal tubular transporters on TFV plasma and urinary concentrations, as well as on markers of tubular function has been widely studied[252, 315, 328, 548]. These studies have provided the scientific basis for the direct or indirect involvement of genes encoding drug transporters in renal proximal tubules on the disposition of tenofovir. Therefore, recognising predictors that identify patients at higher risk of developing renal complications would be beneficial in preventing nephrotoxicity associated with TDF-containing cART. Moreover, over the last decade, several studies have reported their findings from candidate gene screening of variants suspected to be involved in TFV-induced nephrotoxicity in predominantly Caucasian and Asian populations. These include *SLC22A6* (hOAT1) and *SLC22A8* (hOAT3); *ABCC2* (MRP2), *ABCC4* (MRP4)[298, 397, 400] and *ABCC10* (MRP7)[253, 396, 401]. Apart from genes encoding drug transporters,

genes such as the oculocerebro-renal lowe protein 1 (*OCRL1)* has shown to be associated with Mendelian FS and has independently been associated with increased SCr levels in patients with TDF-FS[398]. Although pharmacogenetic studies of tenofovir have not been completely reproducible, SNPs in genes encoding TFV proximal tubular transporters have been reported to possess the potential to alter the TFV renal transport thereby altering its renal clearance and causing subsequent nephrotoxicity. Furthermore, recently, unpublished data from a genome-wide association study (GWAS) in a cohort of UK HIV patients with TDF-FS identified some genetic markers of interest. These suggested SNPs from the GWAS are represented in the Manhattan plot shown in figure 3.1. The suggested SNPs were mainly found in transmembrane genes that include the transmembrane protein 120A (*TMEM120A*) gene, intersectin1 (*ITSN1*) and Peroxisomal Membrane Protein *(PEX14*). *TMEM120A* is a transmembrane gene while *INSN1* encodes a cytoplasmic membrane-associated protein that indirectly coordinates endocytic membrane traffic with the actin assembly machinery. PEX14 encodes a peroxisomal membrane protein (proxin14), an essential component of the peroxisomal import machinery that is vital for peroxisome movement through the direct interaction with tubulin.

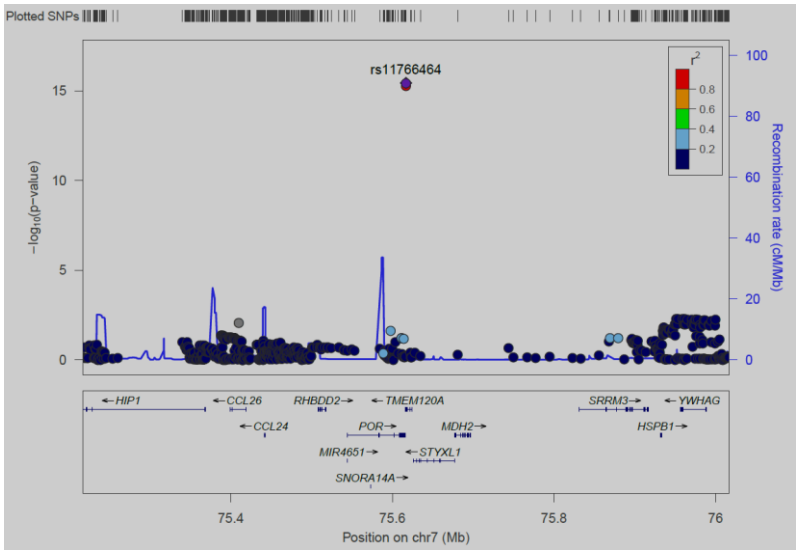


Figure 3.1. The Manhattan plot of Genes and SNPs from GWAS of TDF-FS

In the present study, we investigated the role of selected candidate gene variants in developing TDF-RT in a cohort of HIV positive patients receiving recruited from Africa; Zambia. Zambia is part of the Sub Saharan African region which is home to the highest global burden of HIV infection (29 million) where TDF may remain in use for the foreseeable future. So far, to our knowledge, only one single centre study from Ghana, comprising a cohort of sixty-six participants[396] has investigated the role of SNPs in TDF-RT in the African population.

3.1.1 Research Hypothesis

Single nucleotide polymorphisms in genes encoding tenofovir efflux drug transporters hOAT4 (*SLC22A11*), *ABCC2, ABCC4* and *ABCC10* previously found associated with TDF-RT in other populations, as well as genes associated with mendelian Fanconi syndrome (*OCRL1, TMEM1201, PEX14* and *ISTN*) may be associated with TDF-RT in Zambian HIV patients.

3.1.2 Study Objectives

1. To determine the association between TDF-RT with previously reported genetic variants in genes encoding drug transporter proteins in a cohort of Zambian HIV positive patients.
2. To explore the association of genes involved in mendelian Fanconi syndrome (OCRL1) and transmembrane genes with TDF-RT.

3.2 METHODS

3.2.1 Study Design and Recruitment

Unrelated HIV-1 infected subjects of African ethnicity were retrospectively recruited from the University Teaching Hospital in Lusaka, Zambia. The recruitment process, inclusion/exclusion criteria and characteristics of the cohort have been described in Chapter 2. We used a candidate

gene-based case-control design for investigating the contribution of SNPs in TDF-RT. Definitions for cases and controls are described in Chapter 2. Candidate gene selection is described below. Apart from demographics and clinical data, each recruited participant donated a whole blood sample (9ml) which was collected in EDTA bottles for DNA extraction for the genetic study.

3.2.2 Ethics Considerations

The protocol to conduct pharmacogenetic research and biomaerker was approved by The University of Zambia Bioresearch Ethics Committee, Ref.NO.013-05-17, appendix A. Permission to obtain research data and material was obtained from the local health institution in collaboration with The National Health Research Authority, appendix B. The Material Transfer Agreement to transfer human whole blood samples from Zambia to Liverpool was given by The National Health Research Authority (MH/101/23/10/1) see Appendix E.

3.2.3 Experimental Materials and Reagents

The genomic DNA extraction kits consisting of Chemagen paramagnetic beads, protease, lysis buffer, wash buffers, binding and elution buffers and disposable plastic tips were sourced from PerkinElmer LAS (UK) LTD (Beaconsfield, Bucks, UK. A nanodrop N8000 from Thermo Fisher Scientific (Wilmington, DE, USA) was used for the quantification of extracted DNA and quality control. PicoGreen® dsDNA quantitation reagents (PicoGreen reagent, 20XTE and lambda DNA standard) were purchased from Thermo-Fisher Scientific (Loughborough, UK). The two multiplex (18 plex and 6 plex) SNP assays were designed by the Agena software and iplex genotyping reagents (iPLEX® Gold, PCR, extension and termination reagents and SpectroCHIP® Kit) were sourced from Agena Bioscience GmbH (, Hamburg, Germany). 30 base primer pairs for amplification and extension of the selected SNPs were supplied by

Metabion International AG (Planegg Germany). For TaqMan genotyping; Genotyping Master Mix and assays for ABCC2 -24T (rs717620), ABCC2 (rs3740066) and ABCC10 (rs2127539) were purchased from Thermo Fisher Scientific (Paisley, Renfrewshire, UK).

3.2.4 Selection of single nucleotide variants in candidate genes

All SNPs were selected based on previous research evidence of their association with TDF induced nephrotoxicity due to their possible role in proximal tubular transportation and/or impact on tenofovir excretion. Firstly, we selected SNPs in genes encoding tenofovir transporter proteins based on previously reported studies and their potential functional significance. We selected those SNPs with a minor allele frequency of >3% in the African population; where this was not met, we searched for SNPs that were in linkage disequilibrium (R^2~0.8 and D'= 1) with the reported SNP and selected that. A summary of the selected twenty-four (24) SNPs from eight genes showing the basis of their selection is given in table 3.1.

3.2.5 Experimental Methods

The genetic analysis comprised of various steps leading to the downstream statistical analysis. To ensure good clinical and laboratory practices, all the steps leading to the final analysis was carried out in compliance with the University's Health and Safety guidelines and are described below.

Table 3.1. Summary of selected the SNPs

GENE - rsID	Chr : BP, SNP Consequence	Evidence of TDF-RT	Population Reported	Involved Allele	AFR	CAU	ASI	REF
ABCC2 rs717620 C>T	10: -24 5' prime UTR	↑TDF-RT. CC genotype ↑ plasma TFV, phosphorus wasting, β_2-MG excretion and ↓eGFR	Spanish, Thai	C	3	21	22	298, 335, 339, 397, 549
rs2273697 G>A	10: 1249 missense	Higher MAF in TDF-RT(42.3% vs 17%). ↑amino acid excretion and risk of stopping TDF treatment within 1 year in carriers of the allele. Part of high-risk haplotype	Swiss	A	19	20	10	334, 398
rs3740066 C>T	10 :3972 missense	Associated with RT, β_2-MG excretion and phosphorus wasting. Part of high-risk haplotype	Spanish/ Japanese	C	22	37	25	298
rs8187707 C>T	10: 4488 synonymous	↑risk of TDF-FS and altered GFR. Allele A overrepresented in patients with TDF-RT.	North American	G	3	7		397, 398
rs79174032 C> A	10: 1032 synonymous	Independently associated with changes in SCr in TDF-FS	North American	A	4	0	0	398
rs7899457 C>T	10: 4110 Synonymous	Significantly associated with TDF-FS	Caucasian	T	11	0	0	398
rs17222519 G>A	10: 99 Intron			G	3	0	0	298, 397
rs17216177 T>C	10: 3742 non-coding			T	18	7	0	398

	exon							
ABCC4 rs899494 A>G	13: 669 synonymou	↑in TDF-RT; phosphorus wasting; ↑Allelic frequency T in patients with TDF-RT	Spanish /Thai	A	20	16	19	298, 334, 397
rs3742106 A>C	13: 4131 3'prime UTR	Altered uric acid excretion and genotype TG/GG associated with ↑TFV plasma	Spanish /Thai	G	31	38	50	402, 403
rs1751034 C>G	13: 3463 missense	High intracellular TFV-DP. SNP alters messenger RNA (mRNA) splicing in MRP4 expression	Thai	G	27	19	22	403
rs1059751 G>A	13:879 3'prime UTR	Predisposition AA genotype ↑ TDF-RT, ↓TFV excretion, ↑ β_2-MG excretion	Thai ,	G	28	48	49	403, 404
rs11568658 C>A	13: 559 missense	Predicted by SIFT likely to affect MRP4 function		A	0	3	12	SIFT
rs2274409 C>T	13:819 Intron	Associated with TDF-FS	Caucasian	T	4	10	19	398

Table 3.1. Continued.

GENE - rsID	Chr : BP, SNP Consequence	Evidence of TDF-RT	Population Reported	Involved Allele	AFR	CAU	ASIA	REF
ABC10 rs9349256 G>A	6:1875 Intron	Allele G sig associated with TDF-RT (72.2%), ↑ urinary excretion of phosphorus and B2MG in the TDF-RT. ↓ TFV excretion in GA/AA genotype treated with PIs.	Spanish, Italian, African	A	3	46	59	253, 404, 405
rs2125739 T>C	6:2759 Intron	the SNP (also part of the haplotype) has been associated with TDF-KTD. Significantly assoc with poor kidney function and low CrCl)		C	31	23	8	253, 396
OCRL rs7057639 C>T	X :2677 intron	Assoc with mendelian Fanconi syndrome Significantly associated with ↑ΔSCr in TDF-FS	American	T	15	2	34	398 OMIM 300535
rs113165732 C>T	X:1719 intron	Significantly associated with ↑ΔSCr in TDF-FS		T	9	1	10	
SLC22A11 rs11231809 T>A	11: 266 intergenic	The gene encodes Hoat4. TT genotype alters uric acid excretion[9]. Interference with torsemide excretion could affect TFV clearance	Caucasian (Spanish)	T	6	31	31	298, 550
TMEM 'rs11767816(A>T	7:285	Indirectly regulates genes that may have a role in kidney disease.	Caucasians	T	3	37	31	**
PEX14	1: 155-1074	Peroxisomes expression in kidneys likely to be involvement nephropathy		A	57	13	18	**

rs284267 C>A								
rs284265 T>C	1: 155 - 2944	Encodes FOXA1, albumin enhancing protein; overexpression of albumin may result in histological changes of progressive tubulopathy.		C	42	87	88	**
rs284301 G>A	1:2671	Involvement in tubulin motility with a role in mitochondrial cellular organelle biogenesis and may be implicated in renal tubular disease.		A	32	15	5,	**
ITSN rs2834254 C>T	21:186	Indirect involvement through endocytic membrane traffic		T	33	11	15	**

AFR = Africa, CAU = Caucasian, ASI = Asia; ↑ High /Increases; ↓low / Decreases; ΔSCr = changes in SCr; ** SNPs suggested in an unpublished GWAS of TDF-FS in a Caucasian cohort and met the cut-off

3.2.6 Collection, Shipping and viral inactivation of Whole blood samples

We collected 9mls of a whole blood sample from each participant in K3-EDTA tubes. Once collected, samples were stored at -80 degrees in Lusaka at the adult infectious laboratory until they were shipped on dry ice to the University of Liverpool. Since the samples contained active HIV, the samples were stored at the Bioanalytical Facility (BAF), a containment level 3 laboratory in the Royal Liverpool Hospital until we carried out the HIV inactivation.

3.2.6.1 **Inactivation of the HIV Infected Samples**

All the risks associated with the manipulation of HIV samples were assessed prior to carrying out any laboratory work. All necessary health and safety requirements for handling infectious samples such as wearing double gloves, protective goggles and disposable laboratory coats and good laboratory practices were adhered to throughout the process. Additionally, standard operating procedures were followed to prevent contamination of surfaces and for appropriate disposal of contaminated materials. Inactivation of HIV infected samples was carried out using the BAF protocol (BAF LOG030 version 1). The workflow and main steps are illustrated in Figure 3.2.

The inactivation process involved the preparation of samples that included sorting checking of labels and left to thaw, a tack that was done in a laminar flow cabinet Category 3. The water bath was filled with sufficient water levels to cover the rack with samples and was preheat to 58ºC. Once samples had thawed, the tubes containing blood were loaded in a metal rack and placed in the water bath while ensuring the water level was above the blood level in the tubes. Samples were incubated to allow HIV RNA inactivation for 40 minutes with temperature maintained at 58ºC. The temperature was monitored and documented at 20 minutes and the

end of the inactivation process. Once inactivation had finished, samples were removed and left to cool at room temperature. The inactivation log-sheet was completed by recording the number of tubes for each inactivated sample IDs and the inactivation conditions. Thereafter, a transfer log-sheet was completed to indicate the number of tubes /sample ID to be transferred. Three compartment bio-safe containers were used to transfer HIV inactivated samples from the BAF to the WCPM molecular laboratory where they were stored at -20 degrees until DNA was extracted.

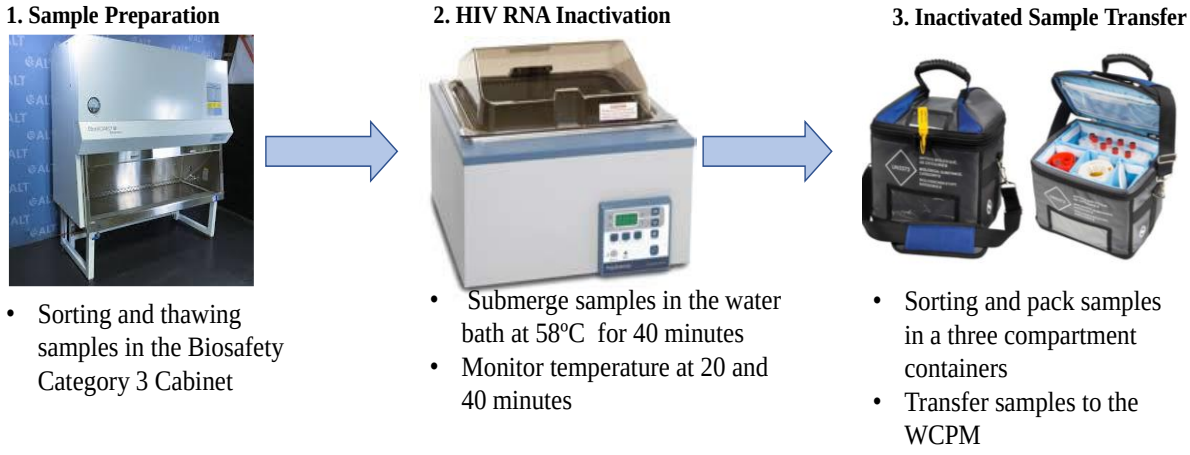


Figure 3.2. Illustration of the work environment and steps for inactivation of HIV RNA

3.2.7 DNA extraction

DNA extraction was conducted in the designated DNA extraction room in the molecular lab of the Wolfson Centre for Personalised Medicine. Handling and manipulation of the samples were done in the Class II Biosafety cabinet. Genomic DNA was extracted from whole blood samples using the automated benchtop Chemagic Magnetic Separation Module I (MSM1) extraction robot and the extraction kits (Chemagen, Perkin Elmer, UK). The principle of the DNA extraction method is based on the isolation and purification of genomic DNA using advanced magnetic beads technology. The Chemagen robot is an 8-position machine; seven of these

(positions 2 -8) taking 12 x 50 ml universal tubes per rack while position 1 is filled with disposable plastic tips to cover the magnetic metal rods. The Chemagen machine, set-up and summarised steps of DNA extraction are illustrated in Figure 3:3. The main steps of the protocol are detailed below.

3.1.1.1 Sample preparation and Chemagen machine set-up

To commence DNA extraction, frozen blood samples were sorted and thawed at room temperature for 30 minutes in the biosafety cabinet. The DNA extraction log sheet (Appendix F) was then completed with sample IDs and protocol conditions for tracking and quality control. The Chemagen workstation was set-up, by ensuring position 1 was filled with disposable plastic tips which were necessary to avoid contamination through direct contact of the magnet metal rods with the sample. Tubes in position 2 and position 8 were pre-label with samples IDs to be extracted in the predetermined order.

- **Lysate Mixture preparation**

Once the Chemagen machine was set, then the blood lysate mixture preparation was done by pipetting 7.5 ml lysis buffer and 20µl protease enzyme to tubes in rack 2. Thereafter, at least 2.5ml of the blood sample of the corresponding prelabelled ID on the tube was added to each tube (a procedure done in the wood safe cabinet). 500µl elution buffer was added to each tube in rack position 8. The lysate mixture was prepared by running “lysate mixture protocol A” (Step 1) from the computer software for approximately 10 min.

- **DNA isolation, purification and Elution**

DNA isolation and purification were conducted after setting up the rest of the machine positions, filling tubes in rack positions 3 to 7 with the specified buffer and the corresponding

volume according to the Chemagen DNA extraction protocol. To each tube containing the lysate mixture, 19.5 ml binding buffer followed by 500µl of magnetic beads were added. The beads and binding buffer were essential for binding and later capture of the DNA to the disposable tips. Protocol B "Chemagen DNA Blood5k elution" was automatically run from the computer software illustrated step 2 for approximately 45 minutes. During this protocol, the lysate was resuspended and mixed with the beads by a brief vigorous mixing. This was followed by the application of an electromagnetic field to isolate and captures the DNA from the mixture with high specificity, then, the beads with the bound DNA are attracted to the metal rod through the plastic tips. The magnetic metal rod then went through an automated purification in 4 serial washing buffers in tubes racked in positions 3 to 7. Finally, the purified, DNA was resuspended and released in pre-labelled tubes containing 500µl of elution buffer (rack position 8) (illustrated Step3), Once the protocol had completed, the extracted DNA was transferred into prelabelled Eppendorf and immediately proceeded to quality assessment by NanoDrop® spectrophotometry before storage at -20ºC.

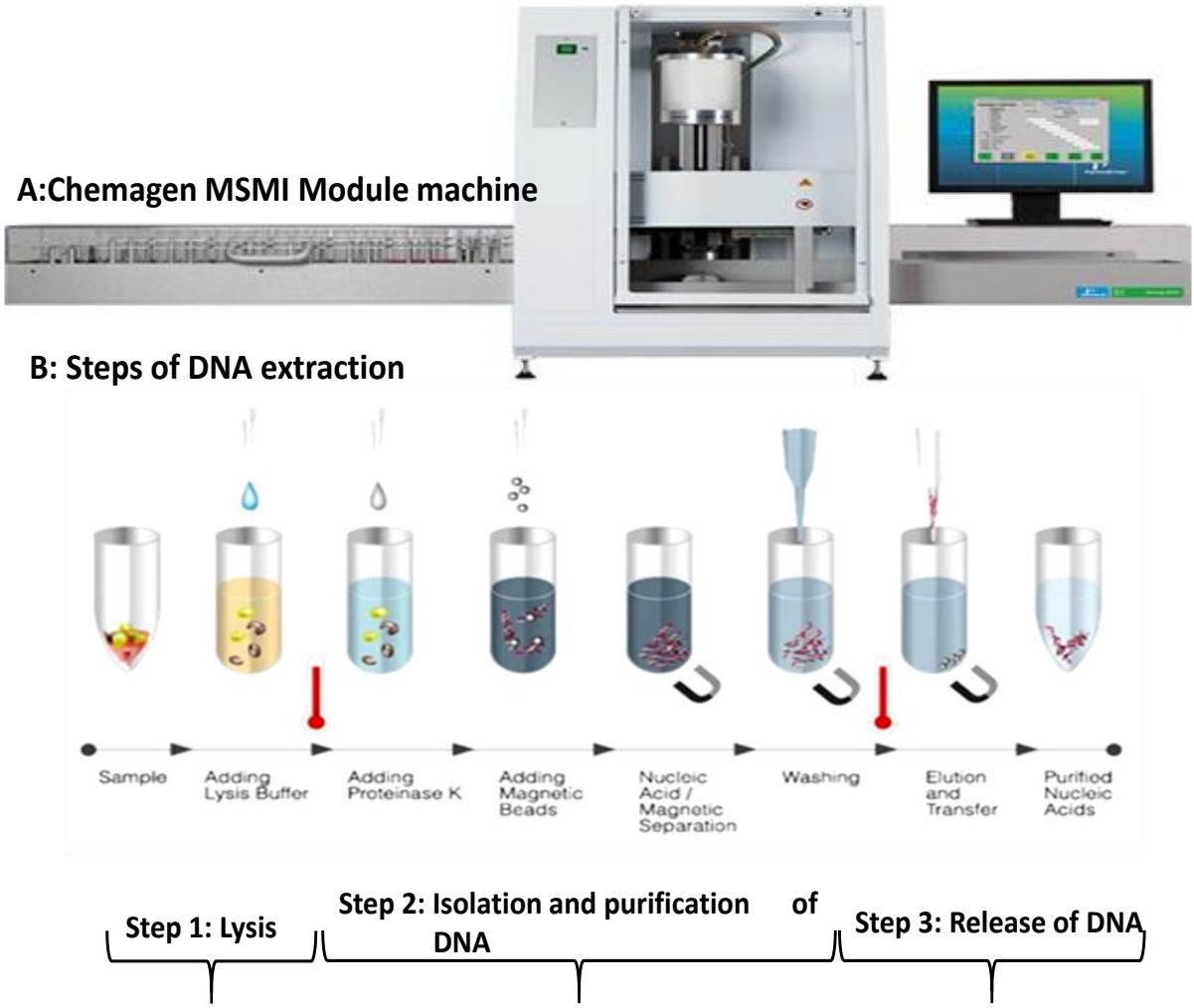


Figure 3.3. Schematic procedure for nucleic acid isolation and purification by magnetic bead technology.
The Chemagen robot (A) is set up by loading 12 X 50 ml tubes in position 2 - 7 rack positions. Position 1 is loaded with plastic tips over which the magnetic metal rod attracts the magnetic beads bound DNA and goes through the washing process in buffers from racks 3 to 7. In the final step (Step3), the purified magnetic beads bound DNA is released in the tubes containing elution buffer.
Diagrams adapted from https://chemagen.com/chemagic-msm-i / and chemagen Biopolymer-Technology AG, Germany

3.2.8 DNA Quality and quantity assessment

- **Nanodrop DNA quantitation**

The quality and purity of the nucleic acid were immediately assessed and estimated using the NanoDrop™ 8000 Spectrophotometer from ThermoFisher Scientific. This was performed by initially calibrating and blanking the spectrophotometer with 1.5 µl of the elution buffer, and

thereafter adding 1.5µl of each DNA followed by reading the absorbance of nucleic acid. The in-built software displayed the calculated DNA concentration, nucleic acid purity ratios, and spectra of each sample. DNA quality was assessed by considering the characteristic absorbance spectra together with the absorbance ratios: A260/A280 ~1.8 and A260/A230 = 2.0-2.2 were used to rule out protein and solvent contaminants. Where necessary, DNA samples falling outside the expected absorbance ratios were cleaned using 7.5 M Ammonium acetate solution for molecular biology.

- **DNA quantitation using PicoGreen fluorometry**

Quantitation of double-strand DNA (dsDNA) concentration was measured by the PicoGreen protocol using the Invitrogen™ Quant-It™ PicoGreen™ fluorometry dsDNA Assay Kit 25pg/ml sensitivity of. PicoGreen reagent is an ultra-sensitive fluorescent that selectively stains nucleic acid which absorbs at 260nm (A_{260}). The unknown DNA concentration extrapolated from four orders of magnitude linear standard curve in DNA concentration from 1 ng/mL to 1000 ng/mL. The protocol involved the initial preparation of a five-point lambda DNA the standard curve. Followed by dispensing 100ul of each standard DNA concentration in duplicate into a 96 well flat bottom UV plate. Then 1ul of each stock DNA sample diluted with 99ul of 1X TE was pipetted in duplicate into the rest of the wells of the 96 well plate. Then, 100ul of the PicoGreen reagent, prepared with 1X TE according to the protocol was added pipette in all the wells of 96-well. The plate was then incubated for 2 to 5minutes away from light before reading the fluorescence absorbance at 260 nm using the Beckman Coulter DTX 880 Multimode detector microplate reader. The concentration of each sample was then extrapolated against the standard curve. DNA was normalised to 10ng/ul as required for the genotyping methods and protocols.

3.2.9 Genotyping by iplex MassARRAY® System

SNP genotyping was conducted by two methods; All SNPs were genotyped by Agena Bioscience iplex MassARRAY® System; a platform integrating matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry with end-point PCR iplex technology. SNPs that failed iplex genotyping as well as SNPs that required validation, were genotyped by a TaqMan Real-time PCR using validated SNP genotyping assays (Assays-on-demand; Applied Biosystems, Loughborough, UK).

3.2.9.1 **The principle of Genotyping by iplex MassARRAY® System**

Genotyping by MassARRAY System combines the robust multiplexed primer extension chemistry of the iPLEX® assay with highly sensitive MALDI-TOF mass spectrometry to precisely, rapidly, and effectively analyse multiple genotypes. The chemistry involves a homogeneous reaction format with a single extension primer to generate allele-specific products with distinct masses, multiplexed PCR reactions, a single termination mix and universal reaction conditions for all SNP.

The whole genotyping process involves a process of distinct reactions that include a PCR amplification reaction and posts PCR shrimp alkaline phosphatase (SAP) dephosphorylation of excess nucleotides followed by an integrated iPLEX base extension reaction which uses a mix of oligonucleotide extension primers, extension enzyme and mass modified dideoxynucleotide terminators. In this reaction, the base adjacent to each SNP is extended and terminated by a single complementary base. After desalting the extension products with Clean Resin, the final product is then transferred by a nano dispenser onto a SpectroCHIP Array where they crystalize with a MALDI matrix and the MassARRAY analyser separates molecules by time-of-flight based on the mass. The time of flight for each extension product is

recorded and a Typer software generates reports by identifying the SNP alleles (homozygous or heterozygous) in each sample represented by the intensity of the peaks. The process is illustrated in Figure 3.4.

For our selected SNPs, two multiplex assays (18- plex and 6-plex) were designed by the Agena biosciences software to interrogate the DNA samples. The forward and reverse primers for PCR amplification and iPLEX extension reactions were ordered as desalted, unmodified with standard purification (see Appendix G for the primers used).

3.2.9.2 **PCR: Preparation and Reaction**

To run PCR, a 384-well plate reaction; 2µl (10ng/µl) of normalised DNA was pipetted into the 384-well plate and was left to evaporate and dry down at room temperature. Plex primer mixes for 18-plex and 6-plex assays were prepared separately. The frozen forward and reverse primers were sorted and thawed accordingly. They were then resuspended by vortexing.

For each assay (18-plex and 6 plex); the primer mix was prepared to a working concentration of 0.5µM for each primer,.by adding 5µl of each Forward + 5µl of each Reverse primer into an Eppendorf and diluted with nanopure water to a total volume of 500µl.

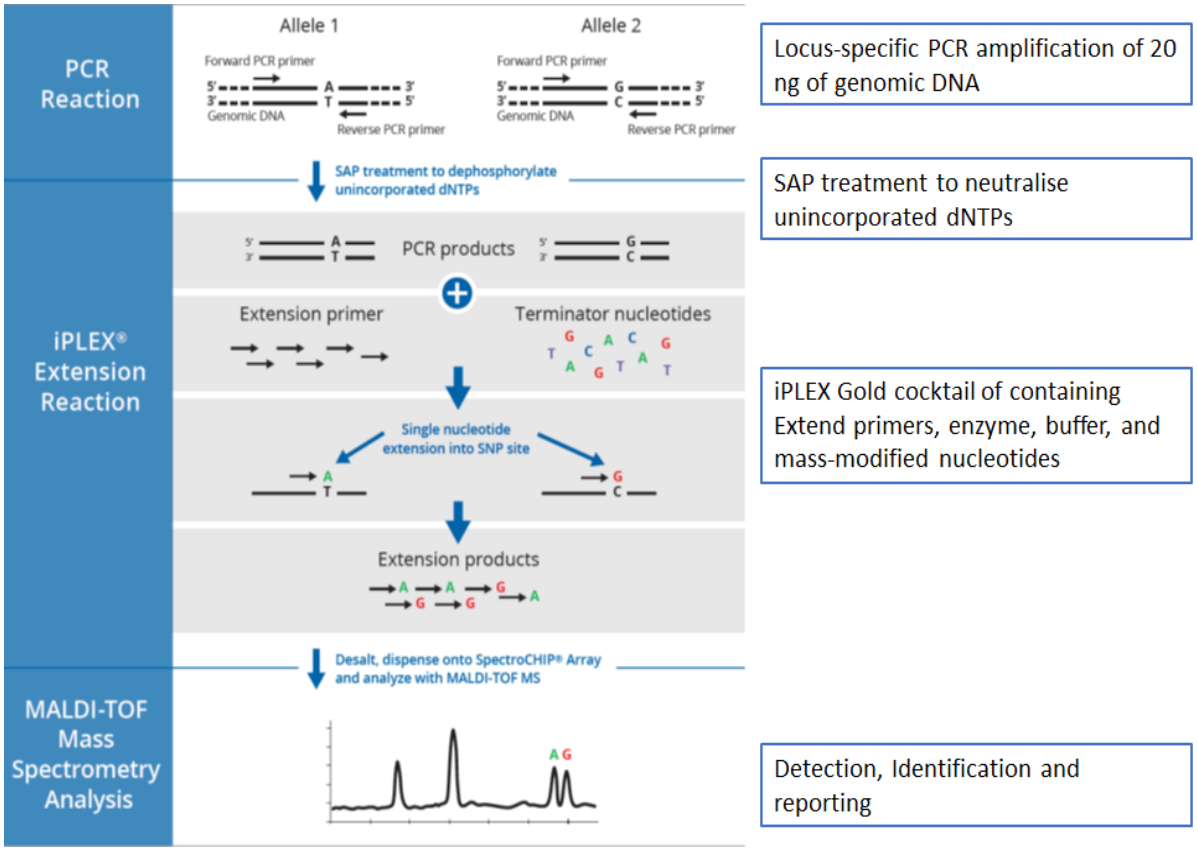


Figure 3.4. Illustration of the workflow and overview of iPLEX MassARRAY genotyping assay chemistry. Figure adapted from Agena bioscience IPLEX application brochure.

3.2.9.3 **Plex PCR mix Preparation**

To run a locus-specific polymerase chain reaction and amplification of genomic DNA, a plex PCR mix was prepared based on 5µl of PCR reaction mix required to run one sample reaction (one well). This was prepared with 0.625µl PCR buffer (10X) + 0.325 µl $MgCl_2$ (25mM) + 0.1µl dNTP mix (25mM) +1µl primer mix (0.5µM) + 0.1µl Hot Star Taq polymerase enzyme (5 U/µl) +2.85 µl H_20. These volumes were then extrapolated for a 384-well plate by multiplying the volume of each component by 384. Then, 5µl of the plex PCR mix was pipetted into each well of a 384 well plate containing dried DNA. The plate was then sealed with the adhesive PCR Seal, vortexed and centrifuged at 3000rmp for 2 min. The iPLEX PCR reaction

was then run on the Thermocycler for 2 hours 30 minutes.

3.2.9.4 **SAP Reaction: Preparation and Addition of SAP Enzyme Solution**

The SAP reaction was run by preparing and adding a SAP Enzyme solution of 2 µl in each well prepared with 0.30µl SAP + 0.17µl hME buffer +.53 µl nano water. These volumes were multiplied by 384 to run a full 384 well plate. 2 µl was then added to each well using an eight-channel pipettor following by running the SAP iPLEX programme on the thermocycler for 1 hour.

3.2.9.5 **Preparation and Addition of iPLEX Gold Reaction Cocktail**

Finally, the extension process was achieved by adding the iPLEX Gold Reaction Cocktail which composed of the extend primer mix, iPLEX Buffer (10x),iPLEX-Termination mix and the iPLEX-Enzyme.

The extend primer mix was prepared using a 4-step mass adjustment. Primers groups and volumes per primer were determined by the Typer software. Table 3.2 shows the preparation of 1200µl of the extended primer mix prepared at 400uM for each primer to run a 384 well plate. The iPLEX Gold Reaction Cocktails for the 18-plex and 6-plex assays were prepared separately. The iPLEX Gold Reaction Cocktail was prepared as shown in Table 3.3 .

2µl of the iPLEX cocktail was then added to each well of the PCR amplicons. The plate was sealed and slightly vortexed and centrifuged at 3000 RMP for 2 minutes. The SAP iPLEX extension programme was run for approximately 4 hours. The iPLEX extended products were then cleaned with cationic resin (Clean Resin) to optimize the mass spectrometry analysis by removing salts such as Na+, K+, and Mg2+ ions to reduce background noise. To achieve this

6mg of resin and 16µl nano-water was added into each well and the extension product was treated for approximately 1hour.

Table 3.2. Preparation of extension primer mix for the 18 plex and 6 plex assays using the 4-Step adjustment method.

Extend Primer Group	Concentration / primer (µM)	Primer (µl)	Total vol / group) (µl) -18 plex 5-5-4-4 groups	Total vol/group) (µl) -6 plex 2-2-1-1 groups
1	7	21	105	42
2	9.3	27.9	139.5	55.8
3	11.66	34.98	139.92	34.98
4	14	42	168	42
Total Primer mix Vol (µl)			552.42	174.78
Water			647.58	1025.22
TOTAL(µl)			**1200**	**1200**

Table 3.3. iPLEX Reaction Cocktail Solution Preparation for 384 well plate

Reagent	Volume for Single reactions x	Volume for 384 wells reactions
H_2O	0.755 µl	400.10µl
iPLEX-Buffer (10x)	0.2 µl	105.98µl
iPLEX-Termination mix	0.2 µl	105.98µl
Primer mix	0.804 µl	426.06µl
iPLEX-Enzyme	0.04 µl	21.20µl
Total	**2.0 µl**	**1059.34µl**

3.2.9.6 **Spotting Primer Extension Products on SpectroCHIPs and Detection**

Using an automated nanodispenser, the final product was transferred from the 384 well plates onto a SpectroCHIP® Array. The chip was then placed on the mass spectrometer and the MALDI-TOF mass spectrometry separated and analysed the products in each sample by their

molecular mass to charge ratio.

Detection, identification of SNP alleles and generation of the report was done by the Typer software. The peak intensity demonstrates the allele detected.

3.2.9.7 **Analysing and Visualising Genotyping Call Results**

During chip detection, genotype calls are made in real-time, and the software allows visualising results of all SNPs in the multiplex reaction. Selection of an individual SNP allows visualisation of results for a specific SNP at a time. Assay results were therefore visualised through the Traffic light, Spectrum, histogram and cluster plot all of which provides specific information to aid analysis of the results. Each SNP genotype spectrum and cluster plot were manually checked and scored accordingly, and results were finally exported in an excel file for further sorting and analysis. An example of generated spectrums with peaks identifying specific SNPs is shown in Figure 3.5.

3.2.10 TaqMan SNP genotyping

We selected SNPs, rs3740066, rs717620 and rs2125739 to be genotyped by TaqMan Real-Time PCR allelic discrimination method. For this, we used validated SNP genotyping assays (Applied Biosystems). 10ng/ul DNA samples were mixed with 2.5ul of TaqMan PCR genotyping master mix and 0.25ul of respective TaqMan PCR probes (C___2814642_10C, C__11214910_20 and C__15827253_10, respectively) and were plated in a 384-well plate. The real-time PCR was carried out using a Quant Studio™ 6 Flex Real-Time PCR System (Thermo Fisher, Loughborough, UK) and the resultant genotype data were analysed using QuantiStudio software v1.3 to obtain allelic discrimination plots and genotype calls. In either genotyping method, quality control procedures included the use of replicate samples, negative

and positive template controls.

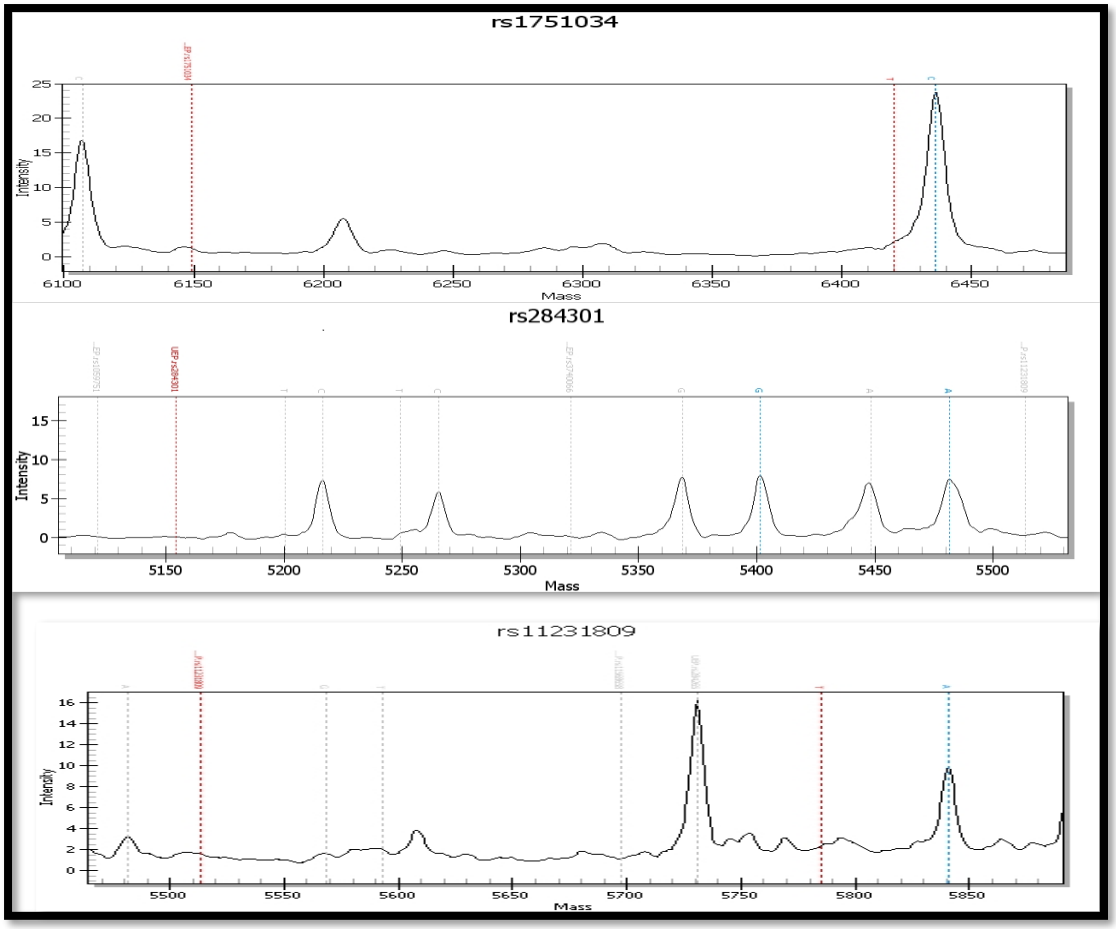


Figure 3.5. The spectrum display showing spectrum of selected well for rs1751034, rs284301 and rs11231809. Illustrated spectrum shows identified peaks for homozygous CC, heterozygous CA and homozygous AA genotypes respectively.

3.2.11 Statistical Analysis: Quality Control and analysis

All valid SNPs were tested for deviation from Hardy-Weinberg equilibrium (HWE) using the Fisher's exact X^2 corrected for multiple testing using a Bonferroni method. The Bonferroni corrected p-value of 0.002 ($p<0.05/ 22$ SNPs) was considered as the threshold of significance. MAF and genotype missingness was also determined. Any sample with < 80% call rate and an assay or SNPs with more than 10% missing genotypes were eliminated from the final analysis. Statistical comparisons for genotype and allele frequencies between cases and controls were

tested by use of Fisher's exact test. An association between each SNP genotype with case and control status was analysed and adjusted for previously identified predictors of TDF-RT (age, sex, baseline BMI and BMI at recruitment, baseline SCr, CrCl and changes in CrCl). The logistic regression model for the genotypic-additive model was used and Bonferroni correction for multiple testing was applied to determine a statistical association for p-value <0.05/n, where n was the number of SNPs analysed. All statistical tests, quality control (QC) and association tests were conducted using PLINK 1.09 software (www.cog-genomics.org/plink/1.9/) version 3 integrated with the RStudio platform. We further performed haplotype analysis using Haploview software version 4.1 to investigate if previously reported haplotypes in genes such as *ABCC2*, *ABCC4* and *ABCC10* were observed in our cohort. D' and r^2 were used for linkage disequilibrium analysis.

3.3 RESULTS

A cohort of HIV positive patients receiving TDF based cART were originally identified and recruited following a signed consent certificate. The main characteristics of the study participants were discussed in chapter 2. A total of 903 participants had their DNA samples extracted from whole blood. After excluding some participants for various reasons (figure 3.5). we finally included 880 participants who had complete genotyping and clinical data in the pharmacogenetic analysis. Of these, 155 (17.6%) were defined as cases having met the criteria for case definition by CrCl-CG <60ml/min and 725 were defined as controls. We evaluated the association of selected SNPs with TDF-RT in this cohort of 155 cases and 725 controls. See the participants included in the study in figure 3.6.

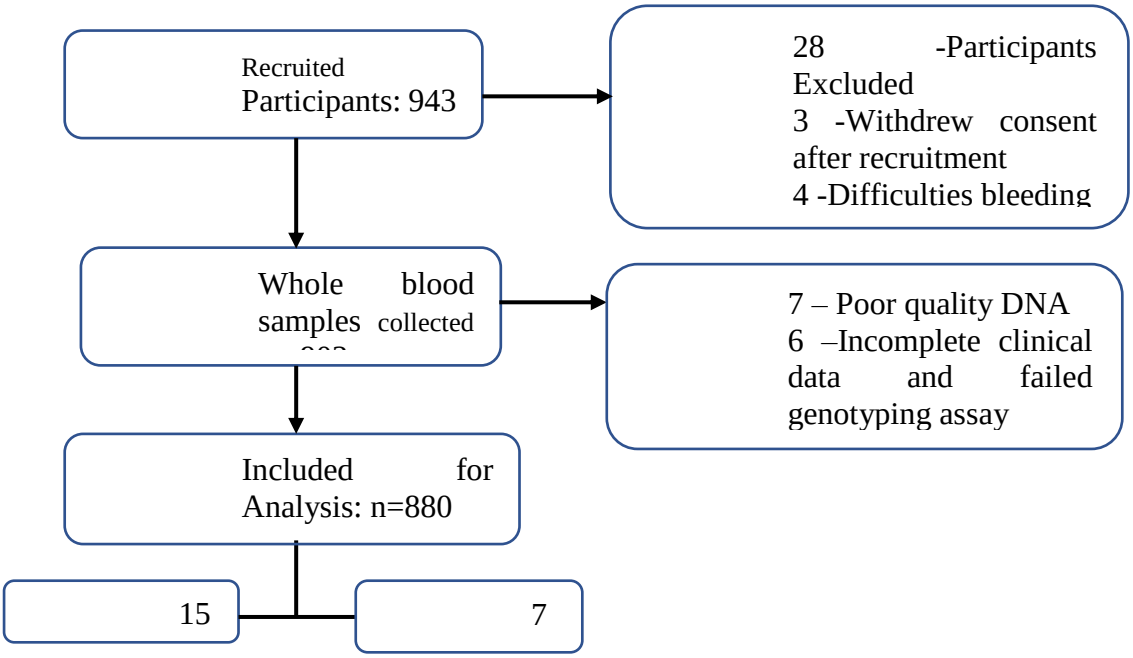


Figure 3.6. Recruitment and inclusion of participants for the Pharmacogenetic study

3.3.1 Single Nucleotide Polymorphism Analysis

Out of the 24 selected SNPs, one SNP failed genotyping (*ABCC4*, rs1059751) and another was found to be monomorphic (*TMEM*, rs11767816); these were excluded from the analysis. Three SNPs (*ABCC4*-rs2274409, *OCRL*-rs113165732 and rs7057639) showed a deviation from HWE although the MAFs of these SNPs in our cohort were similar to those reported in the African population. Even though these 3 SNPs deviated from HWE, we included them in our single SNP association analyses.

3.3.1.1 Characteristics of SNPs and trends in Genotyping Data

A total of 22 SNPs in seven genes: *ABCC2* (8), *ABCC4*(5), *ABCC10*(2), *OCRL* (2), *SCL22A11* (1), *PEX14* (3) and *ITSN* (1) were included in the genetic analysis. The majority of the SNPs were intronic and missense variants. The predicted consequence of the analysed SNPs is summarised in figure 3.7A.

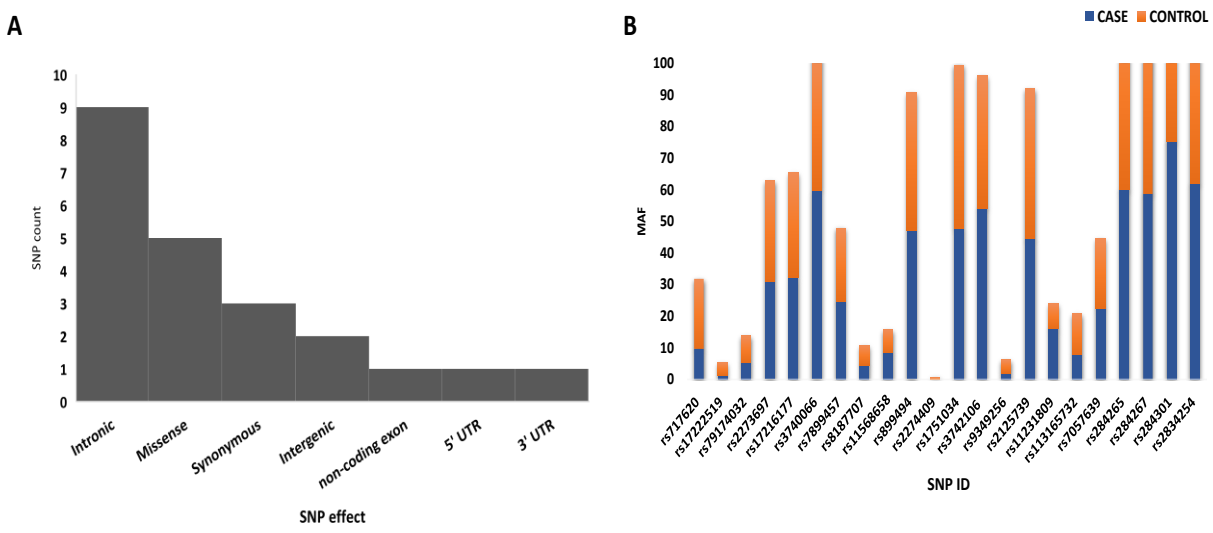


Figure 3.7. The frequency of SNP effects /consequences on amino acids in analysed SNPs (A) and the proportion of MAF in Cases vs Controls per SNP (B).

In comparison to controls, presented genotyping data suggested that a greater proportion of cases had a higher frequency of the mutated allele in some SNPs. These trends were observed in ABCC2 rs374006 C>T (60 vs 43.2), ABCC4, rs3742106 (54.2 vs 41.9) and ITSN rs2834254 (61.9 vs 58.2%) (Figure 3.7B). which are missense, 3' UTR and intronic variants respectively. On the other hand, for ABCC4, rs2274409 C>T, the mutant allele was not represented in the case group and only less than 1% in the control group while neither cases nor controls were homozygous mutant carriers for ABCC2, 8187707 and ABCC4, rs9349256.

3.3.2 SNP association with TDF-RT:

The summary of genotype and allele distribution between cases and controls and results from the association analysis for all SNPs are given in Table 3.3. Using a Bonferroni corrected p-value of 0.002 as the threshold of statistical significance, the single SNP association analysis showed that there were no significant differences observed between cases and controls. Because no single SNP was associated with TDF-RT, we did not proceed to adjust for

covariates previously determined as predictors of TDF-RT.

However, a SNP in *PEX14*, rs284301 G>A, showed a significant association with TDF-RT ($p<0.012$; uncorrected p-value). The variant allele, A showed a frequency of 72.5% in cases compared to 53% in controls even though it did not remain significant after Bonferroni correction for multiple testing ($P < 0.002$).

Table 3.4. Genotypes and allelic frequencies of selected SNPs in Cases and Controls

Gene, SNP / SNP Consequence	**Genotype /Allele**	**Cases: n (%)**	**Control: n (%)**	**P-value**
ABCC2				
rs717620 C>T	CC	140 (90.3)	560 (77.2)	0.073
5 prime UTR	CT	13 (8.4)	153 (21.1)	
	TT	2 (1.3)	7 (1)	
	C	293 (94.5)	1273 (88.4)	
	T	17 (5.5)	167 (11.6)	
rs17222519 G>A	GG	150 (96.8)	676 (93.2)	0.411
	GA	2 (1.3)	30 (4.1)	
	AA	0 (0)	0 (0)	
	G	302 (99.3)	1382 (97.9)	
	A	2 (0.7)	30 (2.1)	
rs79174032 C>A	CC	147 (94.8)	656 (90.5)	0.233
intron	CA	8 (5.2)	60 (8.3)	
	AA	0 (0)	2 (0.3)	
	C	302 (97.4)	1372 (95.5)	
	AA	8 (2.6)	64 (4.5)	
rs2273697 G>A	GG	105 (67.7)	493 (68)	0.555
missense	GA	44 (28.4)	218 (30.1)	
	AA	4 (2.6)	12 (1.7)	
	G	254 (83)	1204 (83.3)	
	A	52 (17)	242 (16.7)	

rs17216177 T>C	TT	103 (66.5)	478 (65.9)	0.274
non-coding exon	TC	44 (28.4)	211 (29.1)	
	CC	6 (3.9)	29 (4)	
	T	250 (81.7)	1167 (81.3)	
	C	56 (18.3)	269 (18.7)	
rs3740066 C>T	CC	61 (40.1)	400 (56.7)	0.760
missense	CT	87 (57.2)	266 (37.7)	
	TT	4 (2.6)	39 (5.5)	
	C	209 (68.8)	1066 (75.6)	
	T	95 (31.3)	344 (24.4)	
rs7899457 C>T	CC	116 (74.8)	553 (76.3)	0.484
synonymous	CT	35 (22.6)	157 (21.7)	
	TT	3 (1.9)	12 (1.7)	
	C	267 (86.7)	1263 (87.5)	
	T	41 (13.3)	181 (12.5)	
rs8187707 C>T	CC	148 (95.5)	674 (93)	0.574
synonymous	CT	7 (4.5)	46 (6.3)	
	TT	0 (0)	0 (0)	
	C	303 (97.7)	1394 (96.8)	
	T	7 (2.3)	46 (3.2)	
ABCC4				
rs11568658C>A	CC	142 (91.6)	672 (92.7)	0.577
missense	CA	13 (8.4)	53 (7.3)	
	AA	0 (0)	0 (0)	
	C	297 (95.8)	1397 (96.3)	
	A	13 (4.2)	53 (3.7)	
rs899494 A>G	GG	80 (51.6)	404 (55.7)	0.060
synonymous	AG	61 (39.4)	273 (37.7)	
	AA	12 (7.7)	43 (5.9)	
	G	221 (72.2)	1081 (75.1)	
	A	85 (27.8)	359 (24.9)	
rs2274409 C>T	CC	153 (98.7)	714 (98.5)	0.999

intron	CT	0 (0)	2 (0.3)	
	TT	0 (0)	3 (0.4)	
	C	306 (100)	1430(99.6)	
	T	0 (0)	8 (0.6)	
rs1751034 C>G	TT	81 (52.3)	341 (47)	0.092
missense	CT	53 (34.2)	290 (40)	
	CC	21 (13.5)	85 (11.7)	
	T	215 (69.4)	972 (67.9)	
	C	95 (30.6)	460 (32.1)	
rs3742106 A>C	AA	66 (42.6)	318 (43.9)	0.181
3' UTR	AC	72 (46.5)	284 (39.2)	
	CC	12 (7.7)	92 (12.7)	
	A	204 (68)	920 (66.3)	
	C	96 (32)	468 (33.7)	

Table 3.4: Continued

Gene/SNP/SNP Consequence	**Genotype /Allele**	**Cases: n (%)**	**Control: n (%)**	**P-value**
ABCC10				
rs9349256 G>A	GG	150 (96.8)	687 (94.8)	0.996
intron	GA	3 (1.9)	31 (4.3)	
	AA	0 (0)	0 (0)	
	G	303 (99)	1405 (97.8)	
	A	3 (1)	31 (2.2)	
rs2125739 T>C	TT	86 (55.5)	380 (52.4)	0.886
missense	CT	64 (41.3)	285 (39.3)	
	CC	5 (3.2)	59 (8.1)	
	T	236 (76.1)	1045 (72.2)	
	C	74 (23.9)	403 (27.8)	
SLC22A11				

rs11231809 T>A	TT	129 (83.2)	660 (91)	0.436
intergenic	TA	22 (14.2)	57 (7.9)	
	AA	3 (1.9)	1 (0.1)	
	T	280 (90.9)	1377 (95.9)	
	A	28 (9.1)	59 (4.1)	
OCRL1				
rs113165732 C>T	CC	141 (91)	609 (84)	0.832
intron	CT	6 (3.9)	62 (8.6)	
	TT	6 (3.9)	32 (4.4)	
	C	288 (94.1)	1280 (91)	
	T	18 (5.9)	126 (9)	
rs7057639 C>T	CC	120 (77.4)	564 (77.8)	0.720
intron	CT	25 (16.1)	159 (21.9)	
	TT	10 (6.5)	1 (0.1)	
	C	265 (85.5)	1287 (88.9)	
	T	45 (14.5)	161 (11.1)	
PEX14				
rs284265 T>C	CC	60 (38.7)	264 (36.4)	0.234
intron	CT	75 (48.4)	342 (47.2)	
	TT	18 (11.6)	113 (15.6)	
	C	195 (63.7)	870 (60.5)	
	T	111 (36.3)	568 (39.5)	
rs284267 C>A	AA	62 (40)	256 (35.3)	0.210
intron	CA	70 (45.2)	348 (48)	
	CC	21 (13.5)	114 (15.7)	
	A	194 (63.4)	860 (59.9)	
	C	112 (36.6)	576 (40.1)	
rs284301 G>A	GG	42 (27.1)	340 (46.9)	0.012*
intergenic	GA	85 (54.8)	293 (40.4)	
	AA	27 (17.4)	86 (11.9)	
	G	169 (54.9)	973 (67.7)	
	A	139 (45.1)	465 (32.3)	

ITSN1				
rs2834254 C>T	CC	54 (34.8)	275 (37.9)	0.200
intron	CT	67 (43.2)	325 (44.8)	
	TT	29 (18.7)	97 (13.4)	
	C	175 (58.3)	875 (62.8)	
	T	125 (41.7)	519 (37.2)	

Statistical significance was assessed for p-values <0.002 adjusted for Bonferroni correction. Data are no. (%) of patients for genotypes and no. (%) of alleles for alleles. *= p-value significant (uncorrected)

The distribution of the genotypes and allele frequency in cases and controls for rs284301 (p<0.012; uncorrected p-value). is presented in Figure 3:8.

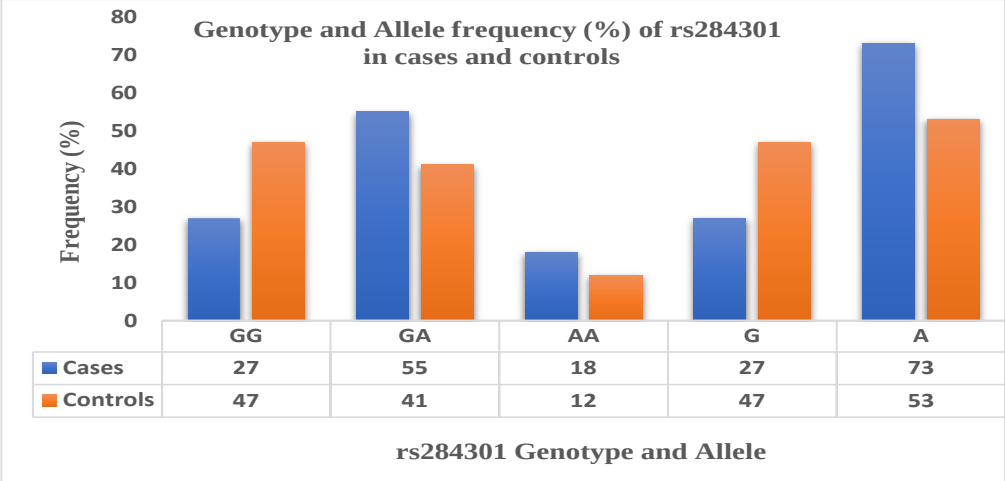
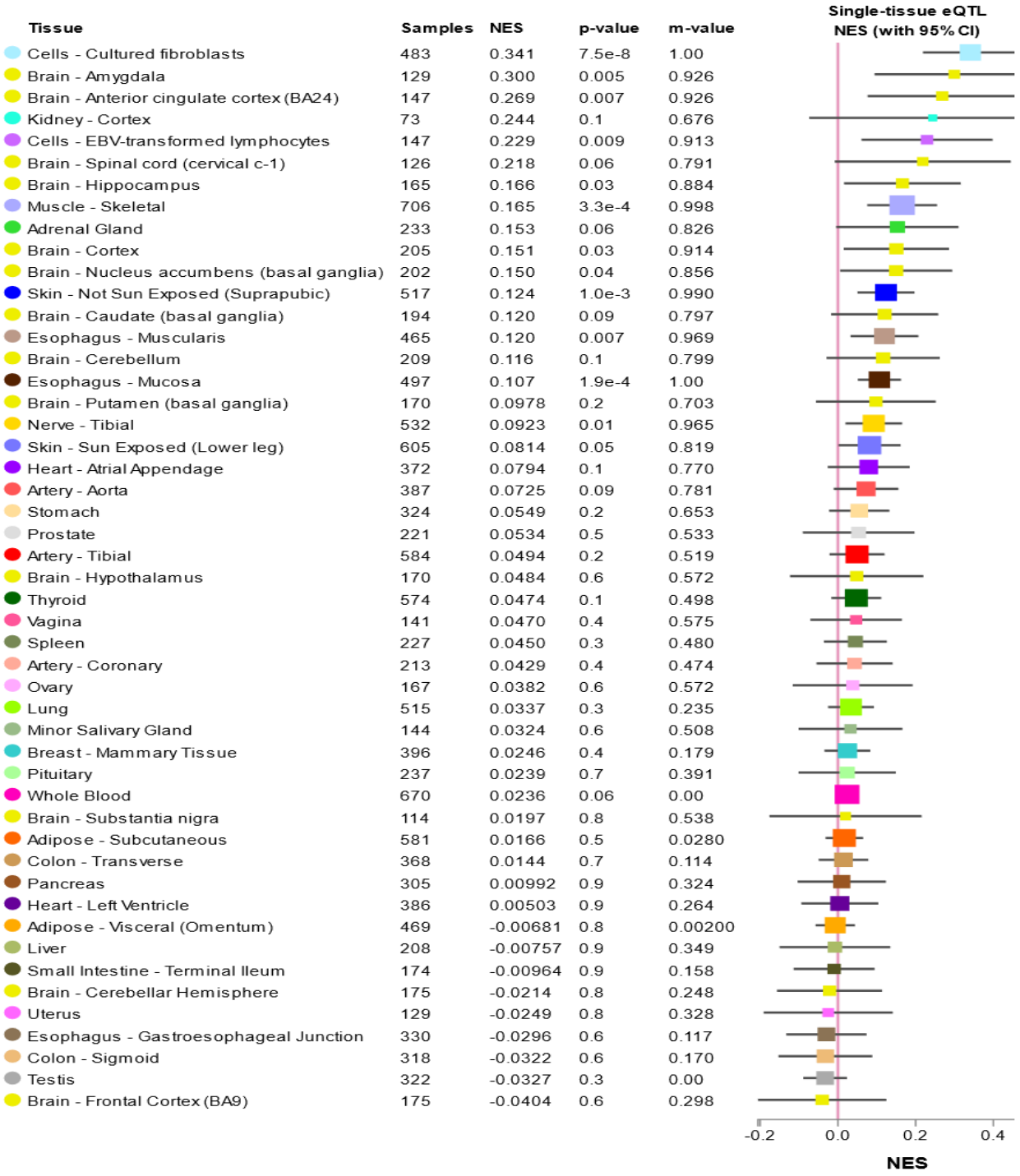


Figure 3.8. Distribution of genotype and allele frequency in cases and controls for SNP rs284301

Although the rs284301 was not significantly associated with TDF-RT after adjusting for multiple measurements, we were, therefore, interested in searching for its possible functional consequences associated with changes in PEX14 expression due to the SNP. PEX14 is highly expressed in tubular and glomerular tissues[551]. It is therefore plausible to assume that alteration in its expression due to SNP would directly or indirectly affect PEX14 functions associated with peroxisomes movement and expression in kidneys and therefore, likely to be involved in nephropathy. It was necessary to show an overview of the effect of the SNP on gene expression

in different tissues. Thus, for our reference, we visualised the eQTL from the Gtex repository to observe how PEX14 expression is associated with rs284301 in different tissues. Figure 3.9.



Tissue	Samples	NES	p-value	m-value
Cells - Cultured fibroblasts	483	0.341	7.5e-8	1.00
Brain - Amygdala	129	0.300	0.005	0.926
Brain - Anterior cingulate cortex (BA24)	147	0.269	0.007	0.926
Kidney - Cortex	73	0.244	0.1	0.676
Cells - EBV-transformed lymphocytes	147	0.229	0.009	0.913
Brain - Spinal cord (cervical c-1)	126	0.218	0.06	0.791
Brain - Hippocampus	165	0.166	0.03	0.884
Muscle - Skeletal	706	0.165	3.3e-4	0.998
Adrenal Gland	233	0.153	0.06	0.826
Brain - Cortex	205	0.151	0.03	0.914
Brain - Nucleus accumbens (basal ganglia)	202	0.150	0.04	0.856
Skin - Not Sun Exposed (Suprapubic)	517	0.124	1.0e-3	0.990
Brain - Caudate (basal ganglia)	194	0.120	0.09	0.797
Esophagus - Muscularis	465	0.120	0.007	0.969
Brain - Cerebellum	209	0.116	0.1	0.799
Esophagus - Mucosa	497	0.107	1.9e-4	1.00
Brain - Putamen (basal ganglia)	170	0.0978	0.2	0.703
Nerve - Tibial	532	0.0923	0.01	0.965
Skin - Sun Exposed (Lower leg)	605	0.0814	0.05	0.819
Heart - Atrial Appendage	372	0.0794	0.1	0.770
Artery - Aorta	387	0.0725	0.09	0.781
Stomach	324	0.0549	0.2	0.653
Prostate	221	0.0534	0.5	0.533
Artery - Tibial	584	0.0494	0.2	0.519
Brain - Hypothalamus	170	0.0484	0.6	0.572
Thyroid	574	0.0474	0.1	0.498
Vagina	141	0.0470	0.4	0.575
Spleen	227	0.0450	0.3	0.480
Artery - Coronary	213	0.0429	0.4	0.474
Ovary	167	0.0382	0.6	0.572
Lung	515	0.0337	0.3	0.235
Minor Salivary Gland	144	0.0324	0.6	0.508
Breast - Mammary Tissue	396	0.0246	0.4	0.179
Pituitary	237	0.0239	0.7	0.391
Whole Blood	670	0.0236	0.06	0.00
Brain - Substantia nigra	114	0.0197	0.8	0.538
Adipose - Subcutaneous	581	0.0166	0.5	0.0280
Colon - Transverse	368	0.0144	0.7	0.114
Pancreas	305	0.00992	0.9	0.324
Heart - Left Ventricle	386	0.00503	0.9	0.264
Adipose - Visceral (Omentum)	469	-0.00681	0.8	0.00200
Liver	208	-0.00757	0.9	0.349
Small Intestine - Terminal Ileum	174	-0.00964	0.9	0.158
Brain - Cerebellar Hemisphere	175	-0.0214	0.8	0.248
Uterus	129	-0.0249	0.8	0.328
Esophagus - Gastroesophageal Junction	330	-0.0296	0.6	0.117
Colon - Sigmoid	318	-0.0322	0.6	0.170
Testis	322	-0.0327	0.3	0.00
Brain - Frontal Cortex (BA9)	175	-0.0404	0.6	0.298

Figure 3.9. The eQTL of the PEX14 rs284301 variant measurable in different tissues

3.3.3 Haplotype Analyses

We performed haplotype analyses to determine whether previously reported haplotypes in other populations were present in our study population. These included *ABCC2* CATC and CGAC haplotypes, that were previously determined in rs717620- -24 C>T, rs2273697-1249 G>A; rs8187694 - 3563 T>A and rs3740066 - 3972 C>T, *ABCC4* haplotypes previously reported in rs899494 - 669 A>G, rs79174032 - 3463,C>G and rs3742196 - 4131 A>C and *ABCC10* GGC haplotype from rs9349256G, rs2487663G, and rs2125739C alleles which were reported in Asian[395, 397] and Caucasian[253, 298] populations. In our analyses, *ABCC2* rs8187694, 3563 T>A and *ABCC10* rs2487663G were not included in the analyses because they did not meet the SNP selection criteria for the study. We did not observe any of the previously reported haplotypes in our study cohort. Due to ethnic differences between our study population and previously reported populations, we assumed that a different LD structure may exist in the genes analysed in our study. We identified two new blocks of LD in our study population; these were a block within the *ABCC2* gene (rs17216177 and rs2273697) and another one in the *PEX14* gene (rs284267 and 284301). These are illustrated in Figure 3:10. Subsequently, novel haplotypes with a frequency of >1% were mapped from these SNPs (table 3.5), although no statistically significant differences were found for the haplotypes between cases and controls.

Table 3.5. Distribution of ABCC2 and PEX14 Haplotypes among the Cases and Controls

Haplotype	Cases n(%)	Controls n(%)	OR	95% CI	p-value
ABCC2					
TG	100 (64.7)	567(64.4)	1.041	(0.52-2.05)	0.911
CG	29(18.6)	165(18.7)	1.04	(0.7-1.55)	0.846
TA	26(17)	148(16.8)	0.778	(0.5-1.21)	0.265
PEX14					
CA	97(62.4)	525(59.7)	1.268	(0.7-2.119)	0.364
TC	55(35.3)	347(39.4)	0.801	(0.5-1.17)	0.251

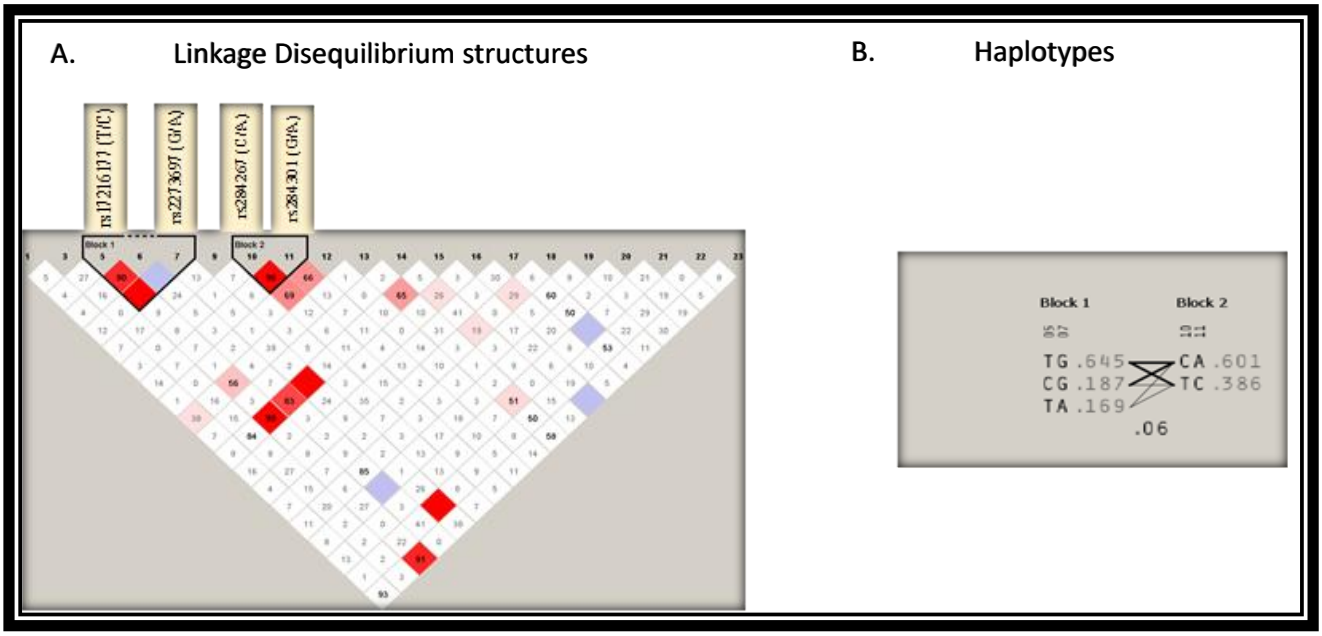


Figure 3.10. A-demonstration of LD patterns and haplotypes in the studied SNPs; intense red and pink colours indicate a strong and weak LD respectively.

Two blocks of determined haplotypes are shown B: block 1 consists of alleles of ABCC2 SNPs 5 rs17216177 and 7 (rs2273697) while block 2 alleles for PEX14 SNPs 10 (rs284267) and 11(284301)

3.4 DISCUSSION

The subclinical and persistent presentations of TDF-RT have been a source of concern in the management of HIV patients receiving TDF cART[295]. The high inter-individual variation in the presentation of TDF-RT suggests a contribution of genetic factors in the development of TDF nephrotoxicity. In the present study, we report our findings from our investigation of the role of genetic variants in the development of TDF-RT in a cohort of stable HIV-infected patients receiving TDF cART from Zambia We believe this is the largest African cohort where genetic susceptibility to develop TDF-RT (as defined by CrCl-CG) has been investigated till date. The study is also the first to explore the association of TDF-RT with genetic variants reported in mendelian Fanconi syndrome and transmembrane genes encoding for peroxisomes and intersectin proteins. Twenty-four (24) SNPs in eight candidate genes were genotyped to investigate their association with TDF-RT in HIV-positive patients recruited from Zambia using a case-control design.

Our study did not find any significant statistical association between previously reported SNPs in drug transporter genes involved in TDF transport (*ABCC2*, *ABCC4*, *ABCC10* and *SLC22A11*). Furthermore, we failed to find any association between variants reported in mendelian FS and transmembrane gene and TDF-RT.

Several studies have reported an association between variants in drug transporters and TDF-RT; however, almost all of these reports consisted predominantly of Caucasians and Asian populations. In particular, *ABCC2* rs717620 C>T has been consistently associated with TDF-RT. Reports include an association with proximal tubular disease[298, 397, 549]; higher expression of the common allele C in patients with TDF-RT[397] and an association with abnormal tubular markers[298]. Although the underlying mechanism for these findings has not been established, a

study by Hulot et al[552] suggested that heterozygous mutations in *ABCC2* may alter MRP2 function leading to the accumulation of nephrotoxic compounds like methotrexate and provided insights into the role of *ABCC2* in drug disposition. In addition, a drug-drug interaction between TFV and protease inhibitors by competing for tubular transporters[345] may further provide the basis to link ABCC2 -MRP2, rs717620 with TDF-RT. However, it is mainly the genotype CC that has been consistently associated with a reduction in eGFR[335] and the development of proximal tubular disease. Interestingly, a more recent study where the tubular disease was defined by urinary RBP/Cr ratio, carriage of genotype *CC* of the *ABCC2* rs7171620 variant was associated with a reduced risk of developing renal toxicty[549] contradicting other studies[298, 395, 397]. In the present study, the proportion of the CC genotype was not different between cases and controls and therefore, no association between this polymorphism and TDF-RT was found in the Zambian population.

MRP4 encoded by *ABCC4* has been demonstrated to have a functional role in the tubular transport of TFV[252]. In both functional[315] and clinical[339, 402] studies have demonstrated that TFV is a substrate of MRP4 and SNP in ABCC4 could potentially cause RT. However, most candidate gene studies conducted have continued to report very conflicting findings, with most studies have reported no association with TDF-RT[298, 397] while others have shown an association with individual tubular markers[298, 403].

We did not observe any association between *ABCC10* SNPs with TDF-RT similar to a recent study in a Ghanaian cohort of HIV-positive patients receiving TDF based where no association was found between *ABCC10* rs2125739 T>C with CKD and tubular disease[396]. Unlike our study, where TDF-RT was defined as CrCl <60ml/min, in this study, TDF-RT was defined as tubulopathy with one tubular abnormality of either hyperphosphaturia (fractional phosphate excretion >18%), normoglycaemic glycosuria, hypophosphataemia <0.8mmol/L or presence

of proteinuria. Interestingly, this study (n= 66 patients), observed a significant association between *ABCC10* rs2125739 T>C with normal baseline kidney function and lower CrCl following 1 year of TDF exposure. While these results may indicate an association between the *ABCC10* variant with declining renal function during TDF treatment, it is not certain whether analysed patients had predominantly CKD or tubular impairment and to what extent patient factors may have influenced this result. TDF-RT is multifactorial and underlying patient factors like comorbidities have previously been reported to influence TDF-RT[298, 339, 345, 402]. On the other hand, we may also speculate that the genetic diversity that exists within the African population is an important factor that with major implications on traits and differences in disease susceptibility[553].

In the present study, we observed an association between a novel investigated gene and variant, PEX14, rs284301T>C although the association was lost once corrected for multiple testing. PEX 14, rs284301 C>T SNP is one of the variants associated with TDF-FS in a GWAS (unpublished) conducted by my supervisors. This was the reason why it was selected for investigation by the present study, and it is interesting to note that this SNP showed an association (uncorrected p-value = 0.012) with TDF-RT in the current cohort. Furthermore, the same SNP was part of a *PEX14* haplotype observed in our study population.

PEX14 gene encodes a peroxin14 protein which is an essential component of the peroxisomal import machinery[554]. Peroxisomes are single-membrane-bound organelles present in most eukaryotic cells with high expression in the liver and kidney[551]**,** and particularly more dense in proximal tubules than in glomeruli, distal tubules, and collecting ducts[555, 556]. The human PEX14 is a multi-tasking protein that forms part of a complex of peroxins that play a key role in facilitating peroxisomal motility and protein import by directly interacting with microtubules. Accordingly, in PEX14 deficient cells, peroxisomes lose their ability to move

along microtubules[557]. In kidney tubular epithelial cells, microtubules play a critical role in maintaining cell homeostasis[558], and their dynamics have an influence on renal function[559]. We can therefore postulate that variants in PEX14 may encode a dysfunctional peroxin 14 protein resulting in loss of microtubule motility and altered kidney function.

Peroxisomes on the other hand are known to possess a variability of more than fifty enzymes which are linked to different multiple biochemical pathways[560]. In relation to kidney function, the most important role involves the β-oxidation of fatty acids (FAO) and detoxification of the resultant hydrogen peroxide and other ROS[561]. These peroxisomal tasks are functionally related to mitochondria although peroxisomes function solely as metabolic organelles compared to mitochondria that are concentrated on sites of antiviral signalling and apoptosis[562]. Recent evidence also indicates that peroxisomes are actively involved in apoptosis and inflammation[563], innate immunity[561], ageing and in the pathogenesis of age-related diseases, such as diabetes mellitus and cancer[564]. Furthermore, their involvement in the pathogenesis of kidney disease has been suggested through two possible pathways. The first hypothesis is based on the nephron's capacity to utilise the fatty acids as a source of energy in addition to the abundant mitochondrial FAO which is widely distributed in the renal tubular segment. Due to the low rate of glycolysis in the proximal tubules, numerous energy-consuming transporters rely on both tubular mitochondrial and peroxisomal FAO as sources of energy[560, 565]. Dysfunctional mitochondrial and/or peroxisomal FAO leads to an increase of unmetabolized fatty acids and subsequently decrease energy production which further inhibits proximal tubule Na+K+-ATPase, destabilize the mitochondrial membrane potential, leading to an increase in inflammatory response[565]. Secondly, peroxisomal FAO together with mitochondrial can generate as well as scavenge ROS and are essential in tightly regulating redox homeostasis. Thus, impaired degradation of H_2O_2 results in the increase of peroxisomal membrane

permeability and leakage of H_2O_2 and peroxisomal matrix components into the cytoplasm. Peroxisomal dysfunction can potentially induce alterations of mitochondrial function and promote the generation of mitochondrial ROS which affects the critical energy-generating pathway for renal tubular cell function and potentially aggravate kidney injury[551]. While to date, most studies have pointed to TFV-induced toxicity on mitochondria in the pathogenesis of kidney disease, studies examining the involvement of peroxisomes are scarce. The marginal association observed in this study between the PEX14 variant, rs284301, and TDF-RT may be worth exploring further to elucidate the functional consequences of the SNP on a renal peroxisomal component. While there are limited studies published on rs284301, perturbed gene expression has been observed based on the eQTL reported in the GTex portal repository. In the context of eQTL parameters, there normalised effect size of rs284301 on PEX14 expression in the kidney was large (0.244), however, the magnitude of this effect and its biological impact and involvement in TDF-RT may need to be confirmed in functional studies.

The contrast in our findings could be explained by several factors. In the first place, our study was conducted in an African population which ethnically presents heterogeneity within the Africans as well as the Caucasian and Asian populations. Secondly, the clinical phenotype in our study may be different to that reported by other studies; this is mainly because there is no universally accepted definition for TDF-RT. Our study used CrCl-CG as the surrogate marker used to define TDF-RT; this is different to surrogate markers such as nondiabetic glucosuria, hyperphosphaturia hypophosphatemia[396, 397] and urine protein markers[298, 549] used by previous studies. Izzedine[397] and Rodríguez-Novoa[298] used at least two tubular abnormalities to define TDF-RT presenting within a month of therapy and median of 42 months respectively. On the other hand, Dajuma[549] and Neary[396] used only one tubular marker for case definition with TDF exposure of at least six months. Clinical characteristics could therefore be another reason for

the lack of association between the SNPs that we investigated and TDF-RT.

3.4.1 Limitations

Although this study used the largest reported samples size to investigate previously reported variants in several genes suggested to play a role in TFV disposition, given RT is a complex phenotype where multiple variants of smaller effect sizes contribute, our study may have lacked sufficient power to uncover any genetic association with TDF-RT. We used surrogate markers that were determined and reported by the clinical laboratory in Zambia, but we could not ascertain the sensitivity of the methods used for measuring these markers. We also acknowledge that the definition of cases using CrCl as a surrogate marker may have missed out on identifying some of the true TDF-RT clinical phenotypes. Ideally, we should have used more than one marker to define the clinical phenotype, but this was not possible. There has been limited reproducibility of TDF pharmacogenetics in the clinical setup and several studies have reported no effect of SNPs. We, therefore, suggest that our findings should be interpreted in the context of these limitations and differences with previous studies.

3.4.2 Conclusion

We have demonstrated that SNPs in TFV transporter proteins previously associated with TDF-RT in other populations may not be associated with a similar risk in the African cohort in the present study. Nevertheless, the validity of our results can be demonstrated only by replicating them in a sufficiently powered independent cohort that would address the highlighted limitations.

CHAPTER 4

SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED WITH TENOFOVIR DISOPROXIL FUMARATE- INDUCED FANCONI SYNDROME: VALIDATION OF A GENOME-WIDE ASSOCIATION STUDY

4.1 INTRODUCTION

Evidence suggests that pharmacogenomics has the potential to improve drug safety and outcomes of the commonly used medications. Genome-wide association studies (GWAS) have been very effective in identifying novel variants associated with a phenotype and or clinical outcome that have been used to identify individuals at high risk of disease, thereby, improving patient outcomes through early detection, prevention or treatment[566]. In recent years, GWAS has also been harnessed to improve drug response by identifying novel and common genetic variants responsible for drug toxicity[567-569]. These advances have contributed to developing practices that aid personalised medicine through appropriate drug selection, patient stratification based on genetic markers, and adjustments to dosing to prevent rare and serious adverse drug reactions[569, 570]. Furthermore, the biological hypothesis generated from GWAS also leads to a better understanding of the mechanisms behind drug-induced toxicity[570].

While offering extensive insights in this regard, GWAS are most often limiting because the causal variants are usually not directly genotyped but are in linkage disequilibrium with the genotyped SNPs, and, a common challenge is a difficulty in distinguishing the causal loci from the many SNPs found associated[571]. For this reason, very stringent significance thresholds (for eg. p-value of $< 5 \times 10^{-8}$) are used to identify associated variants. However, with this approach, some variants with genuine association may be missed. It has also been argued that most associations detected may be false positives and may not directly locate causal variants and genes[566]. One such example is a report by Philstrøm et al[572] where variants thought to be associated with creatinine and graft survival in kidney recipients could not be validated in an independent cohort and this outcome emphasised the importance of validating findings from high-throughput genetics studies. Therefore, most findings require validation by either functional characterisation or recruiting large cohorts to confirm the statistical association and the causal gene variants[570, 572].

In this study, we conducted a candidate gene association study to validate the variants identified from a GWAS of TDF-FS in a UK cohort of HIV positive patients on TDF (unpublished). FS is thought to be a rare but serious adverse effect associated with TDF use either alone or in the presence of other risk factors (See Chapter 1 for a detailed literature review on TDF-FS). Apart from the validation of variants identified by the GWAS using alternate genotyping technology, we

also wanted to i) investigate the association between previously reported gene variants in TDF-FS in our cohort; and ii) investigate whether variants that are associated with the more common TDF-RT play any role in the pathogenesis of TDF-FS.

4.1.1 OBJECTIVES

1. To validate genetic variants identified in a GWAS for their association with TDF-FS using an alternate genotyping method (MALDI-TOF and RT-PCR).
2. To determine whether previously reported SNPs in TDF-FS show any association in our cohort of TDF-FS.
3. To investigate whether variants that are associated with TDF-RT in various populations are associated with TDF-FS.

4.2 METHODS

4.2.2 Study background - Genome-Wide Association Study

A research group that includes my supervisors (Prof Munir Pirmohamed and Dr Sudeep Pushpakom) with collaborators at the Kings College London, prospectively conducted UK-wide multi-centre recruitment of HIV positive patients who presented with Fanconi syndrome while receiving TDF as part of their ART. The aim of that study was to conduct a GWAS to identify genetic variants that may be associated with TDF-FS in a UK HIV population.

4.2.2.1 **Study Cohort**

Sixty-three consenting participants, predominantly of Caucasian ethnicity met the inclusion criteria and were prospectively recruited into the study. The patients were known HIV positive patients who were receiving antiretroviral therapy containing TDF 300mg once daily and developed TDF-FS. Patients were recruited if they had FS attributed to TDF which met the defined parameters for TDF-FS. Fanconi syndrome was defined by biochemical evidence of significant proximal tubulopathy (PT) as evidenced by at least two markers of PT judged to be a result of TDF exposure and resulted in permanent TDF discontinuation. These defining markers included a new onset or worsening of pre-existing proteinuria of at least ≥1+ on urinary dipstick (or uPCR >50mg/mmol), a new onset or worsening of pre-existing glycosuria of at least ≥1+ on urinary dipstick with concomitant serum glucose <11.1 mmol/L, Serum K^+ <3.0 mEq/L, Serum HCO^{3-}

<19 mEq/L in those with a CrCl ≥25 mL/min, Serum H_3PO_4 <0.65 mmol/L.

A patient was excluded from participating if they presented with sepsis and or a severe infection during the presentation of renal toxicity. Any patient with a comorbidity such as cardiac failure, liver failure, cancer chemotherapy and other plausible aetiologies that are risk factors for kidney disease were also excluded from participating. 56 participants had complete data and were included in the DNA analysis for GWAS.

4.2.3 Genome-Wide Association Study and results

Genotyping of the Kings College TDF-FS cohort was conducted by UK Biobank Affymetrix Axiom Arrays. This array is a high-throughput and powerful tool for translational research in the fields of epidemiology, human disease, and population genetics designed by leading researchers for use by UK Biobank. The platform contains one 96-array plate that allows for genome-wide genotyping of a large number of sample cohorts to explore the genetics of complex disease traits and translational research cost-effectively. Affymetrix Axiom SNP chip allows interrogation of 820,967 genetic variants.

Genotyping data for controls were obtained from the Wellcome Trust Case Control Consortium to undertake GWAS analysis. Four SNPs: two SNPs s in *TMEM120A* (Chr 7), one SNP in Chr 14 (gene not annotated) and another SNP in *MIR3976HG* (Chr 18) were found associated with TDF-FS at genome-wide significance ($p=5x10^{-8}$). In addition, the GWAS also identified a total of 8 variants in multiple genes such as *PEX14* (Chr 1), *IK2F1* (Chr 3), *STMN2* (Chr 8) *and ITSN1* (Chr 21) at $p< 5 \times 10^{-5}$. The genes for these are shown on the Manhattan plot, Fig 4.1.

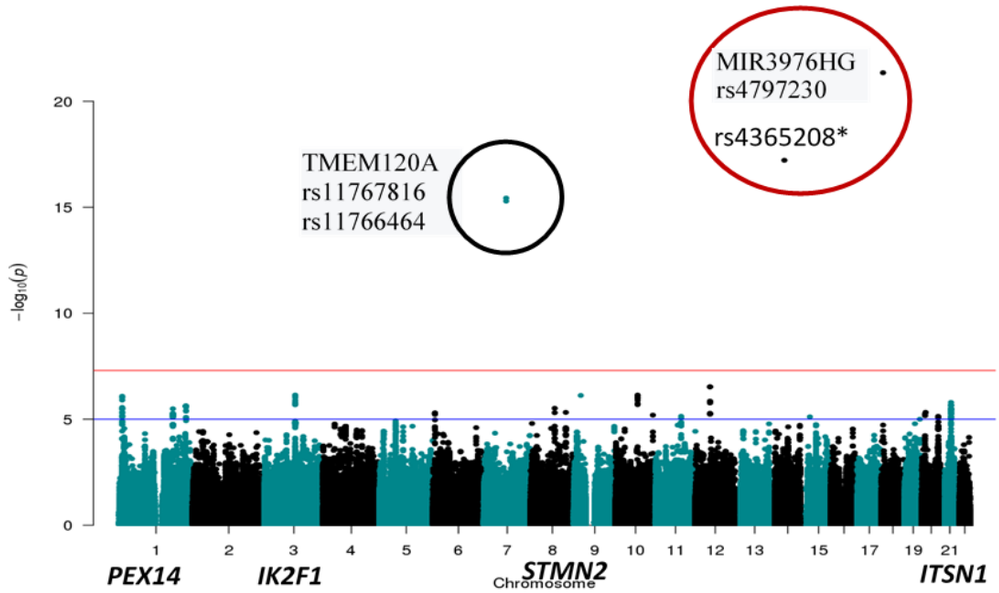


Figure 4.1. Manhattan plot of identified SNPs and genes after filtering for SNPs with $p<10 \times 10^{-5}$ on the genome. The gene on Chr 14 in which rs4365208 falls is not annotated.

Table 4.1. Genes and SNPs identified by the GWAS and their p-values.

Gene	Chr	rsID	BP	p-value
PEX14	1	rs284267	10656351	8.1855×10^{-7}
		rs284301	10694125	7.4196×10^{-6}
		rs284265	10598164	9.6186×10^{-7}
	3	rs4856157	103237932	7.2856×10^{-7}
TMEM	7	rs11767816	75987460	5.1125×10^{-16}
		rs11766464	75616779	3.6743×10^{-16}
STMN	8	rs7001019	80565018	3.0367×10^{-6}
-	14	rs4365208	51743970	6.0795×10^{-18}
MIR3976HG	18	rs4797230	5785518	4.4893×10^{-22}
ITSN	21	rs2834254	35105542	1.614×10^{-6}
		rs9975466	35039977	8.072×10^{-6}
		rs2834251	35084921	2.2735×10^{-6}

A dash (-) represents unavailable information about the gene; Chr-chromosome, rsID-SNP ID, BP-Base pair position

4.2.4 SNP selection for association studies.

We conducted a case-control candidate gene association study to i) validate the GWAS selected SNPs and, ii) to investigate other reported SNPs that are relevant in either TDF-FS, Mendelian FS or TDF-RT[398]. To validate the GWAS-associated SNPs, we further consulted RegulomeDB, an online repository (http://www.regulomedb.org/) to determine whether these SNPs had any

regulatory function of biological processes in the respective genes involved. We consolidated the information extracted with published literature and selected variants in genes with plausible association with renal function.

In addition, we selected 17 SNPs in drug transporter genes and 2 SNPs in the OCRL gene which were previously reported to be associated with TDF-RT and TDF-FS respectively as well as the MAF in Caucasians, Asians and Americans.

4.2.5 Control cohort

The ideal control cohort for comparison against TDF-FS cases would be a cohort of HIV-positive, TDF-treated individuals with no evidence of TDF-FS and are matched for ethnicity and other variables. However, as this was not available, we used genotyping data for healthy individuals who were part of the National Blood Services Biobank (NBS) dataset obtained from the WTCCC. We searched for genotyping data for the selected SNPs for validation. Based on the reported incidences of Fanconi syndrome at ≤1.7%, we used 1:5 cases: control ratio to determine a control cohort for comparison with high statistical power. We filtered the database for the SNPs in our study and the selection of individuals for the control cohort was conducted using the systematic random sampling method.

4.2.6 Genotyping of selected SNPs

We conducted SNP genotyping by two methods; all SNPs were genotyped by Agena Bioscience iPlex MassARRAY® System; a platform integrating mass spectrometry (MALDI-TOF) with end-point PCR iplex technology and where MALDI-TOF failed, those SNPs were genotyped by a TaqMan Real-time PCR using validated SNP genotyping assays (Assays-on-demand; Applied Biosystems, Loughborough, UK). SNPs, rs3740066, rs717620 and rs2125739 were selected to be genotyped by TaqMan Real-Time PCR allelic discrimination method using a Quant Studio™ 6 Flex Real-Time PCR System (Thermo Fisher, Loughborough, UK). A detailed step by step description of the procedure and genotyping methods is given in chapter 2 subsection 3.3.9.

4.2.7 Statistical Analysis

Quality control was conducted to test all SNPs for deviation from Hardy-Weinberg equilibrium (HWE). A single SNP genotype association between case and control groups was analysed using the logistic genotypic model of 2 degrees of freedom (2df). Where the allele frequency was too

low to determine an association using the genotypic model, we used the allelic model determined by Fisher's test. We adjusted all statistical significance using the Bonferroni correction for multiple testing. We used MS Excel to sort and organise data which was analysed conducted using PLINK 1.9 software (www.cog-genomics.org/plink/1.9/) version 1 integrated with the RStudio platform. We further performed haplotype analysis using Haploview software version 4.1 to investigate linkage disequilibrium and haplotype blocks.

4.3 RESULTS

In total, 24 SNPs: 5 SNPs from GWAS, 17 SNPs in drug transporter genes associated with TDF-RT; and 2 SNPs in the OCRL1 gene were selected for further validation.

4.3.1 GWAS SNPs selected for further investigation.

4.3.1.1 SNPs with Genome-Wide significance of $p< 5x 10^{-8}$

As shown in Table 4.1 and figure 4.1 in the methods section, 4 SNPs had genome-wide significance ($p=5x10^{-8}$). However, although two SNPs (rs4365208, Chr 14; rs4797230, Chr 18) appeared to be genome-wide significant, these were considered as artefacts because they were singletons that lacked a well-defined LD in their respective gene locus, and therefore were excluded from further analysis.

2 SNPs in chromosome 7 in the *TMEM120A* gene were statistically significant at the genome-wide significance level by the GWAS (Figure 4.2). The lead SNP rs11766464 had a p-value of 5×10^{-16} while the neighbouring SNP, rs11767816 showed significance at $p< 3x10^{-16}$. Although the lead SNP had an eQTL for aorta, brain, cerebellum and cells transformed fibroblasts, preliminary assessment with RegulomeDB annotation showed that both SNPs had a very low ranking suggesting that they have minimal evidence of regulatory function. Further, GeneAtlas by the Roslin institute did not determine a plausible association with the phenotype. *TMEM120A* SNPs are supported by the biological plausibility based on *TMEM120A* expression in fat cells induced by adipocyte differentiation but without direct association with kidney disease. The gene's role in the expression of other genes necessary for adipocyte differentiation *GATA3, GLUT4* and *FASN* could be indirectly implicated in kidney disease associated with impaired fatty acid metabolism. Although an evidence of direct association with kidney disease was lacking, we selected both the *TMEM120A* SNPs for validation.

For our genotyping validation, we were not able to design a compatible assay for rs11766464; attempts to custom-make a TaqMan assay also failed and therefore this SNP was excluded from any analysis. Therefore, only one SNP, rs11767816 was selected.

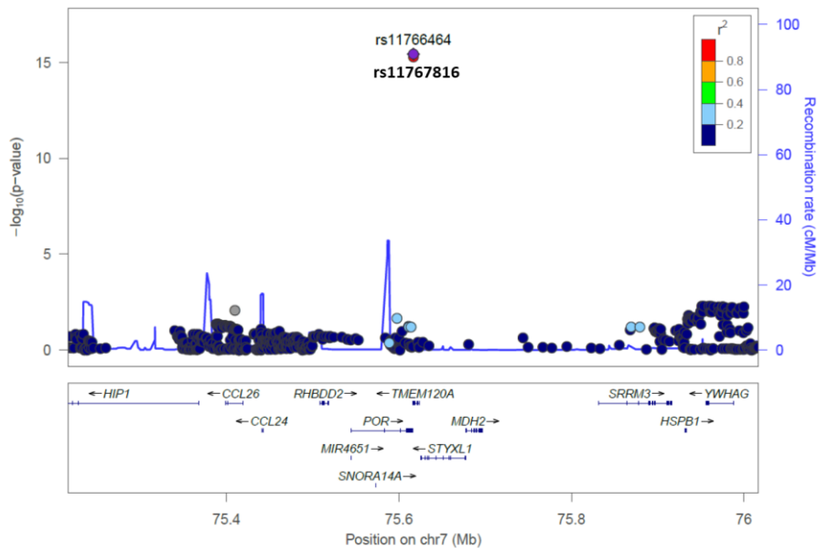


Figure 4.2. Manhattan plot of Chromosome 7 displaying rs11766464 and rs11767816 in a circle.

4.3.1.2 **SNPs that were not genome-wide significant but with biological plausibility.**

GWAS highlighted another 8 SNPs to be significant at $p<5x10^{-5}$ (See Table 4.1 in Methods). We selected 4 out of these 8 SNPs: PEX14 (3 SNPs) and ITSN1 (1), for further validation. When a causal association was not well defined, only the lead SNP was selected. SNPs on chromosomes 3 and 8 were excluded due to a poorly defined stack of SNPs in LD in their respective gene locus (Figure 4.3).

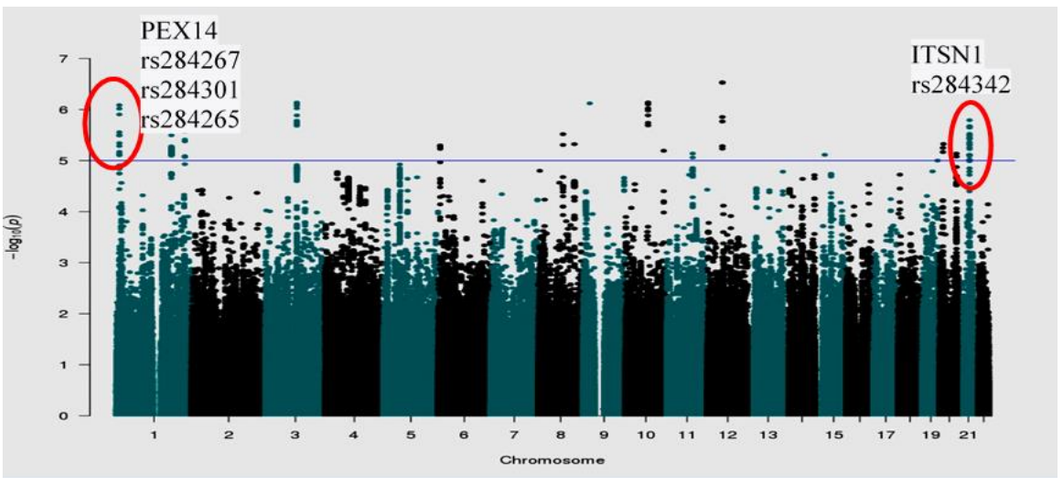


Figure 4.3. Display of GWAS SNPs with defined clusters (circled) considered for further investigation and the excluded SNPs in un circled clusters.

The lead SNP rs284267 in Chr 1 falls in a region with two genes, *PEX14* and *CAS21* and may

explain the likely association with kidney function. Literature shows that *PEX14* encodes peroxisome proteins which are highly expressed in kidneys, and it plays a key role in peroxisome movement through direct interaction with tubulin, while, on the other hand, CAS21 has been associated with blood pressure variation which could support a plausible involvement in kidney disease for rs284267. Two other SNPs in *PEX14*, rs284301 and rs284265 are shown to have high and moderate regulatory evidence in RegulomeDB, respectively. Moreover, rs284265 is linked to the regulation of *FOXA1*, an albumin enhancing protein, encoded by *PEX14*. Overexpression of albumin may result in histological changes of progressive tubulopathy. Finally, rs2834254 in chromosome 21 was selected on the basis that it falls on the *ITSN* gene which indirectly coordinates clathrin-mediated endocytic membrane traffic with the actin assembly machinery. This interaction may further influence the proximal tubular endocytic receptors megalin and cubilin that play a significant role in LMWP endocytosis.

Therefore, we selected a total of 5 SNPs based on the GWAS data. – these are rs11767816 (TMEM120A); rs284267, rs284301, rs284265 (PEX14), and rs2834254 (ITSN1).

4.3.2 SNPs in drug transporters and OCRL1 genes

In particular, *OCRL1* (responsible for Lowe Oculocerebrorenal Syndrome and Dent Disease) variants were initially reported to be associated with TDF-FS by Dahlin et al[398] and has also been associated with the Mendelian Fanconi syndrome. SNPs that were previously associated with TDF-RT included those in genes encoding for influx and efflux drug transporters. The evidence and detailed rationale for the selection of SNPs in drug transporter genes and *OCRL1* gene has previously been summarised and shown in Chapter 3, table 3.1. The selected SNPs include those in transporter genes *SLC22A11* (rs11231809*) and ABCC2* (rs717620, rs2273697, rs8187707, rs3740066, rs17216177, rs17222519, rs7899457 and rs79174032), *ABCC4* (rs1059751, rs3742106, rs2274409, rs899494, rs11568658 and rs1751034) and *ABCC10* (rs9349256 and rs2125739) respectively. Selected SNPs in the OCRL gene are rs7057639 and rs113165732. Table 4.2 below gives the final list of all the 24 SNPs selected for the case-control association study to validate GWAS and candidate genes.

Table 4.2. List of the 24 selected SNPs for the validation study, includes 5 from GWAS, 17 SNPs from transporter genes and 2 from the OCRL gene.

GENE	Chr	rs ID	Position	Allele A	Allele B
PEX14*	1	rs284267	10656351	A	C
	1	rs284265	10658221	C	T
	1	rs284301	10694125	A	G
ABCC10	6	rs9349256	43404511	A	G
	6	rs2125739	43412865	C	T
TMEM120A*	7	rs11767816	75616778	T	A
ABCC2	10	rs717620	101542578	T	C
	10	rs79174032	101560106	A	C
	10	rs2273697	101563815	A	G
	10	rs17216177	101603522	C	T
	10	rs3740066	101604207	T	C
	10	rs8187707	101610533	T	C
	10	rs17222519	99794393	G	A
	10	rs7899457	99845746	C	T
SLC22A11	11	rs11231809	64302950	A	T
ABCC4	13	rs1059751	95672950	G	A
	13	rs3742106	95673791	C	A
	13	rs1751034	95714976	C	T
	13	rs2274409	95860214	T	C
	13	rs899494	95861804	A	G
	13	rs11568658	95863008	A	C
ITNS1*	21	rs2834254	35105542	T	C
OCRL	X	rs7057639	129540176	T	C
		rs113165732	129576236	T	C

*: GWAS SNPs. Chr: Chromosome; Allele A: minor allele; Allele B: Common allele.

4.1.1 Control cohort and genotype data.

338 individuals with genotype data for the selected SNPs were determined and selected for the comparison study. In the NBS database, control genotype data was available for 22 out of the 24 SNPs genotyped in cases. Among the 22 SNPs, 13 SNPs were directly genotyped and 9 were imputed. All imputed SNPs had an imputation quality of 89.4% to 99.9% except for two SNPs in the ABCC2 (rs17222519 and rs7899457) that had an imputation quality of ≤2.9%, and were, therefore, excluded in the downstream analyses. Thus, a total of 13 directly genotyped and 7 imputed SNPs (Total = 20 SNPs) were included in the final case-control association analysis. A

summary description of the SNPs extracted from the NBS database is given in Table 4.3.

Table 4.3. Table 1. Summary of SNPs data for controls included in the analysis.

	Gene	Chr	rsID	BP
Genotyped SNPs	PEX14*	1	rs284267	10656351
	PEX14*	1	rs284301	10694125
	ABCC10	6	rs9349256	43404511
	ABCC10	6	rs2125739	43412865
	ABCC2	10	rs717620	101542578
	ABCC2	10	rs2273697	101563815
	ABCC2	10	rs8187707	101610533
	ABCC4	13	rs1059751	95672950
	ABCC5	13	rs3742106	95673791
	ABCC6	13	rs2274409	95860214
	ABCC7	13	rs899494	95861804
	ABCC8	13	rs11568658	95863008
	ITSN*	21	rs2834254	35105542
Imputed SNPs	PEX14*	1	rs284265	10598164
	TMEM*	7	rs11767816	75987460
	ABCC2	10	rs3740066	99844450
	ABCC2	10	rs17216177	99843765
	ABCC2	10	rs79174032	99800349
	ABCC4	13	rs1751034	:95062722
	SLC22A11	11	rs11231809	64535478
Excluded SNPs				
Poor imputation quality	ABCC2	10	rs17222519	99794393
	ABCC2	10	rs7899457	99845746
Unavailable	OCRL1	X	rs113165732	129576236
	OCRL1	X	rs7057639	129540176

*SNPs from GWAS

4.3.3 Association of selected SNPs with TDF-FS

A total of 20 SNPs were analysed for the association of SNPs with TDF-FS. The distribution of genotypes at the *PEX14, TMEM120A, ITSN1, ABCC2, ABCC4, ABCC10, and SLC22A11* genes is given in table 4.4. Five (5) SNPs that were associated by the GWAS were found to be once again significantly associated with TDF-FS after correcting for multiple testing. These include three in the *PEX14 (* rs284267, rs284301, rs rs284265 one in *ITSN1 (*rs2834254) and one in TMEM120A

(rs11767816) genes. Our current results confirm what was observed in the GWAS. None of the SNPs in the genes encoding for influx and efflux transporter proteins were associated with TDF-FS either at unadjusted or adjusted significance levels.

Table 4.4. Association of SNP Genotype and Allele with TDF-FS

GENE (PROTEIN)/ SNP	**Genotype /Allele**	**CASES n=56**	**CONTROLS n=338**	**p-value**
		n (%)	n (%)	
***PEX14* (Peroxins)**				
rs284267	CC	27 (48.2)	258 (76.3)	**<0.001**
	CA	23 (41.1)	75 (22.2)	
	AA	6 (10.7)	5 (1.5)	
	C	77 (68.7)	591 (87.4)	
	A	35 (31.3)	85 (12.6)	
rs284265	TT	28 (50)	259 (76.5)	**<0.001**
	TC	22 (39.3)	75 (22.3)	
	CC	6 (10.7)	4 (1.2)	
	T	77 (68.75)	593 (87.7)	
	C	35 (31.25)	83 (12.3)	
rs284301	GG	28 (50)	252 (74.6)	**<0.001**
	GA	21 (37.5)	81 (24)	
	AA	7 (12.5)	5 (1.4)	
	G	77 (68.7)	585 (86.5)	
	A	35 (31.3)	91 (13.5)	
***ABCC10* (MRP7)**				
rs9349256	GG	12 (21.4)	91 (26.9)	0.55
	GA	29 (51.8)	164 (48.5)	
	AA	10 (17.8)	83 (24.6)	
	G	65 (57)	346 (51.2)	
	A	49 (43)	330 (48.8)	
rs2125739	TT	34 (60.7)	179 (53)	0.35
	CT	19 (33.9)	126 (37.2)	
	CC	3 (5.4)	33 (9.8)	
	T	87 (77.7)	484 (71.6)	
	C	25 (22.3)	192 (28.4)	
***TMEM120* (TMEM 120A)**				
rs11767816**	AA	56 (100)	127 (38)	**<0.001**

	AT	0 (0)	154 (45.6)	
	TT	0 (0)	54 (16)	
	A	112 (100)	675 (99.9)	
	T	0 (0)	1 (0.1)	
***ABCC2*(MRP2)**				
rs717620	CC	36 (64.3)	216 (63.9)	0.820
	CT	18 (32.1)	110 (32.5)	
	TT	2 (3.6)	12 (3.6)	
	C	90 (80.4)	542 (80.2)	
	T	22 (19.6)	134 (19.8)	
rs79174032	CC	56 (100)	337 (99.7)	0.683
	CA	0 (0)	1 (0.3)	
	CC	0 (0)	0 (0)	
	AA	112 (100)	675 (99.8)	
	C	0 (0)	1 (0.1)	
	A	0(0)	0(0)	
rs2273697	GG	37 (66.1)	205 (60.7)	0.524
	GA	17 (30.4)	116 (34.3)	
	AA	2 (3.6)	17 (5)	
	G	91 (81.3)	526 (77.8)	
	A	21 (18.8)	150 (22.2)	
rs17216177*	TT	48 (85.7)	294 (87)	0.838
	TC	8 (14.3)	43 (12.7)	
	CC	0 (0)	1 (0.3)	
	T	104 (92.9)	631 (93.3)	
	C	8 (7.1)	45 (6.7)	
rs3740066	CC	29 (51.8)	131 (38.7)	0.145
	CT	20 (35.7)	174 (51.5)	
	CC	7 (12.5)	33 (9.8)	
	T	78 (69.6)	436 (64.5)	
	C	34 (30.4)	240 (35.5)	
rs8187707**	CC	49 (87.5)	295 (87.3)	0.831
	CT	7 (12.5)	43 (12.7)	
	TT	0 (0)	0 (0)	
	C	105 (93.8)	633 (93.6)	
	T	7 (6.25)	43 (6.4)	

p-value adjusted for multiple corrections.** Association determined by Fisher’s exact allelic model.

Table 4.4 Continued.

GENE (PROTEIN)/ SNP	Genotype /Allele	CASES n=56	CONTROLS n=338	p-value
		n (%)	n (%)	
***SLC22A11* (hOAT4)**				
rs11231809	TT	22 (39.3)	117 (34.6)	0.561
	AA	22 (39.3)	156 (46.2)	
	TA	12 (21.4)	65 (19.2)	
	T	66 (58.9)	390 (57.7)	
	A	46 (41.1)	286 (42.3)	
***ABCC4* (MRP4)**				
rs1059751	AA	21 (37.5)	112 (33.1)	0.572
	AG	21 (37.5)	162 (47.9)	
	GG	14 (25)	64 (18.9)	
	A	63 (56.3)	386 (57.1)	
	G	49 (43.7)	290 (42.9)	
rs3742106	AA	20 (48.2)	110 (32.5)	0.499
	AC	30 (41.1)	167 (49.4)	
	CC	6 (10.7)	61 (18)	
	A	70 (68.8)	3877 (93.1)	
	C	42 (31.2)	289 (6.9)	
rs1751034	TT	36 (50)	236 (69.8)	0.9
	CC	19 (39.3)	95 (28.1)	
	TC	1 (10.7)	7 (2.1)	
	T	91 (68.75)	567 (83.9)	
	C	21 (31.25)	109 (16.1)	
rs2274409	CC	47 (83.9)	271 (80.2)	0.700
	CT	8 (14.3)	61 (18)	
	TT	1 (1.8)	4 (1.2)	
	C	102 (91.1)	603 (89.7)	
	T	10 (8.9)	69 (10.3)	
rs899494**	GG	40 (71.4)	262 (77.5)	0.435
	AG	16 (28.6)	71 (21)	
	AA	0 (0)	4 (1.2)	
	G	96 (85.7)	595 (88.3)	
	A	16 (14.3)	79 (11.7)	
rs11568658**	CC	55 (98.2)	324 (95.9)	0.708
	CA	1 (1.8)	14 (4.1)	
	AA	0 (0)	0 (0)	

	C	111 (99.1)	662 (97.9)	
	A	1 (0.9)	14 (2.1)	
***ITSN1* (Intersectin-1)**				
rs2834254	CC	42 (75)	288 (85.2)	<0.001
	CT	7 (12.5)	46 (13.6)	
	TT	7 (12.5)	4 (1.2)	
	C	91 (81.3)	622 (92)	
	T	21 (18.7)	54 (8)	

p-value adjusted for multiple corrections.** Association determined by Fisher's exact allelic model.

4.3.4 Haplotype association with TDF-FS.

The haplotype frequency and IDs for each estimated haplotype block with their estimated positions were initially determined in Plink. The LD position was calculated for SNPs within 200kb. The Plink-determined haplotype blocks were then confirmed with Haploview, and the associated alleles and their LD structure were generated. We reported haplotypes with a frequency of >1%. Two haplotypes CT and AC were determined in PEX14, rs284267C>A and rs284265 C>T. The association with TDF-FS was statistically significant (CT<0.001) and AC (<0,001). Table 4.4 shows haplotype association results and the distribution of the frequency of haplotypes in the cases and Controls. The LD structure for PEX 14(block 1) is given in Figure 4.5.

Table 4.5. Haplotype Association with TDF-FS.

Haplotypes	Cases (n,%)	Controls **(n,%)**	p-value
PEX14			
CT	39 (68.7)	295 (87.1)	<0.001
AC	18 (31.2)	41 (12.9)	<0.001

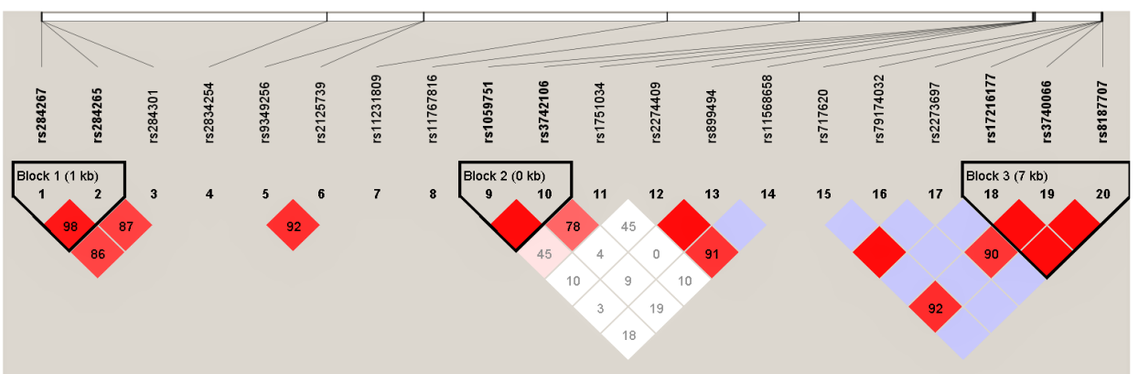


Figure 4.4. LD structure for haplotypes in PEX14 (block1)

4.4 DISCUSSION

Genetic variations may be responsible for the observed variability in drug response and toxicity in individuals. Variations in drug response have been observed in efficacy, susceptibility to adverse drug effects, toxicity and variability in optimising the effective dosage for an individual[568]. Single variant association studies have identified very important associations that have driven the possibility of precision medicine. In this study, we report the results of a case-control candidate gene association study of a cohort of 56 HIV positive patients with a confirmed diagnosis of TDF-FS and a population control group of 338 participants. We aimed to confirm SNPs identified in a GWAS using a different genotyping method. In addition, we also explored if genetic variants associated with the more common TDF-RT are implicated in susceptibility to TDF-FS. We confirmed the association of five variants in *TMEM120A*, *PEX14* and *ITSN1* genes that were previously identified in the GWAS with TDF-FS.

In the preliminary results from GWAS, TMEM120A rs11767816 SNP was found associated with TDF-FS at a genome-wide significance level. Validation of this result by alternate technology might further strengthen its involvement in kidney injury processes. *TMEM120A* codes a nuclear envelope transmembrane protein which is highly expressed preferentially in fat and plays a significant role in adipocyte differentiation and metabolism[573]. According to RegulomeDB annotation, there is very low evidence of *TMEM120A* involvement in regulatory function[574]. In addition, there appears to be no direct functional association between *TMEM120A* and kidney disease based on available literature. *TMEM120A* has been reported to be associated with steroidogenesis due to cytochrome P450 oxidoreductase deficiency (https://www.genecards.org/cgi-bin/carddisp.pl?gene=TMEM120A). Therefore, the role of *TMEM120A* in TDF-FS and kidney disease in general still need to be ascertained further through functional studies to confirm whether this is a valid association.

Experimental studies have shown that the knockdown of the *TMEM120A* gene alters the expression of several genes that are essential for adipocyte differentiation. These affected genes such as GATA Binding Protein 3 (*GATA3)*, fatty acid synthetase (*FASN*) and Glucose Transporter 4 (*GLUT4*) have been independently linked to kidney disease through various mechanisms[575, 576]. Whether adipocyte induction of toxic pro-inflammatory cells such as interleukins that in turn cause damage to many organs and promote insulin resistance leading to type 2 diabetes is responsible for the association with kidney disease is yet to be investigated[577]. However, there is a strong suggestion of its involvement through adipocyte metabolism. Thus, we hypothesise an indirect involvement of *TMEM120A* in kidney disease through defective adipogenesis and regulation of *GATA3, FASN, and GLUT*4.

High levels of energy are required to maintain the acid-base homeostasis and reabsorption processes in proximal renal tubules and the abundance of mitochondria in proximal tubules make them metabolically active due to the reabsorption of most glomerular filtrate. Moreover, Mitochondrial FAO serves as the preferred source of ATP in the kidney and its dysfunction results in ATP depletion and lipotoxicity that in turn elicits tubular injury and inflammation and subsequent fibrosis due to progressive tissue injury[578-580]. Variations or depleted proximal tubular ATP have been shown to be a contributing factor in the development and progression of AKI, CKD, diabetic and glomerular nephropathy. We, therefore, assume that *TMEM120A* variants may encode a dysfunctional protein that affects adipogenesis and adipocyte metabolism leading to depleted fatty acids that may affect mitochondrial function either by reduced energy generation or defective mitochondrial fatty acid oxidation. Thus, tubulopathy may occur through multiple mechanisms, the additive effect of dysfunctional *TMEM120A* and other adipogenic genes, *GATA3, GLUT4* and *FASN* as well as cytotoxicity due to accumulation of TDF. This may explain the characteristic of generalised tubulopathy observed in the TDF-

FS phenotype. Our hypothesis could be supported by the use of Peroxisome Proliferator-Activated Receptor (PPARα) agonist in the treatment of defective FAO in AKI and CKD due to its role in peroxisomal FAO[578, 581, 582]. The function of peroxisomes and their association in many aspects of lipid metabolism and involvement in proximal tubular impairment has been reported[551, 583]. An investigation of fatty acid composition in various stages of CKD reported alterations in plasma fatty acids composition in patients with CKD which was associated with an increase in fatty acid synthase enzyme demonstrating reduced adipogenesis or functional fatty acids in CKD[581].

It is plausible to suggest that *GLUT4*, *GATA3* and *FASN* pathways are also important indirect possibilities for TDF-RT. Although *GLUT4* is highly expressed in fat tissues and expressed in very low levels in the kidneys, studies have shown an intensive *GLUT4* expression in renal afferent vasculature in the renal glomerulus of normal rat kidneys[584] and through all the segments of proximal tubules and in the epithelial cells of the thick ascending loop of Henle[585]. Moreover, inhibition of *GLUT4* may be responsible for angiotensin 2 induced systemic and renal haemodynamic changes due to reduced renal blood flow and glomerular filtration rate[586]. Is has also been reported in insulin resistance and diabetes displayed in heterozygous GLUT4+/- mice with decreased GLUT4 both of which are risk factors of kidney injury and may lead to glomerular hyperfilteration[584]. On the other hand, GATA3 is a transcription factor known to regulate the expression of a wide range of clinically important genes[575]. The role of GATA3 in the neonatal development of kidneys is well known[576], but very little is known about its role in the adult kidney and regulatory pathways. Moreover, *GATA3* is thought to have a protective role in glomerulonephritis through unknown molecular mechanisms, although a study has demonstrated that high expression of *GATA3* in immune-mediated kidney injury is vital for the recruitment of regulatory T cells (Tregs) that are necessary for recovery following

renal tissue injury[575]. It is not known whether this renal protective role of *GATA3* is lost in individuals with mutated *TMEM120A* making them susceptible to any type of renal injury. If *TMEM120A* knockdown affect the expression of *FASN* gene, then its variant rs11767816 may attenuate the catalytic role in biosynthesis of long-chain saturated fatty acids that are vital for PT mitochondrial energy [577, 587]. The importance of mitochondrial FAO in proximal tubular epithelial cells is essential for tubular metabolic function[580, 581]. It may also be possible that the association we with a SNP in *TMEM120A* is not genuine. Functional studies are now required to effectively ascertain the role of this gene in kidney disease.

PEX14, rs284267 (C/A), rs284265 (T/C) and rs284301 (G/A) association with TDF-FS is an important confirmation in this study because it further adds strength to the earlier association for PEX14observed with TDF-RT in African patients (Chapter 3; unadjusted p-value). We have elaborated the possible pathways of peroxisomal proteins in kidney disorders in chapter 3, discussion section. Peroxisomes are well known ubiquitous, single membrane-bound cell organelles with a large variety of metabolic functions. Although originally, peroxisomes were not considered of physiological importance relative to other organelles, recent advances in understanding their role have demonstrated that they play a crucial role in human physiology[588-591]. While SIFT dbSNP database may not have yet identified predictions on the specific consequence of the SNPs in this study on amino acid substitution, the physiological significance of *PEX14* has been demonstrated by several inborn diseases caused by defects in peroxisome function[589]. A reduction or absence of functional peroxisomes in human cells is characterised by a rare but severe congenital disorder known as the 'spectrum of Zellweger syndrome' often leading to infancy death in the first few months of life[589].

PEX14 has a recognised vital role in microtubule-based peroxisome motility in human cells

that involves the cooperation of a collective group of various peroxisome proteins known as peroxins. These proteins possess distinct functions in the different stages of the import machinery for matrix proteins[561]. The process involves the crucial activity of *PEX3, PEX16* and *PEX19* in the targeting and insertion of peroxisomal membrane proteins[592] and recognition of the matrix proteins by the import receptor peroxins, *PEX5* and *PEX7* through their peroxisomal targeting signal of type 1 (PTS1) at the extreme C-terminus or a PTS2 close to the N-terminus, respectively. Then finally *PEX14* and *PEX13*-mediates the docking of the complex cargo-loaded PTS1/2-receptors complex to the peroxisomal membrane where it dissociates. The export of peroxisomal protein import machinery is a complex process that involves ubiquitylation which is highly dependent on ATP dislocation by ATPases[593]. It is no wonder peroxisomes are highly expressed in most mammalian cells, the highest being in the liver and kidney, because of the high metabolic activities that take place in these organs.

Indeed, very important metabolic pathways have been identified to exclusively or partially depend on peroxisomes. These include oxidation of α and β long fatty acid chains (FAO), synthesis of cholesterol and bile acids, metabolism of amino acids, purines, ROS and reactive nitrogen species (RNS). The success of these metabolic pathways requires a reliable and functional cofactor system which has become the main source of several peroxisomes associated disorders[594, 595]. With nearly over 50 years since their characterisation, there is substantial evidence that peroxisomes can function as a subcellular source, drainage sink, or target of ROS/RNS which can lead to chronic or severe alteration in the redox homeostasis and promote cell proliferation or trigger cell death[590, 596].

The source of peroxisome associated disorders is also highly explained by the a close functional interplay exhibited by peroxisomes with other organelles like mitochondria to sustain most of

their metabolic functions in mammals[596-598].This is consequently important because the role of mitochondria in TDF associated nephrotoxicity has already been demonstrated[374, 377]. Both organelles contain FAO machinery systems that catalyse the stepwise oxidation of acyl-CoAs to produce acetyl-CoA and propionyl-CoA (when a 2-methyl-acyl-CoA is oxidized). However, although the organelles share identical steps of FAO that include dehydrogenation-hydration-dehydrogenation-thiolytic cleavage, there are distinct differences that complement their interplay function. These include (i) different catalytic enzymes;(ii) Mitochondrial FAD-dependent dehydrogenase electron transfer into the respiratory chain compared to peroxisome FAD-dependent acyl-CoA oxidases indirectly donating electrons to molecular oxygen(O_2); (iii) the difference in the forms of fatty acids transported by both organelles across their respective membranes; (iv) the absence of the carnitine mediated uptake of peroxisomal fatty acids but crucial in mitochondria uptake of peroxisomal FAO products for their full oxidation to CO_2 and H_2O through the citric acid cycle and respiratory chain reactions[597, 598]. In addition, whereas peroxisomes are only capable of oxidising long to short-chain fatty acids, mitochondria complete the oxidation by converting peroxisome FAO products to acetyl-CoA and eventually to CO_2 and H_2O which is vital in maintaining cellular ROS homeostasis[598]. The absence of peroxisomal respiratory chain enzymes means that peroxisomes are indirectly dependent on the final mitochondrial anaplerotic oxidation of FAO for the generation of ATP and H_2O_2. Peroxisome-mitochondria interplay is important for various physiological cellular functions, particularly, the role of peroxins bound organelles throughout the nephron and especially the proximal tubules have been recognised as an important primary source of proximal tubular energy and detoxification of the resultant H_2O_2 and other ROSs[551, 594]. Thus, this pathway and organelle interplay are a crucial target and source of tissue damage[555, 556 561] . In addition, these pathways are also very critical in functions that consist of genetic disorders

of organelles biogenesis and many other nongenetic origins of metabolic disturbances which has linked both organelles to several diseases including[594]. From this organelle interplay, peroxisomal associated metabolic impairment leading to complete FAO may be a primary source of renal tubular injury because of inadequate ATP biogenesis which is vital for tubular metabolic function and failure of catalase antioxidants transport to neutralise the H_20_2 which if left unopposed, may cause tissue damage. Studies have demonstrated that mutations in the structural genes encoding proteins with a physiological role in FAO results in inhibited or dysfunctional FAO leading to adverse effects on the kidney tissue[592, 599, 600].

On the other hand, a functional FAO prevents the accumulation of fatty acids, their peroxidation, and the formation of lipid aldehydes that can further aggravate renal injury. Lipid peroxidation described generally as a process under which oxidants (ROS/RNS) attack unsaturated lipids such as polyunsaturated fatty acids have been demonstrated as the main source of peroxisome induced kidney injury[590, 591]. Moreover, Peroxisomes are also vital in the cellular metabolism of ROS/RNS through the ROS-producing multienzymes including acyl-CoA that are present in all peroxisomes irrespective of tissue and cell[601]. Other oxidases have been identified to produce H_2O_2 in various peroxisomal metabolic functions. These include the D-amino-acid oxidase and D-aspartate oxidase enzymes in amino acid metabolism, the enzyme 2-hydroxy acid oxidase (HAO1) also known as glycolate oxidase, which is vital in glyoxylate detoxification, L-pipecolate oxidase (PIPOX), a unique oxidase to peroxisomes for lysine metabolism[598]. Furthermore, there is a large network of peroxisomal enzymatic and non-enzymatic antioxidant activities that are vital in the protection of the organelle from oxidative stress and cell damage[602]. In kidney, peroxisomal metabolism of ROS/RNS alone or through its cooperation with mitochondria[594, 601, 602] may explain the pathogenesis of lipid bilayer damage and cell apoptosis induced in PTEC leading to severe impairment of metabolic dysfunction such as those observed TDF-FS. Functional

peroxisomes also contain the catalase enzyme which neutralises the harmful effects of H_2O_2 either by hydrolysis (converting it to water) or by using it as a co-factor in oxidation reactions[603]. However, genetic variants in genes like *PEX14* may to lead to dysfunctional peroxisomes and therefore impaired FAO and unregulated oxidative stress resulting from an imbalance between pro-oxidants and antioxidants[602, 603].

In addition, H_2O_2 decomposing enzymes (catalase) are dependent on the matrix protein *PEX5*-PTS1 receptor recognition for their membrane-bound translocation. A recent study has demonstrated that in response to oxidative stress, PEX14 which is vital in the matrix protein import machinery that transports catalase is phosphorylated at multiple sites by H_2O_2 leading to different physiological outcomes[603]. A *PEX14* ser232 phosphorylation suppressed peroxisomal mediated catalase movement in mammals, while in vitro, this impaired the interaction of the PEX5-PEX14 complex thus, function as an anti-oxidative stress response[603]. Further, the same study established that elevated levels of cytosolic catalase and conferred cell resistance to H_20_2 in the presence of a PEX14-S232D variant[603]. Similar studies have also shown that genetically overexpression or knockdown of catalase may be involved in various physiological and pathological processes of kidney injury[604]. Thus, depending on the sensitivity of individual cells, peroxisomal impairment may promote an initial cell survival, but persistent redox imbalance and catalase disfunction may lead to chronic oxidative stress and damage of tissue, cell and organs[602]. The PTEC are extremely sensitive to alterations in the integrity of tubular morphology and the energy generating capacity of the tissue which may lead to impairment of the highly energy dependent metabolic functions[594, 605, 606].

Based on a detailed link and consequences of the peroxisomal metabolic pathway as well as its link with well-known mitochondria metabolic processes, we postulate that TDF-FS in patients

exhibiting SNPs in rs284267, rs284265 and rs284301 in the *PEX1*4 gene is a result of additive effects including metabolic and drug toxicity. Thus, (i) TDF toxicity through mitochondria DNA inhibition and drug accumulation, (ii) failed peroxisome β-oxidation of fatty acids a primary precursor of mitochondria ATP biogenesis, (ii) generation of excessive ROS/NOS through the peroxisomal-mitochondria interplay and functions and (iv) depleted mitochondria due to unopposed ROS may be the main drivers of PTEC damage resulting in generalised tubular impairment observed in FS. As a result of impaired peroxisomal catalase causing alterations of mitochondrial membrane proteins and stimulation of generation of mitochondrial ROS, mitochondria damage has been described as a hallmark for the progression of tubular damage in acute kidney injury (AKI)[597]. This may be observed throughout the mitochondrial abundant proximal tubular cells as well as the glomerulus and endothelial cells which present as ischemic AKI, progressive CKD with eventual apoptotic cell death. Equally, peroxisomes are actively involved in apoptosis and inflammation, innate immunity, ageing and in the pathogenesis of age-related diseases like diabetes, cancer and kidney disease[594].

ITSN1 variant, rs2834254 C>T is another SNP with confirmed TDF-FS association. The *ITSN* (*ITSNs*) represent a family of multi-domain adaptor proteins that regulate endocytosis and cell signalling[607]. Several studies have recognised that *ITSN1* plays an important role in endocytosis and vesicle trafficking in mammals particularly through indirect regulation of endocytic membrane traffic with the actin assembly machinery and regulating the formation of clathrin-coated vesicles. The possible involvement of *ITSN1* with TDF-FS is strongly linked to clathrin-dependent proximal tubular endocytosis[607, 608]. This is because the functional integrity of *ITSN1* is highly associated with its modular protein structure that contains three of these EH domains (9Eps15 Homology), a CC domain (Coiled-coil) and five SH3 domains (Src Homology 3) that are specifically responsible for mediating its scaffolding function of interacting with the

endocytic machinery to regulate actin-dependent endosomes movements and morphology[608-611]. Thus, the association of *ITSN1*variants with TDF-FS may be explained by the Clathrin dependent endocytosis pathway which is exclusively responsible for the apical endocytosis in the proximal tubules and is initiated by ITSN1 interaction with actin receptor CDC42 which facilitates budding and fission[612-614]. This leads to uncoating of Clathrin-coated vesicles and subsequent pH-dependent dissociation resulting in a highly regulated actin-assembly machinery trafficking[608, 615]. This may explain why variants in *ITSN1* may affect proximal tubule endocytosis through failed regulation of the actin assembly machinery and the formation of clathrin-coated vesicles. It is understood that this cascade process is also likely to govern the progression of the proximal tubular apical endocytic process[616]. Many splicing events in genetic variants of *ITSN1* cause frameshifts in *ITSN*s mRNA and introduce premature termination codons that lie more than 50 nucleotides upstream of an exon-exon junction[617, 618]. This results in truncated proteins with conformational changes in the functional domains and affects interaction with the actin receptors[616]. This has been demonstrated in a study where both silencing and overexpression of *ITSN1* decreased uptake of biomolecules and inhibits endocytosis, phenomena that suggest a concentration-dependent effect of the protein[609]. Moreover, endocytosis is a tightly controlled process that allows the delivery of proteins from the plasma membrane into the cells to maintain cellular homoeostasis[619]. Shen and colleagues[620] have demonstrated that null mutants have morphological defects due to failed differentiation resulting in abnormal actin distribution and defective endocytosis[620]. Moreover, studies in models of proximal tubule epithelial cells treated with inhibitors of Clathrin-dependent endocytosis or with inhibitors signalling of GTPase regulation of vesical fission from the membrane showed inhibition of LMWP uptake in proximal tubules[619].

There is also an emerging role of *ITSN1* in proximal tubular endocytosis through the regulation

and expression of protein endocytic receptors (megalin and cubilin) in proximal tubular epithelial cells (PTEC) that is associated with proteinuria[612, 614]. PTEC are functionally specialised for apical endocytosis of filtered proteins and small bioactive molecules from the glomerular ultrafiltrate to prevent loss of LMWP through urine[612]. The convoluted segment of the proximal tubule cells expresses a highly specialised large transmembrane multiligand receptors, megalin and cubilin that mediate the efficient uptake of LMWP and other molecules from the glomerular filtrate[614, 621, 622]. Because the interaction of cubilin-megalin is responsible for ligand binding and internalisation, experimental studies in rat models have demonstrated that the absence of megalin is associated with LMW proteinuria[610, 612, 614, 621, 623]. Cubilin and megalin are co-dependent receptors whose interaction has also been demonstrated to facilitate transportation of some drug toxins such as aminoglycosides, gentamicin and polymyxin B, all of which are nephrotoxic. Studies have shown that PTEC mediated endocytosis is a dynamic highly regulated process that is adversely affected by different conditions that can affect reabsorption efficiency[622, 624]and it is also highly dependent on the integrity of the actin cytoskeleton which relies on *ITSN* regulation[608, 609, 621, 625]. Thus, impaired tubular endocytosis resulting from genetic mutations manifests as urine loss LMWP or tubular proteinuria a prominent characteristic of TDF-FS.

This effect of *ITSN1* has been well characterised in brain disorders where *ITSN1* is almost exclusively expressed and plays a role in synaptic vesicle endocytosis in brain neurons responsible for down syndrome, schizophrenia and Alzheimer's disease[616, 626]. We have a plausible cause to suggest that variants in *ITSN1* by either up or downregulating its expression may significantly affect endocytosis although there has been no direct link of *ITSN1* to specific renal abnormalities. A better understanding of the proximal tubule endocytic pathway and the consequences of its dysfunction may provide insights on whether it can be an alternative route

for tenofovir renal transport and, therefore identify new interventional targets to prevent or limit kidney disease.

4.4.5 Limitations

While we have demonstrated very important findings of novel SNPs involved in a rare but serious phenotype of TDF-RT, our findings need to be interpreted with caution due to some limitations. We recognise that GWAS identified SNPs may not be the primary SNPs explaining the causal effect of TDF-FS. In our study, the case-control analysis used a non-conventional control group which was neither exposed to HIV nor the drug and had no clinical or demographical data to enable adjusting for covariates. Therefore, these may not be the most ideal cohort for controls. However, it should be noted that TDF-FS is an extremely rare phenomenon that occurs in less than 1% of TDF exposed patients and we presume that there would be very minimal differences in the results even with an ideal control group. However, we acknowledge that this is a major limitation of our validation. We also used imputed SNP data for some of the SNPs in the control groups, particularly two of the SNPs that were significantly associated were imputed. While genotype imputation has been demonstrated to be an important tool in analysing genetic data, imputed SNPs, if not accurately imputed, may impact and limit the interpretation of the results .

4.4.6 Conclusion

We have validated GWAS results in a UK TDF-FS cohort using a case-control candidate gene design and alternate genotyping technologies. We have confirmed that SNPs in *PEX14, TMEM and ITSN1* genes may be associated with TDF-FS in HIV positive patients, and this may be potentially through different mechanisms. We have also demonstrated that SNPs in tenofovir transporter genes that have been previously associated with TDF-RT in various populations

may not play a significant role in TDF-FS. Furthermore, we have reported a set of haplotypes that may be significantly associated with TDF-FS in HIV patients receiving TDF. We conclude that *PEX14* role in proximal tubular β-oxidation of fatty acids (FAO) and detoxification of the resultant reactive oxygen species may explain alternative pathways responsible for a rare but serious phenotype of TDF-RT. However, we recommend further replication of these findings in a separate cohort of TDF-FS and compared against appropriate controls that are HIV-positive, on TDF but without FS. Furthermore, biological functional studies are required to validate the proposed mechanisms of the identified SNPs.

CHAPTER 5

THE CORRELATION OF URINE PROTEIN BIOMARKERS WITH EXPOSURE TO TENOFOVIR IN TREATMENT NAÏVE HIV PATIENTS: AN EXPLORATORY STUDY

5.1 BACKGROUND

Early detection of kidney injury regardless of aetiology is key in the provision of timely medical intervention and therefore, prevent any associated complications[627]. Despite dominating the diagnosis of kidney injury, use of traditional markers (serum creatinine, urea nitrogen and proteinuria) is influenced by nonrenal factors that limit their effectiveness in detecting small but significant declines in GFR often observed with TDF exposure[295, 628, 629]. Moreover, the use of SCr, currently considered the "gold standard" of nephrotoxicity, as a marker for routine monitoring and detection of kidney impairment in patients receiving TDF is limited by the lack of sensitivity and specificity[439, 627]. As a result, early detection of TDF-RT in HIV patients has remained a challenge. Kidney injury markers that are highly sensitive and specific may provide an important milestone in early detection and timely management of TDF-RT, which could prevent morbidity and complications that may adversely affect life-long ART.

Results of experimental studies comparing the diagnostic performance of the low molecular weight protein, urinary kidney injury molecule-1 (KIM-1) with traditional biomarkers in predicting tubular abnormalities has demonstrated better performance of KIM-1 against SCr[439]. A correlation of urine biomarkers including KIM-1 with the subsequent decline in kidney function has also been reported in HIV patients[630]. A study in treatment-experienced HIV patients showed that each year of TDF exposure is significantly associated with an increase in KIM-1 levels by 3.4% (1.1–5.77%)[631]. Similar studies have reported that the marker is predictive of both tubular and glomerular dysfunction[446] and mortality[447]. Recently, Danjuma et al observed that in treatment-naïve and experienced patients (> 7 years TDF exposure), there was a significant increase in KIM-1/Cr and a high baseline KIM-1/Cr ng/mg compared to

values reported in normal subjects[632]. In another study, HIV patients treated with TDF, without evidence of glomerular dysfunction, had a higher median RBP/Creatinine ratio (214µg/g; Normal range < 159 µg/g) than those on TDF-free regimens (111.6 µg/g) or naive patients (92.5 µg/g)[295].

While these studies provide important insights on the utility of these biomarkers in HIV, and in particular in TDF based cART, the previously studied cohorts were predominantly treatment-experienced and these outcomes could have been influenced by several factors including HIV infection, cumulative TDF exposure (median 7 (4-11) years), comorbidities and concomitant use of unreported potentially nephrotoxic drugs. We, therefore, set out to conduct an exploratory study to prospectively determine the association of urine biomarkers with TDF-RT in treatment naïve patients.

5.1.1 STUDY OBJECTIVE

1. To prospectively profile the correlation of low molecular weight protein (KIM-1 and RBP4) biomarkers with TDF treatment in the treatment naïve HIV positive patients initiated on TDF at the University Teaching Hospital in Zambia.
2. To determine the correlation between longitudinal measurements of KIM-1 and RBP4 following 6 months of TDF and renal disease outcomes.

5.2 METHODS

5.2.1 Study Design

This was an exploratory prospective study that aimed to recruit and follow-up treatment naïve HIV-positive patients who were to be initiated on TDF based regimens at the University

Teaching Hospital in Lusaka, Zambia. We designed a longitudinal collection of urine samples for the measurement of KIM-1, RBP4 and creatinine at predetermined time points. The measured protein biomarkers were expressed as a ratio of urine creatinine and the participants were subsequently followed up for a maximum period of 6 months for any evidence of renal toxicity attributed to TDF. The study design is summarised in Figure 5.1.

5.2.2 Ethical Approval

The research protocol was approved by the University of Zambia Biomedical Ethics Research Committee under Ref. No 013-05-10 (Appendix A). The protocol included a detailed patient information leaflet and consent form before any participant was recruited in the study. Research participants were anonymised with a study ID once the prospective follow-up had concluded. The collection of research data and samples from the University Teaching Hospital and transfer of research materials to the University of Liverpool; UK was authorised and approved by the National Health Research Authority of the Ministry of Health Research Directorate (Appendix B).

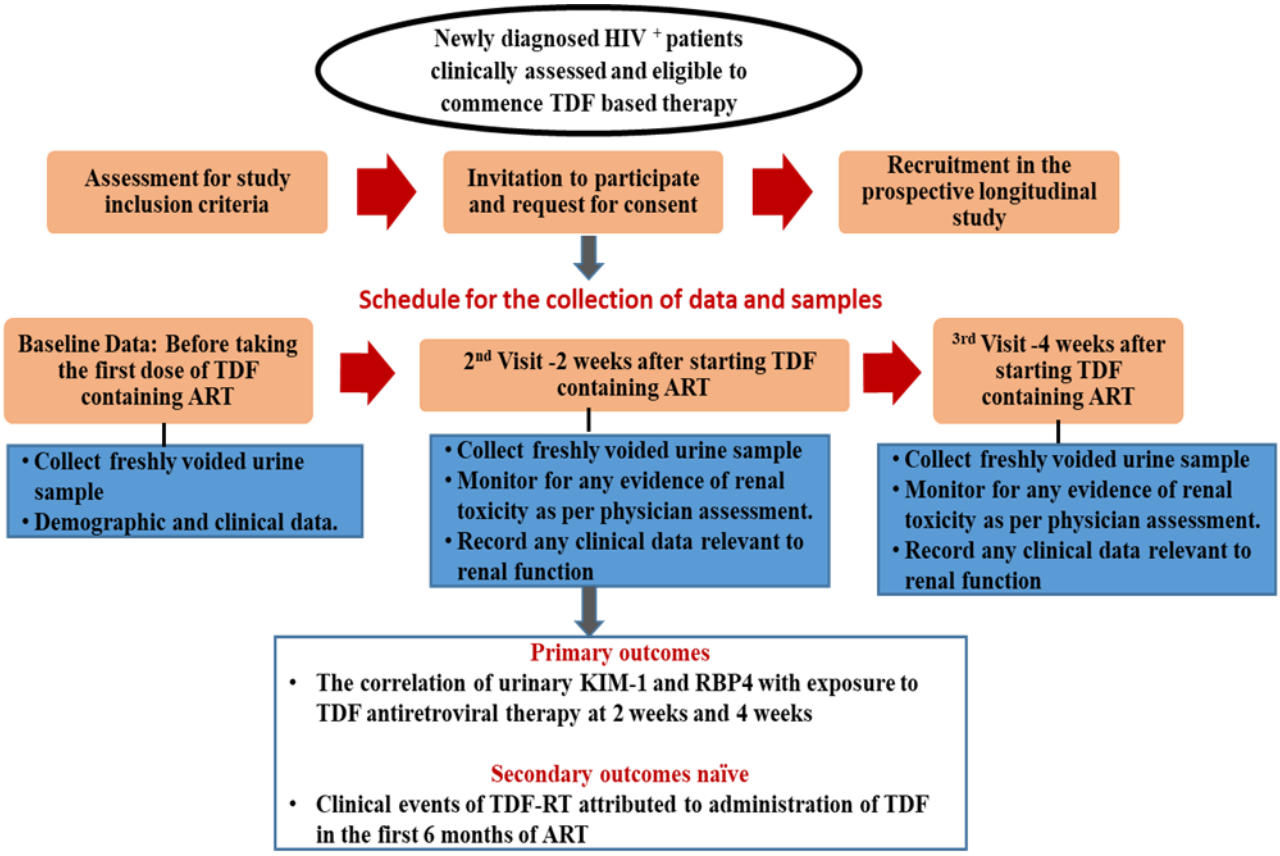


Figure 5.1. Study design: flow chart for recruitment and data (clinical and samples) collection.

5.2.3 Inclusion and exclusion criteria

- The study recruited newly diagnosed HIV-infected patients aged ≥18 years who had never been on ART treatment but were clinically assessed and qualified to commence an ART regimen containing TDF 300mg once daily. In addition, the recruited patients needed to have a prior assessment and documented baseline renal function before commencing treatment. Participants also needed to be able to provide a signed informed consent certificate.
- Any participants with a history of renal disease of any cause in the last four weeks prior to TDF initiation were excluded. We also excluded participants that were hypertensive, diabetic or presenting with AIDS-defining illnesses. Inability to provide informed

consent or unwillingness to turn up for subsequent follow-up visits was also an exclusion criterion.

5.2.4 Recruitment Procedure

5.2.4.1 Identification and obtaining Consent

Newly diagnosed patients were identified through the linkage unit and were approached once they were assessed for eligibility to commence antiretroviral therapy. Potential participants were assessed for eligibility to take part in the study based on the inclusion criteria. If the inclusion criteria were met, patients received the patient information leaflet specific for the biomarker study and were invited to participate in the study (Appendix H). Further counselling was given by explaining and clarifying the purpose of the study and the need for subsequent visits for the prospective collection of samples and follow-up. Upon agreeing to take part in the study, participants signed the consent certificate and were then recruited into the study.

5.2.4.2 Samples collection and storage

On the day of recruitment, participants were requested to submit a freshly voided urine sample which was transferred into pre-labelled 2.8 ml cryovials and stored at 80^0 Baseline medical information was recorded from each recruited participant. All participants were then given an appointment date for the subsequent collection of prospective urine samples for biomarker determination. Subsequent appointment visits were given for the collection of the second and third samples at 2 and 4 weeks after commencing TDF containing antiretroviral therapy respectively. Reminders through phone calls were made a few days before the appointment to ensure participants did not miss their appointment. A return transport fee refund was given for every visit made. All the collected samples were stored in pre-labelled 2.8 ml cryovials

indicating the sample ID, time, date and corresponding visit when the sample was collected. All samples were centrifuged and stored at -80^{0}C in the Adult Infectious diseases Centre's laboratory. Figure 5.1 illustrates the study design and procedure for recruitment. Once all participants were recruited, samples were shipped to the University of Liverpool according to the International Air Transport Association (IATA) and WHO guidelines for the transport of infectious substances. Since the samples contained active HIV, the samples were stored at the Bioanalytical Facility (BAF), a containment level 3 laboratory in the Royal Liverpool Hospital until we carried out the HIV inactivation.

5.2.4.3 **Patient data collection**

Data collected included demographics such as age and gender. At baseline, body weight and height were collected, together with available clinical data such as CD4 cell count, Viral Load, urinalysis results, serum creatinine, blood urea nitrogen (BUN) and other biochemistry tests that included liver function tests (alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)), albumin, potassium and serum phosphate. Comorbidities (Hepatitis B and Hepatitis C co-infection, hypertension, Diabetes) and any concomitant use of potentially nephrotoxic drugs was also collected. After 3 months of recruitment, all the data that was collected at baseline was updated with particular attention on renal function for evidence of nephrotoxicity.

5.2.4.4 **Participant follow-up**

We planned to follow up with all participants for a maximum of 6 months to document renal toxicity attributed to TDF as assessed by physicians. Patients who presented with renal toxicity were followed up to the time when they presented with TDF-RT. The period of TDF exposure

up to the occurrence of TDF-RT was documented and SCr was collected at the end of the follow-up for both participants with and without TDF-RT. Any other relevant clinical data were also collected.

5.2.5 Description of clinical data

- TDF-RT was defined clinically and by a creatinine clearance of <60ml/min calculated by the Cockcroft Gault formula.
- CD4 cell count was determined by the UTH pathology laboratory by flow cytometry. For optimal accuracy, blood specimens were often processed within 18 hours of collection. Approximate corresponding values for absolute CD4 count and CD4% of the lymphocytes are: >500 (>29%), 200-500 (14-28%) and <200 (<14%). In our study, we recorded the absolute values as this was always recorded.
- Viral Load (VL) values were determined at the AIDC lab using the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Qualitative Test, version 2.0 (TaqMan 96) protocol. This instrument only determines HIV-1 RNA copies reported as copies/ml, and therefore, viral loads for patients with HIV-2 were not determined. Viral load tests were done at baseline and 6 months of treatment. Absolute determined values that were reported as 'not detected' (ND) indicated a viral load below the detection range. For the sake of this research, we categorised viral load as high (VL <100000 copies/ml), Low (<10 000 copies/ml) and Very Low (VL <50copies/ml). Note that at the time of data collection, VL results measured at 6 months of treatment were not determined for most patients because the machine had broken down, and thus VL at 6 months was excluded from data analysis.

5.2.6 Biomarker Analysis.

All the risks associated with the manipulation of HIV samples were assessed before carrying out any laboratory work. All necessary health and safety requirements for handling infectious samples and good laboratory practices were adhered to throughout the process. Prior to undertaking biomarker analysis, we carried out the inactivation of HIV infected samples. Additionally, standard operating procedures were followed to prevent contamination of surfaces and for appropriate disposal of contaminated materials. Two different methods for HIV inactivation were used depending on the method for biomarker analysis. For sample aliquots intended for KIM-1 and RBP4 analysis, we used the inactivation of HIV positive samples using Polysorbate 20 (Tween 20) protocol (BAF protocol version 1.0) while for samples intended for urine creatinine analysis, we used the Inactivation of HIV-infected Samples using the Triton X-100 protocol (BAF PRO 026 version 1.0). The inactivation procedure was performed in the cat 3 labs of the Bioanalytical Facility. The procedure for each method is detailed under the respective subtitle below. All inactivated samples were recorded in the Sample inactivation log **(LOG 007).**

5.2.6.1 **Inactivation of HIV positive samples using Polysorbate 20 (Tween 20).**

Tween 20 is a non-ionic detergent that is effective in the inactivation of enveloped viruses like HIV Vaccinia, Hepatitis C and B viruses and several other viruses. It effectively inactivates viruses at a concentration of 0.005% to 1% following incubation from 15 seconds to 1 hour[633]. In this study, we took advantage of the 0.5% Tween 20 detergent which was supplied within the protein analysis kit for the MSD platform as a blocking agent and wash buffer (a mixture of 20x Phosphate-buffered saline and 10X Tween 20). The HIV inactivation was integrated with the MSD protein analysis protocol detailed under KIM1 and RBP4 analysis. The samples

were incubated in the Tween-20 containing wash buffer at room temperature (25°C) for 3 minutes after which it was washed once. This process was then repeated a further two times at which point HIV was considered inactive and samples were safe to be manipulated and measured on any plate reading machine.

5.2.6.2 **Inactivation of HIV-infected Samples using Triton X-100**

HIV was inactivated using 1% Triton X-100 solution for 60 minutes (1 hour). This treatment is effective in the inactivation of HIV. The procedure was performed in the Category 3 lab of the Bioanalytical Facility (Willian Henry Duncan building room 133). The inactivation procedure involved thawing the frozen urine samples on ice in the biosafety cabinet followed by pipetting 90ul of each urine sample into each well of a 96 well plate. To achieve 1% Triton solution, 10 µl of 10% Triton was added to the wells containing 90 µl of urine making a total volume of 100ul with 1% Triton-100 effective for HIV inactivation. The plate was sealed with a self-adhesive plate seal and incubated using the plate shaker for 1 hour at 700rpm after which the samples were deemed inactivated and were transferred to the Molecular lab in the Wolfson Centre for Personalised Medicine where they were stored at -80℃ until they were analysed. All procedures were carried out with adherence to the Health and Safety requirements for handling and disposal of infectious samples.

5.2.7 Determination of urine KIM-1 and RBP4

KIM-1 and RBP4 analysis was conducted using the Meso-Scale-Discovery platform which consists of rapid and convenient detection of either single target protein (single-plex) or multiple targets (multiplex) in one single sample using the principle of sandwich immunoassay reactions utilizing electrochemiluminescence detection technology. In our protocol, we used the standard single-plex reaction MSD platforms for KIM-1 and RBP4 assays. To run the assays, the protocol

and MSD kits for both KIM-1 and RBP4 assays were supplied by Meso Scale Discovery® (MSD), (Rockville, Maryland, USA). Each assay kit was supplied with an MSD plate precoated with a specific capture antibody adhered to the base of the plate for KIM-1 and RBP4, respectively. The sandwich reaction was formed when a sample volume was added to the MSD plate followed by an incubation period to promote the capturing and binding of the analyte to the capture antibody immobilised on the working electrode. The sandwich was complete after the addition of the biomarker detection antibody which is conjugated with the electro-chemiluminescent labels MSD SULFO TAG™. A 1-hour incubation allowed the detection antibody to be recruited to the initially formed analyte-capture antibody sandwich. In the presence of a reading buffer, the plate was loaded into the SECTOR® imager (plate reader) where a voltage applied causes the captured labels to emit light whose intensity was measured by the instrument to provide the equivalent quantity of the biomarker. A stepwise procedure is detailed below. Figure 5 illustrates the principle of the MSD biomarker assay.

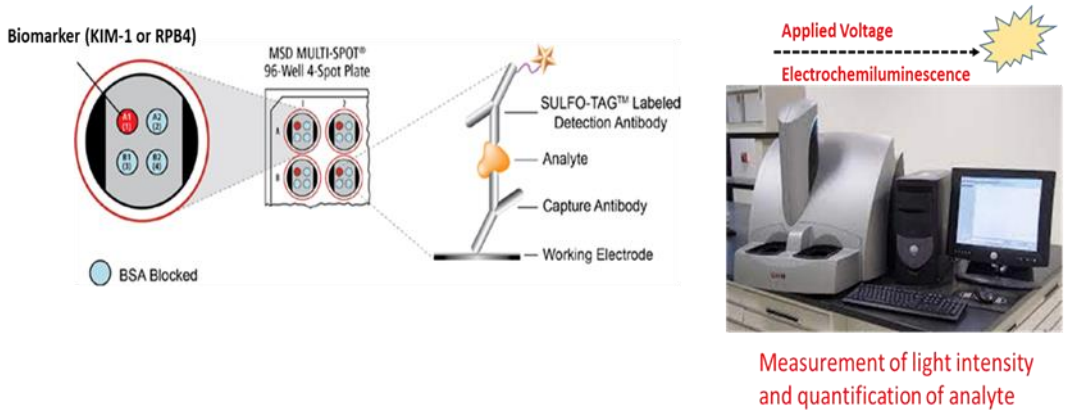


Figure 5.2. Generic illustration of the MSD platform for the measurement of KIM-1 and RBP4 biomarkers. (Figure adapted from MSD online brochure)

5.2.7.1 **Procedure for the determination of KIM-1 and RBP4**

Determination of KIM-1 and RBP4 from urine samples was based on the MSD manufacturer's protocol provided in the standard assays for human KIM-1 and RBP4 kits. The protocols for determining the two biomarkers were similar apart for the difference in sample dilution and preparation of the standard curves. Overall, both MSD protocols consisted of a three-step procedure detailed below. Any significant differences between the two protocols will be highlighted.

- **STEP 1: Preparation of Standard Curve**

We prepared an 8-point standard curve with 4-fold serial dilution steps which included a zero calibrator (blank). Previously frozen calibrators were thawed on ice and a 4-fold serial dilution undertaken to make solutions of concentrations ranging from 20 000 pg/ml to 4.9 pg/ml for KIM-1 and 50 000 mg/ml to 12pg/ml for RBP4, respectively. The first standard solution for KIM-1 was prepared by adding 15ul of the stock calibrator provided at 400 000pg/ml to 285ul of diluent 37 and the same preparation was done for the first standard of RBP4 stock calibrator provide at 1 000 000pg/ml. The rest of the standards were prepared by following a 4-fold serial dilution using diluent 37 until the seventh standard solution was prepared. The eighth standard solution was a blank prepared from diluent 37.

- **STEP 2:Preparation of Samples and MSD experimental plate**

We performed a 10-fold and 50-fold dilution of KIM-1 and RBP samples using diluent 37 respectively. To prepare the plates, we initially prepared and added 150µL of Blocker A solution to each well of the KIM-1 and RBP4 plates. The plates were then sealed and incubated with vigorous shaking at 700rpm for 30min. The plates were then washed three times with

300µl/well of Polysorbate saline-Tween20 (PBS-T) buffer solution and were dried ensuring that no residual buffer solutions were adherent to the bottom of the wells. To create the first sandwich analyte-capture antibody, 50ul of standard solution and urine sample was then added to the plate in duplicate; the plate was sealed and incubated with vigorous shaking at 700rpm for another 30min. Afterwards, the plates were again washed three times with 300µl/well of PBS-T buffer solution drying out all residue buffer followed. Then, 25µL of 1X detection antibody solution was pipetted into each well of the plate, properly sealed and incubated for 2 hours with shaking at 700rpm at room temperature for 2 hours. KIM and RBP4 plates were barcode labelled and only KIM-1 and RBP4 detection antibodies would therefore only work for the respective plates. At the end of the incubation, the plates were again washed with 300µl/well of PBS-T buffer three times and dried for the residue buffer accordingly. Finally, 150ul of 2X Read Buffer was added to each well of the plate and without incubation, the plates were immediately analysed on the MSD Sector Imager (Rockville, MD, USA).

- **STEP 3: Analysis of MSD results:**

The signals of electrochemiluminescence were read using the MSD DISCOVERY WORKBENCH® version 4.4 software. The software also fitted the algorithm to generate a standard curve that was used to extrapolate and calculate the concentration of analyte in the samples. For both KIM-1 and RBP4 assays, we used the software 4-parameter logistic model (or sigmoidal dose-response) with a $1/Y^2$ weighting function to provide a better fit of data over the upper and lower limits of quantification (ULLQ and LLOQ) on the standard curve. For KIM-1, the mean LLOQ for the assay was 0.98 (0.39-3.1) pg/ml and 1.8pg/ml for RBP4. Sample plots for the standard curves for KIM-1 and RBP4 and the plots for the unknown samples are shown in figures 5.3 A and B, respectively. The values derived for KIM-1 and RBP4

were normalized to urinary creatinine **(**determined spectrophotometrically) and expressed as KIM-1/Cr (ng/mg) for the final analysis.

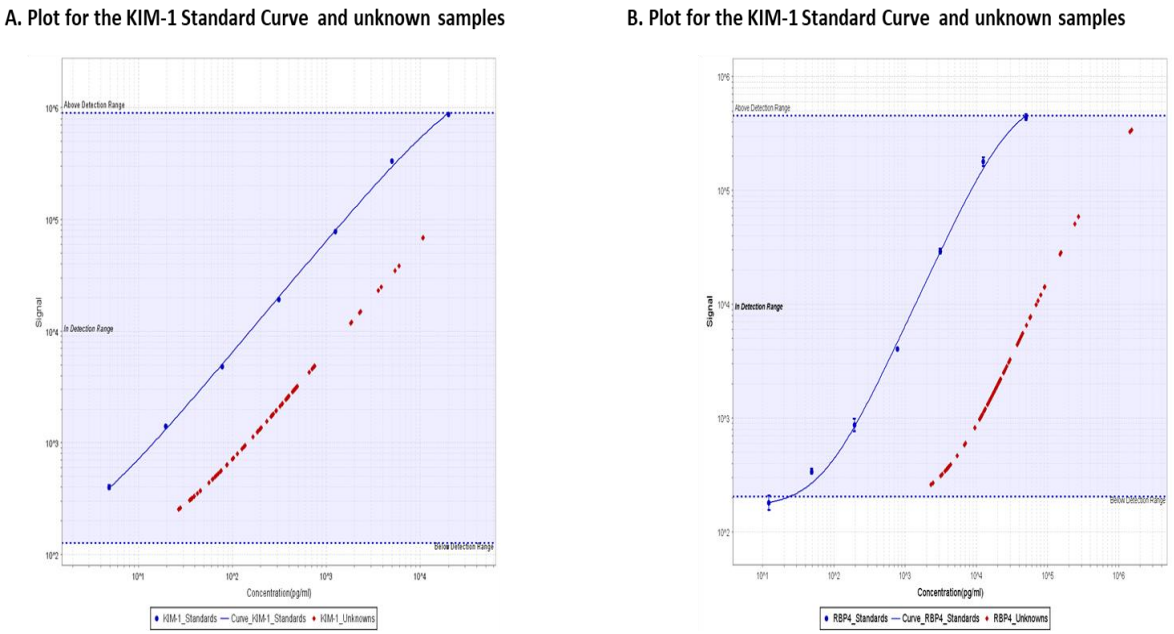


Figure 5.3. Standard curves plots for KIM-1 (A) and RBP4 (B) with unknown samples

5.2.8 Determination of urinary creatinine concentration

The Invitrogen™ Creatinine urinary detection Kit consisting of creatinine standard (100 mg/dL creatinine 96 well plates, and Creatinine reagent (Picric acid) was purchased from Thermo Fischer Scientific (Loughborough, UK). The detection kit is designed to quantitatively measure creatinine present in urine samples based on the Jaffe calorimetric reaction. In this reaction, at alkaline pH, creatinine in the urine sample reacts with picric acid to form a creatinine-picrate complex whose absorbance at 490nm is directly proportional to the concentration of creatinine in the sample. The assay procedure consisted of the preparation of creatinine standard solutions for the standard curve according to the manufacturer's protocol (Thermo Fischer Scientific, Loughborough, UK). 50ul of the standard solution and urine samples diluted 20-fold were pipetted into a clear microtiter plate. Thereafter, 100ul of the colour generating creatinine

detection reagent was added to each well and incubated for 30 min at room temperature. The absorbance was then read at 490nm in the Beckman Coulter DTX 880 Multimode Detector microplate reader. The background absorbance was subtracted from each sample absorbance read before plotting. A standard curve was generated using a GraphPad Prism software version 7. A four-parameter algorithm was used to extrapolate the creatinine concentrations for the corresponding absorbance and the final concentration was corrected for the dilution factor (multiplying by 20). The analytical assay sensitivity was reported as 0.019 mg/dL creatinine and has a performance of detecting creatinine from human random urine samples with a mean of 90.7mg/dL ranging from 17.2 to 168.9mg/dL. Figure 5.4 shows a 96 well plate after measurement of absorbance in the micro-plate reader and generated creatinine standard curve.

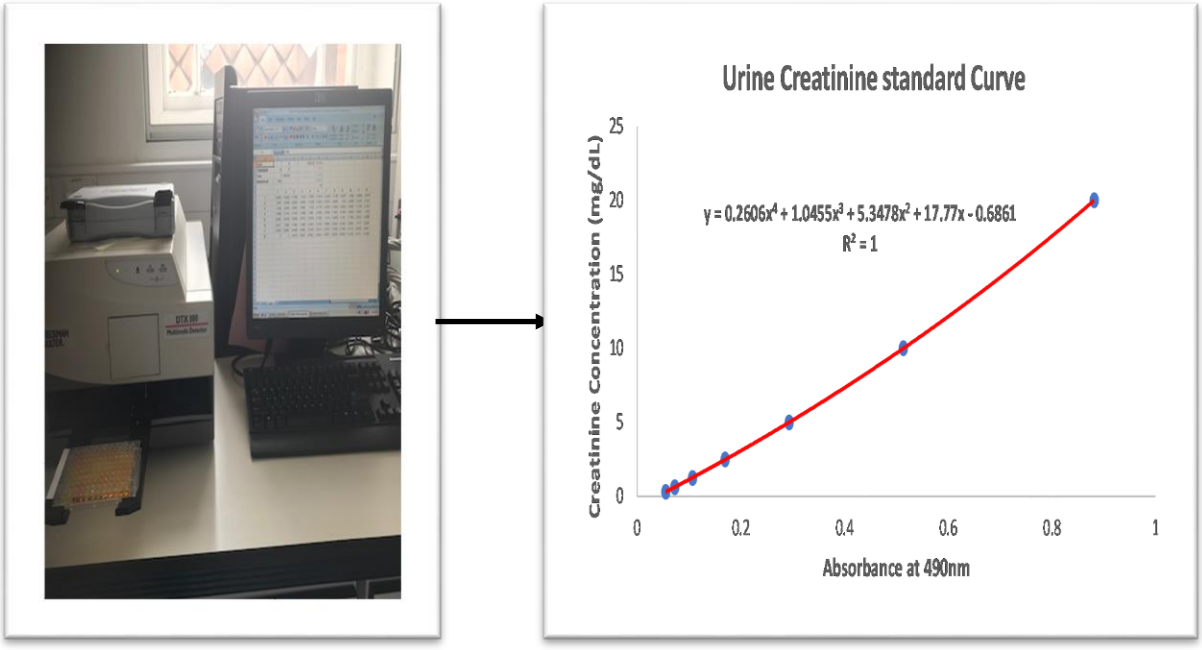


Figure 5.4. Measurement of Creatinine in Beckman Coulter DTX 880 Multimode Detector microplate reader and generated standard curve

5.2.9 Statistical Analysis

We explored the continuous data variables to determine distribution using the Shapiro Wilk

test for normality. Descriptive analyses of participants' demographics were reported as frequencies (counts) and percentages (%) for categorical data. Continuous measurements were described as means (± standard deviation) and median (interquartile range) depending on the distribution of the data. Data transformation was performed using the inverse transformation to create normally distributed data for variable deviation of normality (Shapiro Wilk $p<0.05$). Note, the logarithmic transformation could not generate a normal distribution on some variables. For the longitudinal data of KIM-1 and RBP4, we generated spaghetti plots to profile the trends of the biomarkers measured over the three-time points for the TDF-RT outcome groups (TDF-RT and NO TDF-RT). Univariate analysis was also conducted to determine the difference between the repeated measures of biomarkers in the TDF-RT outcome groups. Statistical significance (significance $p<0.05$) and 95% confidence intervals were reported accordingly. We performed an analysis of variance (one way-ANOVA) between the measurements (within-subjects) to determine the differences in the means and a pairwise comparison to determine the specific time points where differences in biomarkers were observed. The assumption of homogeneity-of-variance was tested by Mauchly's test of sphericity, and where the assumption was violated, the correction with the Greenhouse-Geisser test was considered. We further carried out a spearman's correlation test between matrices urine kidney biomarkers and with CrCl and the difference between baseline and serum creatinine at 6 months of RT (Delta CrCl (ΔCrCl). The receiver operator characteristic (ROC) curves were generated and the area under the curve (AUC) was determined to assess the performance, sensitivity and specificity, based on the positive predictive value (PPV) and negative predictive value (NPV) for each biomarker for the prediction of TDF-RT. Excel for windows 10 was used to sort and organise the data and IBM SPSS Statistics 25 was used for the analysis.

5.3 RESULTS

5.3.1 Cohort Description

We prospectively recruited 68 newly diagnosed HIV positive treatment naïve patients who met the inclusion criteria for the study. By the end of the study, we had an attrition rate of 16.2% representing 11 participants. After excluding participants who were lost to follow up and those with insufficient data, 52 were included in the analysis. At the end of the follow up period, 12 (23.1%) presented with RT (CrCl< 60ml/min). The majority of these were female (Demographic and clinical characteristics of the 52 participants are summarised in Table 5.1 and Table 5.2 for the description of markers at 6 months of therapy. The mean age was 35 (± 10 (SD)), years, 67% were female and nearly all patients had HIV 1 infection (92.3%). The prescribed regimens were TDF+3TC+DTG (52%) and TDF+3TC+EFV (48%). At baseline, the median CrCl was 92.91 ml/min (IQR 70.71-124.63) and dipstick urinalysis was generally normal with either a trace or negative for proteins, ketones and glucose. The majority of the participants (67.3%) had a very high viral load (VL) (>10 000) and the median CD4 was 189 cell/cm^3 (IQR, 85.5-388).

Table 5.1. Demographics and Clinical Characteristics of recruited participants

Variable Description			Number Analysed
Baseline Characteristic			
Sex (Female)	N (%)/	35 (67.3)	52
Age (Years)*	Mean (±SD),	40 (10)	
Type 1 HIV	N (%)/	48 (92.3)	52
ART regimen	N (%)/		

TDF+3TC+EFV		24 (48)	52
TDF+3TC+DTG		26 (52)	52
Weight (Kg)	Median (IQR)),	65.3 (56.15-73.2)	
BMI (Kg/m^2)	Median (IQR)	24.59 (21.58-26.87)	52
Urinalysis:			
Ketones (Negative)	N (%)	44(95.7)	46
Glucose (Negative)	N (%)/	46(100)	46
Baseline SCr (umol/L)	Mean (±SD),	76.35 (60.2-92.93)	52
Baseline CrCl (ml/min)	Mean (±SD),	92.91 (70.71-124.63)	52
Baseline CD4 ($cells/mm^3$)	Mean (±SD),	189 (85.5-388)	49
Viral Load (Copies/ml)	Mean (±SD),	40515(2619-165432)	43
<50 Copies/ml	N (%)	13(25)	
≥50 <10000 Copies/ml		4(7.7)	
≥ 10000		35(67.3)	

Table 5.2. Description of urine markers after 6 months follow up

Variable Description			**Number Analysed**
CrCl (ml/min) at 6 months	Mean (±SD)	81.92(66.27-104.35)	52
ΔCrCl min/ml	Mean (±SD)	12.87 (-7.47 – 39.91)	52
KIM1/Cr (ng/mg) *	Mean (±SD)		
Baseline		0.52 (0.68)	52
2 Weeks of TDF treatment		1.05 (1.39)	52

4 Weeks of TDF treatment		2.55 (5.02)	49
RBP4/Cr(ng/mg)*	Mean (±SD)		
Baseline		30.86 (29.42)	52
2 Weeks of TDF treatment		33.58 (29.84)	52
4 Weeks of TDF treatment		50.58 (39.83)	49

Categorical variables are reported as count (%); Continuous variables as mean* (standard deviation) and median**(interquartile range). Missing VL was for patients with HIV type 2. Δ CrCl (difference between Baseline and 6 months CrCl).

5.3.2 Profile and trends of KIM-1/Cr and RBP4/Cr according to RT outcome groups.

The profile and trend of KIM-1/Cr and RBP4/Cr repeated measurements for each patient following TDF exposure and stratified by RT outcome were plotted on spaghetti plots and are presented in Figure 5.6. Overall, the repeated measurements of KIM-1 and RBP4 did not show a uniform trend either in RT (Figure 5.5 A and C) or in the no RT group (Figure5.5 B and D) respectively, However, there was a notable trend towards a rise for both biomarkers after 4 weeks of TDF exposure in both groups. The plotted grand means of both KIM-1 and RBP4, Figure 5.6 showed a linear trend although these were generated by the effects of two measurements.

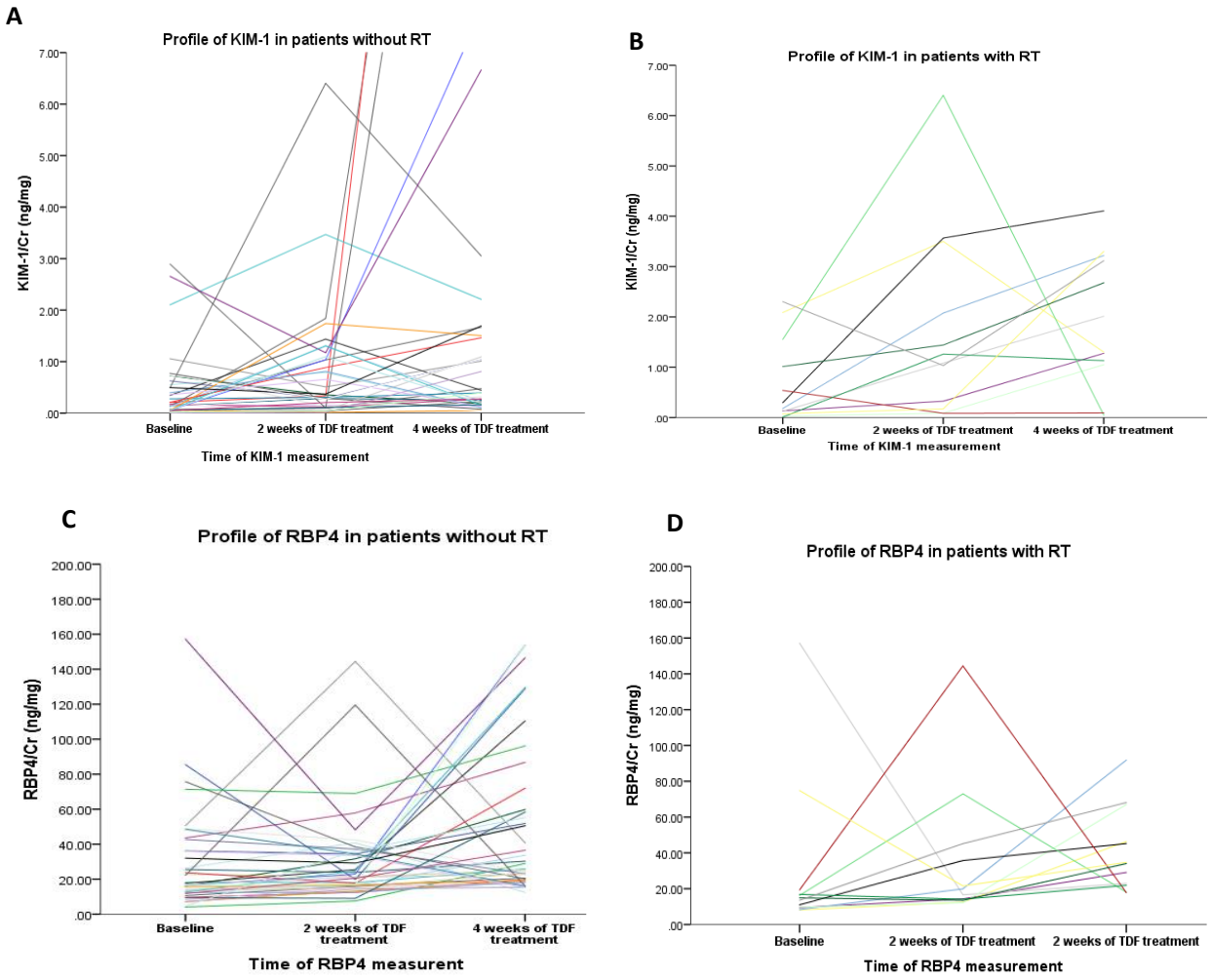


Figure 5.5. Profile and the general trend of KIM-1 (A, B, C) and RBP4 (D, E, F) in HIV positive patients following 4 weeks of TDF treatment

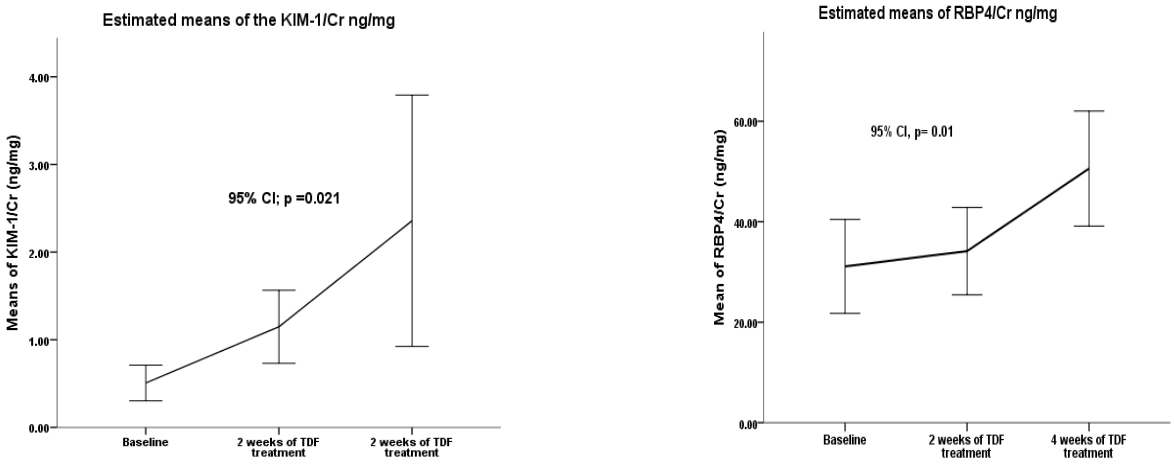


Figure 5.6. Linear tread of KIM-1/Cr and RBP4/Cr following TDF exposure.

5.3.3 Primary Endpoint: Effect of TDF treatment on KIM-1/Cr and RBP4/Cr

The result for the one-way repeated measures analysis of variance is summarised in table 5.3. For KIM-1/Cr measurements, the assumption of sphericity was violated, and we, therefore, used the alternative adjustment with the Greenhouse-Geisser (G-G) test. Following TDF treatment, there was a statistically significant difference between the baseline KIM-1/Cr measurements with KIM-1/Cr at 2 and 4 weeks after TDF treatment (F (1.133,50.69) 5.39, p=0.021). This difference was observed with a large effect size (partial eta-squared=0.105). Similarly, there were significant differences in RBP4/Cr measurements following TDF treatment (F (2, 96) 5.39, p=0.01), with an equally large effect size of 0.104. Although we had a few repeated measurements, significant linear trends were observed for both KIM-1/Cr (p=0.003) and RBP4/Cr (p=0.007) when plotted as means of measurements at different time points during the course of treatment (Table 5.3).

Table 5.3. Results of the effect of TDF exposure on repeated measures of KIM-1 and RBP4

Variables	MS	(df)	F	Effect Size ($\eta 2$)	p-value
KIM-1/Cr (ng/mg)	41.506	(1.13, 50.69)	5.39	0.105	0.021
RBP4/Cr (ng/mg)	6935.374	(2, 96)	5.590	0.104	0.01
Within-Subjects					
Contrasts-Trends					
KIM-1/Cr -Linear		(1, 47)	9.650	0.177	0.003
RBP4/Cr - Linear		(1, 47)	6.757	0.032	0.012

Note: —MS = Mean squares, effect size = Partial Eta Squared (partial $\eta 2$), statistical significance at $p < 0.05$.

In a pairwise comparison using the Bonferroni-adjusted paired t-test (Table 5.4), two significant pairwise differences in KIM-1/Cr (ng/mg) were observed. At baseline, KIM-1/Cr was lower by 0.641 ng/mg (p=0.003) compared to week 2 following TDF treatment, while week 4 KIM-1/Cr levels were higher by 1.851 ng/mg compared to baseline. No significant differences were observed between week 2 and week 4 after TDF treatment. For RBP4/Cr, the only significant difference observed was an increase by 19.48ng/mg from baseline to 4 weeks of TDF treatment by (p=0.022).

Table 5.4. Pair-wise comparison of biomarkers means within-subjects (paired t-tests with Bonferroni correction

Factor (Time of Biomarker measurement)	Mean Differences between biomarkers	95% CI of the difference	p-value
KIM-1/Cr (ng/mg)			
Baseline vs Week 2	-6.41	(-1.01 - 1.83)	0.003
Baseline vs Week 4	-1.85	(-3.62 - 0.85)	0.037
Week 2 vs Week 4	-1.21	(-0.284 - 0.446)	0.228
RBP4/Cr (ng/mg)			
Baseline vs Week 2	-3.05	(-13.63 - 7.53)	1.000

Baseline vs Week 4	-19.48	(-36.76 - -2.202)	0.022
Week 2 vs Week 4	-16.43	(-34.76 - 1.31)	0.078

Significant Statistics p <0.05.

5.3.4 Secondary Endpoint: Renal Toxicity

After 6 months follow up, 12 (23.1%) individuals developed RT; the median CrCl for the RT group was 53.82ml/min (IQR 42.67-57.15) compared to a median CrCl of 89.62 ml/min (IQR, 76.71-108.35) for the RT free group. The average time to RT after initiating TDF therapy was 3.5 months (14 weeks).

5.3.4.1 Distribution and mean differences of KIM-1/Cr and RBP4/Cr stratified by RT outcomes

Table 5.5 presents a stratified summary of the characteristics of the two groups (TDF-RT and no TDF-RT). Comparison of the means of the biomarkers at each time points showed that only KIM-1/Cr measured at the second visit, 2 weeks after TDF exposure was significantly different between the two groups with a difference in the means of 0.97ng/mg, (95%CI, 0.08-1.87, P=0.003) while at week 4, the difference was marginal (p=0.060). Figure 5.6 represents the box plot of the differences in the means of KIM-1/Cr in patients with and without TDF-RT. On the other hand, no significant differences were observed in RBP4/Cr and the variation in CrCl from baseline (Δ CrCl). When baseline patient characteristics were compared between the two groups, no significant differences were observed with gender, age, BMI, CD4 and viral load but there was a statistically significant difference in baseline CrCl in the TDF-RT group (median CrCl, 81.19ml/min (IQR, 67.87-92.21)) compared to no TDF-RT group (median 100.46 ml/min (IQR, 77.48-130.88), p=0.003)

Table 5.5. Characteristics of participants stratified according to TDF-RT outcome

Variable	TDF-RT n=12	NO TDF-RT n=40	Δ means (95%CI)	p
KIM-1/Cr				
Baseline	0.66 (0.79)	0.46 (0.66)	0.16(-0.37-0.7)	0.464
2 Weeks	1.81 (1.54)	0.92 (1.33)	0.97 (0.08-1.9)	0.042
4 Weeks	4.42 (5.04)	1.65 (2.69)	2.48 (1.02-6.0)	0.06
RBP4/Cr				
Baseline	26.19 (33.71)	32.69 (32.53)	-6.08(29.21-17.1)	0.587
2 Weeks	31.17 (36.69)	35.12 (28.39)	-3.13(27.59-21.3)	0.789
4 Weeks	50.58 (39.84)	50.49 (43.44)	0.36(21.37-22.1)	0.973
Δ CrCl	1.29(0.56)	1.36(0.67)	-0.71(-0.48-0.34)	0.730
Sex (Female)	10(83.4)	25(62.5)		0.151
Age (Years)	37.42(9.97)	35.5(9.59)		0.563
ART regimen				
TDF+3TC+EFV	5 (41.7)	20(50)		0.613
TDF+3TC+DTG	7 (58.3)	20 (50)		0.243
BMI (Kg/	23.65(20.45-25.45)	24(22.27 - 27.3)		0.451
BMI (Category)				
Type 1 HIV	10(83.3)	38(95)		0.224
CrCl (ml/min) at 6 months	81.19(67.87-92.21)	100.5(77.48-130.9)		0.003
CD4 (cells/mm)	186(74-400.5)	194(86-393)		0.142
VL (Copies/ml)	16783.5(13668-40431.5)	49503.5(1689-165432)		0.437
Time to TDF-RT (Months)	3.5 (0.75 - 5.5)	NA		

Δ means differences between means of TDF-RT and No TDF-RT groups with the 95%CI (95 % confidence interval), Significance $p<0.05$.

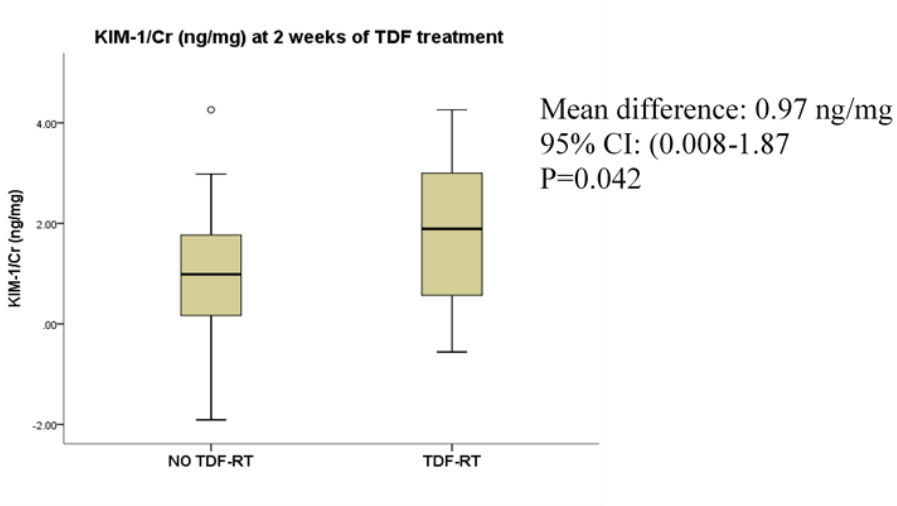


Figure 5.7. Box plot for the gran means (±SD) of KIM-1/Cr at 2 weeks following TDF exposure stratified according to patients with TDF-RT 1.81 (1.54) and patients without TDF-RT 0.92 (1.33).

5.3.5 Correlation of KIM-1/Cr and RBP4/Cr with traditional markers

The correlation of KIM-1/Cr and RBP4/Cr as a function of CrCl at baseline and 6 months was measured (Table 5.5). There was a relatively strong positive correlation between baseline RBP4 and Baseline CrCl. The rest of the variables were not correlated. It is noteworthy that baseline CrCl was also associated with TDF-RT.

Table 5.6. Correlation between LMW Kidney marker as a function of CrCl (baseline and at 6 months)

	CrCl at 6 months		Baseline CrCl	
Kidney Biomarker	r	p-value	r	p-value
KIM-1/Cr				
Baseline	0.433	0.563	-0.080	0.575
2 Weeks	-0.134	0.344	0.039	0.783
4 Weeks	-0.083	0.575	-0.027	0.852
RBP4/Cr				
Baseline	0.047	0.740	.515	**<0.001**
2 Weeks	-0.143	0.313	-0.115	0.415
4 Weeks	0.136	0.35	-0.052	0.724

r= Spearman's rho correlation coefficient. Significance at $p<0.05$

5.3.6 Receiver Operating Characteristic (ROC) Analysis of KIM-1/Cr measurements

The AUC for KIM-1/Cr at different time points was determined using the ROC curve to determine its performance in predicting TDF-RT (Figure 5.7). To determine the sensitivity and specificity, the threshold and cut off was set based on the mean 1.05 (±1.39) of KIM-1/Cr at two weeks because it consistently showed significant differences with baseline and was also significantly associated with the differences observed between the two outcome groups. The AUC for KIM-1/Cr at two weeks was statistically significant (AUC 0.69, 95%CI 0.500-0.878, p=0.049). However, there was no difference in the 4-week value (AUC 0.68, 95% CI,0.478-0.855, p=0.085). The AUCs and their respective sensitivities and specificities are given in table 5.6.

Table 5.7. ROC results for KIM-1/Cr measurements

KIM-1/Cr (ng/mg)	AUC	95% CI	Sensitivity (%)	specificity (%)	p-value
Baseline	0.558	(0.358-0.759)	58.30	55.00	0.543
2 Weeks	0.689	(0.500-0.878)	83.30	70.00	0.049
4 Weeks	0.667	(0.478-0.855)	83.30	78.40	0.085

AUC, the area under the Curve, 95% CI, 95%, confidence interval.

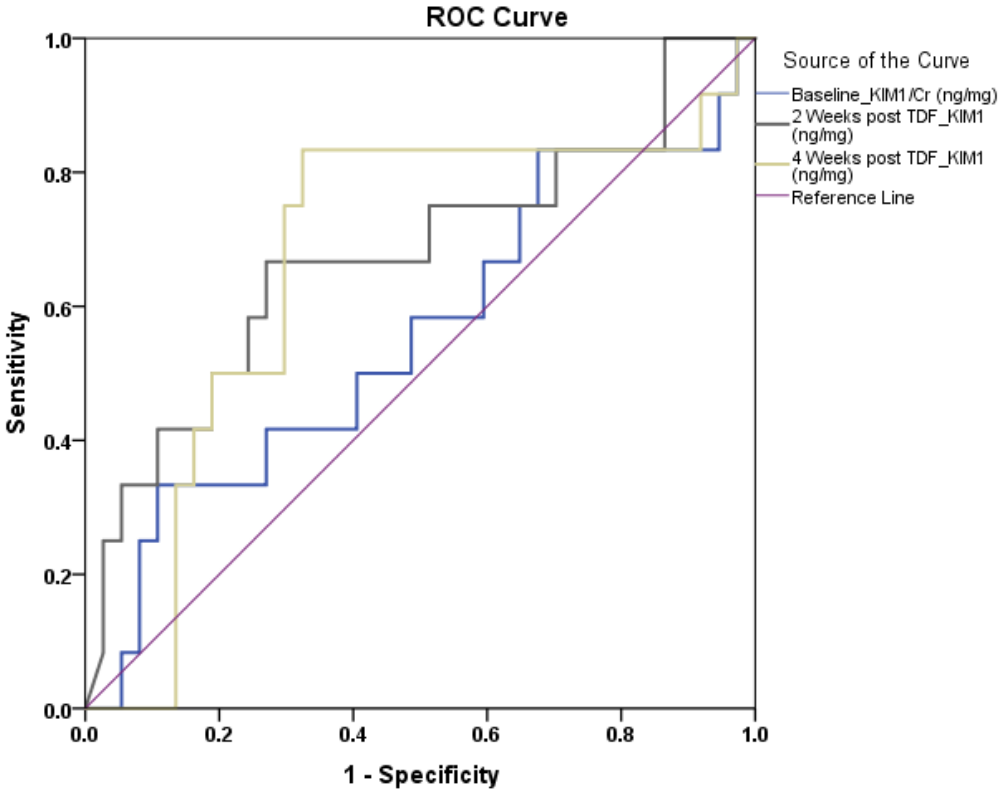


Figure 5.8. ROC analysis of KIM-1/Cr at baseline, 2 and 4 weeks after TDF

5.4 DISCUSSION

Renal toxicity has become an important issue in HIV-infected patients receiving ART containing TDF regimens[225, 242]. Independent of the origin, kidney disease is associated with immediate and long-term morbidity and mortality. Effective management of kidney injury is limited by adequate methods that are capable of accurately detecting significant renal injury on time. None of the markers available for monitoring renal function is validated for use in HIV infected patients, and the serum creatinine-based equations for estimating eGFR have been shown to underestimate GFR[280]. We prospectively investigated KIM-1 and RBP4 in this exploratory study to determine if they have the potential to monitoring and predicting TDF-RT events following cumulative TDF exposure in newly diagnosed HIV treatment naïve patients.

In our cohort, patients had normal baseline renal function and no significant proteinuria was demonstrated by dipstick urinalysis. Before the end of the 6 month follow up, 23.1% of the patients had evident RT (CrCl <60ml/min) after a median of 3.5 months treatment. KIM-1/Cr appeared to significantly predict RT after 6 months of therapy. Most ART guidelines recommend a renal assessment of newly diagnosed HIV positive patients before commencing ART containing potential nephrotoxic drugs like TDF. SCr based methods are used to identify patients at risk of renal impairment. However, the use of SCr, along with proteinuria, essentially screens for markers of glomerular disease and may not effectively detect subclinical of the renal tubules, and earlier stages of renal injury. In these settings, SCr has consistently failed to prevent TDF-RT in patients perceived to have normal baseline renal function[195, 634].

In our study, KIM-1 significantly increased from the baseline after commencing TDF-based ART. This increase was later significantly correlated with the incidence of TDF-RT which occurred between 3 to 14 weeks of TDF exposure. KIM-1 is a transmembrane protein known

to be upregulated in the renal proximal tubule of the kidney after acute kidney injury, and other studies have shown its high expression in patients with confirmed acute tubular injury[432, 434]. Studies have shown that in several cases, renal toxicity may go undetected by clinicians, particularly in those stages where creatinine clearance is >60 ml/min[635].

A steady increase in KIM-1/Cr within 4 weeks of TDF exposure may be evidence of acute proximal tubular injury which is associated with TDF in susceptible individuals. Our study showed that KIM-1/Cr after two weeks of TDF treatment was significantly associated with RT in treatment naïve HIV positive patients. The spike of KIM-1 is specific to the presence of slighted, differentiated and regenerating proximal tubular cells resulting from various origins[636]. Elevation in urinary KIM-1 has been observed in patients with a confirmed diagnosis of acute tubular necrosis compared with those with normal renal function, CKD and AKI of other causes[434].

In our study, patients were diagnosed as having TDF-RT through a CrCl value that had fallen below 60ml/min 3 months after commencing TDF. Although this was associated with higher KIM-1/Cr levels after 2 weeks of treatment, it just narrowly missed significance at 4 weeks, which probably reflects our small sample size. Unlike KIM-1 which is indicative of tissue injury, SCr is a marker of renal function which presents with a nonlinear relationship with GFR and requires a 50% decline of GFR from normal, before evidence of a rise on SCr[636]. Despite well-known limitations in the use of SCr as a marker of kidney function, clinical practice has for over half a century relied on serum creatinine as a gold standard marker of renal function. This may explain why patients develop complications related to TDF-RT because subclinical kidney injury goes undetected, and patients continue to take TDF[295]. Our findings suggest that KIM-1/Cr measurement may have adequate predictive characteristics as assessed by AUC,

sensitivity and specificity after 2 weeks of TDF exposure. These findings were similar to those observed elsewhere[632], although, the cohort comprised patients with a longer duration of HIV infection and a median TDF exposure of 3 years[632]. In another study, urinary KIM-1 was an excellent diagnostic marker for AKI originating from different aetiologies[637]. Another study observed that patients with urinary KIM-1 values in the upper quartile were three times more likely to have a poor prognosis[442]. It should be noted that our cohort comprised treatment naïve patients with normal renal function, yet KIM-1 sensitivity was predictive of tubular impairment more than 5 months before renal dysfunction was observed.

We wish to note that in our cohort, 58% of the participants that developed TDF-RT in the first 6 months of ART were on cART regimens containing dolutegravir (DTG), one of the antiretroviral drugs in the class of integrase Inhibitors (ISTI). According to recent WHO guidelines, ITSIs are an alternative option to efavirenz for first-line ART[638]. DTG is predominantly metabolized by uridine glucuronosyl transferase 1A1 (UGT-1A1) with extensive protein binding (>99%) capacity and excreted primarily in faeces, with only <1% excreted in urine unchanged[639]. For this reason, DTG does not require dose adjustment even in severe renal impairment (CrCl < 30 mL/min)[640]. Although known not to be of any clinical significance, in vitro studies have shown that DTG has a potential of decreasing creatinine clearance due to renal organic cationic transporter-2 (OCT2) inhibition on the basolateral membrane of the proximal tubules[204, 208].This is very important because creatinine is a substrate of OCT2[641], and therefore, like any other inhibitor of OCT2, DTG can block the tubular uptake of creatinine from the blood, leading to a rise in serum creatinine and decreased CrCl, although, without changing true the truwGFR[642]. In clinical studies, both the SPRING2[643] and VIKING [644] studies observed a non-progressive decline of CrCl in the DTG group compared with other regimens. This change was observed during the first 2–3 weeks of treatment and did not warrant

treatment discontinuation due to renal adverse events [642-644]. It has been demonstrated that these increases in serum creatinine seen shortly after commencing DTG based cART do not correspond with a decrease in GFR or glomerular injury[205]. We acknowledge that the initial rise in serum creatinine induced by DTG exposure may synergistically potentiate the overall effect due to TDF resulting in a further decline of CrCl, however, there was not significant difference between participants that developed TDF-RT and those that did not. Perhaps future studies should consider stratifying patient outcomes based on exposure to DTG. Our study did not adjust for the exposure of DTG and therefore our results should be interpreted in this context.

The mean KIM-1/Cr identified in our study (1.65 ng/mg) is lower compared to >4.17ng/mg elsewhere[632] although both were significantly associated with TDF-RT at 3 months and 3 years after TDF exposure respectively[632]. This may indicate cumulative damage caused by TDF-RT based on cumulative exposure to TDF. Our findings indicate that while treatment naïve patients are initiated on TDF ART on the assumption of normal kidney function, following TDF treatment, susceptible individuals present with an increase in KIM-1/Cr which is sensitive to minor tubular injuries that are worsened by prolonged and continued TDF administration. After significant tissue damage and decline in GFR, renal impairment is detected much later in HIV patients during routine reviews. The measurable decline in renal function (CrCl) that may have commenced with acute tissue injury within a month of TDF ART was only confirmed over 3 months later. However, it is important to highlight that this is an exploratory study, and further studies in larger cohorts will be needed. Ultimately a randomised controlled trial may be needed to show the utility of KIM-1 measurement when compared to conventional markers of renal function to make sure that the benefits of TDF treatment are maximised when compared to the potential for renal toxicity.

The mechanisms of damage to the kidney are variable and complex, and therefore may lead to different phenotypes in different individuals, and therefore using a combination of biomarkers may improve the identification of injury compared to using a single marker[645]. Indeed, both KIM-1 and SCr may have their place in the monitoring and detection of renal impairment. Indeed, because of the wide spectrum of pathologies that lead to AKI, no single marker may achieve the characteristics of an ideal biomarker that include high sensitivity to identify with specificity the origin of renal injury and provide vital information about the severity and long term prognosis of the injury[406, 411, 462]. Thus, the development of a panel of biomarkers may be necessary to provide the best clinical information to detect and manage renal injury caused by TDF and indeed by other nephrotoxic agents.

In our study, overall, although there was a significant difference with repeated measurements of RBP4, this difference was not significant when comparing patients with and without TDF-RT. Tubular proteinuria is a sensitive marker of proximal tubule impairment and RBP is believed to be a reliable LMWP. In a study that screened for subclinical renal tubular toxicity in patients with HIV, uRBP/Cr was significantly higher in TDF treated patients than non-TDF and treatment naïve groups[295]. Chan et al[646], in a study where the correlation between TDF and RBP4 was investigated, baseline RBP4 levels were higher in patients with pre-existing proteinuria and an increase in creatinine. Further, RBP4 exhibited a positive correlation with tenofovir AUC. In the ASSERT study, patients randomised to TDF experienced a significant increase in urinary RBP excretion compared to other regimens[647]. Furthermore, a cross-sectional study showed that patients receiving TDF with PI/r had higher urinary RBP concentrations[472]. In these studies, however, there were noteworthy distinctions compared to our study. Unlike our study, in Campbell et al's cohort [472], participants were included irrespective of the presence of pre-existing kidney disease or risk factors for kidney disease.

The ASSERT study assessed renal function from 4 weeks up to a maximum of 12 weeks after commencing treatment compared to 4 weeks in our study. In our study, the cohort did not have proteinuria at baseline and serum creatinine was normal. Collectively, because our patient characteristics were different (we excluded comorbidities such as diabetes and hypertension) from previous studies, this may account for the differences in RBP4 findings. Clearly, most urine protein markers are confounded by pre-existing conditions and patient characteristics, and it will be important to account for this in future studies, and to ensure generalisability of findings to the wider population with HIV. Incidentally, none of our patients were on protease inhibitors and we, therefore, exclusively attribute the effects on the urine biomarkers observed in our study to TDF treatment. It is also important to highlight the differences in findings between KIM-1 and RBP4 in our study – this may indicate better sensitivity of KIM-1 as a biomarker, but of course, may also indicate that these biomarkers are picking up subtly different mechanisms of kidney injury.

The investigation of new kidney injury biomarkers has been at the centre of extensive research[433, 437, 627, 648, 649]. However, despite the enthusiasm about this subject, most of the proposed novel biomarkers have not adopted for use in routine clinical practice in HIV or non-HIV patients. It is generally accepted that no single biomarker will perform adequately to stand alone to function as a diagnostic, severity of the injury, and prognostic marker. Therefore, future research should look at a panel of biomarkers in addition to currently used markers, and this panel will need to not only show better clinical utility but also demonstrate cost-effectiveness.

5.4.7 Limitations

Our reported results need to be interpreted cautiously in the context of some important limitations. This was an exploratory study of participants recruited from Zambia. We cannot rule out the possible degradation of urine proteins during collection and transportation due to the intermittent freeze and thaw processes. Admittedly, our sample size was small because of the high attrition rate in our prospective study. This may have further reduced the power to enable us to compare the differences in the RT outcome group. We may have also missed an opportunity to evaluate biomarker variation during the follow-up period by limiting ourselves to only 2 repeated measurements of biomarkers after TDF treatment. These results, therefore, require validation in a larger sample with more repeated measurements to determine the utility of biomarkers to detect renal injury in patients on TDF treatment.

5.4.8 Conclusions

In this study, we have demonstrated that there was a significant difference in tubular biomarkers from baseline and subsequent measurements following TDF treatment in HIV positive patients. We further demonstrated that these differences particularly in KIM-1/Cr were correlated with TDF exposure. KIM-1/Cr levels may be predictive of renal impairment even in patients without other risk factors after starting TDF-based ART. However, these findings need to be validated in a larger sample size which should include patients with and without other comorbidities to improve generalisability. We have evaluated two biomarkers (KIM-1 and RPB4) in comparison to serum creatinine and creatinine clearance, but future studies should evaluate the clinical utility and cost-effectiveness of a panel of biomarkers.

CHAPTER 6

FINAL DISCUSSION

6.1 GENERAL PERSPECTIVE

Tenofovir disoproxil fumarate (TDF) remains a widely used component of cART, especially in resource-limited countries. In the era of improved survival resulting from successful ART, the primary challenge physicians will constantly face is managing emerging age-related non-HIV conditions. At the core of patient management also is the prevention of medicines-related problems that may affect the patient's quality of life. Among the primary challenges that physicians have constantly faced is to balance between the benefit and risk of prescribing TDF based regimens because of the associated nephrotoxicity of varying severity that has been reported with its use. The main aim of my PhD research was to investigate the pharmacogenetic and urine biomarkers associated with TDF-RT in HIV positive patients receiving antiretroviral therapy containing TDF in Zambia. In the pharmacogenetic sub-study, I sought to investigate the role of SNPs in the pathogenesis of TDF-RT in a cohort of HIV positive patients recruited in Zambia. Evidence about the role of SNPs in TDF-RT in an African population at the time of this research was very limited. The demographic and clinical data from a cohort of participants recruited under pre-defined criteria was collected to investigate the clinical determinants responsible for TDF-RT and the collected blood samples analysed for the association of TDF-RT with SNPs previously reported to be associated in other populations. Within the pharmacogenetic study, I also worked with previously existing data from a GWAS of TDF-FS to confirm the GWAS suggested causal SNPs using a different genotyping platform. The urine biomarker study involved prospective recruitment of a cohort of treatment naïve HIV positive patients who were eligible to be initiated on TDF based ART. In this study, I aimed to explore the utility of urine biomarkers (KIM-1 and RBP4) corrected for urinary

creatinine in predicting TDF-RT by correlating their longitudinal measurements at two and four weeks of TDF exposure with nephrotoxicity after 6 months, i.e., did the biomarkers show a pre-symptomatic change well before there was a fall in creatinine clearance. The integrated findings of my experimental studies (chapters 2, 3, 4 and 5) provide insights into the information gaps relating to the pharmacogenetics of TDF in an African population. The confirmation of an association of SNPs suggested from the GWAS with TDF-FS is exciting because it opens new opportunities to understand the possibility of new pathways in TDF-RT that may not only be relevant to TDF but other drugs that share similar disposition processes. Furthermore, early and accurate detection of TDF-RT is critical to improving patient outcomes. The ageing HIV population faces a higher risk of developing debilitating age-related comorbidities that include diabetes, kidney and cardiovascular disorders, and therefore our findings provide an understanding of the clinical usefulness of renal urine safety biomarkers in the follow-up of patients treated with TDF and if further validated, have the potential to prevent the long-term adverse effects and complications of TDF-RT.

6.1.1 Clinical determinants of TDF-RT

In 18% of patients who developed TDF-RT while receiving TDF containing regimens, baseline factors including sex (female), older age, a low BMI and high serum creatinine (although in the normal range) were significant clinical determinants of TDF-RT in our cohort study.

Many studies have confirmed the nephrotoxicity of TDF in HIV positive patients although the pathogenesis and the pattern of TDF-RT remain poorly understood[202, 240, 276, 293, 299, 650]. Evidence in the literature from case reports, observational studies, and randomised case controls indicate that TDF-RT is a result of multiple factors ranging from the host (age, gender), comorbidities (hypertension, diabetes mellitus, pre-existing renal impairment), clinical (viral

load, $CD4^+$, biochemistry profile), co-infections (hepatitis B or C), co-administration of PIs and other nephrotoxic agents and in some settings, genetics[651]. However, it is also not uncommon to come across reports with contrasting findings because patients with confirmed TDF-RT may have a combination of these risk factors and therefore, a case-by-case analysis may lead to identifying specific determinants. Moreover, the population of HIV positive patients is reported to be very diverse with varied inter-individual characteristics including age, weight, comorbidities and exposure to potentially nephrotoxic agents[652]. In our cohort, there was a higher risk of developing TDF-RT in female patients (OR=13). A similar finding was observed in a prospective study assessing the pharmacokinetics of TFV in HIV positive patients with at least 6 months of exposure to TDF and renal function assessed over the succeeding 7 years. The high baseline plasma TFV AUC tertial was associated with significantly lower eGFR than those in the lower tertial and these differences widened over the seven-year follow-up period[653]. This study shows that variation in TFV drug exposure, especially with higher AUC, may partially explain the observed nephrotoxicity in persons infected with HIV. Although our study did not measure any TFV concentrations, we have identified factors that may significantly influence the pharmacokinetic parameters and potentially affect the CrCl. These include low body weight, increasing age and high baseline but within the normal range of SCr (indicative of lower eGFR). These were also common conditions that were observed to affect the pharmacokinetics of TFV in HIV infected females in another study[652]. Similar studies have reported a significant change in creatinine of 5 to 7% in HIV patients on TDF ART for more than 6 months[262, 320, 654]. Apart from factors determined in our study, other factors likely to affect TDF excretion include drug-drug interactions with PIs[653]. However, our cohort had very few patients on PIs to enable us to measure the impact of this interaction on TDF-RT. On the other hand, TDF-related kidney toxicity seems to be increased in patients with CD4 cell

counts of less than 150 cells/mm^3 suggesting the influence of immunosuppression and the degree of viral infection on the risk of nephrotoxicity[239, 285]. Our cohort was selected from a clinically stable population and there were no significant differences in CD4 cell count between patients with TDF-RT and controls. We acknowledge that despite minor disparities in the risk factors reported from various studies and our study, any factor likely to affect the disposition of TDF can pose a risk of developing TDF-RT. Overall, the safety profile of tenofovir is relatively good and predicting which patient is at risk of developing toxicity is vital for patient stratification and regular monitoring.

In our findings, we showed a set of baseline characteristics as determinants of TDF-RT. Several studies have also reported a cluster of baseline risk factors that include but not limited to our identified risks[261, 283]. However, what seems to be lacking is integrating these findings to inform current clinical practice. What we may ask is whether such findings have added an impact on the body of knowledge or practice. To start with, it is acknowledged that HIV treatment guidelines recommend assessing the renal function of newly diagnosed HIV$^+$ patients before commencing them on TDF based cART[32]. However, the role of this assessment appears to only identify and exclude patients with pre-existing renal impairment from the immediate risk of being exposed to the potential nephrotoxicity effects of TDF and has no impact on the short or long-term effect of TDF administration. Moreover, currently, there are no further assessments or deliberate actions prescribed for a patient exhibiting any reported risk factors for TDF-RT despite having a normal kidney function. Based on previous study reports and indeed actual practice, it would appear that pre-treatment renal assessment has not prevented the potential occurrence of TDF-RT because it has no impact on other independent risk factors. Research findings from the effect of baseline renal function on TDF outcomes, a study of Zambian HIV positive patients, appear to support my assumption[475]. This study showed that

although the proportion of patients with moderate to severe renal impairment at baseline was low in the TDF treated patients compared to non-TDF (1.9% vs 4.0%), the TDF arm had a higher risk of developing moderate and severe declines in eGFR. Interestingly, among patients with moderate or severe renal dysfunction at baseline, renal parameters improved independently of the prescribed ART regimen[475]. These findings are important indicators that TDF-RT is not limited by normal baseline renal function and can manifest following cumulative exposure to TDF. Moreover, TDF-RT is dose-dependent and often occurs from the accumulation of TFV resulting from the influence of multiple factors on its disposition of[318, 653, 655]. In addition, in practical clinical settings, patients may have other co-morbidities, non-ART medications, or background characteristics that may predispose them to TDF-RT leading to significant AKI[238] which may occur following months of treatment and may worsen requiring long-term renal replacement therapy[265, 312].

In the current treatment guidelines, baseline or pre-treatment assessment of renal function is based on urinalysis (for protein) and SCr-based estimation of GFR[32]. There is a real chance of missing background kidney injury using SCr and urinalysis because they cannot detect the subclinical tubular injury that is seen in TDF-RT. Moreover, these are also late markers of glomerular function that signal long-standing renal impairment and therefore, are inappropriate for detecting the subtle risk of tubular impairment that may be relevant to TDF therapy outcomes. Even after commencing TDF based ART, routine monitoring of renal function still relies on SCr which has recognised limitations and lacks the sensitivity to detect tubular injury seen in TDF-RT[377]. Like our study, similar research findings have motivated researchers to recommend intensive and regular monitoring of patients with identified risks for TDF-RT.

Routine monitoring of renal function in HIV patients is recommenced independent of the ART

regimen because HIV infection is an independent risk factor for CKD[656]. Currently, guidelines recommend standard routine monitoring of ART outcomes every 3 to 6 months independent of the patient risk[480]. Among the objectives of this practice is the detection of toxicity to ARV drugs. This, therefore, means that unless renal impairment presents with clinical symptoms that may warrant a patient to seek medical care, as most renal symptoms are insidious, they will be discovered incidentally during routine assessment in which case the period for early intervention to avoid a progressive disease would be missed. A study in Ghana reported an association between TDF with increased proteinuria and asymptomatic tubular dysfunction[290]. Moreover, by the time a clinically evident rise in measurable SCr is observed, there has generally been a loss of at least 75% of functional nephrons and the damage could be potentially progressive and irreversible[125]. In addition, most equations estimating GFR are not validated in HIV patients and may underestimate the extent of renal impairment because of a mismatch between SCr and muscle mass, age and sex which is common in most HIV patients[657]. Notwithstanding the effort of routine monitoring, what is very significant is that it does not promote detection of TDF-RT because TDF-RT targets proximal tubular damage and not glomerular damage which is measured by SCr and urinalysis (proteinuria). Studies have previously shown subclinical tubular injury in HIV patients on TDF[295] while tubular impairment without loss in glomerular function has also been reported[294]. In addition, routine monitoring studies have shown that TDF-RT is characterised by very low urine albumin protein and dipstick urinalysis may be unreliable in TDF-RT[658]. Like our study, Mulenga also reported similar factors that were associated with poor renal outcomes following TDF treatment[475]. In his study, he recommended an assessment of the role of baseline assessment on patients prescribed with TDF cART. To date, we do not know the impact of this assessment and routine monitoring regimens on early prediction or prevention of nephrotoxicity. I recommend

establishing a stratified risk approach to the routine monitoring schedule of patients treated with TDF using at least two tubular markers, and in future, measure the impact of this approach on TDF-RT incidence.

6.1.2 Pharmacogenetics of TDF-RT in Zambian patients

The individual variability observed in the manifestation of TDF-RT has strongly suggested the role of genetic factors in the pathogenesis of TDF-RT. Chapters 4 and 5 highlight our findings of the role of SNPs in Zambian patients with TDF-RT and Caucasian UK HIV positive patients with confirmed TDF-FS. Collectively, these results provide insights to further understand the role of pharmacogenetics in these populations concerning TDF use. Pharmacogenetic studies, in general, have the potential to change the standard treatment approach of one size fits all and all those involved, physicians and patients alike, have high expectations of the precision and personalised approach that pharmacogenetic understanding of commonly used drugs may offer. This is because patients with known genetic predispositions may benefit from receiving treatment that is free of serious adverse effects and on the other hand, clinicians have evidence-based informed guidance for the optimum selection of medicines and dosage regimens tailored to individual need[659]. Findings from our Zambian study sample did not identify any SNPs associated with TDF-RT in Zambian HIV positive patients. However, there are several limitations to our findings, and these should be taken into account when interpreting the results: (a) our sample size was limited, and although we can exclude a SNP with large effect size, we cannot exclude SNPs with a moderate effect; (b) we relied on previously reported associations in different ethnic populations in the hope of replicating these; and (c) we used a relatively insensitive marker of nephrotoxicity rather than more sensitive biomarkers of tubular injury.

We highlighted that rs284302 in PEX14 was significantly associated with TDF-RT (unadjusted

for multiple corrections). We highlighted it as a SNP of interest due to the recognised role of PEX14 in kidney disease. Perhaps testing this SNP in a larger sample of patients would give us more conclusive results. The association with PEX14 rs284301 was confirmed in the Caucasian population with TDF-FS. From our point of view, this was a significant finding because it was a variant identified from the GWAS and it is likely that screening the Zambian population using GWAS may identify unique causal SNPs in this population.

Genetics differences associated with drug metabolism, action and disposition may be responsible for interethnic variability in drug response[660]. These genetic variations have the potential to significantly affect treatment outcomes and increase the risk of developing adverse drug reactions which vary from population to population. This is particularly important because of the existing interpopulation differences in the MAFs, linkage disequilibrium (LD) and haplotype distributions among different populations[660]. Our study also failed to replicate previously reported LD and haplotype distributions. This observation is supported by the evidence that existing heterogeneity in genetics explains why there are differences in the distribution of over 159 drug responses related to SNPs in the global populations[660]. It is possible that alleles related to drug response that are considered as minor in the global population may exist as major alleles in other populations and can distinguish populations in a way that may correspond to a deviation from Hardy Weinberg equilibrium and geographic distributions[660]. These interpopulation genetic variations indicate that genetic markers used for some drug responses in one population may not be appropriate for another population. One such example is rs4917639 in the CYP2C9 gene which is present with ≥0.2 frequency only in the African super-population and requires an ethnicity dependent dosing for warfarin according to the Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline[661]. This highlights the importance of distinct, ethnicity tailored clinical guidelines, especially for the

African populations to avoid ADRs and optimise treatment outcomes. It is known that African-specific pharmacogenetics-based therapeutic recommendations in drug labels are lacking[662]. Additionally, caution should be exercised when attempting to replicate genetic associations previously identified in different ethnic population groups. It is in this knowledge that I firmly believe that there is a possibility that entirely novel variants specific to the African population may be responsible for the genetic predisposition to TDF-RT and therefore, undertaking genome-wide association studies, and even more robustly, whole-genome sequencing, that includes identification of rare and intronic regulatory variants that are relevant to adverse drug reactions specific to Africans[663], will be important. For future studies, adequate statistical power will be important, but cost-sharing is also going to be important for what would be an ambitious undertaking. Collaborative approaches like this already exist, for example, the Human Heredity and Health in Africa (H3Africa) consortium empowers and facilitates research to generate unique data that could be used to improve both African and global health (https://h3africa.org/).

6.1.3 Pharmacogenetics of TDF-FS in Caucasian patients.

In my study, we have confirmed the association of genetic variants in *TMEM120A, PEX14* and *ITSN* genes with TDF-FS. These are very interesting findings because they widen our understanding that TDF-FS is not limited to the previous focus of mitochondrial toxicity, but instead, can be explained by alternative pathways.

Until now, many studies have revealed that the main site for TDF toxicity is the proximal tubule which in severe cases result in the development of Fanconi syndrome, a generalised tubulopathy that is characterized by acute kidney injury, phosphaturia, glycosuria, bicarbonate wasting, tubular proteinuria, and aminoaciduria[384]. Human mitochondrial DNA polymerase

(mtDNA) α has been implicated in the toxicity associated with nucleoside analogues[247]. Pharmacologically, chronic inhibition of mitochondrial polymerase α by TDF has been seen as an instigator of organ-specific nephrotoxicity resulting from depleted mtDNA and failure of the mtDNA encoded proteins in energy biogenesis which is vital for transporter facilitated excretion of TDF[247, 374]. Both human and animal studies have demonstrated that mitochondria are the target organelles of tenofovir cytotoxicity in renal proximal tubular cells[128, 312, 377]. In human studies, kidney biopsy light microscopy images of HIV patients with TDF-RT have revealed abnormalities of intracytoplasmic inclusions in proximal tubule cells while electron microscopy showed widespread structural abnormalities in proximal tubular cell mitochondria of varied size and shape, the disruption of cristae, mitochondrial swelling, and intra-mitochondrial deposits[307, 312]. Furthermore, studies have up to date focused on the role of drug transporters in intracellular TFV accumulation in the pathogenesis of RT. Even genetic studies have explored the role of SNPs in drug transporter genes in TDF-RT, albeit yielding inconsistent and un-replicated results[340, 395, 402, 664, 665].

Other experimental studies have demonstrated that the depleted mitochondrial cellular antioxidant system contributes to TFV induced mitochondrial damage resulting from increased oxidative stress in the kidney[384]. Chronic administration of tenofovir to adult Wistar rats replicated proximal tubular damage similar to that observed in patients with Fanconi syndrome with widespread tubular mitochondrial injury as revealed by electron microscopy of biopsy images. Mitochondrial damage was demonstrated by an increase in protein carbonyl content (low molecular tubular proteins), a reduction in mitochondrial glutathione, loss of superoxide dismutase, glutathione peroxidase and to a larger extent glutathione reductase loss by 150%. In addition, a decline in carbonic anhydrase and succinate dehydrogenase activities further highlighted the reduction in mitochondrial activity induced by chronic TDF administration[384].

The presence of SNPs in drug transporters makes TDF a double-edged sword because not only do individuals with loss-of-function variants lack the capacity to mediate TFV excretion, but they also promote TFV accumulation and subsequent chronic inhibition of mtDNA polymerase α, thus impairing the energy generating capacity of mitochondria.

Due to the levels of ATP required by proximal tubules for the various metabolic processes to maintain cell homeostasis, energy depletion of any origin is critical in the pathogenesis of acute or chronic kidney injury, and diseases such as diabetic nephropathy. Functional mitochondrial β-oxidation of fatty acids (FAO), a primary source of tubular ATP is crucial in preventing ATP depletion and lipotoxicity induced tubular injury, inflammation and tissue fibrosis[666]. It is there possible that SNPs in *TMEM120A*, a gene responsible for energy biogenesis in cooperation with other genes like *GLUT4, GATA3* and *FASN* can indirectly elicit tubular injury due to depleted fatty acid synthesis, an important precursor of ATP synthesis[573, 575, 580, 667].

Human *PEX14* is a multi-tasking protein that not only facilitates peroxisomal protein import but is also required for microtubule-based peroxisome motility in human cells[557]. In addition, the role of peroxisome in beta-oxidation through multiple enzymatic pathways has been identified[560, 605]. What is more significant in the peroxisomal capacity for mitochondrial FAO that is of equal magnitude in the proximal and the distal nephron as it is in liver cells[605]. This is particularly important because energy biogenesis for the proximal tubular function is not limited to mtDNA ATP synthesis but also includes peroxisomal mt FAO, both of which contribute to efficient tubular metabolic function. What we may be interested to know is which of these pathways when knocked down would significantly affect the energy required for metabolic function. This is probably a question to be explored in future work.

The molecular role of *PEX14* in mitochondria FAO in the development and progression of

kidney injury has been reported[551]. In what seems to be functional cooperation with *TMEM120A,* adipogenesis is required for the action of *PEX14* in mitochondrial FAO to generate ATP. Peroxisome bound organelles in proximal tubules, glomeruli, distal tubules, and collecting ducts play an important role in FAO synthesis of ATP and detoxification of the resultant hydrogen peroxide and other ROS that prevents mitochondrial tissue damage[555, 556, 561]. Collectively, our findings and those of other studies suggest that further investigations are needed to understand the biological functional role of *PEX14* and *TMEM120A* pathways in TDF-RT.

Another interesting finding in the association of SNPs with TDF-FS is the link between transmembrane and PTEC receptor endocytosis and TDF-RT. Several processes involved in the mechanism of endocytosis are ATP dependent. The exchange of free and bound clathrin in clathrin-coated pits requires ATP[668]. On the other hand, an intact actin cytoskeleton, whose polymerisation is also ATP dependent is important for endocytosis[616, 618]. The clathrin-dependent trafficking of endocytosis vesicles within cells along microtubules by a complex of proteins is also dependent on ATP[608]. Conversely, the expression of megalin and cubilin, highly specialised large transmembrane multiligand receptors in proximal tubular epithelial cells (PTEC) that mediate the efficient uptake of low molecular weight proteins (LMWP) and other biomolecules from the glomerular filtrate[614, 621, 622] is dependent on the integrated regulation of clathrin-dependent endocytosis[608, 616, 617]. Our results show a significant association between TDF-FS with a variant in the *ISTN* gene which plays a key role in coordinating endocytic membrane traffic with the actin assembly machinery and the formation of clathrin-coated vesicles which are critical in endocytosis[608, 609, 615]. In summary, although there has been a focus on mitochondrial toxicity and SNPs in drug transporters in the pathogenesis of TDF-RT, multiple mechanisms are likely to play a role including those associated with receptor-mediated

endocytosis in the PTEC[614, 622, 669]. Based on these findings, we hypothesise that impaired tubular endocytosis may be important for two reasons: i) In the presence of ATP deficiency, there is inhibition of clathrin-dependent endocytosis which further inhibits the activation of megalin-cubilin receptor uptake of LMWP; and ii) genetic variants in *ITSN* by either up or down-regulating its expression may significantly affect tightly regulated endocytosis membrane traffic which further inhibits the cascade of signalling required for the recruitment of PTEC megalin-cubilin mediated receptor endocytosis resulting in tubular impairment and urinary loss of LMWP, a phenomenon observed in FS.

6.1.4 The correlation of urine kidney injury biomarkers with exposure To TDF in HIV treatment Naïve Patients.

We have demonstrated that there is a statistically significant correlation of KIM-1/Cr and RBP4/Cr measured at baseline and subsequently following TDF treatment in HIV positive patients. However, only KIM-1/Cr measurements were predictive of TDF-RT in HIV positive patients within 6 months of therapy.

Studies have revealed that patients with HIV infection are likely to have background proximal tubular disease due to invasion of glomerular cells by the virus[295]. Moreover, traditional markers have limited value in detecting early tubular impairment. This becomes very significant when there is a need to rule out any pre-existing tubular impairment before prescribing potentially nephrotoxic drugs like TDF. As previously discussed under predictors of TDF-RT (section 6.1.1), failure to detect subclinical tubulopathy increases the risk of subjecting patients to a greater effect of clinically significant nephrotoxicity. Unfortunately, for over 50 years now, the measurement of kidney function in clinical settings has depended on the use of fairly unreliable traditional markers of renal function including SCr and urine

dipstick (for albumin)[627, 670]. Just recently, a systematic review of TDF associated kidney disease in Africa reported that out of 31 studies, 60% reported renal outcomes based on serum creatinine estimated GFR by using the Cockcroft-Gault, MDRD or CKI-EPI formulae. Other studies also reported absolute SCr levels, and urinalysis, clinical signs and symptoms as measures of renal dysfunction[240]. None of the studies utilised tubular markers as indicators of TDF-RT. These reports are in settings where HIV infection is very prevalent and due to limited resources, prescribing TDF will remain common for the foreseeable future.

My research aimed to explore an area of growing research interest in the identification of more sensitive and specific biomarkers which may detect subtle tubular injury at an earlier stage than traditional markers. Implementation of these markers may benefit patients by identifying those at risk and prevent exposure to potentially nephrotoxic drugs but may be advantageous where early diagnosis and intervention may be required to change outcomes. Whether the continued use of SCr and urinalysis is leading to significant glomerular impairment which is likely to increase the incidence of advanced and irreversible kidney disease in HIV patients is an area that needs investigating.

KIM-1 has been widely studied in different forms of AKI[93, 637]. KIM-1 has been identified as a potential biomarker for tubular injury because it is exclusively reabsorbed by proximal tubular cells, and therefore, has a basal low level of expression in the normal kidney. However, following ischaemic-reperfusion injury, KIM-1 is upregulated and is highly expressed in proliferating dedifferentiated PTEC 48 hours after injury[432]. The shedding of membrane extracellular component of KIM-1 in a matrix metalloproteinase (MMP)-dependent manner[671] is responsible for the rising levels of uKIM-1 after tubular injury[432, 434]. On the other hand, KIM-1 expression may also signify phagocytosis of apoptotic bodies and necrotic debris[672] and

may explain why as a biomarker, it could play a role in tubular recovery and regeneration and is consistent with KIM-1 elevation 2-3 days of AKI[672, 673]. In addition, KIM-1 shedding is driven by mitogen-activated proteinase signalling following growth factor expression, cell proliferation and recovery, and therefore, uKIM-1 may be vital in distinguishing between the extension and maintenance phase of injury[672]. Therefore, its interpretation should be made in the context of assumed injury and as complementary to other clinical and biochemical signs.

Other studies have explored the correlation and probable diagnostic utility of KIM-1/Cr in treatment-experienced HIV positive patients and found higher baseline levels in TDF treated patients[632] compared to non-HIV healthy subjects[440] and patients with other forms of AKI[674]. It has also been suggested that KIM-1 levels can offer prognostic insights about kidney injury[675]. Some studies have shown that baseline urinary KIM-1 concentrations may increase in the presence of evident proteinuria as a potential prognostic factor[675]. A rise in KIM-1 along with Urine Liver-Type Fatty Acid-Binding Protein (uLFAB)[445].and arise in KIM-1 and albuminuria in patients on TDF cART with tubular injury has been independently associated with TDF use. This was also correlated with IL-18 in another study[631]. KIM-1 is a hallmark of injury, while albuminuria indicates progressive tubular injury and IL-18 is a cytokine with a prominent pro-inflammatory role with the potential to worsen tissue injury during the extension phase of AKI[676]. Therefore, in the presence of these biomarkers, KIM-1 may serve as a prognostic marker. Importantly, however, it will be important to include KIM-1 in a panel of biomarkers that allows assessment of injury to different parts of the nephron, and indicates different types of pathological processes in the diseased kidney. In clinical practice, KIM-1 may not be useful in the absence of other differential markers, and KIM-1 levels by themselves cannot discriminate between acute, severe or recovering injury. However, by testing a range of biomarkers, some qualitative and quantitative information may provide a further understanding

of the pattern of kidney disease taking place as well as its prognosis.

RBP has been shown to correlate with TDF administration and is a predictor of AKI[646]. However, it is also significantly associated with renal function in patients with CKD and which potentially expands its role beyond proximal tubulopathy as a prognostic marker[677, 678]. In addition, compared to SCr, RBP4 showed the potential to be a more sensitive marker of TDF induced renal tubular injury in the presence of risk factors such as co-administration of LPV/r and low body weight[303] Despite a lot of interest from researchers on novel biomarkers, none of the biomarkers has been validated for use in TDF-RT in HIV patients. The challenge with the continued use of TDF in HIV patients may remain unresolved if vital questions to prevent renal outcomes are not answered. Because subtle kidney function decline affects long-term morbidity and mortality, the balance between efficacy and probable adverse effects requires further study[320].

For future studies, I recommend considering validating the clinical findings in a larger cohort, with an extended period for longitudinal measurements of KIM-1 and RBP4, and other renal biomarkers. It would also be interesting to determine whether subjecting KIM-1 collected from HIV subjects with different disease states to the proteomic investigation may identify different KIM-1 isoforms. The field of urinary proteomics appears to promise to expand the number of biomarkers for the diagnosis and prognosis of several human diseases[679]. Thus, urine proteomic screening for novel biomarkers may be useful in evaluating and preventing the potential nephrotoxicity of drugs such as TDF and several others.

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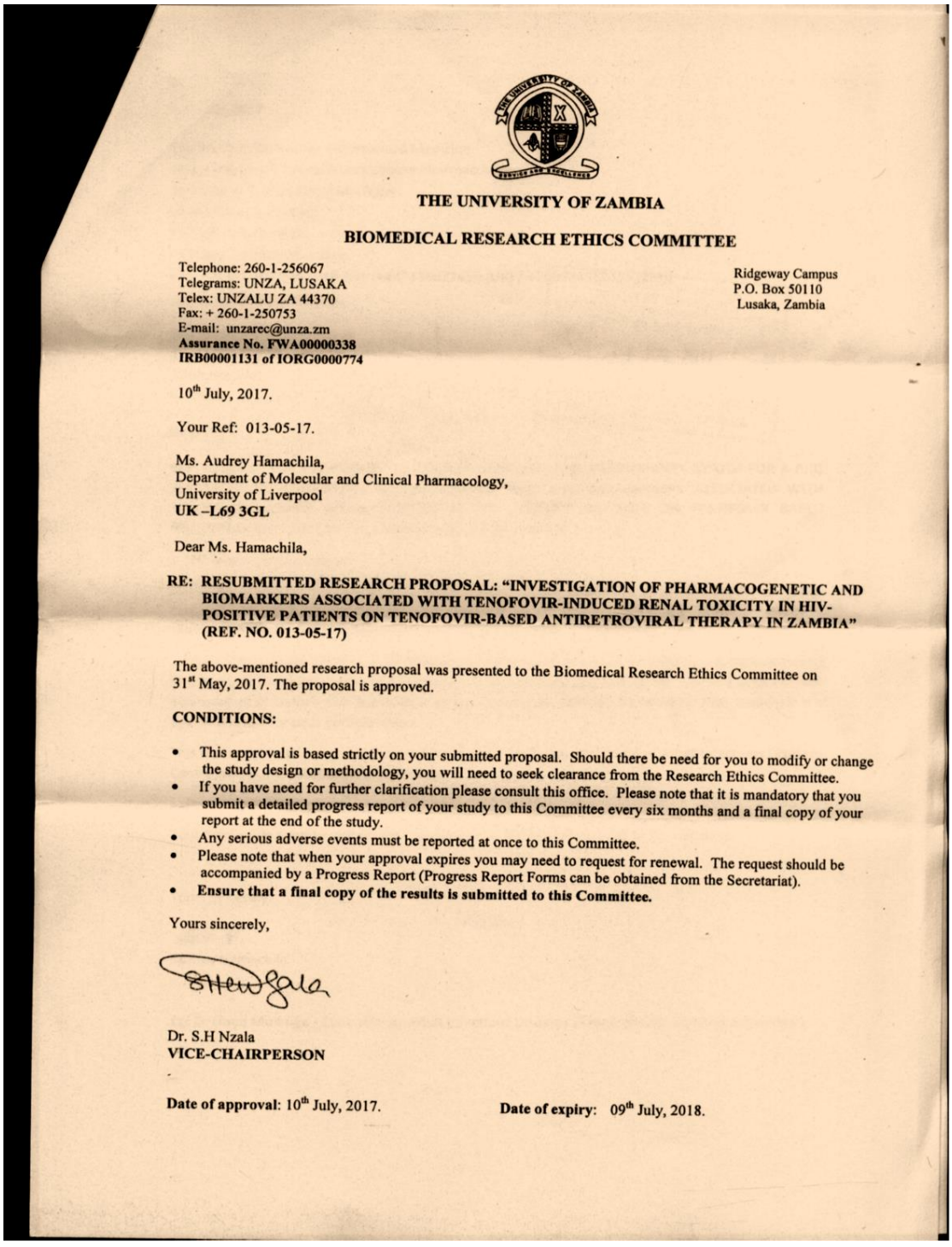
APPENDICES

APPENDIX A RESEARCH ETHICS APPROVAL REF. NO 013-05-17 (RENEWED COPY BELOW)



THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@unza.zm
Assurance No. FWA00000338
IRB00001131 of IORG0000774

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

10th July, 2017.

Your Ref: 013-05-17.

Ms. Audrey Hamachila,
Department of Molecular and Clinical Pharmacology,
University of Liverpool
UK –L69 3GL

Dear Ms. Hamachila,

RE: RESUBMITTED RESEARCH PROPOSAL: "INVESTIGATION OF PHARMACOGENETIC AND BIOMARKERS ASSOCIATED WITH TENOFOVIR-INDUCED RENAL TOXICITY IN HIV-POSITIVE PATIENTS ON TENOFOVIR-BASED ANTIRETROVIRAL THERAPY IN ZAMBIA" (REF. NO. 013-05-17)

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 31st May, 2017. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. S.H Nzala
VICE-CHAIRPERSON

Date of approval: 10th July, 2017. **Date of expiry**: 09th July, 2018.

UNIVERSITY OF ZAMBIA
BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
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Federal Assurance No. FWA00000338

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia
E-mail: unzarec@unza.zm
IRB00001131 of IORG0000774

25th February 2019.

Your Ref: 013-05-17.

Ms. Audrey Hamachila,
University of Liverpool,
Department of Molecular and Clinical Pharmacology,
L69 3GL, Liverpool,
United Kingdom.

Dear Ms. Hamachila,

RE: **RENEWAL OF RESEARCH PROPOSAL: "INVESTIGATION OF PHARMACOGENETIC AND BIOMARKERS ASSOCIATED WITH TENOFOVIR INDUCED RENAL TOXICITY IN HIV POSITIVE PATIENTS ON TENOFOVIR BASED ANTIRETROVIRAL THERAPY IN ZAMBIA" (REF. No. 013-05-17)**

We acknowledge receipt of your request for renewal and enclosed progress report therewith.

Your request was reviewed and Renewal is hereby granted for the periods:
31st May 2018 to 30th May 2019 and;
31st May 2019 to 30th May 2020.

Yours sincerely,

Munsaka.

Sody Mweetwa Munsaka, BSc., MSc., PhD
CHAIRPERSON
Tel: +260977925304
E-mail: s.munsaka@unza.zm

APPENDIX B AUTHORISATION TO CONDUCT RESEARCH

THE NATIONAL HEALTH RESEARCH AUTHORITY
C/O Ministry of Health
Haile Selaisse Avenue,
Ndeke House
P.O. Box 30205
LUSAKA

MH/101/23/10/1

14 July 2017

Audrey Hamachila
Department of Molecular and Clinical Pharmacology
University of Liverpool
UK-L69 3GL

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Investigation of Pharmacogenetic and Biomarkers Associated with Tenofovir-Induced Renal Toxicity in HIV-Positive Patients on Tenofovir-Based Antiretroviral Therapy in Zambia."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been **approved** to carry out the above mentioned exercise on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, University leadership, and all key respondents.

Yours sincerely,

Sandra Chilengi-Sakala
For/Director
National Health Research Authority

APPENDIX C PATIENT INFORMATION LEAFLET AND CONSENT FOR THE PHARMACOGEENTIC STUDY.

Title of The Research: "A Pharmacogenetic and Biomarkers of Tenofovir- Induced Renal toxicity in HIV-Positive Patients in Zambia."

Name of Researcher: Miss Audrey Hamachila

University of Liverpool, Department of Molecular and Clinical Pharmacology

Institute of Translational Medicine; 1-5 Brownlow Street, Liverpool L69 3GL

Mobile: +260974962365 / +4497436617459

haudrey@liverpool.ac.uk

This informed consent form has two parts;

1. Information Sheet (to share information about the research with you)

2. Certificate of Consent (for signatures if you agree to take part)

PART I: INFORMATION LEAFLET.

My name is Audrey Hamachila, and I am a PhD student at the University of Liverpool, UK. I am doing a research on kidney disease caused by tenofovir, a drug commonly used for treating HIV-infection in Zambia. The information given here is for you to use in deciding whether or not you may want to participate in the research. Please ask any questions you feel like either to me or your doctor for clarification.

PURPOSE OF THE RESEARCH:

HIV-infection is a common disease in our country. The medicines that are currently used to treat HIV are good but they are causing some side effects in some patients. For example, tenofovir a drug found in 'Atripla' and 'Truvada' is known to cause kidney side effects. This research wants to find out the differences in the genes in patients which will make them more likely to develop a kidney side effect. If we are able to find those genes as part of this research, we may be able to identify those patients who are more likely to get a kidney side effect when administered tenofovir in the future. We will also look at specific proteins in the blood and urine which can help us in the diagnosis of kidney side effect.

We are inviting everyone who have been on tenofovir for at least 3 months to participate in this

research study. Your participation in this research is entirely voluntary. Your choice to participate or not to will not affect any future services that you receive at this hospital.

What will happen if I chose to participate in the study?

If you have had a kidney side effect whilst you have been on tenofovir, you will be asked to donate a sample of blood (10ml or 2 teaspoonfuls) by your treating doctor when you visit the clinic.

If you have NOT had any kidney side effects whilst you have been on tenofovir, you will be asked to donate two samples of blood (20ml or 4 teaspoonfuls) and a sample of urine by your treating doctor when you visit the clinic.

Your samples will be coded so that your personal details will not be known to anyone outside the research team. The samples will be transferred to the University of Liverpool, United Kingdom, to measure specific proteins in your blood and urine; we will also obtain DNA (the chemicals which make up your genetic characteristics) from your blood sample and will be analysed for genes that are important in kidney side effects caused by tenofovir. This may help us to identify people who are most likely to develop kidney side effects with tenofovir. Your samples will be stored safe and under secure conditions for any follow up tests or analysis that may be necessary during this research study and any remaining samples will be destroyed according to the human material disposal protocol.

Research results will be communicated to the clinicians who may use it for their clinical intervention. Individual patients who may want to know the results can see them through their clinicians.

You are free to withdraw your blood sample if you do change your mind about participating by contacting the researcher on contact details stated above.

PART II: CERTIFICATE OF CONSENT

I have read the information about the research / the information has been read or explained to me in my local language (if illiterate). I have had the opportunity to ask questions about it and all questions that I have asked to have been answered to my satisfaction. I therefore voluntarily consent to participate in this research study and donate blood and /or urine samples which can be used for this study and any follow up tests or analysis that may be necessary. I have also been informed that the upon completion of the study, research results will be shared with my clinicians.

Print Name of Participant / Witness*_____ Signature _____

Date _____

*If participant is unable to read or write, a witness if possible selected by the participant and not connected to the researcher must sign.

APPENDIX D

Research Title: "Tenofovir induced renal toxicity; A Pharmacogenetic and Biomarker investigation in HIV positive patients in Zambia"											
STUDY CODE: ZTDF-PGx-000N					CASES SHEET						
Checklist for inclusion criteria of TDF-RT study group:					TDF: 300mg ≥18 yrs Baseline SCr (µmol/L): TDF-RT 3mo after starting TDF				Hx of TDF-RT SCr >120umol/L or eGFR Continued (C) or discontinued (D) TDF		
Study-ID	Age	Sex F/M	Baseline SCr µmol/L	Tx Duration at TDF-RD	SCr at TDF-RT	TDF Tx	Wt at RT (Kg)	Age at RT	Other ARVs	Non-ARV drugs	Other medical conditions
ZTDF-PGx-0001											
ZTDF-PGx-0002											
ZTDF-PGx-0003											

APPENDIX E MATERIAL TRANSFER AGREEMENT

Serial number: ***E01617***

NATIONAL HEALTH RESEARCH ETHICS COMMITTEE

Material Transfer Agreement Form
To be filled-in if exporting biological material

Section A.

1. Name of requesting organisation/individual: **Miss Audrey Hamachila**
2. **Physical Address:**
   The Wolfson Centre for Personalised Medicine
   Department of Molecular and Clinical Pharmacology
   Institute of Translational Medicine.
   University of Liverpool; L69 3GL- Room A129. UNITED KINGDOM
   Tel: +44 151 794 5557; Mobile (+447436617459 (UK) / +260974962365 (ZM))
3. **Contact details:**
   Telephone No: Tel: +44 151 794 5557;
   - ✓ Fax No:
   - ✓ Cell phone No: +447436617459 (UK) / +260974962365 (ZM))
   - ✓ Email address: haudrey@liverpool.ac.uk.

Name/s of Principal Investigator: Audrey Hamachila
Title: **Miss**
Title of Study: Investigation of the pharmacogenetics and biomarkers associated with tenofovir- induced renal toxicity in HIV-positive patients on TDF based antiretroviral therapy in Lusaka-Zambia

Signature: _____

Date: 21st December, 2017.

Please return a filled in copy of this Agreement to:

The National Health Research Ethics Committee, C/O Ministry of Health, P.O Box 30205, LUSAKA.
NHREC/MOH will be maintaining signed originals and the official list of signatory organizations.

The purpose of this agreement is to provide a record of the biological material transfer, to memorialize the agreement between the PROVIDER SCIENTIST (identified above) and the RECIPIENT SCIENTIST (identified below) to abide by all terms and conditions of the Material Transfer Agreement (MTA) and to certify that the RECIPIENT (identified below) organization has accepted and signed a copy of the MTA. All materials collected from Zambia shall remain the property of the Government of the Republic of Zambia. For any commercial product derived from the exported materials, the person from whom the samples were collected shall receive royalties. The RECEIPIENT agrees to acknowledge the source of the material in any publications reporting use of it. The RECEIPIENT agrees to use the MATERIAL in compliance with statutes and regulations.

1

Section B.

1. MATERIAL TO BE TRANSFERRED (full description and quantities)	
For the Pharmacogenetics study: 1000 X 2 whole blood samples in 4 ml vacutainers For the Biomarker study: 272 serum and 272 Urine samples in 2ml cryovials	
2. PURPOSE OF EXPORT: For research and analysis as part of the PhD study for the researcher, protocol Miss Audrey Hamachila	**3.DESTINATION OF MATERIAL (full details of institution and person responsible)** **Institution:** The Wolfson Centre for Personalised Medicine Department of Molecular and Clinical Pharmacology- Institute of Translational Medicine. University of Liverpool; L69 3GL- Room A129. UNITED KINGDOM **Person Responsible: Miss Audrey Hamachila.**
4. SOURCE OF MATERIAL (Study site): The University Teaching Hospital; AIDC. Lusaka	
5. RECIPIENT ORGANIZATION CERTIFICATION (Organization receiving the ORIGINAL MATERIAL)	
I hereby certify that the RECIPIENT organization has accepted and signed a copy of the MTA and will not outsource the material to another organization/laboratory without written approval from NHREC. I have read and understood the conditions outlined in this Agreement and I agree to abide by them in the receipt and use of MATERIAL.	
a. Name:	MS SHARON GRANT
b. Title:	HEAD OF OPERATIONS
c. Organization/Laboratory:	UNIVERISTY OF LIVERPOOL
d. Physical Address:	THE FOUNDATION BUILDING, 765 BROWNLOW HILL
e. City and Country:	LIVERPOOL, L69 7ZX ENGLAND
f. Email and website:	sgrant@liverpool.ac.uk / www.liverpool.ac.uk
g. Signature:	SC Grant Date: 27/11/17

STATUS: **Recommended / ~~Not Recommended/Deferred~~**

REPUBLIC OF ZAMBIA
NATIONAL HEALTH RESEARCH AUTHORITY
08 FEB 2018
OFFICE OF THE DIRECTOR/CEO
P.O. BOX 30075
LUSAKA

2

VICE-CHAIRPERSON NHREC: Dr Lonia Mwape

Signature:

Date: 09.02.2018

CHAIRPERSON NHREC: Prof P Musonda

Signature:

Date: 08/02/2018

Official Stamp:

REPUBLIC OF ZAMBIA
NATIONAL HEALTH RESEARCH AUTHORITY
08 FEB 2018
OFFICE OF THE DIRECTOR/CEO
P.O. BOX 30075
LUSAKA

APPENDIX F DNA DATA EXTRACTION LOG-SHEET

DNA EXTRACTION LOG SHEET

EXTRACTIONS…….. **Date** ……

STUDY CODE	**Mls Used**	**Back-Up Sample**
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		

NOTES:

Position of samples corresponding to Prelabelled tubes in rack 2

10	11	12
7	8	9
4	5	6
1	2	3

Checked by ……

Chemagen machine used:……

Comment

……

……

……

……

……

APPENDIX G FORWARD AND REVERSE PCR PRIMERS 50 (µM) AND EXTENSION IPLEX PRIMERS (400 µM)- DESALTED AND UNMODIFIED USED FOR IPLEX MASSARRAY GENOTYPING

Sequence name	5' -3' Forward primer Sequence	3'-5' Reverse Primers sequence	Extension Primers
PRIMERS FOR THE 18-PLEX ASSAY			
rs899494	ACGTTGGATGTTCTTACACTTCCTGTGGGC	ACGTTGGATGCCCAGCAAGGCACGATATTC	ACCACTGCAGGCGAT
rs7899457	ACGTTGGATGATGATGGTCAGCTTCTCTCG	ACGTTGGATGCTCTTCAGAATCTTAGAGGC	gCTCTCGGAGGTCGTG
rs2274409	ACGTTGGATGTGTGCCTGCCGCTGATAAG	ACGTTGGATGGGCATCCGTGAAAGTTGCAG	gGGGCAGGCTGTGATC
rs1059751	ACGTTGGATGCATTTATGGAAGGCTAACCC	ACGTTGGATGAGCAGAGCCTATTCATGCAA	GCTAACCCTCTGTTGAC
rs284301	ACGTTGGATGTAACGCATCTGGATGCCAAG	ACGTTGGATGTCCACGACAGATACTGGAAG	TAAAGCCATGACCCTGT
rs3740066	ACGTTGGATGCCTTCACTCCACCTACCTTC	ACGTTGGATGTTAACAACTACCAAGTGCGG	tcCCTTCTCCATGCTACC
rs11231809	ACGTTGGATGGCAAATACATGAGGTGGTGG	ACGTTGGATGCTCACCTGTTCCGTGATTTC	ATCGTTTGTAAGGACTCA
rs11568658	ACGTTGGATGGATTGACTATCTGGCCTGTG	ACGTTGGATGTTTCAGGCACTTCGTCTTAG	cccCCTGTGGTTGTCTTCC
rs284265	ACGTTGGATGAGTCTGCAAAAGACCACTCG	ACGTTGGATGTTAAAAGCTCTGTGTTGCGG	tgcaAGCCCTTGCTGTTCC
rs11767816	ACGTTGGATGGTAAGTCGTGCCTCTGTGTC	ACGTTGGATGGCAGGGAGGAGGCATTTTAC	tgcgCCAGCAGGGACTGGG
rs3742106	ACGTTGGATGGACAGCACTGTGAATCCAAC	ACGTTGGATGCAATGTGGTTTACATAGTCC	cttaCGTTCCGAAGGCATTT
rs9349256	ACGTTGGATGTCCTGGCAATAAGCCAACTC	ACGTTGGATGAGAATGCCGGGTTGAGCTGT	cttaACTCTCTCCTGACCTTT
rs284267	ACGTTGGATGATGTCCTCATCTTGTTCTCG	ACGTTGGATGGAGGAAGATGACACTCAAGG	CTCGTTCTAATATTCTAACCC
rs2273697	ACGTTGGATGTGGTCACATCCATGAGCTTC	ACGTTGGATGTATCCAACTTGGCCAGGAAG	catccGGTTCACTGTTTCTCCAA
rs2834254	ACGTTGGATGTCAGTCAGAACAGATAGTCC	ACGTTGGATGCCCTCTTCTTCTCACTGAAC	GAACAGATAGTCCAGAAATAGTT
rs17222519	ACGTTGGATGAGTCCCATGAAGTTCCTGTC	ACGTTGGATGTGTAGGTAATGCTACTCAGG	aTGTCTCCAATTGGTTTACATTTC
rs113165732	ACGTTGGATGATAGGAAAACCTCCAGTGGG	ACGTTGGATGTGTCTCTTCCCCCTCATTGC	tcGAGTATAAGAACCAACAGAAAA
rs17216177	ACGTTGGATGTCAGGGTTTGTGTGATCTAC	ACGTTGGATGGGTTTGAGTGGTTGAGTTGG	cAACCAGAAGACTGAAAATCATCAT
PRIMERS FOR THE 6-PLEX ASSAY			
rs2125739	ACGTTGGATGAGTTTTGGTTACCGACAGCC	ACGTTGGATGGGTAGAAACGGATGTCTGAG	CCTCACCACCCAGCA
rs717620	ACGTTGGATGAGCATGATTCCTGGACTGCG	ACGTTGGATGCCTGTTCCACTTTCTTTGATG	GACTGCGTCTGGAAC
rs7057639	ACGTTGGATGTGCGGTCAAACTGCGGAAC	ACGTTGGATGAAACCCATTCCTTGCTGGAC	CGGAACTGCAGCGAGA
rs79174032	ACGTTGGATGTAGTGTAGTCTAGCTGGCTG	ACGTTGGATGCAATCTGCCAGAGAAAAGCC	AGAGGCATCCTTGGAGG
rs8187707	ACGTTGGATGACACTCACTTGTCACTGTCC	ACGTTGGATGACCATCCAAAACGAGTTCGC	cggCACTGTCCATGATGGT
rs1751034	ACGTTGGATGCAACTGAAATTGGACTTCACG	ACGTTGGATGTAAAAGGGGCAGGTAAGGAC	CTTCACGATTTAAGGAAGAA

APPENDIX H PATIENT INFORMATION LEAFLET AND CONSENT FORM FOR THE BIOMARKER STUDY

Title of The Research: "A Pharmacogenetics and Biomarkers Study of Tenofovir - Induced Renal Disease in HIV-Positive Patients in Lusaka, Zambia."

Name of Researcher: Miss Audrey Hamachila

University of Liverpool, Department of Molecular and Clinical Pharmacology

Institute of Translational Medicine; 1-5 Brownlow Street, Liverpool L69 3GL

Mobile: +260974962365 / +447563353229

haudrey@liverpool.ac.uk

This informed consent form has two parts;

1. Information Sheet (to share information about the research with you)
2. Certificate of Consent (for signatures if you agree to take part)

PART I: INFORMATION LEAFLET.

This research is being conducted by Audrey Hamachila a PhD student at the University of Liverpool, UK. The research on kidney disease caused by tenofovir, a drug commonly used for treating HIV-infection in Zambia. The information given here is for you to use in deciding whether you will participate in the research or not. Please ask any questions you feel like either to me or your doctor for clarification.

PURPOSE OF THE RESEARCH:

HIV-infection is a common disease in our country. The medicines that are currently used to treat HIV are good but some may cause some side effects in some patients. For example, tenofovir a drug found in 'Atripla' and 'Truvada' is known to cause kidney side effects. This research wants to find out if once a patient is started on Tenofovir, measuring specific proteins in the blood and urine would help to predict the possibility of developing kidney side effects.

We are inviting everyone who will be started on tenofovir to participate in this research study. Your participation in this research is entirely voluntary. Your choice to participate or not to will not affect any future services that you receive at this hospital.

What will happen if I chose to participate in the study?

If you choose to participate, you will be asked to donate a sample of blood (10ml or 2 teaspoons full) and a sample of urine at three (3) time points: 1. before you start treatment, 2. at two (2) weeks after you have started treatment and 3. at 4 weeks after starting treatment. These times correspond to the times that you would normally come for your reviews.

Your samples will be coded so that none of your personal details will be known to anyone outside the research team. The samples will then be transferred for analyses to the University of Liverpool, United Kingdom. Your samples will be stored safe and under secure conditions for this study and any follow up tests or analysis that may be necessary during this research study. Any remaining samples will be destroyed accordingly.

This research may help us to identify people with a risk of developing kidney side effects while taking tenofovir and provide measures to prevent the occurrence. Research results will be communicated to the clinicians who may use it for future clinical interventions.

You are free to free to ask further questions now or later by contacting the researcher on the contact details stated above.

PART II: CERTIFICATE OF CONSENT

I have read the information about the research / the information has been read and or explained to me in my local language. I have had the opportunity to ask questions about the research, and all questions that I have asked have been answered to my satisfaction.

I therefore voluntarily consent to participate in this research study and donate blood and urine samples that will be used for this study and any follow up tests or analysis that may be necessary. I have also been informed that upon completion of the study, research results will be shared with my clinicians.

Print Name of Participant / Witness*_____**Signature _____**

Date _____

*If the participant is unable to sign /write; a witness selected by the participant and not connected to the researcher can sign.