A pilot investigation on plasma tenofovir levels and possible side effects in HIV-infected women

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Abstract

Tenofovir disoproxil fumarate (TDF) is a nucleotide reverse transcriptase inhibitor and a prodrug of tenofovir (TFV). It is the currently recommended first line combination treatment of human immunodeficiency virus (HIV) infection in adults. Various clinical studies have associated treatment with a TDF-containing antiretroviral therapy (ART) regimen with reduced bone mineral density (BMD) and renal dysfunction. Hardly any studies to date have correlated plasma TFV concentration with markers of renal function and bone turnover (BTM). This knowledge is also unavailable in the South African public health care system. Hence, the correlations between plasma TFV concentration and renal function markers and BTM in HIV-infected women were investigated. Renal function markers and BTM in HIV-infected women were compared with those in HIV-uninfected control women.

A pilot cross-sectional sub-study within the Prospective Urban and Rural Epidemiology (PURE) South Africa study was conducted. Sixty women participated, of which 30 HIV-infected women were matched for age and body mass index with 30 HIV-uninfected ones. Ethics approval was obtained from the North-West University, Human Research Ethics committee (NWU-00016-10-A1) on 12 April 2013 to conduct this sub-study and the North West Department of Health, Mmabatho on 08 August 2013 to access patient health information.

A validated high-performance liquid chromatography tandem mass spectrometry method was developed to analyse TFV in plasma. Renal markers measured were the estimated glomerular filtration rate (eGFR), creatinine clearance (CrCl), albuminuria, serum creatinine (SCr), serum urea, serum uric acid, glucosuria, urine sodium (UNa) and maximum tubular reabsorption of phosphate ($TmPO_4$/GFR). The BTM markers measured included C-terminal telopeptide (CTx), alkaline phosphatase (ALP), parathyroid hormone (PTH), total vitamin D (VitD), serum calcium (SrCa), serum phosphate (SrP) and BMD. BMD was assessed using the DTX-200 peripheral DXA system (Osteometer MediTech, Hawthorn, California, USA). Renal and bone markers were analysed on Elecsys® 2010 and COBAS INTERGRA® 400 plus (Roche

Diagnostics, Switzerland). Baseline data for HIV-infected participants with regard to CD4+ cell count, SCr prior to TDF initiation, time since TDF initiation, weight prior to TDF initiation and time since HIV diagnosis were collected retrospectively from participants' public health care files. Statistical analyses applied were linear regression, analysis of covariance, the Mann-Whitney U test, paired t-test and unpaired t-test. IBM® SPSS® Statistics software 22 was used to perform all the statistical analyses.

The median and interquartile range of plasma TFV concentration was 113 (74-139.4) ng/mL (n=25) and no TFV was detected in five participants' plasma. Adjusted analyses showed TFV concentration to be associated with albuminuria (adjusted r^2 = 0.339; p = 0.001). Values of CrCl, eGFR and albuminuria (p = 0.032; p = 0.038; p = 0.048, respectively) were significantly higher in HIV-infected women compared to HIV-uninfected women. CrCl [112 (84-137) mL/min] and eGFR [134 (93-153) mL/min/1.73m^2] values were abnormally high in HIV-infected women. There was also an increase in both CrCl and eGFR (p = 0.008; p < 0.001, respectively) from baseline to median follow-up of 16.6 (8.8-23.4) months in HIV-infected women. At a TFV plasma concentration of ≥ 120 ng/mL, CTx and ALP correlated positively (r = 0.704; p = 0.016). ALP (112 ± 28 U/L; p < 0.001), CTx (0.68 ± 0.4 ng/mL; p = 0.027) and PTH (56.3 ± 32 pg/mL; p = 0.050) were higher in HIV-infected women compared to HIV-uninfected women. CD4+ cell count increased from baseline to follow-up in HIV-infected women (+250 cells/mm^3; p = 0.001).

In HIV-infected women on a TDF-based regimen, TFV plasma concentration is associated with an increase in albuminuria, while perturbations in BTM equilibrium occur at ≥ 120 ng/mL of TFV plasma concentration. Abnormally higher CrCl and eGFR are present in HIV-infected women, seen as glomerular hyperfiltration compared with HIV-uninfected women. There was immunological improvement with TDF-based ART in HIV-infected women. Longitudinal studies with larger sample sizes are needed to confirm these findings.

Keywords: TDF, HIV infection, plasma tenofovir, renal dysfunction, bone turnover, ART

Opsomming

Tenofovirdisoproksielfumaraat (TDF) is ’n nukleosied trutranskriptase-remmer en ’n voorloper van die geneesmiddel, tenofovir (TFV). Dit word tans aanbeveel as die eerstelinie antiretrovirale behandeling teen die menslike immuniteitsgebrekvirus (MIV) in volwassenes. Verskeie kliniese studies het behandeling met antiretrovirale (ARV) kombinasie geneesmiddels wat TDF bevat, met verlaagde beenmineraaldigtheid (BMD) en nierdisfunksie geassosieer. Baie min studies het tot dusver TFV-plasmakonsentrasie gekorreleer met merkers van nierfunksie en beenomset (BO). Hierdie kennis is ook nie in die Suid-Afrikaanse openbare gesondheidsorgstelsel beskikbaar nie. Daarom is die korrelasies tussen plasma TFV-konsentrasie met nierfunksiemerkers en BO in MIV-geïnfekteerde vroue vergelyk met dié in MIV-ongeïnfekteerde kontrolevroue in hierdie studie.

’n Loods dwars deursnit substudie is uitgevoer as deel van die *Prospective Urban and Rural Epidemiology (PURE) South Africa*-studie. Sestig (60) vroue het aan die studie deelgeneem. Dertig (30) MIV-geïnfekteerde vroue is vergelyk met dertig (30) MIV-ongeïnfekteerde kontrolevroue met dieselfde ouderdom en liggaamsmassa indeks. Etiese goedkeuring is op 12 April 2013 van die Menslike Navorsing Etiekkomitee van die Noordwes-Universiteit verkry (NWU-00016-10-A1) om hierdie substudie uit te voer. Verdere goedkeuring is ook ontvang van die Noord Wes Departement van Gesondheid, Mmabatho op 08 August 2013 om inligting oor pasiënte se persoonlike gesondheid te bekom vanuit hulle rekords.

’n Gevalideerde vloeistofchromotografie-tandem massaspektrometriese metode is ontwikkel om TFV in plasma te analiseer. Die volgende niefunksiermerkers is gemeet: beraamde glomerulêre filtrasietempo (eGFR), kreatinienopruiming (CrCl), albumienurie, serumkreatinien (SCr), serum-ureum, serum-uriensuur, glukosurie, urien-natrium (UNa) en maksimum-tubulêre herabsorpsie van fosfaat ($TmPO_4$/GFR). Die BO-merkers wat gemeet was, sluit in C-terminale telopeptied (CTx), alkaliese fosfatase (ALP), paratiroïedhormoon (PTH), totale serum vitamien D (VitD), serumkalsium (SrCa), serumfosfatase (SrP) en BMD. BMD is met die DTX-200 perifere DXA-sisteem bepaal

(Osteometer MediTech, Hawthorn, California, USA). Nier- en beenmerkers is op Elecsys® 2010 en COBAS INTERGRA® 400 plus (Roche Diagnostics, Switserland) geanaliseer. Basislyndata vir MIV-geïnfekteerde deelnemers betreffende hulle CD4+ seltelling, SCr en gewig voor aanvang van TDF-terapie, tydsverloop sedert aanvang van TDF-terapie en sedert die diagnose van MIV was retrospektiewelik verkry uit deelnemers se publieke gesondheidsorg-lêers. Die volgende statistiese analises is uitgevoer: lineêre regressie, kovariansie analise, die Mann-Whitney U-toets, gepaarde en onafhanklike t-toetse. IBM® SPSS® Statistics-sagteware 22 was gebruik om al die statistiese analises uit te voer.

Die mediaan- en interkwartielverspreiding van die TFV-plasmakonsentrasie was 113 (74-139.4) ng/mL (n=25) en in vyf deelnemers se plasma is geen TFV gevind nie. Aangepaste analises het aangetoon dat TFV-plasmakonsentrasie verband hou met albuminurie (aangepas r^2 = 0.339; p = 0.001). Waardes van CrCl, eGFR en albuminurie (p = 0.032; p = 0.038; p = 0.048, respektiewelik) was beduidend hoër in MIV-geïnfekteerde vroue in vergelyking met diegene wat nie met MIV geïnfekteer was nie. Die CrCl [112 (84-137) mL/min] en eGFR [134 (93-153) mL/min/1.73m^2] waardes was abnormaal hoog in MIV-geïnfekteerde vroue. Daar was ook ’n toename in beide CrCl en eGFR (p = 0.008; p < 0.001, respektiewelik) van die basislyn na mediaan opvolg van 16.6 (8.8-23.4) maande in MIV-geïnfekteerde vroue. In TFV-plasmakonsentrasie ≥ 120 ng/mL het CTx en ALP positief gekorreleer (r = 0.704; p = 0.016). ALP (112 ± 28 U/L; p < 0.001), CTx (0.68 ± 0.4 ng/mL; p = 0.027) en PTH (56.3 ± 32 pg/mL; p = 0.050) was hoër in MIV-geïnfekteerde vroue vergeleke met MIV-ongeïnfekteerde vroue. CD4+ seltelling het toegeneem vanaf die basislyn tot opvolg in MIV-geïnfekteerde vroue (+250 cells/mm^3; p = 0.001).

In MIV-geïnfekteerde vroue op TDF-gebaseerde behandeling is TFV-plasmakonsentrasie geassosieer met ’n toename in albumienurie, terwyl versteurings in BO-balans voorgekom het by TFV-plasmakonsentrasie ≥ 120 ng/mL. Abnormaal hoër CrCl en eGFR het voorgekom in MIV-geïnfekteerde vroue, wat beskou word as glomerulêre hiperfiltrasie, vergeleke met MIV-ongeïnfekteerde vroue. Immunologiese verbetering het voorgekom met die gebruik van TDF-gebaseerde ARV behandeling in MIV-geïnfekteerde vroue. Longitudinale studies met groter steekproefgroottes is nodig om hierdie bevindinge te bevestig.

Sleutelwoorde: TDF, MIV-infeksie, plasma-tenofovir, nierdisfunksie, beenomset, ART

Conference Proceedings

Parts of the results obtained from the current study were presented as follows:

a) MULUBWA M., RHEEDERS M., KRUGER I., VILJOEN M. **Associations between plasma tenofovir concentration and bone metabolism markers in HIV-infected women**. Presented as a podium presentation at the Provincial Research Conference, Department of Health, North West Province, held in Mafikeng, South Africa on 2nd October 2014.

b) MULUBWA M., RHEEDERS M., FOURIE C., KRUGER I., DU PLESSIS L., GROBLER A., VILJOEN M., **Tenofovir plasma concentration is a strong predictor of albuminuria in HIV-1-infected women**. Presented as a poster presentation at the 17th World Congress of Basic and Clinical Pharmacology, held in Cape Town, South Africa 13-18 July 2014.

c) MULUBWA M., RHEEDERS M., FOURIE C., KRUGER I.,VILJOEN M., **Bone density and renal function markers in tenofovir exposed and non-exposed black South African women**, *Basic & Clinical Pharmacology & Toxicology,* 115 (2014) 54-91, Issue Supplement s1 (Abstract).Presented as a poster presentation at the 17th World Congress of Basic and Clinical Pharmacology, held in Cape Town, South Africa 13-18 July 2014. Awarded second prize in Clinical Pharmacology.

The abstract, posters presentations and certificate of award are provided in Addendum A.

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List of Abbreviations

ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
ALP	Alkaline phosphatase
ANCOVA	Analysis of covariance
ART	Antiretroviral therapy
AZT	Zidovudine
ATV/r	Ritonavir boosted atazanavir
α1MG	Alpha 1 microglobulin
BALP	Bone specific alkaline phosphatase
BMI	Body mass index
BMD	Bone mineral density
BTM	Bone turnover markers
β2MG	Beta 2 microglobulin
CAS	Chemical abstract service
CG	Cockroft-Gault
CKD-EPI	Chronic kidney epidemiology collaboration
CKD	Chronic kidney disease
C_{max}	Maximum concentration
C_{min}	Minimum concentration
CrCl	Creatinine clearance
CSF	Cerebro-spinal fluid
CTx	C-terminal telopeptide

CV	Coefficient of variation
DNA	Deoxyribonucleic acid
D4T	Stavudine
EDTA	Ethylenediaminetetra acetic acid
EFV	Efavirenz
eGFR	Estimated glomerular filtration rate
FTC	Emtricitabine
GFR	Glomerular filtration rate
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HPLC-MS/MS	High performance liquid chromatography tandem mass spectrometry
HQC	High quality control
ISTD	Internal standard
IQR	Interquartile range
KTD	Kidney tubular dysfunction
LPV/r	Ritonavir boosted lopinavir
LLOQ	Lower limit of quantification
LQC	Low quality control
M-CSF	Macrophage colony-stimulating factor
MDRD	Modified diet in renal disease
MRP4	Multidrug resistance-associated protein 4
MRP7	Multidrug resistance-associated protein 7
mtDNA	Mitochondrial deoxyribonucleic acid
MQC	Medium quality control
NGAL	Urine neutrophil gelatinase-associated lipocalin
NHLS	National Health Laboratory Services

NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NTX	N-terminal cross-linked telopeptide of type 1 collagen
NVP	Nevirapine
NWU	North-West University
OAT1	Baso-lateral membrane organic anion transporter 1
OAT3	Baso-lateral membrane organic anion transporter 3
OPG	Osteoprotegerin
OR	Odds ratio
PHC	Primary Health Care
PI	Protease inhibitor
PURE	Prospective Urban and Rural Epidemiology
PURE-SA	Prospective Urban and Rural Epidemiology-South Africa
P1CP	C-terminal propeptide of type 1 collagen
P1NP	N-terminal propeptide of type 1 collagen
PTH	Parathyroid hormone
QC	Quality control
RANK	Receptor activator of nuclear factor κβ
RANKL	Receptor activator of nuclear factor κβ ligand
RI	Renal impairment
RNA	Ribonucleic acid
RSD	Relative standard deviation
RTV	Ritonavir
SCr	Serum creatinine
S-Cys C	Serum cystatin C
SNP	Single nucleotide polymorphism
SrCa	Serum calcium

SrP	Serum phosphate
TDF	Tenofovir disoproxil fumarate
TDP	Tenofovir diphosphate
TFV	Tenofovir
$TmPO_4/GFR$	Maximum tubular reabsorption of phosphate
3TC	Lamivudine
UCr	Urine creatinine
UNa	Urine sodium
UP	Urine phosphate
VitD	Vitamin D
VL	Viral load

SYMBOLS AND UNITS

α	Alpha
β	Beta
κ	Kappa
γ	Gamma
k	Constant
kg	Kilograms
kDa	Kilo dalton
mg	Milligrams
U/L	Units per litre
L/h	Litre per hour
mmol/L	Millimoles per litre
mg/L	Milligrams per litre
L/kg	Litre per kilogram
µmol/L	Micromole per litre
mL/min	Millilitre per minute
pg/mL	Picograms per millilitre
µg/mL	Microgram per millilitre
mg/dL	Milligrams per decilitre
ng/mL	Nanograms per millilitre
µg•hr/mL	Microgram-hour per millilitre
kg/m^2	Kilograms per square metre
g/cm^2	Grams per square centimetre
$mL/min/1.73m^2$	Millilitre per minute per 1.73 square metres

Introduction | Chapter 1

1.1 PROBLEM STATEMENT

By the end of 2012, 34 million people worldwide had been infected with human immunodeficiency virus (HIV) and by the end of 2013 close to 12.9 million people were receiving antiretroviral therapy (ART). Sub-Saharan Africa is the most severely affected, accounting for 69% of the HIV-infected people globally. Scaling up of ART has generated health gain, with a drop in HIV/acquired immunodeficiency syndrome (AIDS) mortality and a decline in incidence (Ortblad *et al.*, 2013; UNAIDS-Global Report, 2014; UNAIDS-Gap Report, 2014) "Despite the recent declines in global HIV/AIDS mortality, today, HIV/AIDS remains one of the leading global causes of both mortality and burden" (Ortblad *et al.*, 2013).

The World Health Organisation recommends ART regimens comprising either a combination of tenofovir disoproxil fumarate/emtricitabine (TDF/FTC) or tenofovir disoproxil fumarate/lamivudine (TDF/3TC) and efavirenz as first line therapy in adults (WHO, 2013).

The South African Department of Health published new clinical guidelines for the management of HIV and AIDS in adults and introduced TDF, a prodrug of tenofovir (TFV) as part of first line ART in 2010. The number of South Africans receiving ART increased to 33% of the HIV-infected population in 2013. The guidelines recommend initiation of a TDF-based regimen in patients with a creatinine clearance (CrCl) greater than 50 mL/min (Brennan *et al.*, 2011; NDoH, 2010; UNAIDS-Gap Report, 2014).

TDF has however been linked to renal failure (Gara *et al.*, 2012; Mathew & Knaus, 2006; Menezes *et al.*, 2011) and reduction in bone mineral density (BMD) in the literature (Haskelberg *et al.*, 2012; Liu *et al.*, 2011; McComsey *et al.*, 2011). Furthermore, hypophosphatemia in patients on a TDF-based regimen has been reported and is the clinically most important parameter that can predict early symptoms of renal dysfunction (Hall, 2012). It is a priority in South Africa to continue

pharmacovigilance monitoring of the safety of TDF as part of the first line ART regimen with regard to its nephrotoxicity and bone toxicity risk (Mehta *et al.*, 2014).

Despite TDF treatment being associated with nephrotoxicity in a recent study conducted in South Africa (Brennan *et al.*, 2011), no plasma TFV concentrations were determined. Hence, no associations between plasma TFV concentration and the observed nephrotoxicity could be investigated. Besides, no other significant parameters of renal tubular function or bone metabolic markers were measured apart from CrCl. It has been recommended in the literature to examine the correlation between plasma TFV concentrations and probable adverse effects on the kidney and bone metabolism in patients (Rodríguez-Nóvoa *et al.*, 2010; Scherzer *et al.*, 2012).

In light of the above, comprehensive investigations of the relationship between plasma TFV concentration and renal tubular function and bone metabolic markers are urgently called for.

1.2 STUDY OBJECTIVES

The primary aim of this study was to investigate the correlation between plasma TFV concentration and markers of renal function or bone turnover in HIV-infected black South African women receiving a TDF-based ART regimen.

1.2.1 Primary objectives

- To develop and validate a high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method to determine TFV concentrations in human plasma.

- To determine plasma TFV concentrations in HIV-infected female participants on a TDF-based ART regimen in the PURE-SA sub-study.

- To investigate the correlation between plasma TFV concentrations and renal function markers [estimated glomerular filtration rate (eGFR), CrCl, albuminuria, maximum renal tubular reabsorption of phosphate ($TmPO_4$/GFR), serum urea, serum creatinine (SCr), serum uric acid, glucosuria or urine sodium (UNa)] in HIV-infected female participants.

- To investigate possible correlations between TFV concentration and bone turnover markers [C-terminal telopeptide (CTx), alkaline phosphatase (ALP), parathyroid hormone (PTH), serum phosphate (SrP), serum calcium (SrCa) or total vitamin D (VitD)] in HIV-infected female participants.

- To compare renal function and bone turnover markers (BTM) between HIV-infected female participants on a TDF-based ART regimen and matched female HIV-uninfected controls within the PURE-SA sub-study.

1.2.2 Secondary objectives

- To track immunological recovery/status by comparing CD4+ cell count values between baseline and follow-up in HIV-infected female participants on a TDF-based ART regimen.

- To evaluate renal function status by comparing CrCl and eGFR between baseline and follow-up in HIV-infected female participants on a TDF-based ART regimen.

1.3 STRUCTURE OF THIS DISSERTATION

This dissertation is presented in article format. All chapters in this dissertation have their own reference list provided at the end of each chapter and are arranged as follows:

Chapter 1: Introduction.

Chapter 2: Literature review.

Chapter 3: Materials and methods.

Chapter 4: Manuscript A: Development and validation of high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for determination of tenofovir in small volumes of human plasma (submitted for publication).

Chapter 5: Manuscript B: Associations between plasma tenofovir concentration and renal function markers in HIV-infected women (pending submission).

Chapter 6: Manuscript C: Associations between plasma tenofovir concentration and bone turnover markers in HIV-infected women (pending submission).

Chapter 7: Additional results and discussion not covered in Manuscripts A-C.

Chapter 8: Conclusions and recommendations.

Addendum A: Conference proceedings: abstract, two poster presentations and certificate of award (2nd prize Clinical Pharmacology).

Addendum B: Instructions to the Author: Journal of Pharmaceutical and Biomedical Analysis, AIDS Research and Human Retroviruses and European Journal of Clinical Pharmacology.

Addendum C: Study protocol and GLP laboratory protocol.

Addendum D: Ethics clearance letters, informed consent forms for original PURE study at recruitment for 2005, bone sub-study from 2010 to 2013, TDF data and access to patients' records from 2012 to 2013 and medication questionnaire.

Addendum E: Summary statistics of all variables, self reported information on alcohol consumption and smoking status, participants' plasma TFV concentrations, plasma dilutions and correlations between TDF treatment exposure and VitD status.

1.4 CONTRIBUTION OF AUTHORS TO THE MANUSCRIPTS PRESENTED IN THIS DISSERTATION

The roles and responsibilities of the authors who were involved in this study and manuscripts presented in this dissertation are provided in **Table 1.1**.

Table 1.1 **Contributions from authors**

AUTHOR	AFFILIATION	ROLE
Mr M Mulubwa (MSc Student)	Pharmacen, Division of Pharmacology, NWU.	First author, preparation of laboratory protocol for HPLC-MS/MS method development and validation of plasma TFV, clinical sample collection, statistical analyses in consultation with Statistical Consultation Services, NWU, writing manuscripts and dissertation.
Dr M Viljoen (Supervisor)	Pharmacen, Division of Pharmacology, NWU.	Supervisor of M Mulubwa, PURE-SA data collection, protocol and informed consent, liaison with Ethics (NWU and NW DoH), liaison with Tlokwe / Ganyesa PHC and hospitals, liaison with Lancet and NHLS, guidance on writing of manuscripts and dissertation.
Dr M Rheeders (Co-supervisor)	Pharmacen, Division of Pharmacology, NWU.	Co-supervisor of M Mulubwa, guidance with writing of protocol, manuscripts and dissertation
Dr C Fourie	HART, School for Physiology, Nutrition and Consumer Science, NWU.	Co-author, advice on aspects of renal physiology (Manuscript 2).
Dr I M Kruger	Africa Unit for Transdisciplinary Health Research, NWU.	Co-author, advice on aspects of bone metabolism (Manuscript 3), PURE-SA project leader.
Mrs L du Plessis	DST/NWU Preclinical Drug Development Platform, NWU.	Co-author (Manuscript 1), HPLC-MS/MS operations, technical aspects and validation.
Prof. A Grobler	Director: DST/NWU Preclinical Drug Development Platform, NWU.	Co-author (Manuscript 1).

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Literature Review | Chapter 2

2.1 INTRODUCTION

This literature review will explore the clinical pharmacology (pharmacokinetics, mechanism of action, pharmacodynamics and toxicity) of TDF. It will also address in general the physiology and mechanisms associated with renal dysfunction and bone loss. Furthermore, this Chapter examines the influence of TDF and plasma TFV concentration on renal function and bone turnover. Gaps in literature are identified.

2.2 CLINICAL PHARMACOLOGY OF TDF

2.2.1 Pharmacokinetics

TDF is a water-soluble prodrug (characterised by greater bioavailability and cellular penetration), which is converted to the active ingredient TFV in plasma. The oral bioavailability of TDF is approximately 25% in the fasting state and enhanced by a high-fat meal (Gagnieu *et al.*, 2008) with a first-order absorption rate constant of 1.03 h^{-1} (Baheti *et al.*, 2011). A single oral dose of 300 mg TDF achieves maximum serum TFV concentrations (C_{max}) of 0.30 ± 0.09 µg/mL after 1.0 ± 0.4 hours with an area under the curve of 2.29 ± 0.69 µg•hr/mL in adults (Stöppler, 2013). TFV pharmacokinetics is similar in male and female subjects; it is dose-proportional over a dose range of 75 to 600 mg and is not affected by repeated dosing (Stöppler, 2013).

Binding to human plasma or serum proteins is less than 0.7 and 7.2%, respectively, over the TFV concentration range of 0.01 to 25 µg/mL (Stöppler, 2013). TFV concentrations in the cerebro-spinal fluid (CSF) are only 5% of plasma concentrations, suggesting limited transfer into the CSF, and possibly active transport out of the CSF (Best *et al.*, 2012). In adults, TFV has a volume of distribution of 0.813 L/kg (Fung *et al.*, 2002) and is best described by a two-compartment model (Baheti *et al.*, 2011). Apparent and inter-compartment clearance is 42 L/h and 181 L/h respectively (Baheti *et al.*, 2011).

Studies have indicated that neither TDF nor TFV is a substrate for cytochrome P-450 enzymes. Following a single oral dose, the terminal elimination half-life of tenofovir is approximately 17 hours. If TFV concentrations are measured between seven and 14 days after a single oral dose, the elimination half-life is 47 hours. TFV is eliminated by a combination of glomerular filtration and active tubular secretion and is mainly excreted unchanged (70%–80%) in urine. There may be competition for elimination with other compounds that also undergo renal elimination (Fung *et al.*, 2002; Patterson *et al.*, 2011; Stöppler, 2013). The renal clearance of TFV is higher than calculated creatinine clearance (CrCl), as they are both filtered and actively secreted in the tubules (Antoniou *et al.*, 2003). Renal toxicity and bone loss are the two major side effects of TFV and will be discussed in depth later in this chapter.

2.2.2 Mechanism of action

TDF is a nucleotide reverse transcriptase inhibitor, which undergoes initial diester hydrolysis in plasma for conversion to TFV. After intracellular uptake, TFV is phosphorylated by cellular enzymes to form tenofovir diphosphate (TDP), an active metabolite. TDP inhibits the activity of HIV-1 reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate for incorporation into deoxyribonucleic acid (DNA). This leads to termination of DNA synthesis, since it lacks the hydroxyl group in the 3'-position, which acts as the point of attachment for the next deoxyribonucleoside triphosphate. TFV is a weak inhibitor of mammalian DNA polymerases α, β, and mitochondrial DNA polymerase γ (Fung *et al.*, 2002; Kearney *et al.*, 2004; Stöppler, 2013).

2.2.3 Pharmacodynamics

TDF has a virological and immunological effect by decreasing HIV-1 and HIV-2 ribonucleic acid (RNA) levels in plasma and increasing the CD4+ cell count. The effect is optimal at a daily dose of 300 mg. TDF is also active against hepatitis B virus, simian immunodeficiency virus and feline immunodeficiency virus (Barditch-Crovo *et al.*, 2001; De Clercq, 2011; Esser *et al.*, 2011).

2.3 RENAL PHYSIOLOGY

The kidney is a body organ of which the function is mainly to excrete metabolic waste. It also regulates arterial blood pressure, body fluid osmolality, electrolyte concentration

and acid-base balance and has an endocrine function. The excretory function of the kidney is performed by a nephron, a basic functional unit that consists of the glomerulus and renal tubule. Metabolic waste products that are excreted by the nephron include urea (protein metabolic waste), creatinine (muscle metabolic waste), uric acid (nucleic acid metabolic waste), hormone metabolites, drugs and toxins. The quantities of these substances in the urine reflect the integrated functions of the nephron, which are glomerular filtration, tubular reabsorption and secretion. A larger portion of essential electrolytes, such as sodium, potassium, chloride, bicarbonate and compounds such as vitamins and glucose are reabsorbed in the proximal tubules via various transport mechanisms. Some peptides, small proteins and peptide hormones are also reabsorbed in the proximal tubules by the process of endocytosis. Other compounds are either secreted or reabsorbed in the renal tubules by passive or facilitated diffusion down electrical gradient, chemical gradient or active transport (Barrett *et al.*, 2010). These coordinated mechanisms can be altered in certain disease conditions, such as drug-induced nephropathy, in which nitrogenous substances that are normally cleared by the kidneys, such as urea and creatinine, accumulate in the plasma. Increased plasma creatinine concentrations are an indication of a reduced glomerular filtration rate (GFR). Substances that are normally reabsorbed in the renal tubules also appear in large quantities/numbers in urine, which is an indication of tubular damage (Longo *et al.*, 2012).

2.3.1 Mechanism of TFV associated renal toxicity

The actual mechanism by which TFV causes renal dysfunction is not well understood. A direct effect of TFV on the renal tubular cells has been proposed. The proximal tubule is intrinsically vulnerable to mitochondrial dysfunction because of limited anaerobic adenosine triphosphate-generating capacity. TFV undergoes renal elimination by active tubular secretion and glomerular filtration. It is taken up into proximal tubule cells via baso-lateral membrane organic anion transporters (OAT1 and OAT3). TFV subsequently exits across the apical membrane via multidrug resistance-associated protein 4 (MRP4) and possibly also via MRP2. It causes renal proximal tubular mitochondrial ultra-structural abnormalities and depletes mitochondrial deoxyribonucleic acid (mtDNA) in the same cells. Genetic polymorphisms in proximal tubule transporters might predispose certain individuals to accumulating high intracellular TFV levels and theoretically could increase the risk of developing tubular toxicity. This could help to

explain why toxicity occurs in some individuals but not in others. A single nucleotide polymorphism (SNP) on the ABCC4 gene, which encodes for MRP4, was associated with a higher plasma TFV level, while SNP on the ABCC2 gene, which encodes MRP2, has been associated with tubular dysfunction in patients taking TDF. More recently, MRP7, encoded by the ABCC10 gene, has also been associated with TDF transport in the proximal tubules and risk of tubular toxicity (Hall, 2012; Hall *et al*., 2011; Kohler *et al*., 2009).

2.4 MARKERS OF RENAL FUNCTION

2.4.1 Markers of glomerular function

Glomerular function is normally evaluated by the use of SCr levels, blood urea nitrogen, eGFR and CrCl (Herget-Rosenthal *et al.*, 2007; Urbschat *et al.*, 2011). Creatinine is a derivative of an amino acid with a molecular weight of 113 kDa. It is freely filtered by the glomerulus and is also secreted by proximal tubular cells (Stevens *et al.*, 2006). Production of creatinine varies according to race, age, gender, muscle mass, muscle metabolism, body weight, nutritional status and hydration, thus it is not an ideal endogenous marker of glomerular function (Herget-Rosenthal *et al.*, 2007; Urbschat *et al.*, 2011). GFR is widely accepted as the best index of kidney function (Levey *et al.*, 2005).

There are three equations from which the GFR in adults is estimated from SCr:

- The Cockroft-Gault (CG) equation [$CrCl = \frac{(140-\text{Age}) \times \text{Weight} \times 0.85 \text{ (if female)}}{(0.814 \times \text{SCr})}$] was derived from a study that included 236 hospital inpatients in Canada (96% male, ethnicity not stated) with good kidney function. It includes body weight, age and SCr as variables (Cockcroft & Gault, 1976).

- The Modification of Diet in Renal Disease (MDRD) equation [$eGFR = 30849 \times SCr^{-1.154} \times Age^{-0.203} \times 0.742\ (if\ female) \times 1.212(if\ black)$] was derived from 1628 patients in the USA (651 women, 195 African-American) and includes age, SCr, gender and ethnicity as variables. For African-Americans, an ethnicity factor of 1.212 was established (Levey *et al.*, 2007).

- The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [$eGFR = 141 \times \min(\frac{SCr}{k}, 1)^{\alpha} \times \max(\frac{SCr}{k}, 1)^{-1.209} \times 0.993^{Age} \times 1.018\,(if\ female) \times 1.159(if\ black)$] was developed in the USA using data from 8 254 people (3 606 women, 1 728 African-Americans) and validated using data from 3 896 people (1 767 women, 384 African-American). The essence of developing the CKD-EPI equation was to create an equation that was more accurate than MDRD. The variables used in CKD-EPI are age, gender, ethnicity and SCr (Levey *et al.*, 2009).

- The World Health Organisation recommends the use of the CG and MDRD equations to estimate GFR as a way to monitor renal function in HIV-infected people on ART regimens (WHO, 2013). The MDRD and CKD-EPI values for eGFR were found to be more accurate and closer to the measured CrCl in a cluster-randomised trial (n = 944, 589 women) performed in Ghana if the ethnicity factor of 1.212 was omitted (Eastwood *et al.*, 2010). Similarly, a study that was performed in South Africa among blacks (n = 100, 49 women) showed that the MDRD equation was more accurate in calculating eGFR if the black ethnicity factor of 1.212 was omitted. The use of an ethnicity factor of 1.212, as suggested for African-Americans, overestimated eGFR in black South Africans (Van Deventer *et al.*, 2008).

- Another endogenous marker of glomerular function is cystatin C. It is a non-glycosylated low molecular weight (13 kDa) basic protein synthesised by most nucleated cells and is freely filtered by the glomerulus. Cystatin C is completely reabsorbed in the renal tubules and is not secreted (Dharnidharka *et al.*, 2002). Cystatin C production, unlike creatinine, is not affected by age, gender or muscle mass, hence serum cystatin C concentration is proposed to be an excellent surrogate marker of GFR (Knight *et al.*, 2004). Some studies have compared SCr-based predicting equations with serum cystatin C-based (S-Cys C) predicting equations. A study conducted among black South Africans (n = 100, 49 females) found an S-Cys C-based eGFR equation more accurate than a SCr-based eGFR equation (Van Deventer *et al.*, 2011). In another study of 5352 participants (40% black and 42% female), S-Cys C-based equation performed better than SCr-based equation. Overall the combined SCr-S-Cys C equation performed better than equations based on either S-Cys C or SCr alone (Inker *et*

al., 2012). However, S-Cys C in HIV-infected people correlates positively with HIV RNA and inversely with CD4+ cell count, thus S-Cys C decreases after suppression of HIV replication and leads to overestimation of eGFR. Hence the use of S-Cys C to estimate GFR in HIV-seropositive individuals is not recommended (Mauss *et al.*, 2008).

2.4.2 Markers of renal tubular dysfunction associated with TDF

Urine biomarkers specific for tubular injury provide an earlier indicator of impairment. Urine neutrophil gelatinase-associated lipocalin (NGAL) is a member of the lipocalin family of proteins that is secreted into the urine by the thick ascending limb of Henle and the collecting ducts of the kidney. Increasing concentrations of NGAL in urine are seen in the presence of epithelial injury and inflammation. N-acetyl-β-D-glucosaminidase is a proximal tubule lysosomal enzyme which, when present in the urine, suggests proximal tubular damage. β-2-microglobulin (β2MG) is a low molecular weight protein (13.7 kDa), found in all nucleated cells, freely filtered by glomeruli, and catabolised by the proximal tubules. α-1-microglobulin (α1MG) is a protein synthesised by the liver and readily bound to serum immunoglobulin A. Only its free unbound forms are filtered by the glomerulus and reabsorbed in the proximal tubules (Lisowska-Myjak, 2010; Oboho *et al.*, 2013). Some of the markers of tubular injury associated with TDF are outlined in the following studies:

- In a single-centre cross-sectional study, Japanese patients (n=190) with HIV infection were treated with TDF for a median duration of 71.5 weeks. Both urinary β2MG and α1MG were identified as good screening markers with high diagnostic accuracy among the tubular markers examined. These two markers were potentially suited for screening TFV-induced kidney tubular dysfunction (KTD) (Nishijima *et al.*, 2013).

- A cross-sectional study was conducted among HIV-infected women (n=132) to evaluate changes in urinary biomarkers over time in a group of women initiating a TDF-based ART regimen, compared with women not initiating TDF or not on ART. The proportion of women with elevated β2MG (> 0.5 µg/mL) increased from 7% to 40% among TDF ART users ($P < 0.01$), from 11% to 16% among non-TDF ART users ($P = 0.29$), and 13% to 18% among non-ART users. β2MG was more elevated among TDF users than among non-TDF users, indicating that this

marker may be an important indicator of TDF-related renal dysfunction (Oboho *et al.*, 2013).

In the above two studies, α1MG and β2MG were urine biomarkers of TDF-associated KTD.

- An early symptom of renal tubular dysfunction is hypophosphatemia caused by reduced phosphate reabsorption and excessive loss of phosphates into urine. A prospective cohort of patients initiating either a TDF-based ART regimen (TDF-exposed group; n=165) or a TDF-sparing ART regimen (TDF-unexposed group; n=90) had normal baseline SrP and SCr values. The TDF-exposed and TDF-unexposed groups had comparable median follow-up times (10.9 versus 8.8 months, respectively). During follow-up, 12.7% of TDF-exposed vs 6.7% of TDF-unexposed patients developed grade 2 hypophosphatemia (2.0–2.4 mg/dL), and 2.4% of TDF-exposed patients vs 0% of TDF-unexposed patients developed grade 3 hypophosphatemia (1.0–1.9 mg/dL). The incidence of hypophosphatemia was somewhat elevated in patients who took TDF-based ART compared with those who took TDF-sparing ART during the first two years of observation, but the difference was not statistically significant (Buchacz *et al.*, 2006).

- Another prospective observational study conducted in the United Kingdom investigated SrP, serum urea and SCr as measured in HIV-infected individuals. There were four treatment groups: TDF-based ART (group A), TDF-sparing ART (group B), ART naive (group C), and off ART but treatment experienced (group D). Groups A, B, C, and D comprised 101, 86, 51, and 14 patients respectively, with a mean age of 39 years (89% male). The frequency of hypophosphatemia in groups A, B, C, and D was 31%, 22%, 10%, and 14%, respectively (Day *et al.*, 2005).

In both of these cohort studies, TDF was associated with a higher prevalence of hypophosphatemia.

2.5 TDF ASSOCIATED RENAL DYSFUNCTION

The following is a summary of documented studies on the effect of TDF on renal function. They are categorised into randomised, longitudinal and cross-sectional studies.

2.5.1 Randomised controlled studies: TDF and renal function

- A 48-week randomised study by Campo *et al.* (2013) evaluated the efficacy and safety of switching to TDF/FTC in North America. Subjects on the abacavir/lamivudine (ABC/3TC) + protease inhibitor (PI) + ritonavir (RTV) combination were randomised to ABC/3TC or TDF/FTC. Three hundred and eleven subjects were treated in this study (PI + RTV + TDF/FTC, n=155 and PI + RTV + ABC/3TC, n=156). Baseline characteristics were similar between the arms: 15% female, 28% black, median age of 46 years. A modest decline in eGFR calculated by MDRD occurred in both arms but was significantly greater in TDF/FTC-treated subjects. CrCl calculated with the CG method produced similar results to the MDRD calculated values.

- HIV-infected participants (n = 385) with a median age of 37 years (20% female and 15% blacks) were randomised to ABC/3TC or TDF/FTC co-administered with efavirenz (EFV) for 96 weeks. The study was conducted in 76 centres across 13 European countries. Baseline demographics were similar across the treatment arms. At week 48, the adjusted mean change from baseline in eGFR by MDRD was +0.22 mL/min/1.73m^2 and +1.18 mL/min/1.73m^2 for the ABC/3TC and TDF/FTC arms (p = 0.435), respectively (Post *et al.*, 2010).

- A multicentre randomised, placebo-controlled, double-blind trial performed in South America, Europe and USA enrolled 600 patients. Their median age was 36 years (20% black) and they were predominantly male. Three hundred and one patients were randomised to receive stavudine/lamivudine/efavirenz (D4T/3TC/EFV), while 299 were randomised to receive TDF/3TC/EFV combination therapy. The mean CrCl of the two treatment groups, calculated using the CG method, remained essentially unchanged from baseline (122 mL/min for the TDF arm and 125 mL/min for the D4T arm) at 144 weeks (+1.5 mL/min for the TDF arm and +6.7 mL/min for the D4T arm). In both groups, there were slight decreases at week 144 in mean SrP values from baseline. The

incidence of grade 1, 2 and 3 hypophosphatemia in the TDF arm at 144 weeks was 4%, 3% and <1 % respectively (Izzedine *et al.*, 2005).

- Two double-blind, placebo-controlled studies reported 19 cases of proximal renal tubular dysfunction diagnosed at 6.89 ± 5.51 months after starting TDF (Izzedine *et al.*, 2004).

- Pooled data from two randomised, controlled trials of Izzedine *et al.* (2005) and Gallant *et al.* (2008) were analysed to evaluate the effect on renal function of long-term treatment with TDF compared with D4T or zidovudine (AZT) in combination with EFV and either 3TC or FTC. A small but statistically significant decrease in eGFR was observed in the TDF group at 144 weeks. A significant increase in eGFR was seen in the control group using both CG and MDRD formulae. At 144 weeks, no clinically relevant changes in SCr and SrP were seen in either the TDF or control groups. The incidence of SCr elevation or hypophosphatemia was less than 1% in both treatment groups. Likewise, a similar incidence of proteinuria (5–6%) was seen in both treatment groups.

In summary, randomised controlled studies on TDF have shown varied significant small effects or no effect on the renal function. The factor that seemed to affect the results from these studies was race, in that a change in eGFR, although not always significant, was commonly seen in studies where the percentage of blacks was at least 30%, with no change in the markers of KTD. In contrast, in studies in which Caucasians formed a greater proportion, markers of KTD such as β2MG and hyperphosphatemia were commonly seen with no significant change in eGFR.

2.5.2 Longitudinal studies: TDF and renal function

Results reported in the literature on longitudinal studies are just as varied as in the controlled studies.

- In a retrospective analysis of 1 647 HIV-infected patients in the USA, 964 patients (24.5% black and 14% female) with a median age of 43 were on a TDF-containing and 683 patients on a TDF-sparing regimen. Renal function parameters were similar at baseline. The TDF-exposed patients experienced statistically significantly greater declines in eGFR (MDRD) through 52 and 104 weeks compared to the TDF-sparing patients. The decline in eGFR was more

pronounced if the baseline eGFR was greater than 80 mL/min/1.73m^2, but also significantly decreased if the eGFR was between 50 and 79 mL/min/1.73m^2. The TDF-exposed patients had a greater development of tubular dysfunction over the period of 104 weeks (Horberg *et al.*, 2010).

- The same results were observed in another retrospective cohort analysis of 324 HIV-infected adults where patients starting a TDF-containing regimen (n=201, 72.1% male) were compared with those starting a TDF-sparing (n=123, 74% male) regimen. The TDF-exposed patients experienced significantly greater declines in eGFR (MDRD) through 24 months compared with the TDF-sparing patients. This decline was also more significant among TDF-exposed persons with a higher baseline eGFR (> 80 mL/min/1.73 m^2). Increases in SCr were also significantly greater among TDF-exposed patients compared with TDF-unexposed ones. A significantly higher percentage (7.8% versus 1.3%, p <0.001) of TDF-exposed subjects met the criteria for proximal tubular dysfunction at the end of a 24-month follow-up compared with TDF-unexposed ones. Tubular dysfunction was diagnosed when two or more of the following abnormalities were present: proteinuria, glucosuria, hypouricemia, hypophosphatemia and hypokalemia (Calza *et al.*, 2011).

- In a cohort analysis in South Africa (n = 890) of HIV-infected adults with a median age of 37.1 years (96.3% black, 73.5% women), 190 were TDF-naïve, 700 switched to TDF and were exposed for a median period of 10.8 months. After 48 months of follow-up, 21 (2.4%) experienced nephrotoxicity and five had mild (60–89 mL/min) and moderate (30–59 mL/min) renal dysfunction calculated by the CG method. It is worth noting that 271 (30.5%) had mild (60–89 mL/min) renal dysfunction and 46 (5.2%) had moderate (30–59 mL/min) renal dysfunction at TDF initiation and that the analysis was restricted to CrCl as a measure of nephrotoxicity, since urine phosphate (UP) and SrP were not routinely measured (Brennan *et al.*, 2011).

- A Swiss HIV cohort study was designed to compare eGFR over time in HIV patients initiating treatment with TDF and EFV or ritonavir-boosted lopinavir (LPV/r) or ritonavir-boosted atazanavir (ATV/r). A total of 940 patients with a median age of 39 years (14% black, 22% female) were enrolled. Patients starting TDF with EFV, LPV/r and ATV/r were followed-up for a median of 1.7, 1.2 and

1.3 years, respectively. Mean eGFR, calculated using the CKD-EPI equation, and declined over time for patients on all three TDF-based therapies. TDF in combination with either LPV/r or ATV/r led to a greater initial decline in eGFR than TDF with EFV. There was no evidence that this decrease would then either reverse or continue beyond six months (Young *et al.*, 2012).

- Through four years of observation in a post-marketing safety surveillance programme conducted in the European Union, Australia, Canada and the USA, 10 343 patients were enrolled. The most common serious adverse drug reactions reported for TDF were renal failure, Fanconi's syndrome and SCr increase in 2.2% of the patients (Nelson *et al.*, 2007).

- In a large analytic cohort of HIV-infected patients (n=10841), assembled from electronic medical records in the USA, 4 303 patients were exposed to TDF while 6 538 were not. Of those exposed to TDF, with a median age of 45 years, 46.9% were black and 2.5% were women. After a median follow-up range of 3.9 and 5.5 years, the results showed that exposure to TDF was associated with increased proteinuria and rapid decline in kidney function, defined as an annual decline of 3 mL/min/1.73m^2 or more for two consecutive years. These kidney disease events did not appear to be reversible (Scherzer *et al.*, 2012)

- A single-centre, retrospective cohort study was designed to determine the incidence of TDF-associated renal dysfunction in Japanese patients. HIV-infected (n=495) patients with a median age of 38 years (95.2% men) were initiated on a TDF-containing regimen. The eGFR baseline was 120.9 (104.8–138.2) mL/min/1.73m^2, calculated by the MDRD formula. TDF-associated renal dysfunction, defined by more than 25% decrease of eGFR from baseline, occurred in 97 patients (19.6%). The median (interquartile range - IQR) time from commencement of TDF to occurrence of TDF-associated renal dysfunction was 39 weeks (13.5–99.4 weeks). Multivariate analysis identified smaller body weight and smaller body mass index (BMI) as significant factors for TDF-associated renal dysfunction (Nishijima *et al.*, 2011).

Generally, in the above longitudinal studies TDF therapy was associated with decreased eGFR and CrCl with evidence of renal tubulopathy. The decrease in eGFR

or CrCl was more severe when TDF was co-administered with boosted protease inhibitors and also when the baseline eGFR was greater than 80 mL/min/1.73 m^2.

In the following studies, some changes observed with regard to CrCl and eGFR and other markers in participants treated with TDF were different from those in other longitudinal studies discussed earlier.

- A cohort of ART-experienced HIV patients who had been switched from either a TDF/FTC regimen (n = 73, 69.9% male) or an ABC/3TC regimen (n = 28, 71.4% male) was evaluated to assess early markers of renal toxicity. Markers of mitochondrial (cytochrome c) or cytosolic (α-glutathione S transferase) toxicity together with common indicators of renal damage were assessed at baseline after one, three, six and 12 months of patient exposure to ART. Treatment with a TDF-based regimen compared to an ABC-based regimen did not produce alterations of classical renal pathophysiologic parameters such as eGFR, proteinuria or microalbuminuria and serum phosphate after a 12-month treatment period. Comparison of median values between the two groups at different time points showed that at one and three months, patients taking TDF excreted significantly higher median urinary levels of cytochrome c than patients treated with ABC (p = 0.015 and p = 0.032 at one and three months, respectively) (Maggi *et al.*, 2012).

- Similarly, an observational cohort in the USA enrolled 432 subjects (baseline eGFR > 50 mL/min/1.73m^2), of which 201 (78% black and 59% male) with mean age of 40 years, who were initiated on a TDF-based regimen. The remaining group (n=231) was on alternative nucleoside reverse transcriptase inhibitors (NRTIs). The TDF and NRTIs groups both experienced an initial decline in eGFR during the first 180 days of therapy, then eGFR stabilised between 180 and 720 days. No significant changes in eGFR from baseline value at six, 12 and 24 months were observed in the TDF and alternative NRTI group (Gallant & Moore, 2009). Furthermore, in this study, baseline eGFR was defined as the average of the two eGFR values obtained closest to and preceding the start of treatment, not as a single value recorded at baseline.

- Cohorts in Lesotho of 566 patients with a median age of 40.6 years (47.9% male) were initiated on a TDF-based regimen. All patients had baseline CrCl ≥ 50

mL/min using the CG method. Renal function improved during follow-up with a median change in CrCl of +5 mL/min at six months and +7 mL/min at 12 months (Bygrave *et al.*, 2011).

In these last three longitudinal studies, there were no changes in CrCl or eGFR in participants who were exposed to TDF, while in another study CrCl increased from baseline. This observed disagreement with other longitudinal studies is attributed to the differences in clinical and social demographic characteristics.

2.5.3 Cross-sectional studies: TDF and renal function

Cross-sectional studies that were conducted with regard to TDF treatment and association with renal function are summarised below.

- In Spain 284 (86% male) HIV patients were included in a cross-sectional study. At recruitment, 154 were on a TDF-based regimen (group 1), 49 on other ART regimens and never exposed to TDF (group 2) and 81 were ART-naïve (group 3). The median age was 44, 46 and 37 years for groups 1, 2 and 3, respectively. Kidney glomerular function as measured by SCr levels, CrCl or both, was within normal limits and comparable among study groups. TDF was associated with signs of proximal tubular kidney damage (β2-microglobinuria, hypophosphatemia, non-diabetic glucosuria or aminoaciduria) after median exposure of 36 months (Labarga *et al.*, 2009).

- In a French hospital-based cohort of 2 588 patients, a cross-sectional survey was conducted to estimate the prevalence of renal impairment (RI) among HIV-infected adult patients and to investigate the associated factors. The median (IQR) age was 41.9 years (36.9–48.1) and the gender of the group predominantly male (74.3%). The prevalence of mild, moderate, severe and end-stage RI was 34.2%, 4.4%, 0.3% and 0.2% respectively when the CG method was used or 49%, 5.5%, 0.3% and 0.3% respectively using the MDRD method. The prevalence of RI was significantly associated with TDF exposure, female gender and age between 40 and 50 years (Déti *et al.*, 2010).

- Similar results were obtained when patients treated with TDF (n = 82) in a cross-sectional study were compared with patients on ART who were TDF-naïve (n = 92). The patients' mean age was 42.6 years and they were predominantly male

(90%). Patients on TDF showed a lower mean (SD) GFR calculated by CrCl (97 ± 49 mL/min/1.73m^2) or cystatin C clearance (86 ± 21 mL/min/1.73m^2) compared with control patients (107 ±39 mL/min/1.73m^2) and (97 ± 20 mL/min/1.73m^2). Thirty per cent of the patients had proteinuria greater than 130 mg/day (Mauss *et al.*, 2005).

In all three studies above, the ethnicity of the participants was not indicated and hence not considered. Higher prevalence of RI was common in patients exposed to TDF. Female gender and age between 40 to 50 years also influence RI.

In summary, treatment with TDF in all three study types (randomised controlled, longitudinal and cross-sectional studies) was associated with renal dysfunction, except in some longitudinal studies where no change in eGFR or CrCl was observed.

2.5.4 Association between plasma TFV concentration and renal function

In the previous sections (**2.5** to **2.5.3**) the influence of TDF treatment on renal function was discussed. In this section, the possibility of correlation between plasma TFV concentration and a degree of renal dysfunction is presented.

- The relationship between TDF exposure and KTD was examined prospectively in 92 HIV-infected individuals. The median exposure to TDF was 33 months. KTD was determined on the basis of non-diabetic glucosuria, altered resorption of phosphorus, hyperaminoaciduria, β-2-microglobulinuria and abnormal uric acid excretion. KTD was defined when at least two of these abnormalities were present, with at least one being a Fanconi's syndrome criterion (glucosuria in non-diabetics, hyperaminoaciduria or hyperphosphaturia). Median TFV plasma levels (mid-dose concentration) were higher in patients with KTD than in those with normal tubular function, 182 (105–220) vs 106 (75–148) ng/mL, respectively. The best TFV plasma concentration threshold to discriminate KTD was 160 ng/mL. Multivariate analysis showed that female gender [OR=71 (95% CI=33–111), P<0.01] and the ratio of body weight/SCr [OR=-1.19 (95% CI= -2.2-0.56), P<0.01] were independently associated with higher TFV plasma levels (Rodríguez-Nóvoa *et al.*, 2010).

- A retrospective study designed to evaluate the correlation between TFV trough concentration and eGFR included 163 patients on ART. Patients were divided

into three subgroups according to TFV trough concentration: Low-level < 40ng/mL, (n = 19), normal-level, 40-90 ng/mL (n = 60), and high-level > 92 ng/mL (n = 84) groups. RI was defined as GFR < 60 mL/min. Median (IQR) TFV trough concentration in the whole population was 92 ng/mL (61-135) and was not influenced by gender [men, n = 99, 100 ng/mL (72-135); women, n = 45, 106 ng/mL (74-164); *p* = 0.465]. Trough concentration of 90 ng/mL was significantly associated with a decrease in eGFR in women but not in men, independent of the time of exposure to TDF. After 12 months of longitudinal assessment of eGFR changes, similar results were obtained for both genders where a higher decrease in eGFR was significant in patients with a TFV trough concentration of > 90 ng/mL, whichever formula was used (MDRD, CG or CKD-EPI) (Poizot-Martin *et al.*, 2013).

- Similarly, a cross-sectional analysis was conducted in HIV-positive patients (n=195, 68.2% male, 85.1% Caucasian), median age of 45 years and on chronic TDF-based ART. Samples were collected at steady state and after a median uninterrupted time of TDF intake of 22.2 months. The median (IQR) TFV trough plasma concentration was 50 (35 to 77) ng/mL. TFV trough concentrations were significantly associated with CrCl (CG), BMI and weight/SCr ratio (*p* = 0.012, 0.025 and 0.001, respectively). The TFV median (IQR) trough concentrations were higher in unboosted ATV recipients [65 (41.7-84.2) ng/mL] compared to patients on boosted PIs [51.5 (34-77) ng/mL], non-NRTIs (NNRTIs) [43 (28.2-50) ng/mL], and raltegravir [37 (28-79) ng/mL]. Receiving a PI, whether boosted or unboosted, was associated with higher TFV plasma trough concentrations [54.5 (37-80 ng/mL) vs 43 (28-50) ng/mL] in the NNRTI and raltegravir group (*p* = 0.007) (Calcagno *et al.*, 2013).

- In another study of participants aged between 18 and 25 years on TDF-based ART, a plasma TFV concentration of 68.8 ±33 ng/mL was negatively correlated with eGFR (*r* = -0.29, *p* = 0.002). However, plasma TFV did not significantly correlate with markers of tubular dysfunction, such as glucosuria and β-2-microglobulinuria (Havens *et al.*, 2013).

From the above literature, levels of plasma TFV higher than 90 ng/mL were associated with decreased CrCl and eGFR, especially in those taking PIs in the presence or absence of indicators of KTD. BMI affected CrCl and eGFR.

Contrasting to the above statement, different observations were made in plasma TFV concentrations and correlation with CrCl. The following two studies show these observations.

- A prospective 48-week trial carried out in France was aimed at determining the correlation between plasma TFV concentration and CrCl in TDF-based ART-experienced patients (96% male, median age 41 years). The median (IQR) trough concentration of TFV was stable over time 53 (23-232) ng/mL, 61 (16-211) ng/mL, 55 (13-393) ng/mL and 60 (27-642) ng/mL at week 4, 8, 16 and 24, respectively. No difference in CrCl was observed at 24 weeks between patients with average TFV trough levels of ≤ 58 ng/mL and > 58ng/mL. There was no significant difference in CrCl when analysis was performed at 100 ng/mL cut-off, a more clinically relevant level. No correlation was observed between peak level of TFV at week 4 and change in CrCl (r = 0.47; p= 0.20). No difference in SCr was observed at week 24 between patients with TFV peak levels ≤ 217 ng/mL and patients with TFV peak levels > 217 ng/mL, but two patients discontinued TDF as a result of RI. Trough TFV plasma concentration was particularly increased in one patient (215 ng/mL) and greater than the median value of the entire cohort in the second patient (66 ng/mL) (Gérard *et al.*, 2007).

- Similar observations were made in a retrospective analysis that investigated the relationship between PI/r co-administered with TDF and changes in CrCl. Comparator groups included HIV-infected patients receiving TDF and NNRTI and non-TDF regimens. Steady-state (week 2) TFV plasma exposures were determined to evaluate the association between TFV concentration and longitudinal changes in renal function. Patients (n = 51; 81% male, age range 32-47 years) receiving a PI/r had TFV plasma concentrations and clearance rates similar to those of NNRTI treated individuals (C_{max}, 255 vs. 225 ng/mL; C_{min}, 76 vs. 63 ng/mL; CL/F, 96 vs. 108 L/h). No association was found between week 2 steady-state TFV plasma exposure and change in CrCl over time even after adjusting for baseline renal function. Treatment with TDF and PI/r was, however, associated with greater declines in CrCl (defined as a > 15% decrease from baseline) over 48 weeks compared with TDF-NNRTI-based regimens (Goicoechea *et al.*, 2008).

In the two studies above, no correlation was found between plasma TFV concentration and CrCl.

In summary, plasma TFV concentration in some studies showed correlation, while in other studies there was no correlation between TFV concentration and CrCl or eGFR. The difference could be non-adjusting for possible confounding factors such as BMI and weight/SCr.

2.5.5 Other factors associated with renal dysfunction

HIV may cause renal dysfunction in HIV-infected persons by a direct effect of the HIV-1 virus itself, which actively replicates within renal cells. HIV-associated nephropathy, a focal segmental glomerulosclerosis, has now become a common disease in the HIV-seropositive population, causing rapid deterioration of renal function. It is the most common cause of chronic renal disease in HIV patients and occurs almost exclusively in black patients (Herman & Klotman, 2003). Renal impairment may be a complication of pre-existing co-morbidities such as diabetes and hypertension (Hall *et al.*, 2014). Certain drugs cause renal failure through different mechanisms. Nonsteroidal anti-inflammatory drugs (NSAIDs) are renowned for inducing haemodynamic renal failure by reducing renal prostaglandins and hence renal blood flow and GFR. NSAIDs may induce glomerular disease, such as membranous nephropathy, which is clinically complicated by nephrotic syndrome. Therapy using cyclosporine A or tacrolimus causes afferent renal vasoconstriction, which is aggravated by NSAIDs, resulting in a further decline in renal blood flow and GFR (Ashley, 2011; Hörl, 2010).

2.6 BONE PHYSIOLOGY

Bone is a dynamic connective tissue that is remodelled constantly throughout life. Its function is to provide mobility and protection to vital organs. Bone also provides a reservoir for phosphorus, magnesium, calcium and other ions vital for homeostasis. The bone remodelling process is achieved by two distinct cell types: osteoclasts that resorb the bone matrix and osteoblasts that synthesise bone matrix. Extracellular components of bone are made up of organic matrix (type 1 collagen) and a solid mineral phase combined with organic matrix. Bone remodelling is regulated by tightly coupled activities of osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation or mineralisation. Parathyroid hormone (PTH) and vitamin D stimulate osteoblasts to

secrete factors such as macrophage–colony stimulating factor (M-CSF) which induce stem cells to finally differentiate into multinucleated osteoclasts. PTH stimulates bone resorption indirectly by binding to receptors on osteoblasts. This leads to stimulation and release of factors (see **Figure 1.1**), such as interleukin 6 and receptor activator of nuclear factor κβ ligand (RANKL), and the expression of RANK receptors. These factors promote bone resorption by osteoclasts (Barrett *et al.*, 2010; Longo *et al.*, 2012).

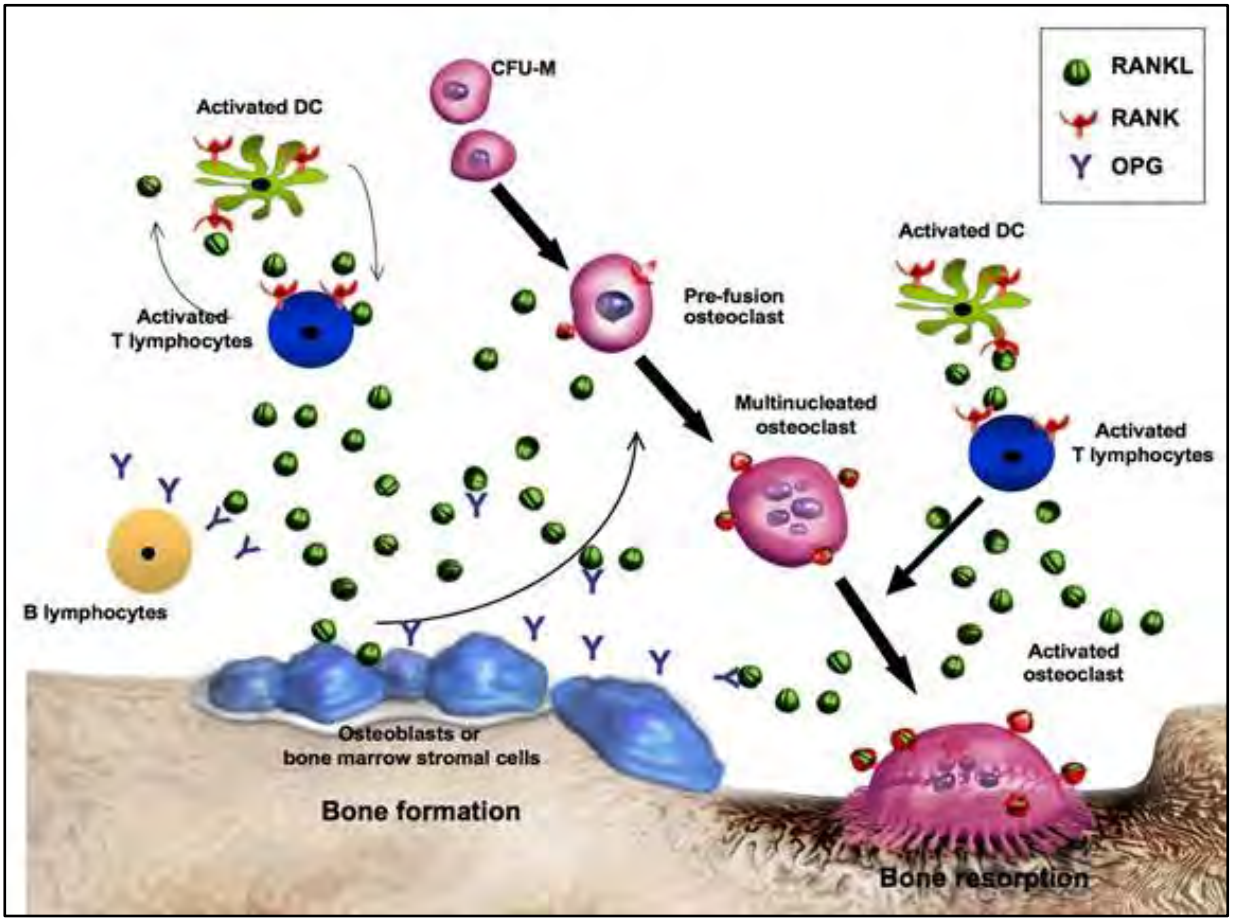

Figure 1.1 Bone remodelling process: involving RANKL, RANK and Osteoprotegerin (OPG) molecules. RANKL is an essential mediator of osteoclast formation, function and survival and is produced by osteoblasts, bone marrow stromal cells and T cells. RANKL acts by binding to the RANK receptor that is expressed by monocytes and dendritic cells. OPG is expressed by mature B cells and osteoblasts, which inhibits RANKL-induced osteoclastogenesis. (Adapted with permission from Macmillan Publishers Ltd: IBMS BonekEy, Ferrari-Lacraz S. & Ferrari S., 2009 March; 6(3):116-126, copyright 2009).

2.6.1 Mechanism of TFV-associated bone loss

The exact mechanism of TFV-induced bone loss is unknown but the following explanation was proposed. Bone is formed and maintained by osteoblasts (which form bone) and osteoclasts (which resorb bone). An extensive cell signalling network between osteoblasts and osteoclasts is required for maintaining a balance in the activities of these two cell types in bone remodelling and bone health. Osteoblasts regulate osteoclast differentiation by expressing two factors that are necessary for osteoclast formation: M-CSF and RANKL. M-CSF is required for survival and proliferation of early osteoclast precursors. Binding of RANKL onto RANK receptor on osteoclasts stimulates expression of genes necessary for osteoclast differentiation, cellular fusion and bone resorption. In addition, osteoblasts express osteoprotegerin (OPG), which inhibits activation of RANK by RANKL. TFV, a phosphonate, is selectively taken up by osteoclasts by a mechanism similar to that of bisphosphonates, ultimately causing cellular stress. The resulting cellular stress perturbs cellular DNA synthesis (nuclear and/or mitochondrial) by altering gene expression that is involved in signalling osteoblast activity, resulting in decreasing bone formation (Grigsby *et al.*, 2010).

2.6.2 Bone turnover markers

Components of the bone matrix and those from osteoblasts and osteoclasts have recently been identified and categorised as either bone formation or bone resorption markers. These markers indicate the metabolic activities of osteoblasts or osteoclasts and are measured in either urine or plasma. Bone formation markers are either enzymes or metabolic products of osteoblasts expressed during bone turnover processes. Bone formation markers that are widely used include carboxy- and amino-terminal propeptides of type 1 collagen (P1CP, P1NP), bone-specific alkaline phosphatase (BALP) and osteocalcin. Bone resorption markers are breakdown products of bone collagen. These include C-terminal telopeptide of type 1 collagen (CTx), amino-terminal cross-linked telopeptide of type 1 collagen (NTX), type 1 collagen alpha 1 helicoidal peptide, pyridinoline, deoxypyridinoline, RANKL and OPG (Wheater *et al.*, 2013). Sustained decrease of 1,25-dihydroxy vitamin D or increase of SrCa, SrP and PTH is indicative of bone resorption. BMD is used to assess bone mass, which is usually low in the presence of high plasma levels of bone resorption makers (Childs *et al.*, 2010; Kumar *et al.*, 2013).

2.7 TDF-ASSOCIATED BONE LOSS

The next section offers a summary of studies published on the effect of TDF on bone turnover. These studies are divided into randomised, longitudinal and cross-sectional studies.

2.7.1 Randomised controlled studies: TDF and bone turnover

- In a phase IV, 96-week trial, 350 eligible participants with a mean age of 45 years (85% Caucasian and 98% male) were randomly allocated to switch existing NRTIs to either the TDF/FTC or the ABC/3TC fixed-dose combination. At week 96, the absolute change from baseline in hip BMD in the ABC/3TC group was 0.004 g/cm^2 and in the TDF/FTC group it was 20.007 g/cm^2. For the lumbar spine, the absolute BMD change in the ABC/3TC group was 0.008 g/cm^2 and in the TDF/FTC group 20.005 g/cm^2. A baseline covariate significantly associated with greater decline in hip and spine BMD was TDF/FTC randomisation (p = 0.013). Significant differences in absolute changes in bone resorption and formation markers were seen after baseline between treatment groups. CTx, a marker of bone resorption, increased significantly at week 12 in TDF/FTC compared to the ABC/3TC arm. Similarly, increases in bone formation markers (P1NP and BALP) were greater with TDF/FTC than with the ABC/3TC arm. There was no significant difference between groups in OPG or RANKL (Haskelberg *et al.*, 2012).

- Similar results were obtained in a multicentre, randomised, open-label study that was conducted in Europe to evaluate the bone safety of ABC/3TC and TDF/FTC administered with EFV in ART-naïve subjects. HIV-infected adults (n=385) with a median age of 37 years (15% black and 81% male) received treatment with either ABC/3TC (192 subjects) or TDF/FTC (193 subjects) for 96 weeks. At week 48, a greater proportion of subjects in the TDF/FTC group had a decrease from baseline in both total hip and lumbar spine BMD of ≥ 6% (hip, 13%; spine, 10%), compared with the ABC/3TC group (hip, 3%; spine, 5%). Increases in all bone turnover markers (BTM -BALP, osteocalcin, P1NP and CTx) were significantly greater in the TDF/FTC group than in the ABC/3TC group at week 24. These increases remained significantly different between the treatment groups at week 48 for all but CTx (Stellbrink *et al.*, 2010).

- A phase two randomised trial (pre-exposure prophylaxis of TDF) among 184 HIV-uninfected men in the USA was conducted to determine the effects of TDF on BMD followed longitudinally. Ninety-four men in the TDF arm had a median age of 40 years and 81% were Caucasians. TDF exposure resulted in a statistically significant decrease in BMD at the total hip and femoral neck relative to baseline when compared to the pre-treatment/placebo group (Liu *et al.*, 2011).

- In another clinical trial in the USA and Puerto Rico, 269 HIV-infected treatment-naïve patients were randomised and blinded to receive ABC/3TC or TDF/FTC with open-label EFV or ATV/r. Overall, 85% of the participants were male and 47% were non-Hispanic white persons. The median age was 38 years. At week 96, the mean percentage changes from baseline in spine and hip bone mineral density for ABC/3TC and TDF/FTC were -1.3% and -3.3% and -2.6% and -4.0% respectively; and for EFV versus ATV/r were -1.7% and -3.1% and -3.1% and -3.4%, respectively. TDF/FTC led to greater decreases in spine and hip BMD than did ABC/3TC and ATV/r induced a significantly greater decrease in spine BMD than did EFV (McComsey *et al.*, 2011).

- Changes in BMD after discontinuing TDF were evaluated in a two-centre randomised pilot study in Spain of HIV-infected patients with low BMD and a mean age of 49 years (predominantly male and 96% Caucasian). Fifty-four patients were randomly assigned to switch from TDF (n = 26) to ABC or to continue with TDF (n = 28). Switching from TDF to ABC after 48 weeks led to a slight improvement in femoral BMD but no differences were detected between groups. No significant difference in mean PTH and 25 hydroxyvitamin D levels were found between groups (Negredo *et al.*, 2014).

In clinical trials, treatment with TDF led to decreased BMD and increased serum markers of bone turnover. These effects were only observed in the first 96 weeks of treatment. The trend in BMD and change beyond 96 weeks of treatment is not known yet.

2.7.2 Longitudinal studies: TDF and bone turnover

The following longitudinal studies are analysed to determine the possible relationship between TDF therapy and bone turnover.

- In a longitudinal study in the USA designed to assess the changes in BTM, 87 participants, predominantly male, were enrolled; 44 were on a TDF-based regimen and 43 on a non-TDF one. The median age was 34 and 37 years for those exposed to the TDF and non-TDF regimen, respectively. At six and 12 months of follow-up, osteocalcin was significantly higher in the TDF-exposed group than the non-TDF one. Furthermore, osteocalcin (bone formation marker) was independently associated with TDF use. Protease inhibitor use, tumour necrosis factor and advanced HIV disease were also associated with changes in BTM. CTx was similar in both groups (Brown *et al.*, 2011).

- An observation cohort analysed ALP dynamics relative to TDF use. The study included 162 treatment-naïve patients starting TDF-based ART, 495 treatment-naïve patients starting TDF-sparing ART, 168 patients reinitiating TDF-containing ART and 193 reinitiating TDF-sparing ART. TDF was associated with a significant increase in ALP in both treatment-naïve and treatment-experienced patients, but not in patients on TDF-sparing regimens. There was a strong correlation between TDF use and increased ALP (Fux *et al.*, 2008). TDF-induced osteomalacia in this cohort was evidently seen as hypophosphatemia and raised ALP enzyme.

In these two longitudinal studies, bone formation markers (osteocalcin and ALP) were associated with TDF exposure.

2.7.3 Cross-sectional studies: TDF and bone turnover

- A cross-sectional analysis in a hospital outpatient-based cohort in Australia enrolled 153 patients who were treatment-experienced (median age 48 years, 98% male), of which 87 were on a TDF regimen. Treatment with TDF was significantly associated with increased osteoblast and osteoclast activity (increased bone turnover). Protease inhibitors were significantly associated with low BMD. The frequency of low BMD was higher in patients who were treated with TDF or PI (Calmy *et al.*, 2009).

In this cross-sectional study, treatment with TDF was associated with increased bone turnover and higher frequency of low BMD than PI use.

In summary, TDF exposure in randomised, longitudinal and cross-sectional studies was associated with high bone turnover.

2.7.4 Association between plasma TFV and bone turnover

In sections **2.7** to **2.7.3**, studies relating to TDF treatment were presented. In this section, a study on TFV plasma concentration and possible correlation with BTM is presented.

- Baseline data from a multicentre randomised trial in the USA was used to measure associations of TDF use with BTM in a cross-sectional manner. A hundred and fifteen participants on a TDF regimen (age range 18 - 25 years, 73% male) had their blood drawn between 14.4 - 23.8 hours post TDF dose. The mean (SD) plasma TFV concentration was 68.8 ±33 ng/mL. Plasma TFV correlated negatively with free 1,25 dihydroxyvitamin D (r = -0.34, p = 0.0001). However, there was no significant correlation between plasma TFV concentrations and SrCa, PTH, BALP, CTx or SrP. When these markers were compared at a TFV concentration ≤ 39.5 ng/mL and > 95.3 ng/mL (lowest and highest quintile), SrCa and 25-hydroxyvitamin D were significantly higher at > 95.3 ng/mL of TFV while free 1,25 dihydroxyvitamin D was significantly lower at > 95.3 ng/mL of TFV. PTH, CTx, BALP and SrP were similar between the lowest and highest quintile of TFV plasma concentration (Havens *et al.*, 2013).

In this study, there was a relationship between plasma TFV concentration and 1,25 dihydroxyvitamin D. At high TFV concentrations (> 95.3 ng/mL), SrCa was high and free 1,25 dihydroxyvitamin D low, which indicated increased bone turnover activity. However, not many studies have investigated the relationship between plasma TFV concentration and BTM.

2.7.5 Other factors associated with bone loss

In untreated patients, HIV infection itself may increase demineralisation of bone, as osteopenia or osteoporosis prevalence was higher in untreated HIV-infected patients compared to an HIV-uninfected control group (Gutiérrez & Masiá, 2011). The mechanisms that result in bone demineralisation are not well understood. It has been postulated that this may be due to osteoclast activity that is stimulated by pro-inflammatory cytokines such as tumour necrosis factor α, interleukins 1 and 6, which

occur in high concentrations in HIV patients (Anastos *et al.*, 2007). Multiple endocrine and metabolic consequences of HIV infection exist that alter bone metabolism in patients with acquired immune deficiency syndrome (AIDS). Osteopenia in AIDS patients has been associated with ART, particularly with PIs (Teichmann *et al.*, 2003).

Treatment with selective serotonin reuptake inhibitors such as paroxetine, sertraline or citalopram was found to be associated with decreased BMD in postmenopausal women (Ak *et al.*, 2014). Cigarette smoking and snuff have been reported to increase the odds of developing osteoporosis among women of 40 years and older (Ayo-Yusuf & Olutola, 2014).

2.8 CONCLUSION

From the literature above, it has been shown that TDF-based ART has been associated with reduced eGFR or CrCl. The decrease in eGFR or CrCl was more severe when TDF was co-administered with boosted protease inhibitors and the baseline eGFR was greater than 80 mL/min/1.73m^2. In most of the clinical trials, TDF treatment had little significant effect on renal function. The concentrations of TFV associated with markers of renal tubular and glomerular dysfunction have not been explored much, unlike associations with TDF exposure status.

TDF exposure has been associated with reduced BMD and also increased markers of bone turnover, but the concentration of TFV at which these changes occur has not been evaluated. The mechanisms of TFV bone and renal toxicity have only been proposed and not fully studied. Therefore, there is a need to establish the relationship between plasma TFV concentration and markers of renal function and bone turnover in HIV-infected women, as this information is lacking in the South African public healthcare system.

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Materials and Methods | Chapter 3

3.1 INTRODUCTION

This Chapter addresses the study design, ethics, study population, blood sample collection, medication history recording, baseline (when TDF was initiated) data collection and statistical methods used. The analytical method validation and the calculation of renal parameter procedures are stated briefly, as they are addressed in Manuscripts A and B for publication.

3.2 STUDY DESIGN

This was a pilot cross-sectional study, which formed part of the already approved sub-study (PURE: Bone density project, NWU-00016-10-A1, 23/02/2010) within the multinational longitudinal Prospective Urban and Rural Epidemiology (PURE) study which took place at the Tlokwe and Ganyesa study sites in November 2012 and continued from April until the end of August 2013. Tlokwe is a sub-district of Dr Kenneth Kaunda district, which consists of 11 health care facilities (one district hospital and 10 primary healthcare clinics - PHC). Ganyesa district comprises Kagisano and Molopo sub-districts, which have 21 health facilities (one district hospital and 20 PHC). Participants in Tlokwe primarily accessed health care at Potchefstroom hospital and eight PHC, namely Boiki Tlhapi, Mohadin, Steve Tshwete, Potchefstroom, Top City, Lesogo, Gateway and Promosa. Participants in Ganyesa primarily accessed health care services at Ganyesa District Hospital and three PHC, namely Tlakgameng, Ganyesa and Tlapeng.

The PURE study is a large-scale epidemiological study that set out to recruit approximately 140000 individuals residing in more than 600 communities in 17 low-, middle- and high-income countries around the world (Teo *et al.*, 2009). The PURE study in South Africa (PURE-SA) commenced in 2005 in North West Province. The longitudinal study follow-ups are scheduled for every five years. The overarching aim of

the PURE study is to examine the relationship of societal influences on human lifestyle behaviours, cardiovascular risk factors and the incidence of chronic non-communicable diseases. Individual data collection included lifestyle behaviours, medical history, blood collection and storage (biochemistry and future genetic analysis), electrocardiogram and anthropometric measurements.

3.2.1 Ethics approval

This sub-study was approved by the Ethics Committee of the North-West University, Potchefstroom (NWU-00016-10-A1) on 12 April 2013 and North West Department of Health (Policy, Planning, Research, Monitoring and Evaluation), Mmabatho on 08 August 2013, specifically to obtain permission to access study participants' related health information from their public health care records. Refer to the approved study protocol and informed consent form in Addenda C and D, respectively.

The study was conducted in accordance with the Declaration of Helsinki 2013 and according to the National Guidelines of Good Clinical Practice, Department of Health. Written and signed informed consent was sought from each participant.

3.2.2 Study population

The sub-study population included 462 participants who were followed up during the PURE-SA sub-study in November 2012, April 2013 and August 2013.

The sample size for this investigation included 47 HIV-infected women (see **Figure 1.2**), of which 30 were taking a TDF-based ART regimen, nine were taking non-TDF ART regimens and eight were ART-naïve (not eligible for ART initiation). Thirty HIV-infected women on TDF-based ART were matched with 30 HIV-uninfected controls (see section **3.2.5**).

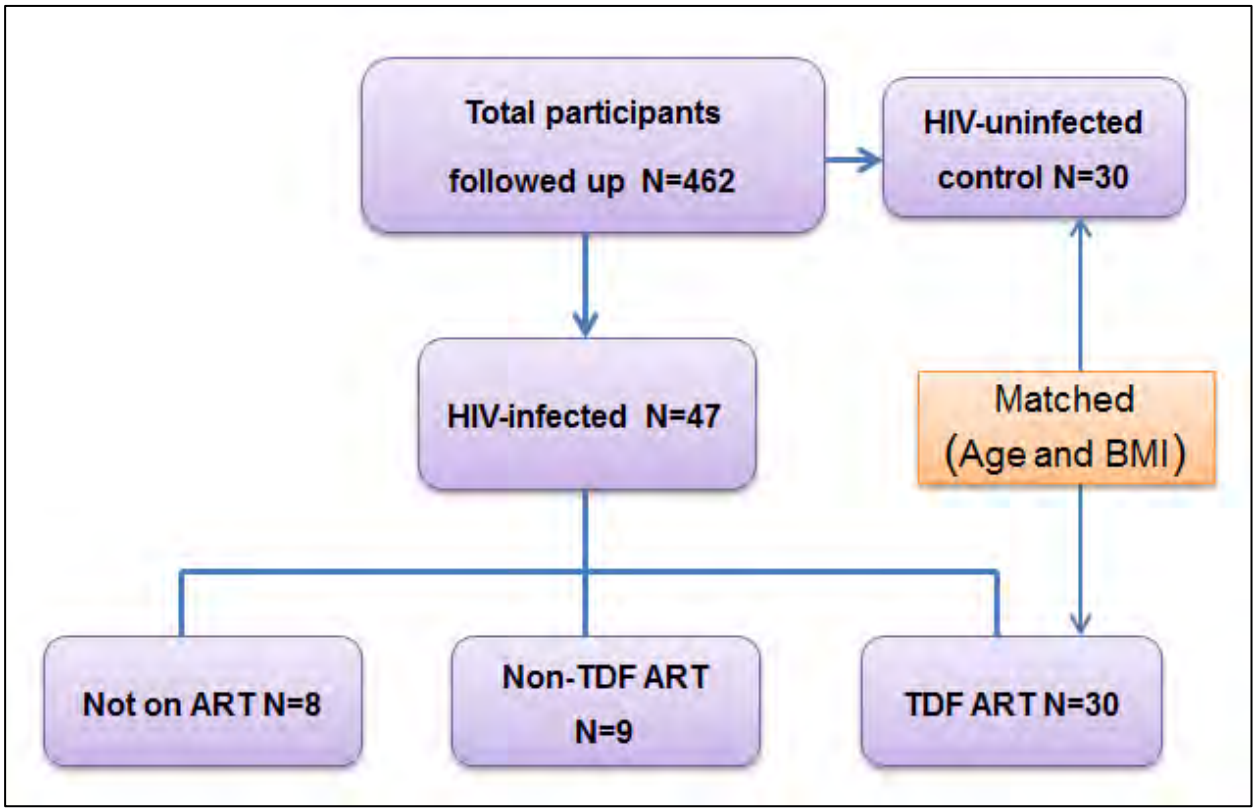

Figure 1.2 Schematic diagram of the sub-study layout.

3.2.3 Inclusion criteria

- All the HIV-infected women, ART-experienced or naïve, who were being followed-up during the duration of the PURE-SA: Bone density project.
- An equal number of HIV-uninfected women of similar age and BMI within the PURE-SA: Bone density project, identified retrospectively to act as a control group for the HIV-infected women taking a TDF-based ART regimen.
- Concomitant medication use for hypertension, diabetes, asthma, epilepsy and tuberculosis was acceptable, but the specific medication history was to be available and recorded.
- Signed informed consent.

3.2.4 Exclusion criteria

- Amputation.
- Known renal disease stated in the medical records.

- History of osteoporosis stated in the medical records.
- Calcium/vitamin D supplementation and hormonal replacement therapy.
- Drugs (streptomycin, lithium, sulfadiazine, phenytoin, allopurinol, amphotericin B deoxycholate, methotrexate, statins, mesalazine), owing to known renal damage associated with usage.

3.2.5 Selection of comparative HIV-uninfected control group

HIV-infected participants taking a TDF-containing antiretroviral therapy regimen were matched to HIV-uninfected ones within the PURE-SA study via propensity score matching with a match tolerance of 0.26. Propensity scores were calculated from a logistic multivariate regression model, which contained age and BMI as factors used to model case/control membership. The selection of controls for the cases was performed automatically from the resulting propensity variable using IBM® SPSS® Statistics software, version 22. Sampling of controls was performed without replacement.

3.3 BLOOD COLLECTION FOR TFV ANALYSIS AND CD4+ CELL COUNT DETERMINATION

In addition to the already approved blood sampling as per the sub-study (PURE: Bone density project) protocol, 3.5 mL of whole blood in a sodium citrate tube and 1.0 mL in an ethylenediaminetetra acetic acid (EDTA) microtainer was collected by trained research nurses. The blood-drawing procedure for all participants took place at the same time and no additional needle-pricking was required at any stage. Blood was drawn between 08:00 and 10:00 on each follow-up visit. The participants underwent voluntary counselling and testing prior to blood drawing and after the procedure.

To carry out TFV plasma analysis, 3.5 mL of whole blood was collected in sodium citrate tubes. After centrifugation of blood, plasma was stored at -80ºC until the day of analysis.

The 1 mL of blood collected in an EDTA microtainer was sent to the Lancet Laboratories in Potchefstroom (Tlokwe study site) for CD4+ cell count measurements, using their standard validated in-house method on a flow cytometer. Blood samples for participants at the Ganyesa study site was sent to the National Health Laboratory Service (NHLS) in Vryburg for CD4+ cell count measurements. The NHLS used a

validated national technique and equipment that is standardised and used for testing of all CD4 cell counts of all HIV-infected patients in South Africa that are obtaining treatment and care from the public sector.

3.3.1 Participants' drug history recording

Qualified pharmacists and assistants recorded the medication history of participants using a standardised medication questionnaire (see Addendum D5). Participants were informed and requested to bring along their medication and clinic books during scheduled visits to the North-West University metabolic clinic (Tlokwe) and Sethlare Lodge (Ganyesa) on pre-arranged dates. The information captured was:

- the type of chronic medication prescribed monthly, dosage, frequency and indication,
- the type of acute medication taken, dosage, frequency and indication within 14 days prior to visiting the study site, and
- herbal/traditional medication use, frequency of administration and indication.

The smoking and alcohol data was gathered and recorded by other researchers within the PURE study by using standard questionnaires and the results (refer to Addendum E2) from these recordings were obtained via the PURE-NWU data base with the permission of the collaborated researchers.

3.3.2 Baseline clinical data collection

Baseline (when TDF was initiated) clinical data was collected retrospectively from the hospital and clinical records for participants who were taking a TDF-based ART regimen. Participants gave permission, by signing a written informed consent form, to access their hospital or clinic records from the public health facility where medication was obtained. The information extracted was:

- the HIV diagnosis date,
- other ART regimens and the duration of the treatment,
- the TDF-based ART regimen commencement date,

- the SCr value prior to TDF-based treatment,
- corresponding weight and height measurements for the respective SCr values, and
- CD4+ cell count and viral load (VL) values prior to TDF-based treatment.

3.4 SERUM AND URINE SAMPLE ANALYSES

Analysis of serum and urine samples were performed by PURE research team members at the School for Physiology, Nutrition and Consumer Science, North-West University, Potchefstroom. Participants' serum was analysed on COBAS INTERGRA® 400 plus (Roche Diagnostics, Switzerland) for:

- alkaline phosphatase,
- creatinine,
- phosphate,
- calcium,
- sodium,
- urea, and
- uric acid.

Urine samples were analysed for:

- phosphate,
- sodium,
- glucose,
- creatinine, and
- albumin.

Bone markers, PTH, VitD and CTx, were analysed in serum on Elecsys® 2010 (Roche Diagnostics, Switzerland).

3.4.1 Calculation of renal parameters

Renal parameters such as eGFR, CrCl, fractional tubular reabsorption of phosphate (TRP), $TmPO_4$/GFR and albuminuria were calculated using various formulae (Barth *et al.*, 2000; Cockcroft & Gault, 1976; Levey *et al.*, 2007; O'Seaghdha *et al.*, 2010). Details are presented in Chapter 5 (Manuscript B).

3.4.2 Analytical method development and validation for plasma TFV

The procedure for method development was performed according to the laboratory protocol in Addenda C and E4. European Medicines Agency and Food and Drugs Administration criteria (EMA, 2013; FDA, 2013) were used as a guide for the plasma TFV analytical method validation. The method was validated for sensitivity, lower limit of quantification (LLOQ), selectivity, matrix effect, linearity, accuracy, precision, recovery, carry-over and stability. A detailed description is presented in Chapter 4 (Manuscript A).

The developed and validated method was used to determine TFV concentrations in HIV plasma samples of the participants. The summary table of individual results and descriptive statistics are presented in Addendum E3.

3.5 STATISTICAL ANALYSIS

All variables in HIV-infected women and their matched HIV-uninfected controls were tested for normality using the Shapiro-Wilk test and visual inspection of histograms and normal Q-Q plots. The unpaired t-test (parametric) was used to make comparisons between two groups if data was approximately normally distributed. The Mann-Whitney U test (non-parametric) was used to compare groups where data was skewed. Analysis of covariance (ANCOVA) was used to perform adjusted analyses for potential covariates (smoking status and alcohol use) between the HIV-infected and uninfected groups.

In the HIV-infected group, Pearson correlation was used to determine the linear relationship between plasma TFV concentration and renal function markers or BTM and BMD. Stepwise linear regression analysis was performed to test for associations and

interactions between plasma TFV concentrations and other variables controlling for age and BMI. Correlations of BTM and BMD at ≤ 100 ng/mL and ≥ 120 ng/mL of plasma TFV concentrations were investigated in the HIV-infected group. Correlations among BTM and BMD were investigated according to the duration of TDF therapy and VitD status. The paired t-test was used to make comparisons between paired data (CrCl, eGFR and CD4+ cell count) sets in the HIV-infected group.

Associations between variables and interactions in HIV-infected and uninfected groups were determined using a linear regression model controlling for covariates (smoking and alcohol use).

Statistical analyses were performed using IBM® SPSS® Statistics software, version 22 in consultation with Dr Suria Ellis and Ms Marike Cockeran from the North-West University. A significance testing level of 0.05 was used.

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Manuscript A

Chapter 4

In this chapter, a manuscript titled:

"Development and validation of high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for determination of tenofovir in small volumes of human plasma"

is presented. The manuscript was submitted to *Journal of Pharmaceutical and Biomedical Analysis* as a full length research paper and prepared according to the specific *Instructions to the Author* inluded in this dissertation as Addendum B1.

Title page

Development and validation of high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for determination of tenofovir in small volumes of human plasma

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Abstract

Tenofovir (TFV) is a nucleotide reverse transcriptase inhibitor with activity against human immunodeficiency virus. Determination of plasma TFV concentrations in small plasma volumes and in a short time period is most desirable in a clinical setting. Thus we developed and validated a HPLC-MS/MS method for the determination of TFV. Plasma sample volumes of 10 µL were extracted by protein precipitation. Cimetidine, used as internal standard (ISTD) and TFV were separated on a C18 (Phenomenex Kinetex^{TM} 30 mm x 2.1 mm, 2.6 µm) reversed phase column with a pre-column. The gradient mobile phase consisted of 10 mmol/L ammonium acetate in water and acetonitrile/methanol (50:50, v/v). TFV and ISTD retention times were 0.27 and 0.90 minutes, respectively, with a run time of 2 minutes. Transition of parent to product ion of TFV (m/z 288.072→176.038) and ISTD (m/z 253.13→158.987) were monitored in positive ionization mode. Calibration curves were linear (average r^2, 0.9958) over a TFV concentration range of 12.5-600 ng/mL. Mean recovery was 91.5%. Accuracy, inter and intra assay of three quality controls and lower limit of quantification (12.5 ng/mL) were within the acceptable limit of < 15% relative standard error. TFV was stable at studied conditions and neither matrix effect nor significant carry-over was observed. A distinct, reliable and robust method for determination of TFV in a small volume (10 µL) of human plasma for clinical application has been developed and validated.

Keywords: Tenofovir, Small plasma volume, Clinical, HPLC-MS/MS.

Highlights

- Tenofovir determined in only 10 µL of human plasma with desired sensitivity and accuracy.
- Improved sample preparation by optimizing easy plasma protein precipitation.
- Short chromatographic run time of 2 minutes.
- Validated HPLC-MS/MS method suitable for clinical application.

Abbreviations

TDF: Tenofovir disoproxil fumarate

TFV: Tenofovir

ISTD: Internal standard

CAS: Chemical abstract service

HIV-1: Human immunodeficiency virus type 1

HIV-2: Human immunodeficiency virus type 2

1. Introduction

Tenofovir disoproxil fumarate (TDF) is a prodrug of tenofovir diphosphate (TFV), a nucleotide analogue of adenosine monophosphate with potent activity against human immunodeficiency virus (HIV-1and HIV-2). It is minimally bound to human plasma or serum proteins *in vitro*. The drug is not a substrate of the cytochrome P-450 enzyme system but is mainly excreted unchanged in urine. After intracellular uptake, TFV inhibits the activity of HIV-1 reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate for incorporation into DNA [1, 2]. A dose of 300 mg/day TDF is used in combination with other antiretroviral drugs to manage HIV-1 infection in adults with proven improved efficacy [3]. The maximum plasma TFV concentration ranges from 195.6-306.3 ng/mL and the trough concentrations from 33.5-62.1 ng/mL [4]. Elevated plasma trough TFV concentration (> 90 ng/mL) is associated with renal impairment and bone mineral loss [5, 6] and this suggests the need of monitoring plasma TFV as a way of managing adverse effects experienced by patients.

To this effect, several analytical methods to determine TFV or in combination with other antiretroviral drugs in plasma have been published [7-10]. The analytical methods differ with the steps involved in sample preparation, the cost of the method and the duration or run time of the assay.

The aim of the present study was to develop and validate HPLC-MS/MS method for the determination of TFV in human plasma. Sample preparation was improved by using smaller volumes of plasma and run time was reduced, while maintaining the desired sensitivity and stability for clinical application.

2. Materials and methods

2.1. Chemicals and reagents

Tenofovir monohydrate USP ($C_9H_{14}N_5O_4P.H_2O$) reference standard (see **Fig.1**) was obtained from Industrial Analytical (Pty) Ltd, South Africa (CAS Number 206184-49-8). Cimetidine ($C_{10}H_{16}N_6S$) as internal standard (ISTD) (see **Fig.1**) was sourced from Sigma-Aldrich, USA (CAS Number 51481-61-9). HPLC water was obtained from a Milli-Q water purification system (Millipore SAS 67120 Moisheim, France). Ammonium acetate, LC/MS/MS hyper grade acetonitrile and methanol were sourced from Merck (Pty) Ltd, South Africa. Drug free plasma containing sodium citrate anticoagulant was kindly donated from healthy volunteers and stored at -23°C.

2.2. Instrumentation

An Agilent 1290 Infinity HPLC System consisting of binary pump with two identical high pressure (1200 bar) pumps; a two-channel solvent degasser and four-channel inlet solvent selection valve and CTC PAL HTx-xt auto sampler with a 20 µL sample loop were used for analysis. Mass spectrometric detection was performed on an AB SCIEX 4000 QTRAP® MS/MS system. Quantitation was performed in multiple reaction monitoring mode (MRM) and Analyst® 1.6 software was used to manage and execute analysis. MultiQuant 3.0 was used to quantify results and create reports.

2.3. Chromatographic conditions

Chromatographic separation was carried out on C18 (Phenomenex Kinetex™ 30 mm x 2.1 mm, 2.6 µm) reversed phase column with a pre-column (UHPLC C18, 2.1 mm ID,). Column and auto sampler tray temperatures were maintained at 20°C and 19°C, respectively. A gradient was used to elute TFV and ISTD as shown in **Table 1**. The total chromatographic run time was 2 minutes. Mobile phase A consisted of 10 mmol/L

ammonium acetate in water and phase B consisted of methanol: acetonitrile (50:50, v/v).

2.4. Mass spectrometric conditions

Optimization of the signal was performed by constant injection of high concentration of TFV and ISTD. The transition of parent to product ion was studied with the use of a turbo spray ionization source, operating in the positive ionization mode. The transition of parent→product ion (m/z) for TFV was 288.072→176.038. Dwell time (msec), declustering potential (DP), collision energy (CE) and collision exit potential (CXP) were optimized at 200 msec, 106, 35 and 8 volts, respectively. The parent→product ion (m/z) for ISTD was 253.13→158.987. The dwell time was 200 msec. DP, CE and CXP was 61, 21 and 12 volts, respectively.

2.5. Preparation of stock and internal standard solutions

Stock solution was prepared by dissolving 8.5 mg of TFV reference standard in 50 mL of Milli-Q water to a final concentration of 170 µg/mL. Stock quality control (QC) solution was prepared by dissolving 9.1 mg of TFV reference standard in 50 mL of Milli-Q water to a concentration of 182 µg/mL. This solution was then further diluted with Milli-Q water to a final QC concentration of 170 µg/mL. 10.08 mg of ISTD was dissolved in 25 mL of methanol: water (40:60, v/v) to a final concentration of 403.2 µg/mL. This solution was diluted to 10 ng/ml with Milli-Q water on the day of analysis. All solutions were clearly labeled and stored in Eppendorf tubes at -23°C.

2.6. Preparation of calibration standards and quality control samples

Calibration standards were prepared by spiking 4235 µL of drug free plasma with 15 µL of TFV stock solution which was then diluted with drug free plasma. The nine calibration standard concentrations were 12.5, 25, 50, 100, 200, 300, 400, 500 and 600 ng/mL.

QC samples were prepared at 30, 150 and 450 ng/mL for low quality control (LQC), medium quality control (MQC) and high quality control (HQC), respectively by spiking 4235 µL of drug free plasma with 15 µL of stock QC solution and then diluting appropriately with drug free plasma. Calibration standards and quality control samples were stored in Eppendorf tubes at -23°C in 100 µL aliquots. Prior to analysis, all frozen samples from participants, calibration standards and quality control were left to thaw unassisted until equilibrated with room temperature.

2.7. Sample pre-treatment

Nine calibration standards and QC plasma samples in Eppendorf tubes were heated in a water bath at 57°C for 30 minutes and were left to equilibrate to room temperature. 25 µL (10 ng/mL) of ISTD stock solution was added to 10 µL of the plasma sample. To precipitate plasma proteins, 100 µL of methanol: acetonitrile (50:50 v/v) was added to the sample. The sample was then vortex mixed for 10 seconds, left in a freezer at -23°C for 10 minutes, vortex mixed for a second time and then centrifuged for 4 minutes at 6000 rpm. 50 µL of the supernatant was transferred into a clean auto sampler vial, 120 µL of Milli-Q water was added and vortex mixed. 20 µL of this solution was injected onto the column for analysis.

The same pre-treatment procedure was performed for all human plasma samples. Heating of plasma samples in a water bath ensured inactivation of HIV.

2.8. Method validation

The analytical method was validated for linearity, sensitivity, accuracy, precision, selectivity, carry-over, recovery, matrix effect and stability according to the European Medicines Agency and US Food and Drug Administration guidelines for bio analytical method validation [11, 12]. Each analytical validation run comprised nine spiked

standards, QC samples, zero blank (with ISTD, without TFV) and blank (without ISTD or TFV) samples.

2.9. Clinical application

This method was developed to determine plasma TFV concentrations of 30 HIV-1-infected women in a pilot cross-sectional within the Prospective Urban and Rural Epidemiology-South Africa study (PURE-SA). Women were all on first line antiretroviral regimen, taking a TDF dose of 300 mg *nocte*. This investigation was approved by Human Research Ethics Committee (HREC) of North-West University in Potchefstroom (NWU-00016-10-A1 on 12/04/2013). Participants signed informed consent to have their blood drawn for analysis. Blood was collected in tubes containing sodium citrate anticoagulant between 11-14 hours post TDF dose and plasma was stored at -80ºC until analyzed.

3. Results

3.1. Method development

TFV and ISTD were successfully separated on C18 (Phenomenex Kinetex™ 30 mm x 2.1 mm, 2.6 µm) reversed phase column with a pre-column. Retention times for TFV and ISTD were 0.27 and 0.90 minutes, respectively. Representative chromatographic profiles of upper limit of quantification (600 ng/mL) and zero blank samples are presented in **Fig.2**.

3.2. Linearity and sensitivity

Linearity and sensitivity were determined by a nine-point TFV calibration standard curve on three separate days. The curve was constructed (Y-axis) using peak area ratios of chromatograms (TFV peak area/ISTD peak area) versus (X-axis) nominal concentration of TFV over the concentration range of 12.5-600 ng/mL. A linear regression weighting

factor of 1/x best described the linearity of the calibration curve with regression coefficient (r^2) ranging from 0.9942-0.9970 with an average of 0.9958. The lower limit of quantification (LLOQ) was 12.5 ng/mL and the signal was more than 5 times the signal of the blank sample (see **Fig.3**) using the FDA/ EMA guidelines [11,12]. Back calculated mean concentrations with percent relative standard deviation and accuracy are presented in **Table 2**.

3.3. Accuracy and precision

Accuracy and precision for the analyte were determined by analyzing QCs including lower limit of quantification samples (HQC, MQC, LQC and LLOQ) with the standard calibration curve. Sets of samples were analyzed within a single analytical run (within-run precision) and between different runs (between-run precision) on different days. **Table 3** summarizes the accuracy and precision results.

3.4. Selectivity and carry-over

Selectivity was demonstrated by analyzing six drug free plasma samples obtained from six random individual donors in sodium citrate tubes spiked at LLOQ. Negligible interference in one donor's plasma was observed at the TFV retention time (0.27 minutes); blank and TFV peak area counts were 96.909 and 988.236 (9.8%), respectively [11, 12]. Chromatograms of the blank and spiked samples from five donors showed no endogenous peaks at TFV retention time or internal standard retention time (0.90 minutes). Hence the method was selective and unaffected by interference from endogenous components in the matrix.

Carry-over was assessed by injecting blank samples after the upper limit of quantification (600 ng/mL) of the calibration standard in each analytical run. TFV carry-over for three runs was negligible (0.00, 0.00 and 0.286 ng/mL) with average of 0.095

ng/mL (0.76% of LLOQ). This was within the acceptable range of not greater than 20% of the LLOQ. There was no observed carry-over in the ISTD.

3.5. Recovery

Extraction recovery of TFV from sodium citrate plasma was determined at 4 concentrations (HQC, MQC, LQC and LLOQ) in triplicate by comparing analyte-internal standard area ratio of samples spiked in plasma with those of spiked in Milli-Q water. The mean recovery of TFV at HQC, MQC, LQC and LLOQ was 78.0, 100.1, 92.8 and 95.3%, respectively. The overall mean extraction recovery (91.5%) was high.

3.6. Matrix effect

Co-elution of some endogenous compounds from the matrix can affect sensitivity, precision and accuracy of the bio-assay. The matrix effect was thus determined by assessing variability in instrument response from six lots of plasma of individual donors. Each plasma lot was spiked with TFV at 30, 150 and 450 ng/mL and analyzed in 6 runs. The overall coefficient of variation (CV) for the calculated concentrations at three levels was less than 15% as presented in **Table 4**. No significant differences were observed in peak areas of the ISTD.

3.7. Stability

Stability of TFV was investigated in plasma spiked at LQC, MQC and HQC. Samples were heated in a water bath at 57°C for 30 minutes and analyses were performed in quadruplicate. Coefficient of variation ranged between 2.6-12.8% and accuracy was between 93.3-99.1%. After a second freeze-thaw cycle at -23°C of the same QC plasma samples, analyses were performed in triplicate and accuracy ranged between 90.3-93% with coefficient of variation between 5.1-10.0%. The mean concentration of freeze-thaw samples compared to heated samples decreased by 8.0, 2.6 and 5.9% for

LQC, MQC and HQC, respectively. Hence TFV was stable after heating in a water bath and after two freeze-thaw cycles.

3.8. Clinical application

This validated assay was successfully applied in measuring TFV in plasma samples of 30 HIV-infected women and investigated the association of TFV plasma and its toxicity on renal function and bone metabolism. TFV was not detected in 5 participant's plasma samples. The mean concentration in 25 participants was found to be 114.0 ± 78.9 ng/mL. The lowest and highest concentration was 17.2 and 434.2 ng/mL, respectively corresponding to mid-dose concentration [6]. The concentrations were all in the range of 12.5-600 ng/mL. The typical chromatograms of high and low TFV plasma concentrations from two participants are presented in **Fig.4**.

4. Discussion

In several published analytical methods where TFV was analyzed in the presence of other antiretroviral drugs [8, 9, 13-15] or alone [7, 10, 16, 17], fairly large plasma volumes were used: volumes between 100-200 µL and 200-1000 µL, respectively for protein precipitation or solid phase extraction were documented. This is the first method, to our knowledge, where only 10 µL of plasma was used for extraction of TFV by protein precipitation, making it preferable and suitable for clinical application. Furthermore, this method is convenient for the emerging interest in therapeutic drug monitoring of TDF in patients [5, 18]. A method [10] with a similar run time (1.8 minutes) to this method (2 minutes) was reported in the literature. However, the former required a solid phase extraction procedure which is expensive and more time consuming in sample pretreatment than the current method.

The method developed is robust, fast, accurate, and sensitive with high extraction recovery and it covered the whole range of expected plasma concentration [6]. A

mixture of acetonitrile and methanol (50:50,v/v) made a better precipitating solvent than acetonitrile or methanol alone. Protein precipitation was further enhanced by refrigeration of samples in addition to vortex mixing. Dilution of supernatant with water at neutral pH produced a higher TFV intensity chromatogram than acidic or basic water. Similarly, the intensity of TFV chromatograms decreased with decreasing auto sampler temperatures but was optimal at 19ºC.

TFV was stable after at least 2 freeze-thaw cycles, which was in agreement with previously published methods [19, 20]. Additionally, this method showed that plasma TFV was stable when exposed to a temperature of 57ºC in a water bath for 30 minutes.

5. Conclusion

A simple, fast, reliable and robust HPLC-MS/MS method has been developed and validated for determination of TFV in human plasma. This developed method is suitable for clinical application and was used for analysis of small volumes (10 µL) of plasma TFV in HIV-1-infected women with desired sensitivity and accuracy.

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Table 1. Chromatography gradient elution

Run time (min)	Flow rate (µL/min)	A (%)	B (%)
0.00	250	95.0	5.0
0.10	250	95.0	5.0
0.60	250	5.0	95.0
1.30	250	5.0	95.0
1.40	250	90.0	10.0
2.00	250	90.0	10.0

Table 2. Summary of standard calibration curve concentrations

Nominal conc. (ng/mL)	**[a]Calculated mean conc. (ng/mL)**	**[b]%RSD**	**Accuracy (%)**
12.5	13.2	2.0	105.5
25	25.8	4.7	103.4
50	47.4	6.1	94.8
100	94.9	5.8	94.9
200	199.7	13.3	99.9
300	308.0	3.6	102.7
400	388.5	2.9	97.1
500	503.1	6.2	100.6
600	606.9	6.2	101.1

[a] Mean of 3 injections on three separate days; [b] percent relative standard deviation.

Table 3. Within-run (intra) and between-run (inter) assay performance for TFV in plasma

Nominal conc. (ng/mL)	**Calculated conc. (ng/mL)**	**Accuracy (%)**	**Precision (%RSD)**
Within-run assay			
12.5[a]	12.2	98.2	3.6
30[b]	30.2	100.6	5.9
150[b]	146.4	97.6	7.9
450[b]	453.1	100.7	11.2
Between-run assay			
12.5[c]	11.5	92.0	3.6
30[d]	28.0	93.2	8.8
150[d]	139.0	92.5	14.9
450[d]	399.2	88.7	5.9

[a] n=5, [b] n=6, [c] n=3, [d] n=4.

Table 4. Matrix effect at LQC, MQC and HQC in six lots of plasma

	LQC (30 ng/mL)		**MQC (150 ng/mL)**		**HQC (450 ng/mL)**	
	Calculated conc. (ng/mL)	Accuracy (%)	Calculated conc. (ng/mL)	Accuracy (%)	Calculated conc. (ng/mL)	Accuracy (%)
1	26.64	88.8	149.31	99.5	466.43	103.7
2	33.16	110.5	133	88.7	405	90.0
3	28.9	96.3	130.52	87.0	403	89.6
4	24.1	80.3	117.73	78.5	331	73.6
5	29.65	98.8	146.4	97.6	444.02	98.7
6	28.42	94.7	121.8	81.2	362.2	80.5
Mean		94.9		88.7		89.3
%CV		10.7		9.6		12.5

List of figures

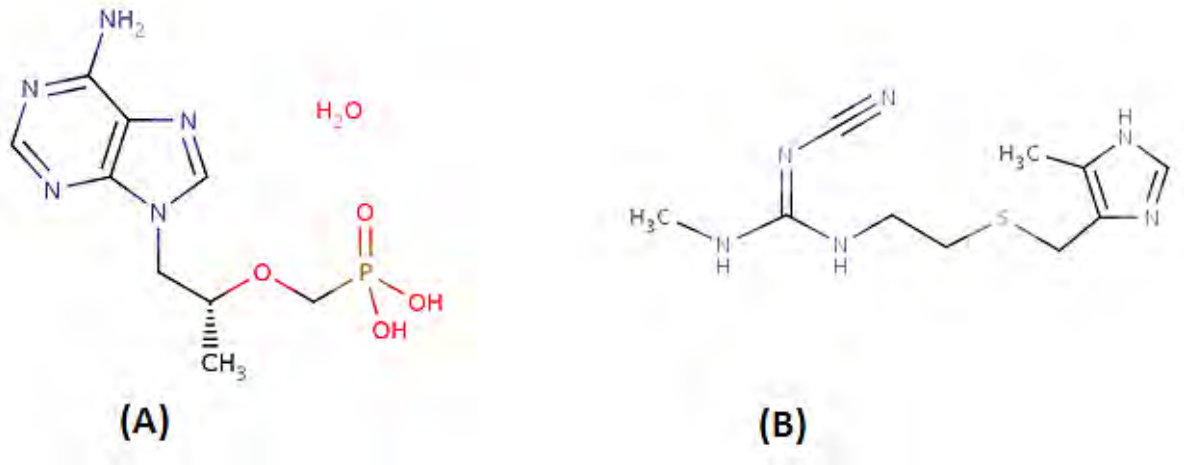
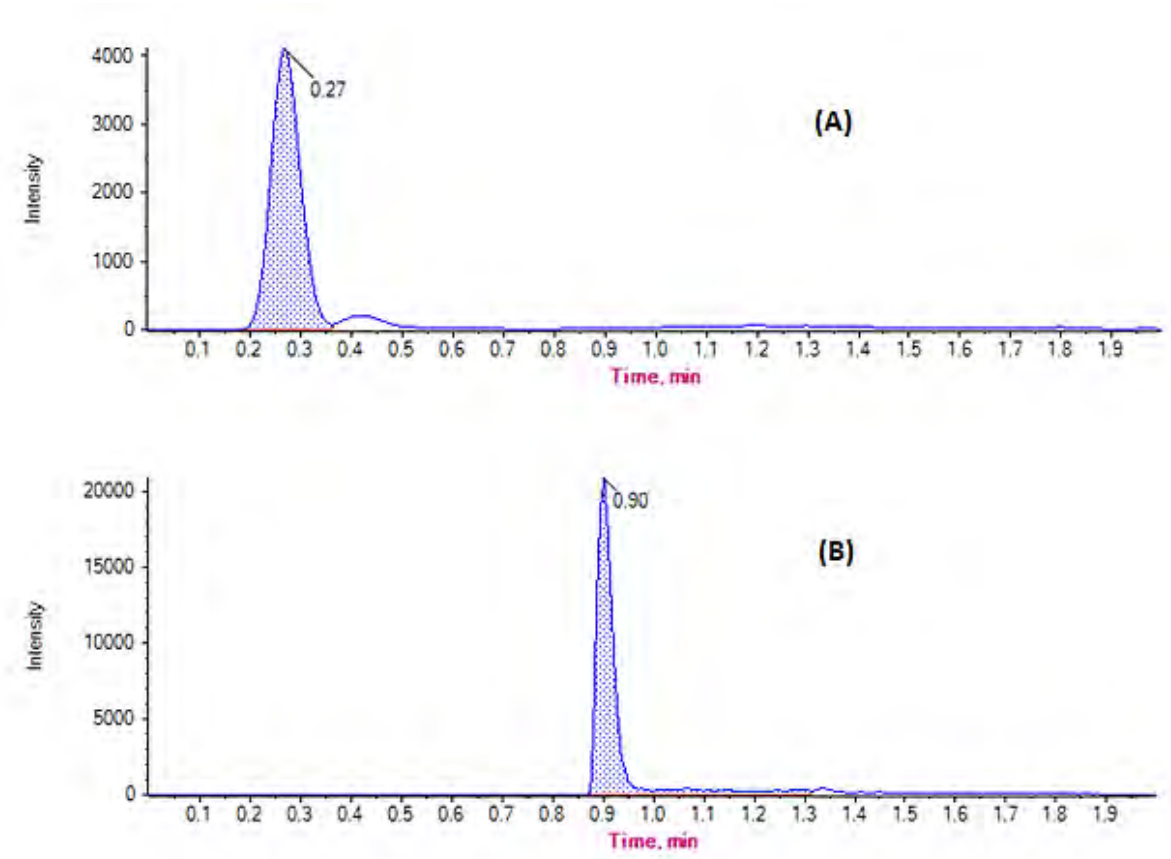


Fig.1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Time, min

Fig.2

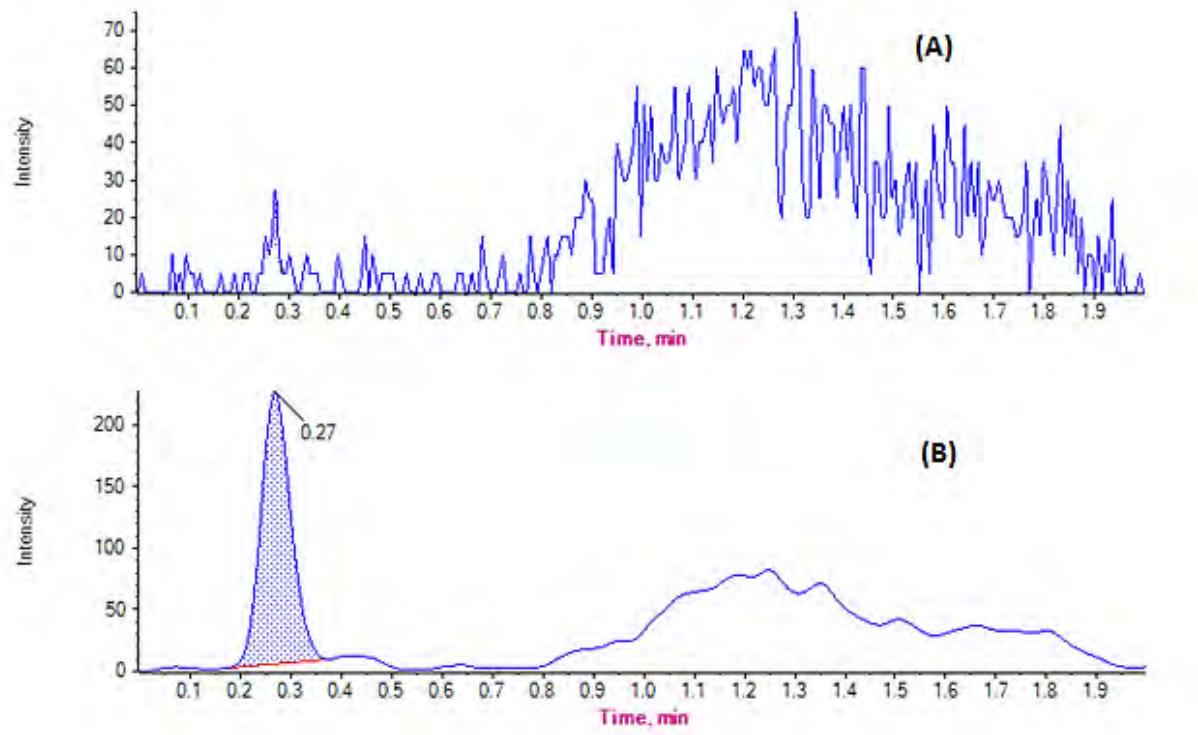

Fig.3

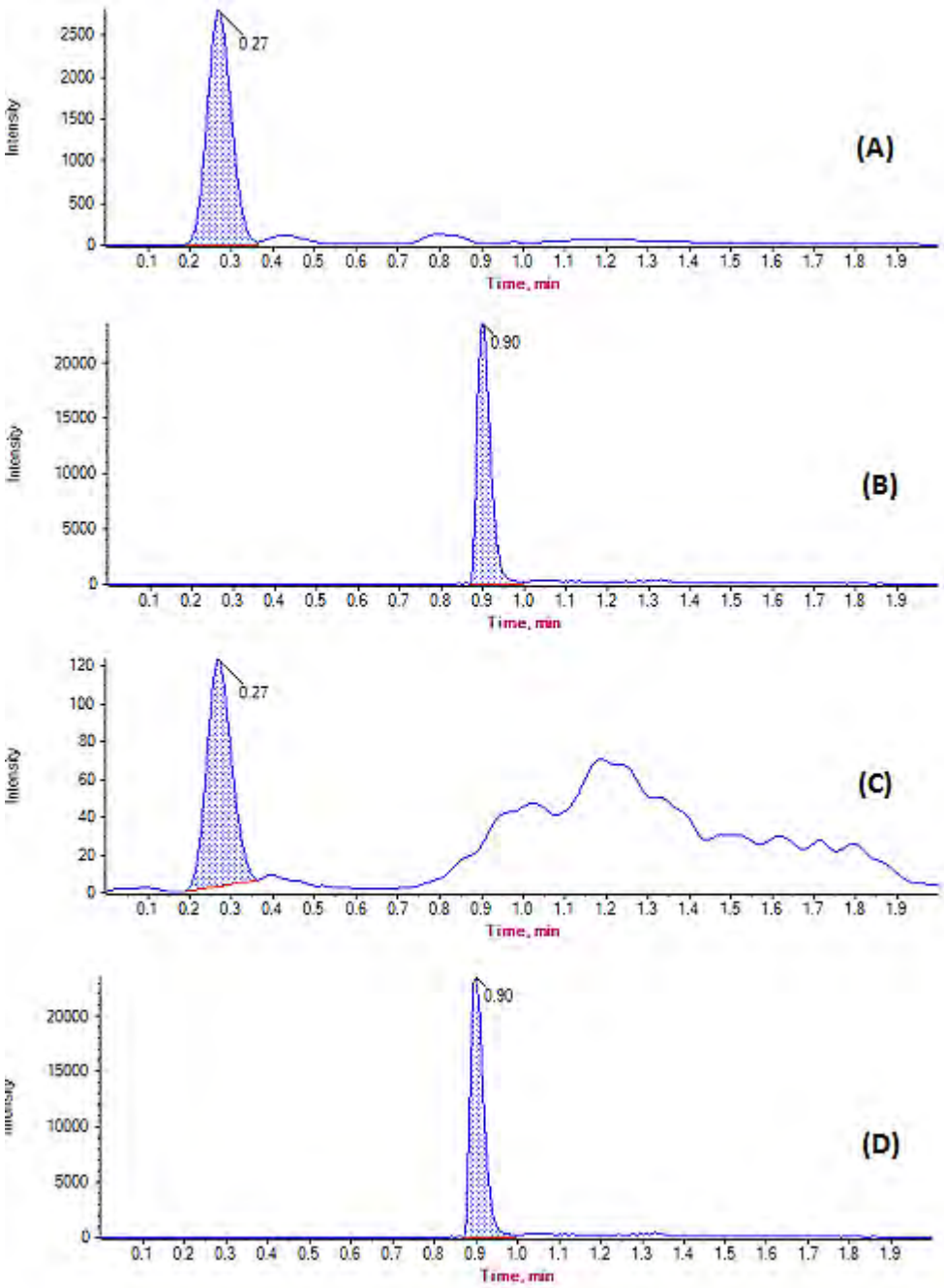
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Fig.4

In this chapter, a manuscript titled:

"Associations between plasma tenofovir concentration and renal function markers in HIV-infected women"

is presented. The manuscript has been prepared for submission to *AIDS Research and Human Retroviruses* as a full communication and prepared according to the specific *Instructions to the Author* inluded in this dissertation as Addendum B2.

Title

Associations between plasma tenofovir concentration and renal function markers in HIV-infected women

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Running title

Tenofovir concentration and renal function

Abstract

Despite tenofovir disoproxil fumarate (TDF) antiretroviral therapy (ART) being associated with renal dysfunction, few studies have investigated the correlation between renal dysfunction and plasma tenofovir (TFV) concentration. In this pilot cross-sectional study (PURE-SA), the correlations between plasma TFV concentration and certain renal function markers in HIV-infected women on TDF were investigated and compared to a control group. Thirty HIV-infected women on TDF-based ART were matched with 30 controls for age and body mass index. Renal markers analyzed were: estimated glomerular filtration rate (eGFR), creatinine clearance (CrCl), serum creatinine, albuminuria, glucosuria, serum urea, serum uric acid, urine sodium and maximum tubular reabsorption of phosphate. Baseline eGFR and CrCl data were obtained retrospectively in the HIV-infected women. Plasma TFV was assayed using a validated HPLC-MS/MS method. Stepwise regression, Mann-Whitney U test, unpaired t-test and paired t-test were applied in the statistical analyses. Only TFV concentration was independently associated with albuminuria (r^2 = 0.339*; p* = 0.001) in HIV-infected women. In the adjusted analysis, eGFR (*p* = 0.038), CrCl (*p* = 0.032) and albuminuria (*p* = 0.048) were significantly higher in HIV-infected than uninfected women, but eGFR and CrCl were abnormally high. Both eGFR (*p* < 0.001) and CrCl (*p* = 0.008) increased from baseline to follow-up in HIV-infected women. The plasma TFV concentration was associated with increased albuminuria in HIV-infected women in this pilot study. eGFR and CrCl were increased in HIV-infected women from baseline. These findings should be confirmed in larger studies and hyperfiltration in HIV-infected women warrant further investigation.

Key words: Plasma tenofovir, albuminuria, HIV, women, renal function, TDF.

Introduction

Tenofovir disoproxil fumarate (TDF) is a prodrug of tenofovir (TFV), a nucleotide reverse transcriptase inhibitor (NRTI). In combination with other antiretroviral drugs, TDF-based antiretroviral therapy (ART) is used as preferred effective first line treatment of adults infected with the human immunodeficiency virus (HIV).[1]

TFV plasma binding is low with less than 1% and 7.2% bound in human plasma and serum, respectively. It is eliminated mainly by renal excretion through a combination of glomerular filtration and active tubular secretion via the apical multidrug resistance protein (MRP4) transporter.[2, 3] In many studies treatment with TDF was associated with kidney tubular dysfunction and reduced renal function, with high prevalence being associated with female gender and age between 40-50 years.[4-6] The main target of toxicity is the proximal renal tubule and in severe cases, patients developed renal Fanconi syndrome.[7] Studies have shown that albuminuria might be a more reliable marker for glomerular and proximal tubular dysfunction.[8] The effect of TFV on glomerular function is less severe than renal tubular function.[6] TFV nephrotoxicity leads to breakdown of solute transport characterized by urine wasting of solutes and proteins like albumin normally reabsorbed in the proximal tubule.[7] Genetic polymorphisms in proximal tubule transporters may predispose certain individuals to accumulate high intracellular TFV levels and could increase the risk of developing proximal tubular toxicity.[9] Chronic kidney disease (CKD) is usually silent until later stages, thus many patients with CKD are detected only shortly before the onset of symptomatic kidney failure.[10] Furthermore, increased exposure to TDF is associated with a higher incidence of CKD.[10] Co-administration of TDF with protease inhibitors (PIs) is associated with even greater declines in renal function.[11] Despite TDF treatment being associated with CKD, few studies [12, 13] have been performed in Europe that related plasma TFV

concentration and renal function. There are no such studies to date that have been performed on Africans and this information is lacking in South Africa.

The objective of this study was to determine the correlations between plasma TFV concentration and renal function markers in HIV-infected women receiving TDF-based ART and to assess changes in renal function from baseline. We also compared these markers with those in a comparative HIV-uninfected control group.

Materials and methods

This was a pilot cross-sectional sub-study within the Prospective Urban and Rural Epidemiology-South Africa (PURE-SA) study.[14] PURE is a large longitudinal epidemiological study taking place in low-, middle-, and high-income countries around the world and since 2005 in the North West province of South Africa. In total, 462 participants with similar socio-demographic characteristics were followed up in November 2012, April and August 2013 in the Tlokwe and Ganyesa districts of the North West province in South Africa. Sixty (60) women participated in this sub-study: 30 HIV-infected women were on full ART taking 300 mg TDF *nocte* (7:00 PM - 09:00 PM, self-reported) and they were matched with 30 HIV-uninfected women for age and body mass index (BMI).

Participants signed written informed consent forms for the PURE-SA and this sub-study and the protocol was approved by the Human Research Ethics Committee of the North-West University (NWU-00016-10-A1) and North West Department of Health. During visits for the follow-up sub-study, participants were seen at NWU metabolic clinic (Potchefstroom Campus in the Tlokwe district) and at Sethlare Lodge (Ganyesa) on pre-arranged dates. Medication history was recorded by qualified pharmacists and assistants using a structured questionnaire.

Participants who had a medical history of renal disease, are amputees or on known renal toxic medication (streptomycin, lithium, sulfadiazine, phenytoin, allopurinol, amphotericin B deoxycholate, methotrexate, statins, mesalazine) were excluded from the study. Concomitant medication use for hypertension, tuberculosis (rifampicin, hydrochlorothiazide, nifedipine and isoniazid) was acceptable if medical history was available.

Sample analysis and outcome variables

Participants were asked to fast for at least 8 hours. Blood and spot urine samples for the participants were collected in the morning between 08:00 AM - 10:00 AM by a registered nurse. Serum creatinine (SCr), serum phosphate (SrP), serum uric acid, serum urea, glucosuria, urine creatinine (UCr), urine phosphate (UP), urine sodium (UNa), urine albumin and were analyzed on a Cobas Intergra 400 plus (Roche Switzerland). Plasma TFV was quantified by a validated high performance liquid chromatography tandem mass spectrometry method (Mulubwa *et al,* under review) for the HIV-infected participants on TDF-based ART. Accuracy and precision was within < 15% of the relative standard error. The mean linear regression coefficient (r^2) of the calibration curves over the concentration range 12.5 - 600 ng/mL was 0.9958 with LLOQ as 12.5 ng/mL.The extraction recovery was 91.5%.

eGFR was calculated using the Modification of Diet in Renal Disease (MDRD)[15] equation without inclusion of the black ethnicity factor for black South Africans as prediction is better without ethnicity factor: [16]

eGFR (mL/min/1.73m^2) = 30849 x SCr $^{-1.154}$ x Age $^{-0.203}$ x 0.742, where SCr is in µmol/L.

The Cockroft-Gault (CG) formula[17] was used to calculate creatinine clearance (CrCl) as follows:

CrCl (mL/min) = (140 – Age) x Weight x 0.85 / (0.814 x SCr), where SCr is in µmol/L.

Maximum tubular reabsorption of phosphate ($TmPO_4$/GFR) was calculated using the following formula[18]:

TmPO4/GFR = 0.3 x TRP / (1 – 0.8 x TRP) x SrP, where TRP = 1 – (UP / SrP) x (SCr / UCr). If TRP was 0.86 or less, then $TmPO_4$/GFR was calculated with the formula: $TmPO_4$/GFR = TRP x SrP.

Albuminuria was calculated as a ratio of urine albumin to urine creatinine (uACR) [19]: [albuminuria = urine albumin (mg/L) / urine creatinine (mmol/L)].

Glomerular hyperfiltration was defined as eGFR > 150 mL/min/1.73m^2 for women.[20]

Baseline information on the weight prior to TDF initiation, SCr, duration of TDF ART and non-TDF ART regimens were recorded retrospectively from the clinic and hospital files of the institutions where these participants accessed their health care services.

Statistical analysis

The selection of HIV-uninfected comparative group was performed by propensity score matching with the HIV-infected women. Propensity scores were estimated from a logistic multivariate regression model containing age and BMI as predictors used to model case/control membership. The selection of controls for the cases was performed automatically from the resulting propensity variable. The optimal match tolerance was 0.26 and sampling of controls was done without replacement.

All variables in the two groups were tested for approximate normality using the Shapiro-Wilk test and visual inspection of respective normal Q-Q plots. Median and interquartile range (IQR) values were computed for the description of variables.

Correlations (Pearson) were performed between plasma TFV concentrations and renal function variables in HIV-infected women. Block stepwise linear regression analysis

was performed to test for associations between variables within the HIV-infected group, controlling for age and BMI.

Mann-Whitney U test or unpaired t-test was used to compare variables between the HIV-infected and the uninfected group if data was not normally or normally distributed respectively. Adjusted analyses for weight were performed using analysis of covariance (ANCOVA) and results were presented in a table. Associations between groups, stratified by TDF exposure (HIV-infected) and non-infected status controlling for age and BMI were performed. CrCl and eGFR were further categorized and presented in a table.

A linear regression model was used to test for interactions. Paired t-tests were performed in the HIV-infected group to determine the mean change of CrCl, SCr and eGFR from baseline to follow-up time. A two-tailed significance testing level of 0.05 was used. All statistical analyses were performed using IBM® SPSS® Statistics software, version 22.

Results

Participants' characteristics are presented in **Table 1**. TFV was not detected in five (5) participants' plasma samples. The median (IQR) TFV plasma concentration was 113 (74-139.4) ng/mL for the twenty five (25) HIV-infected participants. The lowest and highest plasma concentrations were 17.2 and 434.2 ng/mL, respectively.

Plasma TFV concentration and renal function markers in HIV-infected women

No significant correlations were found between plasma TFV concentration and eGFR, CrCl, $TmPO_4$/GFR, SCr, UNa, serum urea or serum uric acid ($p > 0.05$). Nevertheless, a positive correlation was found between TFV plasma concentration and albuminuria ($r = 0.606$; $p = 0.001$). Stepwise linear regression analysis was then performed to test for associations between TFV concentration and albuminuria with age and BMI as fixed

factors. TFV concentration was independently associated with increased albuminuria (see **Fig 1)**.

In the unadjusted analysis, albuminuria (18 vs.8.8 mg/mmol; $p = 0.028$) and eGFR (126 vs. 105 mL/min/1.73m^2; $p = 0.02$) was significantly higher in the HIV-infected group compared to the matched HIV-uninfected group (see **Table 2**). SCr (50 vs. 54 µmol/L; $p = 0.028$) and UNa (76 vs. 102 mmol/L; $p = 0.048$) was significantly lower in HIV-infected than the HIV-uninfected group. No statistical significant difference in $TmPO_4$/GFR, CrCl, serum urea and serum uric acid was found between the groups ($p > 0.05$).

ANCOVA adjusted for weight showed significantly higher CrCl (117 vs. 99 mL/min; $p = 0.024$), eGFR (124 vs. 106 mL/min/1.73m^2; $p = 0.047$) and albuminuria (16.6 vs. 10.2 mg/mmol; $p = 0.048$) in HIV-infected compared to the matched HIV-uninfected control group. eGFR and CrCl were abnormally higher in HIV-infected than HIV-uninfected control group, and percentage categories are presented in **Table 3**. Linear regression analysis showed no significant interaction effect between weight and HIV-infected status (TDF exposure, $p > 0.05$).

Changes in renal function markers retrospectively between baseline and follow-up in HIV-infected women

Baseline values for eGFR, SCr and CrCl were only available for 21 of the HIV-infected participants as information was missing or not included in their medical records. The eGFR significantly increased by 30.7% (+38.5 mL/min/1.73m^2, $p < 0.001$) from baseline (87 ±29 mL/min/1.73m^2) to follow-up. CrCl increased by 25% (+26.5 mL/min, $p = 0.008$) from baseline (80 ±28 mL/min) to follow-up. SCr decreased by 34.5% (-17.9 mmol/L, $p = 0.017$) from baseline (70 ±29 µmol/L) to follow-up. These changes occurred within a median duration of 16.6 months of TDF exposure.

Discussion

In the current study, albuminuria was significantly higher in HIV-infected women on TDF-based ART regimen than the HIV-uninfected controls. Similarly, results from a sub-study of a randomized trial (n = 19) showed significant increased albuminuria in HIV-infected patients who switched to TDF-based ART regimen compared to patients who continued on a non-TDF-based ART regimen from baseline to 48 week follow-up.[21] Albuminuria is an important early sign for progressive renal function loss and a significant risk factor for near term development of overt kidney disease in HIV infection.[22]

Kidney Tubular Dysfunction (KTD) is defined when at least two of the following are present: altered resorption of phosphorus, abnormal uric acid excretion, non-diabetic glucosuria, hyperaminoaciduria or hyperphosphaturia.[12] In a study by Rodriguez-Novoa (n = 92), KTD was associated with median mid-dose plasma TFV concentration of 182 (105-220) ng/mL and normal tubular function with median mid-dose plasma TFV concentration of 106 (75-148) ng/mL.[12] In this study, plasma TFV concentration was found to be a significant independent predictor of albuminuria in HIV-infected participants. The median mid-dose plasma TFV concentration associated with albuminuria was 113 (74-139.4) ng/mL. This suggests the need for monitoring plasma TFV concentration to detect early signs of kidney tubular toxicity, especially with combination therapy. To our knowledge this is the first study to determine an association between plasma TFV concentration and albuminuria in HIV-infected women with a median age of 52 years in South Africa.

We did not find an association between plasma TFV concentration and eGFR or CrCl in HIV-infected women. This is consistent with other findings[11, 23] where trough TFV concentration was used to correlate, unlike the median TFV concentration in our study,

which corresponded to mid-dose. In contrast, high trough plasma TFV concentration had been associated with decreased eGFR.[13]

Glomerular hyperfiltration is common among HIV-infected persons. Although no common definition has been agreed upon, it varies as either abnormally high eGFR, with a threshold ranging from 125 -175 mL/min/1.73 m^2, or increased filtration fraction, or as increased filtration per nephron.[24, 25] Glomerular hyperfiltration can be an early indicator of kidney dysfunction and precedes the onset of impaired renal function and albuminuria; although the length of time it takes to progress to renal impairment is unknown.[24] In this study, eGFR and CrCl were above normal levels in HIV-infected women compared to the HIV-uninfected women. Furthermore, a larger proportion (33.3% vs. 10%) of HIV-infected participants had glomerular hyperfiltration, which could be an early indicator of renal impairment. Our results are in agreement with recent findings from a prospective cohort study with a much larger sample size (n = 367).[24] These authors suggested that glomerular hyperfiltration occur due to HIV infection and ART use.

Renal function had improved in a cohort of HIV patients (n = 566) initiated on TDF at 6 and 12 months follow-up, with CrCl increase of +5 mL/min and +7 mL/min.[26] In another study (n=201), no significant change in eGFR from baseline value at 6, 12 and 24 months was observed in TDF exposed HIV-infected adults, mainly of African-American ethnicity.[27] In this study, the HIV-infected participants on TDF-based ART regimen had an increase in CrCl and eGFR of +26.5 mL/min and +38.5 mL/min/1.73m^2 respectively within a median time of 16.6 months from baseline to follow-up. A complication of HIV disease itself is reduced CrCl, and ART was found to ameliorate renal function in advanced HIV disease [28] and may thus contribute to this increase in CrCl and eGFR that we observed in HIV-infected participants, which also is in agreement with reported literature.[28]

Conversely, in some cohort studies TDF therapy was associated with decreased eGFR[29, 30] that was not completely reversible.[31] This difference could have been due to other factors such as smaller body weight and smaller BMI, which are associated with decreased eGFR in patients exposed to TDF-based ART regimen.[32]

This study has three main limitations to consider. Firstly, the cross-sectional design only provided information at one point in time and hence could not infer causality of the relationship observed with plasma TFV and albuminuria. Secondly, the spot urine sample for the calculation of uACR was used. Thirdly, the sample size of 30 was too small to generalize the findings. Despite that, the strength lays in the fact that there was a control group to compare with HIV-infected participants within the same sub-study population and various renal markers were tested at the same time. Also, there was baseline information on CrCl, eGFR and SCr available to make comparisons within HIV-infected group. Our findings are nonetheless important in managing renal function monitoring in women receiving TDF-based ART regimens.

In conclusion, plasma TFV concentration is independently associated with increased albuminuria in HIV-infected women within this pilot investigation. There is increased eGFR and CrCl in HIV-infected women from baseline. Furthermore, longitudinal studies with larger sample sizes are needed to confirm these findings and to investigate glomerular hyperfiltration in ART-experienced patients, which may be an early sign of kidney dysfunction.

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Author Disclosure Statement

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Table 1. *Characteristics of HIV-infected and uninfected women (control group)*

	HIV-infected (TDF exposed)	*HIV-uninfected (Control)*	*p-value*
Number of participants, (n)	30	30	-
Median age (IQR), years	52 (49-59)	57 (53.7-63.3)	**0.008**
Median BMI (IQR), kg/m^2	20.5 (18.2-28.9)	24.4 (19.4-33.5)	0.333
Median weight (IQR), kg	52.5 (44-68.7)	60.4 (49.1-84.3)	0.179
Other antiretroviral drugs in regimen			
Lamivudine	30 (100%)	-	-
Efavirenz	30 (100%)	-	-
Concomitant medication			
Hydrochlorothiazide	1 (3.3%)	None	-
Nifedipine	1 (3.3%)	None	-
Underlying disease			
Hypertension	2 (6.7%)	None	-
MedianTDF HAART exposure (months)	16.6 (8.8-23.4)[a]	-	-
Median Non-TDF HAART exposure (months)	24.5 (16.8-43.8)[b]	-	-
Median time since HIV diagnosis (months)	31 (22-53.5)[b]	-	-

[a] n = 24 (4 participants declined to provide consent to access their medical records and information from 2 medical records was missing). [b] n = 22 (4 participants declined to provide consent to access their medical records and information from 4 medical records was missing). - = not applicable.

***Table 2**. Comparison between mean values of renal function markers in HIV-infected and uninfected women*

	HIV-infected (TDF exposed) n=30	*HIV-uninfected (Control) n=30*	*p-value*	*Adjusted p-value*[c]
SCr (µmol/L)	50 ±20.3	54 ±12.1	**0.028**[a]	0.441
SrP (mmol/L)	1.05 ±0.2	0.99 ±0.16	0.204[b]	
Serum urea (mmol/L)	3.3 ±1.7	3.5 ±1.32	0.507[b]	
Serum uric acid (µmol/L)	278.7 ±122	311 ±99.6	0.265[b]	
Glucosuria (mmol/L)	0.12 ±0.09	0.19 ±0.27	0.59[a]	
UCr (mmol/L)	4.3 ±3.1	6.0 ±5.8	0.145[b]	
UP (mmol/L)	5.4 ±5.3	7.5 ±9.4	0.524[a]	
UNa (mmol/L)	75.5 ±55.7	101.7±42.3	**0.048**[b]	0.119
Albuminuria (mg/mmol)	18.0 ±78.3	8.8 ±27.9	**0.028**[a]	**0.048**
$TmPO_4$/GFR	1.18 ±0.24	1.15 ±0.27	0.645[b]	
eGFR (mL/min/1.73 m^2)	125.8 ±39.6	105 ±25.5	**0.02**[b]	**0.047**
CrCl (mL/min)	112.5 ±40.5	104.9 ±43	0486[b]	**0.048**
Urine albumin (mg/L)	33.9 ±131	35.5 ±112.4	0.126[a]	

[a] *p*-value calculated from Mann-Whitney U test. [b] *p*-value calculated from unpaired t-test. [c] *p*-value resulting from ANCOVA adjusted for weight.

Table 3. *Percentage eGFR and CrCl categories in HIV-infected and uninfected women*

	HIV-infected (n = 30)	*HIV-uninfected (n = 30)*
eGFR <60 mL/min/1.73 m^2	2 (6.7%)	1 (3.3%)
CrCl <60 mL/min	1 (3.3%)	4 (13.3%)
eGFR 60-150 mL/min/1.73 m^2	18 (60%)	26 (86.7%)
CrCl 60-150 mL/min	25 (83.3%)	22 (73.3%)
eGFR >150 mL/min/1.73 m^2	10 (33.3%)	3 (10%)
CrCl >150 mL/min)	4 (13.3%)	4 (13.3%)

eGFR of > 150 mL/min/1.73m^2 represents glomerular hyperfiltration.

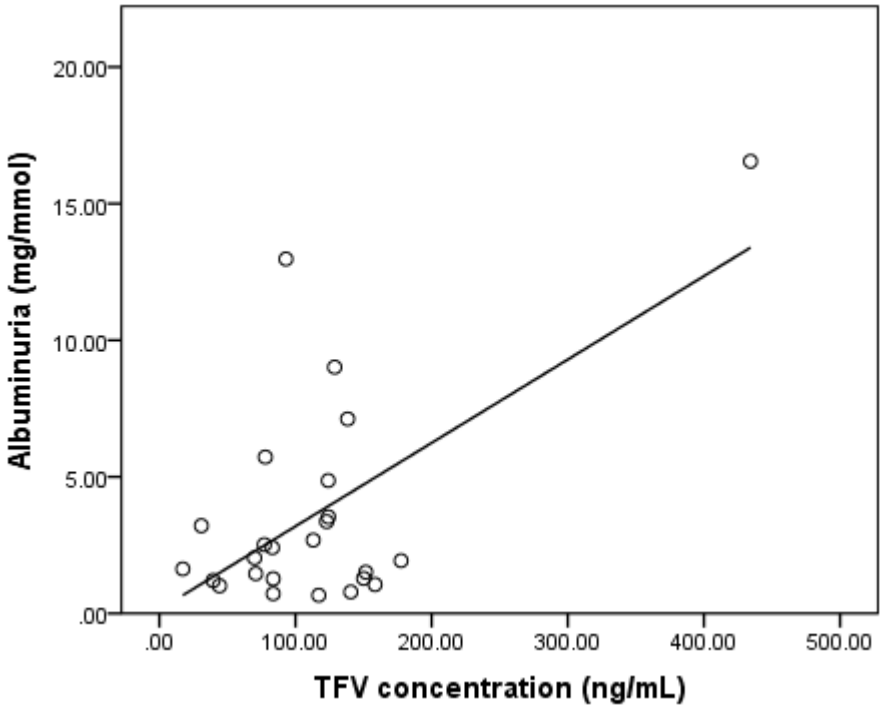

Fig. 1. Regression plot of *association between plasma TFV concentration and albuminuria in HIV-infected women controlling for age and BMI (adjusted $r^2 = 0.339$; $p = 0.001$*).

Manuscript C | Chapter 6

In this chapter, a manuscript titled:

“Association between plasma tenofovir concentration and bone turnover markers in HIV-infected women”

is presented. The manuscript has been prepared for submission to *European Journal of Clinical Pharmacology* as an original research paper and prepared according to the specific *Instructions to the Author* inluded in this dissertation as Addendum B3.

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Abstract

Purpose: The correlation between plasma tenofovir (TFV) concentration and various bone turnover markers and bone mineral density (BMD) in HIV-infected women on tenofovir disoproxil fumarate (TDF) antiretroviral therapy was investigated and compared these parameters with a HIV-uninfected control group.

Methods: A cross-sectional sub-study using 30 HIV-infected women on TDF and 30 HIV-uninfected age and body mass index matched subjects was performed. C-terminal telopeptide (CTx), alkaline phosphatase (ALP), parathyroid hormone (PTH), vitamin D (VitD), serum calcium (SrCa), serum phosphate (SrP) and BMD were measured. Plasma TFV was assayed on HPLC/MS/MS. Mann-Whitney U test, unpaired t-test, ANCOVA and stepwise regression were applied in statistical analyses.

Results: In HIV-infected women, no correlation existed between plasma TFV concentration and CTx, PTH, ALP, SrCa, SrP, VitD or BMD ($p > 0.05$). CTx and ALP correlated positively at plasma TFV concentration ≥ 120 ng/mL ($r = 0.704$; $p = 0.016$). Adjusted analysis showed higher ALP ($p < 0.001$), CTx ($p = 0.027$) and PTH ($p = 0.050$) in HIV-infected compared to HIV-uninfected women. In HIV-uninfected, SrCa correlated negatively with PTH ($r = -0.491$; $p = 0.006$) and positively with SrP ($r = 0.425$; $p = 0.019$) but was not significantly correlated in HIV-infected women.

Conclusion: A positive association was observed between CTx and ALP (surrogate marker of bone formation) at ≥ 120 ng/mL which suggested the possibility of increased bone turnover at higher TFV plasma levels. There was increased bone turnover in HIV-infected women compared to the control group. Larger studies are warranted to confirm these results.

Key words: Plasma tenofovir, bone turnover markers, HIV, antiretroviral therapy, women, TDF

Introduction

Low bone mineral density (BMD) is common among persons infected with human immunodeficiency virus (HIV). In HIV-infected people, causes of osteoporosis, fractures and low BMD are multifactorial, involving traditional risk factors, genetics and HIV infection *per se* [1, 2]. Exposure to antiretroviral treatment (ART), especially nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs) in HIV patients is associated with low BMD [3]. Osteomalacia was reported in patients on tenofovir disoproxil fumarate (TDF) based ART regimen which was characteristic of hypophosphatemia and increased serum alkaline phosphatase (ALP) [4].

In clinical studies, TDF-based ART regimens were reported to be associated with decreased BMD, characterised by the increased serum levels of bone turnover markers (BTM). These include bone resorption marker such as C-terminal telopeptide (CTx) and bone formation markers such as procollagen type 1 N-terminal propeptide (P1NP), bone-specific alkaline phosphatase (BALP) and osteocalcin [5-7]. Increased bone turnover after initiation of TDF therapy was characterised by elevated serum levels of parathyroid hormone (PTH) [8]. The elevated serum levels of PTH were more profound in patients with vitamin D deficiency [9-11].

TDF undergoes initial di-ester hydrolysis in plasma to tenofovir (TFV). A proposed bone loss mechanism associated with TFV, a phosphonate, is its selective uptake by osteoclasts via a mechanism similar to that of bisphosphonates which eventually cause cellular stress. Resulting cellular stress perturbs DNA synthesis by altering gene expression that is involved in signalling osteoblast activity resulting in decreased bone formation [12, 13]. Despite studies [3, 5, 7] performed on the relationship between TDF use and bone turnover, few studies have related plasma TFV levels to BTM and this information is lacking in South Africa. The use of TFV plasma levels to correlate with BTM has more meaning in interpreting the results than mere “TDF-use” status.

The objectives of this study were to determine the relationship between plasma TFV concentration, BTM and BMD in HIV-infected women on TDF-based ART and to compare these BTM and BMD between HIV-infected women and their HIV-uninfected control group.

Methods

This pilot cross-sectional sub-study forms part of Prospective Urban and Rural Epidemiology-South Africa (PURE-SA) study and has been described in detail elsewhere [14]. A follow-up study of 462 participants in total with similar socio-demographic characteristics was performed in November 2012, April 2013 and August 2013 in which 60 women participated in this sub-study. Thirty HIV-infected women on TDF-based ART who reported to be taking 300 mg TDF *nocte* were matched with 30 HIV-uninfected female group for age and body mass index (BMI).

Participants signed written informed consent and this sub-study was approved by the Human Research Ethics Committee of the North-West University (NWU-00016-10-A1) and North West Department of Health. During follow-up visits, study participants were seen at the North-West University metabolic clinic (Tlokwe) and at Sethlare Lodge (Ganyesa) on pre-arranged dates. Medication history was recorded by qualified pharmacists and assistants on a structured questionnaire. The study was performed according to the principles outlined in the Declaration of Helsinki 2013.

Participants with medical history of osteoporosis or taking osteoporosis preventing medication were excluded from this study.

Analysis of blood samples and outcome variables

Blood samples for all participants were collected in the morning between 08:00 -10:00 by registered nurses. CTx, PTH and total vitamin D (VitD) were analysed on an Elecsys 2010. ALP, serum phosphate (SrP) and serum calcium (SrCa) were analysed on a

Cobas Intergra 400 plus (Roche Switzerland). Plasma TFV was quantified by a validated high-performance liquid chromatography method (Mulubwa *et al*, under review) with expected range of 75-148 ng/mL [15]. The lower and upper limit of quantification was 12.5 and 600 ng/mL, respectively. The mean linear regression coefficient (r^2) of the calibration curve was 0.9958 with nine calibration points. Inter- and intra- day precision and accuracy was in the range <15% of the relative standard error. The mean recovery was 91.5%.

Baseline information on the duration of TDF-based ART and non-TDF regimens was obtained from clinic and hospital files retrospectively from where participants received health care services.

Bone mineral density assessment

BMD was assessed at distal forearm using a DTX-200 peripheral DXA system (Osteometer MediTech, Hawthorn, California, USA). BMD was expressed as absolute amount (g/cm^2) of bone mass per unit area (areal density) and T-scores. T-score values were calculated using the Caucasian reference range attributable to a lack of an African reference range database. All measurements were performed on the same Osteometer.

Statistical analysis

The HIV-uninfected control group was selected using propensity score matching with the HIV-infected women. Age and BMI were used as predictors to model case/control membership in a multivariable logistic regression model with optimal match tolerance of 0.26. Sampling of controls was done without replacement.

Variables in HIV-infected and uninfected group were tested for normality using Shapiro-Wilk test and visual inspection of their respective normal Q-Q plots. Unpaired t-test was used to compare variables between the groups if data was normally distributed and Mann-Whitney U test if not. Mean and standard deviation (SD) values were calculated

for the comparison of variables in HIV-infected and uninfected participants (demographic characteristics and bone parameters) using unpaired t-test and Mann-Whitney U test. Adjusted analyses for smoking status and alcohol use were performed using analysis of covariance (ANCOVA). Test for interactions and associations between groups were performed using linear regression model stratified by HIV-uninfected status and HIV-infected (TDF exposure) controlling for smoking and alcohol use.

Pearson correlations were performed between plasma TFV concentration, BTM and BMD in HIV-infected women. Approximately half of the participants had plasma TFV concentrations either ≤ 100 (n = 12) or ≥ 120 ng/mL (n = 11). Thus correlation of BTM and BMD variables within HIV-infected group was also performed at plasma TFV concentration of ≥ 120 and ≤ 100 ng/mL. The means of variables (BTM and BMD) at plasma TFV concentration of ≥ 120 and ≤ 100 ng/mL were computed. Stepwise linear regression analysis was performed to test for associations between variables (BTM and BMD) within HIV-infected group controlling for age, BMI, smoking status and alcohol use.

Pearson correlations between SrCa and PTH or SrP were also performed in HIV-infected and uninfected women.

A two-tailed significance testing level of 0.05 was used and statistical analyses were performed using IBM® SPSS® Statistics software, version 22.

Results

The maximum and minimum plasma TFV concentration in HIV-infected women was 434.2 and 17.2 ng/mL, respectively. The mean (SD) concentration was 114 ±78.9 ng/mL. The median plasma TFV concentration was 113 (74-139.4) ng/mL. In five participant's plasma samples, TFV were not detected hence were excluded from analyses. The ART regimen, in addition to TDF, consisted of efavirenz and lamivudine.

Participants' demographic information and bone variables are presented in **Table 1**. There were no significant difference between the BMI and weight for the HIV-infected and control groups. The age of the uninfected group was statistical significant higher.

ALP (112 ±27.7 U/L; $p < 0.001$), CTx (0.68 ±0.4 ng/mL; $p = 0.027$) and PTH (56.30 ±32 pg/mL; $p = 0.050$) were significantly higher in HIV-infected than matched HIV-uninfected women. HIV-infection status (TDF exposed) was significantly associated with higher ALP (adjusted $r^2 = 0.301$; $p < 0.001$). Mean T-score in HIV-infected women was significantly less compared to the HIV-uninfected counterparts ($p = 0.049$). When analysis was adjusted for smoking status and alcohol use (ANCOVA), no significant difference in mean T-scores were observed between groups.

Correlations between plasma TFV concentration, BTM and BMD in HIV-infected women

There was no statistically significant correlation between plasma TFV concentration and ALP, CTx, absolute BMD, T-scores, SrCa, SrP, PTH or VitD in HIV-infected women ($p > 0.05$). When the HIV-infected group was divided into TFV concentrations ≤ 100 and ≥ 120 ng/mL (**Table 2**), PTH was significantly higher (57.04 ± 26.4; $p = 0.037$) at plasma TFV concentration ≥ 120 ng/mL compared to ≤ 100 ng/mL.

There were significant correlations only among ALP, CTx, VitD and SrP in the HIV-infected divided group. The correlations at TFV concentrations ≤ 100 ng/mL are shown in ***Fig 1*** (ALP and VitD) *and* ***Fig 2*** (SrP and CTx). The correlation at a concentration ≥ 120 ng/mL is presented in ***Fig 3*** (CTx and ALP). CTx was significantly associated with increased ALP controlling for age, BMI, smoking status and alcohol use (adjusted $r^2 = 0.439$; $p = 0.016$).

Fig.1 *Correlation between ALP and VitD at ≤ 100 ng/mL plasma TFV concentrations* (*r = 0.828; p = 0.011)*

Fig.2 *Correlation between SrP and CTx at ≤ 100 ng/mL plasma TFV concentrations* (*r = 0.58; p = 0.048)*

Fig.3 *Correlation between CTx and ALP at ≥ 120 ng/mL of plasma TFV concentrations (r = 0.704, p = 0.016)*

Correlations between SrCa and PTH or SrP in HIV-infected and uninfected women

There was no correlation between SrCa and PTH or SrP in HIV-infected (TDF exposed) women. In HIV-uninfected women, a significant negative correlation was found between SrCa and PTH (r = -0.491, p = 0.006) and a positive correlation between SrCa and SrP (r = 0.425, p = 0.019).

Discussion

In this pilot study, significantly higher ALP, CTx and PTH were observed in the HIV-infected women compared to the uninfected. This was indicative of stimulated bone metabolism in HIV-infected women. A similar phenomenon was observed in the studies conducted by Stellbrink *et al*. and Masiá *et al*. [5, 16], they reported an increase in PTH, BALP, P1NP and osteocalcin values in participants exposed to TDF treatment which were evidence of increased bone turnover [5]. In this study, there was an association between CTx (resorption) and increased ALP (surrogate marker of bone formation) at ≥ 120 ng/mL of plasma TFV which also suggested the possibility of increased bone turnover at higher plasma TFV concentrations. Similarly, a positive correlation was found between CTx and P1NP or osteocalcin (bone formation markers) in predominantly Caucasian patients who were exposed to TDF-based regimen (n = 29, with median age of 45 years) [17]. In this study, significant correlations between ALP and VitD and between CTx and SrP at ≤ 100 ng/mL of plasma TFV were observed. This implied the coupled or regulated bone turnover process at the TFV plasma

concentration ≤ 100 ng/mL. The absence of these correlations at ≥ 120 ng/mL of plasma TFV indicated perturbations in the regulation of bone turnover process.

In order to fully evaluate the mean changes in BTM at different TFV plasma concentrations, Havens *et al* [18] compared BTM at ≤ 39.5 and > 95.3 ng/mL of TFV which represented the lowest 20% and highest 20% (quintile analysis) of the sample size (n = 118), respectively. They found no differences in PTH, SrP, CTx, SrCa, BALP and 1,25 dihydroxyvitamin D. In the current study, there were no significant mean differences in SrCa, CTx, ALP, SrP, VitD, T-score or absolute BMD at ≤ 100 and ≥ 120 ng/mL which represented about lower half and upper half of the sample size (n = 25), respectively. The mean PTH was however higher at ≥ 120 ng/mL than ≤ 100 ng/mL of plasma TFV which suggested the possibility of stimulated bone resorption, as higher plasma PTH levels are associated with low BMD [19].

In the current study, no correlation was found between TFV concentrations, BTM and BMD. Similarly, in a study performed by Havens *et al*. [18] of mostly African American race (n = 118), no correlation was found between plasma TFV concentration and SrCa, PTH, SrP, BALP and CTx. The authors, however, reported a negative correlation between plasma TFV concentrations and free 1, 25 dihydroxyvitamin D [18]. Total vitamin D was measured in this study and not 1, 25 dihydroxyvitamin D as in the study of Havens *et al*.

Higher bone turnover was reported in patients on TDF containing regimens. CTx, P1NP and BALP (iso-enzyme of alkaline phosphatase) were higher in these patient populations of mostly Caucasian males (n = 154, n = 193) than those exposed to non-TDF containing regimens [5, 7]. Exposure to TDF containing regimens was associated with increased total ALP which indicated osteoblast activation [20, 21]. Total ALP had been used to monitor bone metabolism and showed high correlation with bone specific

BALP. The increase in total ALP in the current study was most likely contributed by BALP as no cholestatic side effects of TDF have been described [20].

In a cross sectional study of women (26% African American) age above 50 years (n = 31), mean BMD and T -scores were significantly lower in the HIV-infected ART exposed than uninfected control group at the lumbar spine and total hip [22]. In the current study, BMD and T-scores at the distal forearm in both HIV-infected TDF exposed and uninfected controls were similar. This difference in results may be contributed to the fact that in the former study [22] BMD and T-scores were not adjusted for confounding effects of smoking status and alcohol consumption unlike in this study. The other reason is that, this study evaluated BMD on the distal forearm as opposed to lumbar spine and total hip.

Bone remodelling is a constant process tightly coupled to bone resorption and bone formation mediated by osteoclasts and osteoblasts, respectively. Bone formation and resorption markers are well correlated to one another thus a change in bone turnover signals a disturbance in well controlled bone remodelling process [6, 23]. Furthermore, there is regulated interplay among bone mineral metabolic parameters namely PTH, SrCa and SrP in bone remodelling process [24, 25]. In the current study, we did not find any correlations between PTH, SrCa and SrP in HIV-infected participants. This was owing to the disturbance in the regulation process of bone metabolic parameters likely attributed to ART and HIV infection status [16, 26]. We found significant correlations between PTH, SrCa and SrP in HIV-uninfected participants which indicated synchronization of bone mineral metabolic processes.

This study has some limitations to consider: the cross-section nature of the study rules out assessments of causal relationship between plasma TFV concentration and BTM. The small number of participants does not allow generalising the findings although significant associations observed need to be confirmed in longitudinal studies with

larger sample sizes.The influence of TDF exposure time, vitamin D deficiency and sufficiency as categorisation could not be assessed as it was not statistically sensible owing to a small sample size. Nevertheless, availability of a control group to make comparisons added strength to this study. These findings stimulate the interest of further research into plasma TFV levels and bone metabolism in black women for early detection of bone loss.

In conclusion, the observed positive association between CTx and ALP (surrogate marker of bone formation) at ≥ 120 ng/mL suggests the possibility of increased rate of bone turnover at higher TFV plasma concentrations within this study. Additionally, there was higher bone turnover process in HIV-infected participants (TFV exposed) compared to the HIV-uninfected controls. Further studies with larger sample size are needed to confirm these results.

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Author Disclosure Statement

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Authors' contributions

M. Mulubwa participated in data collection, wrote the manuscript and analysed the data. I.M Kruger revised the manuscript and participated in data collection. M. Rheeders and M. Viljoen interpreted the data and approved final version of the manuscript.

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***Table 1**. Demographic characteristics, BTM and BMD of HIV-infected women compared to the uninfected control group*

	HIV-infected (n = 30)	*HIV-uninfected (n = 30)*	*P-value[f]*	*Adjusted p-value[h]*
Age (years)	53 ±9.3	60 ±11	**0.008**	-
BMI (kg/m^2)	24.2 ±9.5	26.2 ±8.2	0.333	-
Weight (kg)	58 ±22.5	64.7 ±20.5	0.179	-
TDF ART exposure (months)	16.2 ±8.6[a]	-	-	-
HIV disease duration (months)	37.8 ±24.7[b]	-	-	-
SrCa (mmol/L)	2.08 ±0.2	2.12 ±0.2	0.406	0.835
CTx (ng/mL)	0.68 ±0.4	0.56 ±0.4	0.181	**0.027**
ALP (U/L)	112 ±27.7	78.7 ±25	**< 0.001**	**< 0.001**
SrP (mmol/L)	1.05 ±0.2	0.99 ±0.2	0.204	0.165
VitD (ng/mL)	38 ±1[c]	37.6 ±14	0.935	0.676
BMD (g/cm^2)	0.39 ±0.2[d]	0.42 ±0.1[e]	0.366	0.822
T-score	1.49 ±17.7[d]	15.9 ±34.2[e]	**0.049[g]**	0.168
PTH (pg/mL)	56.30 ±32	46.3 ±18	0.383[g]	**0.050**

[a]n = 24 (4 participants unwilling to provide consent to access their medical records and missing information from 2 medical records). [b]n = 22 (4 participants unwilling to provide consent to access their medical records and missing information from 4 medical files). [c]n = 25, [d]n = 18, [e]n = 28, [f]p-value calculated from unpaired t-test, [g]p-value calculated from Mann-Whitney U test and [h]p-value calculated from ANCOVA adjusted for smoking status and alcohol use

Table 2. *Comparisons of mean values (SD) of BTM and BMD at plasma TFV concentration of ≤ 100 ng/mL and ≥ 120 ng/mL in HIV-infected women.*

	TFV plasma ≥ 120 ng/mL (n = 11)	*TFV plasma ≤ 100 ng/mL (n = 12)*	*P value*
SrCa (mmol/L)	2.12 ±0.13	2.01 ±0.19	0.928
CTx (ng/mL)	0.77 ± 0.36	0.65 ± 0.31	0.212
ALP (U/L)	117.93 ± 29.8	105.43 ± 23.6	0.151
SrP (mmol/L)	0.98 ± 0.15	1.01 ± 0.19	0.110
VitD (ng/mL)	36.60 ± 8.6[a]	42.67 ± 4.2[b]	0.330
BMD (g/cm^2)	0.45 ± 0.17[c]	0.39 ±0.13[d]	0.954
T-score	-0.98 ±4.79[c]	5.72 ±24.52[d]	0.630
PTH (pg/mL)	57.04 ± 26.4	56.14 ± 30.9	**0.037**

[a]n = 10, [b]n = 8, [c]n = 6, [d]n = 9. 2 participants had TFV concentration between 101-119 ng/mL.

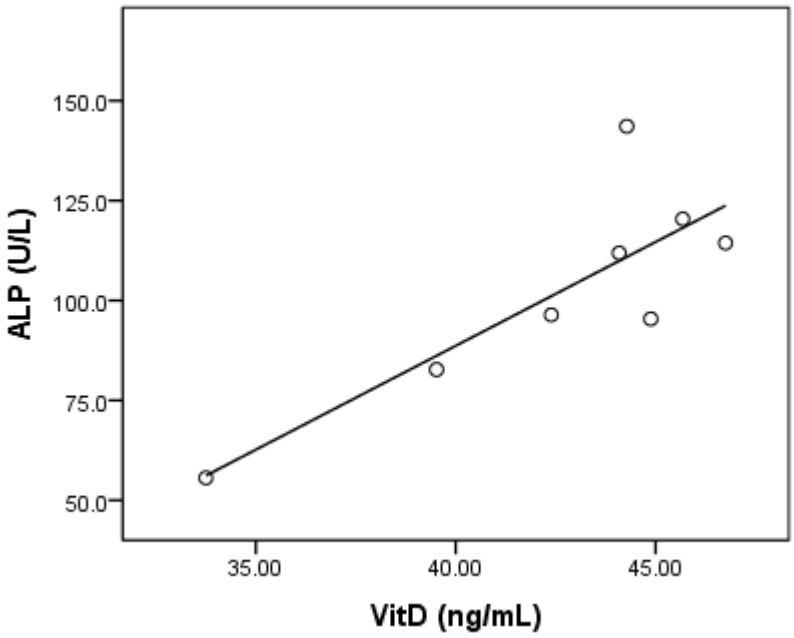

Fig.1

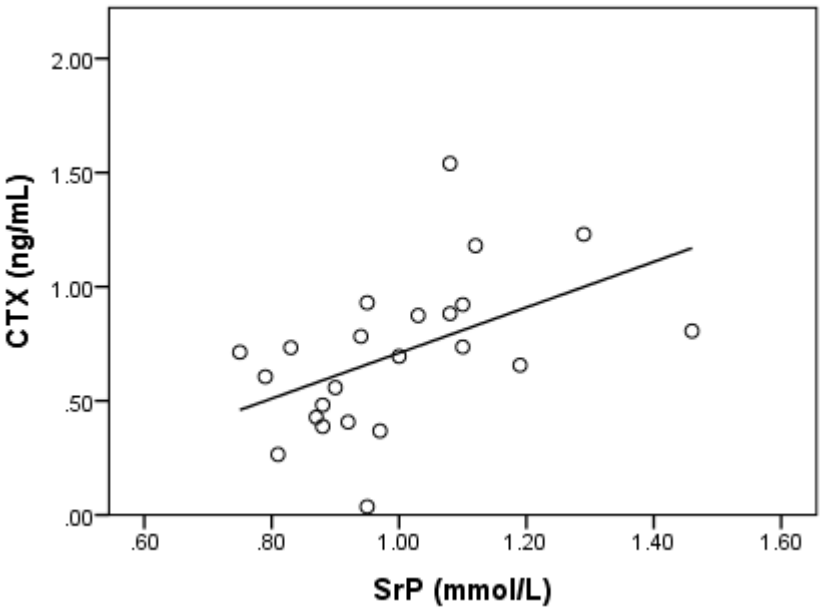

Fig. 2

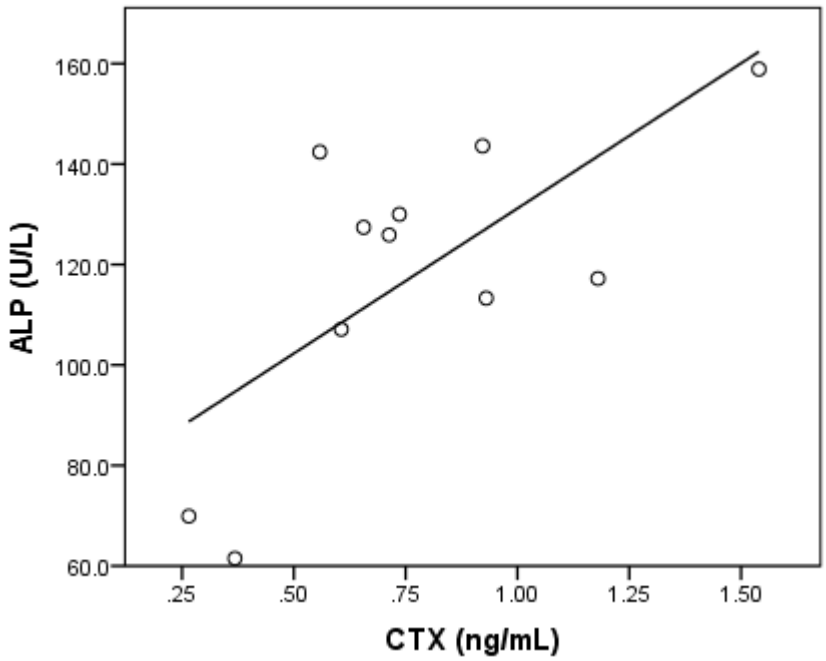

Fig. 3

Additional Results and Discussion | Chapter 7

7.1 INTRODUCTION

This Chapter covers the results and discussion from one of the secondary objectives stated in Chapter 1 that was not discussed in Chapters 4-6 (Manuscript A-C). These results report on the immunological recovery of HIV-infected participants from the time of TDF-based ART initiation to follow-up in November 2012, April 2013 and August 2013 in this sub-study investigation.

A summary of the results of all the variables for renal function and bone turnover is presented in Addendum E1 as well as the individual plasma TDF concentrations (Addendum E3). Information on self reported alcohol consumption and smoking status of participants is presented in Addendum E2.

The two poster presentations, abstract and certificate of award are presented in Addendum A.

BTM and BMD correlations were investigated and participants were categorised according to VitD deficiency, sufficiency, > 12 months and < 12 months or >24 months or < 24 months of TDF exposure. The results, however, were statistically unreliable owing to the small number of participants belonging to either of the category (see Addendum E5).

7.2 RESULTS

The baseline CD4+ cell count was 255±173 cells/mm^3 (maximum 838 and minimum 177) and the follow-up values 505 ±161 cells/mm^3 (maximum 835 and minimum 231) in 16 participants. The difference in mean CD4+ cell count was statistically significantly (p =0.001) different between the baseline and follow-up times. **Table 1.2** shows the paired t-test results of CD4+ cell count at baseline and follow-up for the 16 participants.

Table 1.2 **Comparison of CD4+ cell count at baseline and follow-up**

	n	Baseline	Follow-up	*P*-value[a]
Mean (SD) CD4+ cell count (cells/mm^3)	16	255 ±173	505 ±161	**0.001**
Maximum CD4+ cell count (cells/mm^3)	16	838	835	-
Minimum CD4+ cell count (cells/mm^3)	16	117	231	-

[a]*P-value calculated from paired t-test. - = not applicable.*

Mean CD4+ cell count increased from baseline to follow-up by +250 cells/mm^3.

Table 1.3 **Descriptive statistics of CD4+ cell count values at baseline and follow-up**

	n	Mean (SD)	Median (IQR)
CD4+ cell count (baseline)	23	224 ±154	194 (133-250)
CD4+ cell count (follow-up)	20	485 ±171	488 (341-569)

As stated in Chapter 3 (**Figure 1.2**), there were 30 HIV-infected participants on TDF-based ART. Only 20 CD4+ cell count results were available at follow-up. At baseline, only 23 CD4+ cell count results were available (see **Table 1.3**) from public health care records. Of the 23 CD4+ cell count results, only 16 could be compared between baseline and follow-up for the following reasons:

- three baseline CD4+ cell count values prior to TDF therapy initiation were missing from patients' files,
- four participants did not give permission to gain access to their medical records from the public health care facilities where they collected medication and received their health care services, and
- CD4+ cell count was not measured for 10 participants during the November 2012 follow-up.

The median follow-up time since TDF initiation was 16.6 months (IQR: 8.8-23.4). All participants also took 3TC and EFV in addition to TDF, corresponding to the recommended first line regimen of the National Department of Health. The VL was not

performed at follow-up in these participants owing to limited resources, although it is recommended in the guidelines (NDoH, 2010).

7.3 DISCUSSION

The measurement of the CD4+ cell count has been the prime avenue of monitoring response (efficacy) to ART in resource-limited countries (Ford *et al.*, 2014). In the current results, the mean CD4+ cell count increased statistically significantly (p = 0.001) from baseline to median follow-up time of 16.6 months by +250 cells/mm^3 in 16 participants. This indicated improved immunological recovery. These results are in line with a single-arm multicentre study (n = 67, 33% women) in which the mean CD4+ cell count increased by 176 cells/mm^3 at the end of a 12-month period in those participants who were on a TDF-based regimen (TDF/3TC/EFV) (Esser *et al.*, 2011). The majority of the HIV-infected patients on combination therapy (TDF/3TC/EFV) showed an increase of 93.8% from baseline in their CD4+ cell count after three months in a retrospective study (n = 120, 68% female) in Rwanda (Kadima *et al.*, 2014). In a phase 3 clinical trial (40% female), 85 participants were originally randomised to D4T/3TC/EFV for 36 months then switched to TDF/3TC/EFV. During the 36 months of follow-up after switching, their mean CD4+ cell count increased by +155 cells/mm^3 (Madruga *et al.*, 2007).

One of the major limitations of this sub-study was the unavailability of all the baseline CD4+ cell count values from the participants' medical records. Hence only 53% (16) of the participants' immunological recovery was assessed from baseline to follow-up.

In conclusion, there was an improvement in the immunological status of the participants at the median time of 16.6 months after initiation of TDF-based ART (TDF/3TC/EFV).

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Chapter 8 Conclusions and Recommendations

In this pilot cross-section sub-study of 60 female participants (30 HIV-infected and 30 HIV-uninfected), all the outlined primary and secondary objectives set out in Chapter 1 (**1.2.1** and **1.2.2**) were achieved successfully. These objectives included:

- the development and validation of an HPLC-MS/MS method to determine TFV concentrations in human plasma,
- the determination of plasma TFV concentrations in HIV-infected female participants on a TDF-based ART regimen in the PURE-SA sub-study,
- the investigation of the correlation between plasma TFV concentrations and renal function markers (eGFR, CrCl, albuminuria, $TmPO_4$/GFR, serum urea, SCr, serum uric acid, glucosuria or UNa) in HIV-infected female participants,
- the investigation of possible correlations between TFV concentration and BTM (CTx, ALP, PTH, SrP, SrCa or VitD) in HIV-infected female participants,
- the comparison of renal function (Manuscript B) and BTM (Manuscript C) between HIV-infected female participants on a TDF-based ART regimen and matched female HIV-uninfected controls within the PURE-SA sub study,
- the tracking of immunological recovery/status by comparing CD4+ cell count values between baseline and follow-up in HIV-infected female participants on a TDF-based ART regimen, and
- the evaluation of renal function status by comparing CrCl and eGFR between baseline and follow-up in HIV-infected female participants on a TDF-based ART regimen.

8.1 CONCLUSIONS

The method to measure TFV in human plasma was developed and validated with HPLC-MS/MS. The method was accurate, robust and fast. It was successfully applied to determine TFV in plasma samples of HIV-infected participants on TDF-based ART in this cross-sectional sub-study.

Albuminuria was the only renal function marker that correlated positively with plasma TFV concentration. The TFV plasma concentration in HIV-infected participants on a TDF-based ART regimen was independently associated with increased albuminuria as a marker of kidney dysfunction.

There was a strong positive correlation between CTx and ALP at a plasma TFV concentration of ≥ 120 ng/mL. At plasma TFV concentration of ≤ 100 ng/mL, positive correlations existed between ALP, VitD, CTx and SrP but were absent at ≥ 120 ng/mL. Thus perturbations in the process of bone turnover at ≥ 120 ng/mL of TFV plasma concentration were present in HIV-infected participants on TDF-based ART. The HIV-infected participants had increased bone turnover evidenced by significantly higher PTH, CTx and ALP compared to HIV-uninfected controls.

HIV-infected participants on a TDF regimen compared to HIV-uninfected controls had glomerular hyperfiltration, which was observed as abnormally high eGFR or CrCl. Albuminuria was higher in HIV-infected participants compared to HIV-uninfected ones. ALP, a surrogate marker of bone turnover, was higher in HIV-infected participants compared to HIV-uninfected controls.

Exposure to TDF/3TC/EFV combination therapy in HIV-infected participants improved the immunological status within the median 16.6 months of therapy after TDF was initiated.

Renal function (eGFR and CrCl) in HIV-infected participants on a TDF regimen showed an extreme improvement between the baseline and the median follow-up time of 16.6 months. The possible reason could be recent evidence that ART improves the renal function in HIV-infected patients (Peters *et al.*, 2008).

This study has investigated the relationship between plasma TFV levels, renal function and bone metabolism. Finally, the study has demonstrated that albuminuria, a marker of

renal dysfunction, and perturbations in the bone metabolism are the possible side effects that perhaps need consideration in the TDF-based ART management.

8.2 LIMITATIONS

The type of study design was a limitation in itself for this pilot sub study. The cross-sectional nature only shows results at one point in time, unlike a longitudinal study. Thus the researchers could not establish causality in the relationships that were observed between plasma TFV concentrations and markers of renal function or bone turnover. The sample size of HIV-infected females was too small to generalise the findings owing to recruitment that took place in 2004-2005 that could not be changed in 2012-2013.

The efficacy of the TDF-based regimen was only determined in terms of CD4+ cell count change from baseline. Although VL is the recommended measure of ART efficacy (NDoH, 2010; WHO, 2013), it was not tested in this sub-study because of the high costs involved. It was not possible to match VL testing results and dates performed by NHLS (from patient files retrospectively) with accurate baseline dates when TDF was commenced (baseline) in this study follow-up.

8.3 RECOMMENDATIONS

It is recommended that this study be performed with a larger sample size within a longitudinal design, so as to fully understand the changes in the renal function and BTM with TFV plasma concentration over a period of time. This kind of study will help to generalise the outcomes for implementation purposes. Future studies should study glomerular hyperfiltration in HIV-infected women extensively, as it may be an early sign of renal dysfunction, cardiovascular disease or diabetes (Helal *et al.*, 2012; Tomaszewski *et al.*, 2007).

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Addendum A	Conference Proceedings: Abstract, Poster presentations and certificate of award

Associations between plasma tenofovir concentration and bone metabolism markers in HIV-infected women

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Background

Treatment with tenofovir disoproxil fumarate (TDF), a prodrug of tenofovir (TFV) is associated with reduced bone mineral density. We investigated correlations between plasma TFV concentration and bone metabolism markers in HIV-infected women on TDF antiretroviral therapy (ART) and compared these markers with those in HIV-uninfected controls.

Methods

In this pilot cross-sectional sub-study, 30 HIV-infected (TDF-based ART) were matched with 30 HIV-uninfected women for age and body mass index within the PURE-SA study. Markers measured included: C-terminal telopeptide (CTx), alkaline phosphatase (ALP), parathyroid hormone (PTH), total vitamin D (VitD), serum calcium (SrCa) and serum phosphate (SrP). Plasma TFV was assayed with HPLC-MS/MS. Mann-Whitney U test; ANCOVA and stepwise regression were applied in all statistical analyses.

Results

No correlation was found between plasma TFV concentration and CTx, PTH, ALP, SrCa, SrP or VitD ($p > 0.05$) in the HIV-infected group. Interaction between weight and plasma TFV was associated with increased ALP ($p = 0.035$) at TFV concentration of ≤ 100 ng/mL. CTx and ALP correlated positively at TFV concentration of ≥ 120 ng/mL ($r = 0.704$; $p = 0.016$). Adjusted analysis showed higher ALP ($p < 0.001$) in the HIV-infected compared to the HIV-uninfected group. SrCa correlated negatively with PTH ($r = -0.491$; $p=0.006$) and positively with SrP ($r = 0.425$; $p = 0.019$) only in the HIV-uninfected group.

Conclusion

Disturbances in the bone turnover process at low and high plasma TFV concentrations are present in HIV-infected women and there is an unsynchronised bone turnover process in HIV-infected group compared to HIV-uninfected within this study. These results need to be confirmed in larger studies.

Tenofovir plasma concentration is a strong predictor of albuminuria in HIV-1-infected women

P_1227

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Introduction

Treatment with tenofovir disoproxil fumarate (TDF) has been linked to increased biomarkers of bone turnover, reduced estimated glomerular filtration rate (eGFR)[1] and decreased bone mineral density[2]. Elevated tenofovir (TFV) trough concentration is associated with increased risk of renal impairment and bone mineral loss[3,4]. However, no correlation was found between plasma TFV and creatinine clearance[5]. We investigated the relationship between mid-dose plasma TFV and markers of renal function and bone resorption in HIV-1-infected women and compared these markers with HIV-negative controls.

Methods

Study design & ethics

This was a pilot cross-sectional sub-study within Prospective Urban and Rural Epidemiology-South Africa (PURE-SA). It is a large epidemiological study taking place in low-, middle-, and high-income countries around the world whose overarching aim is to examine the relationship of societal influences on human lifestyle behaviours, cardiovascular risk factors, and incidence of chronic non communicable diseases[6].The sub-study was approved by the Human Research Ethics Committee (HREC) of the North-West University, Potchefstroom (NWU-00016-10-A1) and North West Department of Health, Mmabatho.

Participants

Sixty women participated; 30 HIV-1-infected on 300 mg daily TDF antiretroviral regimen were matched with 30 HIV-1-uninfected for age and body mass index (BMI) via propensity score matching. Plasma TFV was assayed using a validated HPLC-/MS/MS method. Plasma and urine samples were analysed on Elecsys 2010 and Cobas Intergra 400 for the following markers: parathyroid hormone (PTH), C-terminal telopeptide (CTx), alkaline phosphatase (ALP), albuminuria, creatinine clearance (CrCl) and maximal renal tubular phosphate reabsorption ($TmPO_4$/GFR). Blood samples were collected in the morning by registered nurses for all PURE-SA related investigations including TFV analysis (12-18 hours post TDF dose). Medication history was recorded by qualified pharmacists and assistants on a structured questionnaire.

Statistical analysis

Stepwise regression was used to determine relationship between plasma TFV and the markers. Mann-Whitney U test was used to compare markers between groups and then box and whisker plots were used to display significant markers.

Results

Plasma TFV calibration curves were linear (r^2, 0.9942-0.9970) over concentration range of 12.5-600 ng/mL. Mean recovery was 91.5%. Accuracy, inter and intra precision were in the range of ≤15% relative standard error.

Table 1: Participant's comparative results

	HIV-1-infected (n=30)	HIV-1-uninfected (n=30)	P value
Age , years (median)	52 (IQR: 49-59)	57 (IQR: 53-63)	.011
BMI, Kg/m^2 (median)	20.5 (IQR: 18-28.9)	24.4 (IQR: 19-33.5)	.249
ALP, mean rank	40.1	20.9	.000
Albuminuria, mean rank	35.0	25.2	.028

Median tenofovir concentration was 113 ng/mL (IQR: 74-139). No plasma TFV was found in 5 (16.7%) participants. TDF exposure ranged from 2-30 months. Plasma TFV correlated statistically significantly with albuminuria (r= 0.606, p= 0.001, see Fig.1) while PTH, CTx, CrCl and TmPO4/GFR were not significant. Albuminuria and ALP was significantly higher in HIV-1-infected than uninfected women (p<0.05, see Table 1) while PTH, CTx, CrCl and TmPO4/GFR were not significantly different. Median ALP and albuminuria are displayed in Fig.2.

Fig. 1: Regression plot of plasma TFV concentration and albuminuria.

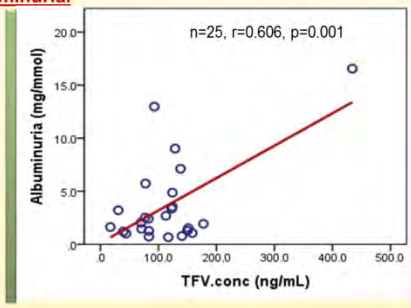
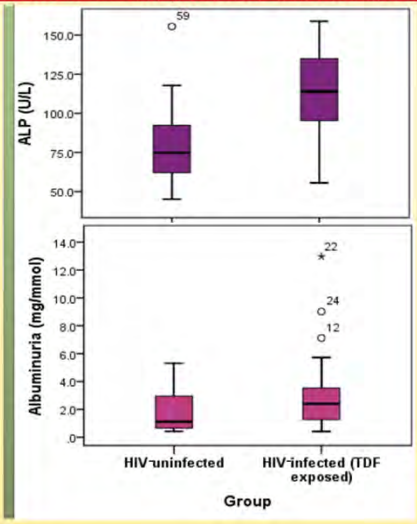


Fig.2: Box and whisker plot of ALP and albuminuria.



Discussion

A strong association was found between TDF treatment and tubular proteinuria[7]. In the current study, albuminuria was significantly higher in HIV-infected than uninfected participants. Moreover, plasma TFV was a significant predictor of albuminuria in HIV-infected participants. Albuminuria in HIV-infected participants was in presence of normal eGFR which was suggestive of tubular rather than glomerular defect. eGFR is insensitive in early renal disease and does not correlate well with tubular dysfunction[8]. PTH, CTx, CrCl and TmPO4/GFR were similar between HIV-infected and uninfected. Total ALP had been used to monitor bone metabolism and showed high correlation with bone specific isoenzyme (BALP)[9]. In the current study, ALP was higher in HIV-infected (TDF exposed) than uninfected participants suggestive of increased bone turn over. Cholestatic side effects of TDF have not been described[10] hence the increase in ALP was most likely caused by bone isoenzyme.

Conclusion

Albuminuria and ALP was higher in HIV-1-infected on TDF treatment than uninfected matched participants and mid-dose tenofovir plasma concentration was a strong predictor of albuminuria in HIV-1-infected women within this pilot investigation.

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Bone density and renal function markers in tenofovir exposed and non-exposed black South African women

P_193

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Introduction

Tenofovir disoproxil fumarate (TDF), a nucleotide reverse transcriptase inhibitor is the preferred effective first line treatment of HIV-1-infection in adults. It has a good safety profile with less metabolic side-effects than nucleoside reverse transcriptase inhibitors (NRTIs)[1]. Exposure to TDF containing regimen is associated with decreased bone mineral density indicated by presence of markers of bone resorption, proximal tubular dysfunction and reduced creatinine clearance[2-4]. In contrast, TDF use has not shown any association with low creatinine clearance[5,6]. Nephrotoxicity associated with TDF leads to breakdown of solute transport characterized by urine wasting of solutes normally reabsorbed in the proximal tubule (PT) of which phosphate loss may result in osteomalacia[7]. Genetic polymorphisms in PT transporters may predispose certain individuals to accumulate high intracellular tenofovir levels and could increase the risk of developing tubular toxicity[8]. TDF was introduced to replace stavudine by South African Department of Health in 2010[9]. We investigated certain markers of bone density and renal function in HIV-1-infected black South African women exposed to (1) TDF, (2) non-TDF regimens and (3) highly active antiretroviral therapy (HAART) naive.

Methods

Study design

A pilot cross-sectional sub-study of HIV-1-infected women within the Prospective Urban and Rural Epidemiology-South Africa study (PURE-SA) was performed. PURE is a large scale epidemiological study taking place in low-, middle-, and high-income countries around the world. The overarching aim is to examine the relationship of societal influences on human lifestyle behaviours, cardiovascular risk factors, and incidence of chronic non communicable diseases[10]. The sub-study was approved by the Human Research Ethics Committee (HREC) of the North-West University, Potchefstroom (NWU-00016-10-A1) and North West Department of Health, (Policy, Planning, Research, Monitoring and Evaluation) Mmabatho.

Patients

Forty four HIV-1-infected women were included; TDF-exposed n=27*, non-TDF exposed n=9 and HAART naive n=8. The markers measured were parathyroid hormone (PTH), alkaline phosphatase (ALP), serum creatinine (SCr), creatinine clearance (CrCl) calculated using Cockroft-Gault method and maximal renal tubular reabsorption of phosphate ($TmPO_4$/GFR). Urine and serum sample were analysed on Elecsys 2010 and Cobas Intergra 400 plus. Blood samples were collected in the morning by registered nurses for all PURE-SA related investigations. Medication history was recorded by qualified pharmacists and assistants on a structured questionnaire.

Statistical analysis

Changes in the markers were assessed by one-way ANOVA and univariate analysis after adjusting for covariates (age and BMI) using SPSS version 22.

Results

Table 1: Demographic characteristics of study participants

	TDF exposed	Non-TDF exposed	HAART Naive	P value
Median age (years)	54 (IQR: 49-59)	55 (IQR: 53.5-57)	55 (IQR: 46.3-59.5)	> 0.05
Median BMI (kg/m^2)	20.6 (IQR: 18.4-28.6)	22.1 (IQR: 20.3-24.2)	25.3 (IQR: 19.4-29.1)	> 0.05

TDF exposure period ranged from 4-23 months. ANOVA and adjusted univariate analysis showed no statistical significant differences in PTH, ALP, SCr, CrCl and $TmPO_4$/GFR among the three groups. Mean PTH and ALP were highest in the TDF exposed group, 53 pg/mL and 108.1 U/L, respectively but was not statistically significant (see Fig.1 and 2).

Fig.1: Median parathyroid (PTH) hormone plasma levels.

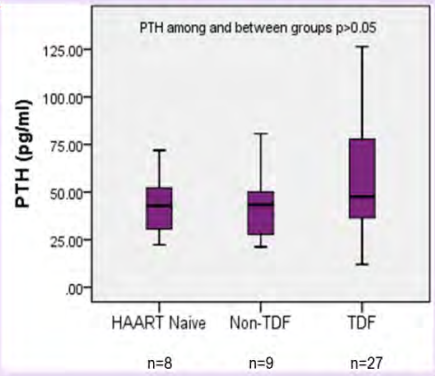
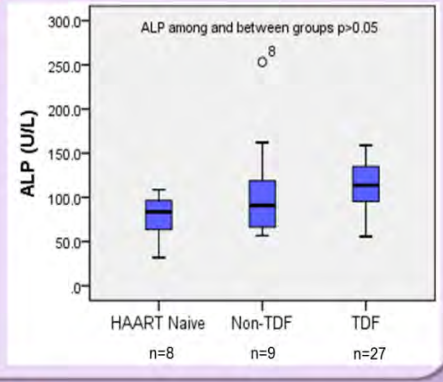


Fig.2: Median alkaline phosphatase (ALP) plasma levels.



Discussion

In agreement with some studies[6,11] we did not find a significant difference in markers of renal function; creatinine clearance, serum creatinine and $TmPO_4$/GFR among HIV-1-infected exposed to TDF, non-TDF and HAART naive. Conversely, TDF exposure had been associated with reduced creatinine clearance and increase in serum creatinine[2,3] . TDF use was associated with bone loss characterized by high plasma PTH[12] and ALP which is used to monitor bone metabolism[13,14]. Although not statistically significant, the increase in PTH and ALP in this TDF group needs further investigation within a larger sample size.

Conclusion

Markers of renal function and bone density were similar among TDF exposed, non-TDF exposed and HAART naive exposure groups in this pilot investigation

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*Results for 3 TDF-exposed participants were not available at the time of abstract submission.

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Addendum B1	Instructions to the Author: Journal of Pharmaceutical and Biomedical Analysis

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Studies from areas of well established and poorly selective methods, such as UV-VIS spectrophotometry (including derivative and multi-wavelength measurements), basic electroanalytical (potentiometric, polarographic and voltammetric) methods, fluorimetry, flow-injection analysis, etc. are accepted for publication in exceptional cases only, if a unique and substantial advantage over presently known systems is demonstrated. The same applies to the assay of simple drug formulations by any kind of methods and the determination of drugs in biological samples based merely on spiked samples. Drug purity/stability studies should contain information on the structure elucidation of the impurities/degradants. Papers dealing with the analytical aspects of traditional folk medicines are acceptable if the results are expected to attract the interest of readers also outside the area of origin. Bioanalytical papers (pharmacokinetic, bioequivalence, protein and DNA binding studies) are accepted if the focus is on innovative analytical methodology.

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AIDS Research and Human Retroviruses is a peer-reviewed journal in the English language. The Journal will publish Full and Short Communications, Letters to the Editor, Perspectives, Sequence Notes, Mini-reviews, and Conference or Workshop Summaries as deemed appropriate.

To help defray the cost of printing, the Publisher requests that page charges of $65 per printed page be paid by all authors who have funds available from research grants or from their institutions. It should be noted that ability to pay page charges is not a prerequisite for publication in the Journal.

Full Communications should be presented in the format described below.

Short Communications, also subject to peer-review, are intended for the presentation of brief observations that do not warrant a full-length text, but also are not preliminary results. Include an abstract; do not use section headings in the body of the text; report methods, results, and discussion in a single section. Reference citations are identical in style to those of full-length papers, but appropriately abbreviated.

Images in HIV Research: Images can tell a research story and capture the imagination as well as provide scientific information about spatial relationships and context. We welcome submissions that contain striking images that illustrate and illuminate a research story, make a timely and relevant point, emphasize a new and emerging concept, or are stunning and beautiful.

Submission Requirements:

- Text should be no more than 500 words
- Limit of four references
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- Images must be in either .TIFF or .EPS files. .JPEG files cannot be used as the resolution of these files is too low.

Images may be considered for use on the Journal cover in the issue the submission is published.

All submissions are subject to peer review. All accepted submissions will be deposited to Medline after final publication.

Letters to the Editor can be on any issue relevant to the field of human retroviruses. They must be brief (one or two journal pages) and appropriately documented by data. Letters can be sent to the Editor-in-Chief or to the basic science or clinical Editors, and may be subject to review.

Mini-reviews and Perspectives may be solicited by the Editors or submitted independently. Mini-reviews are brief summaries of new developments in the field of human retroviruses. Perspectives are more representative of an opinion about an area of the field or a direction of research. The length for either should not exceed seven journal pages, and both may be subject to peer-review.

Sequence Notes offer more rapid publication of papers that present novel sequence data in a brief format. They are generally no more than 3 or 4 journal pages. Include an abstract of not more than 150 words, but do not use section headings in the text, and limit the references to 15. Sequence Notes, like all papers, should be uploaded here.
Allow 2-3 weeks for the initial evaluation.

Vaccines
Michael Betts, University of Pennsylvania, Philadelphia, PA

Animal Models
Ronald C. Desrosiers, New England Regional Primate Research Center, Southborough, MA

Molecular Biology/Other Human Retroviruses
Patrick Green, The Ohio State University, Columbus, OH

Sequence Notes
Brian Foley, HIV Sequence Database, Los Alamos National Laboratory, Los Alamos, NM

Editors - Clinical Investigations

Complications of Therapy
Mariana Gerschenson, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI

Epidemiology
Mauro Schechter, Professor of Infectious Diseases, Head, AIDS Research Laboratory, Hospital Universitario Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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Follow all submission guidelines to avoid delays in processing and publication. Type manuscripts double-spaced. Leave ample margins on the sides, top, and bottom of the page. Manuscripts should be submitted in Microsoft Word.

The title page should include the authors' names and affiliations, and a running title of about 45 characters, including spaces. Please indicate contact information for the corresponding author, including a mailing and/or street address, telephone and fax numbers, and e-mail address. The second page must consist of an abstract of not more than 250 words, which should be self-explanatory without reference to the text. Full communications are structured using the following headings: abstract, introduction, materials and methods, results, discussion, acknowledgments, sequence data, and references. Number pages consecutively. At the end of the manuscript, give the name and address of the individual to whom reprint requests should be directed. The inclusion of that person's e-mail address is also strongly encouraged.

The authors may suggest suitable referees in a cover letter, but must provide accurate contact information for all such individuals, including fax numbers. Please follow the requested style to avoid delays in publication.

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The Journal publishes all accepted papers within 72 hours of acceptance in their unedited, uncorrected format. It is important to note that the information that is published online, and in all indexing services, is pulled directly from the data that is populated into the fields in Manuscript Central – NOT from the manuscript file – when the paper is originally uploaded to the system for peer review. Consequently, any errors contained in the system will remain on our website and all indexing services, including Medline, until the next revision of the article is published. As such, it is critical that authors enter all authors' names correctly into the system at the time of submission.

The next revision will take place after the corresponding author sees page proofs, makes any necessary corrections, and returns the changes to the Publisher. Once the alterations are completed, the revised version will be published on our website, and the newly corrected information will then be released to Medline/PubMed, in addition to any other indexing services in which the Journal is included.

Please note that the typical time between acceptance of a paper and page proof distribution is approximately 4-8 weeks depending on the length and complexity of the paper.

Nomenclature

AIDS Research and Human Retroviruses has adopted the recommendations of the Executive Committee of the International Committee on Taxonomy of Viruses (ICTV) (Ref: Science 232:1486, 1986, and Nature 321:644, 1986) to designate viruses associated with AIDS. The Committee's suggestion was to use HIV (human immunodeficiency virus) or the generic name for this class of human retroviruses. The Committee also recommended maintaining the specific strain names ($LAV\text{-}1_{BRU}$, $LAV\text{-}1_{LOL}$, $HTLV\text{-}III_B$, $HTLV\text{-}III_{RF}$, ARV_1 . . .) in the interest of continuity and appreciation for the biologic and molecular differences of the individual strains. Thus, for the sake of accuracy, the editors encourage the use of specific strain names in experimental details.

Sequence Data

AIDS Research and Human Retroviruses requires submission of accession numbers with any manuscript that uses sequence data. The numbers must be listed in a separate section following acknowledgments. Accession numbers can be obtained from Genbank, EMBL, or DDBJ. Seehttp://www.ncbi.nlm.nih.gov/genbank/index.html for information, or send e-mail to info@ncbi.nlm.nih.gov

Tables

Submit each table with its title in a separate file. Use Arabic numerals to number tables. Each table must stand alone, i.e., contain all necessary information in the caption, and the table itself must be understood independently of the text. Details of experimental conditions should be included in the table footnotes. Table legends and footnotes should be double-spaced. Information that appears in the text should not be repeated in tables, and tables should not contain data that can be given in the text in one or two sentences.

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Figures/images should be presented according to these guidelines:

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- Do not prepare any figures in Word as they are not workable.

- Line illustrations must be submitted at 1200 DPI.
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- Please submit either .TIFF or .EPS files.
- **Color art must be saved as CYMK not RGB or INDEX.** If RGB files are submitted, the files will be converted to CYMK, and some slight color variation may occur.
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Acknowledgments

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References

All references must be cited in the text. Cite references within the text by a superscript Arabic number. Number references in the order that they appear in the text. Double-space the references and triple-space between each listing. When there are more than six authors, it is acceptable to list the first three followed by "et al." Use the appropriate styles:

Journals

(1) surname of author(s), initials, (2) paper title, (3) journal, (4) year of publication, (5) volume number, and (6) first and last page of citation. **Example:** Williams N: T cells on the mucosal frontline. Science 1998;280:198–200.

Books

(1) surname of author(s), initials, (2) chapter title, (3) title of book, (4) editors of book (if applicable), (5) edition of book (if applicable), (6) publisher, (7) city of publication, (8) year of publication, and (9) first and last page reference. **Example:** Bolognesi D: HIV vaccines. In: *Immunology of HIV infection.* (Gupta S, ed.) Plenum Medical, New York, 1996, pp. 561–576.

Patents

(1) country of original registration, (2) patent number, (3) month, day, and year of issuance.

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Addendum B3	Instructions to the Author: European Journal of Clinical Pharmacology

European Journal of Clinical Pharmacology

Coordinating Editor: Rune Dahlqvist
ISSN: 0031-6970 (print version)
ISSN: 1432-1041 (electronic version)
Journal no. 228

Types of papers

- **Original research papers**

  The number of printed pages per article including figures, tables and references should not exceed 8-10 printed pages (one printed page accounts to approx 650 words or 4300 characters, plain text without spaces; figures and tables have to be counted extra as half a page each).

- **Editorials** (without abstract)

- **Letter to the Editor**

  A commentary or a case report or otherwise a brief communication on a specific topic should have no more than 600 words main text (excepting the reference list) and contain no more than one table or one figure. The letter should not contain an abstract and should not be subdivided into sections.

- **Review articles**

  Review articles on various topics are welcome. Both invited and unsolicited submissions are published. The submitted review will be peer-reviewed as other submissions. A word limit is no specified for reviews. The Journal welcomes "full-sized" reviews of up to 4,000 words (main text) as well as "condensed" reviews or "occasional updates" of around 1,000 words.

- **Reporting Clinical Trials**

  The Editors believe that it is important to foster a comprehensive, publicly available database of clinical trials. In compliance with the guidelines of the International Committee of Medical Journal Editors (ICMJE), EJCP therefore requires that authors must register clinical trials before the first subject is enrolled. This policy goes into effect on June 1, 2007. Trials that were under way before that date and not registered and that are submitted to EJCP no later than June 1, 2008 will not be forced under the new guideline. For EJCP, clinical trial is defined as any research project that prospectively assigns human subjects to a pharmacological intervention or concurrent comparison or control groups to study the cause-and-effect relationship between this intervention and a health outcome. The ICMJE policies on registration of clinical trials can be found at:
  http://www.icmje.org/clin_trialup.htm.

  EJCP does not advocate one particular registry. Appropriate registries (such as www.clinicaltrials.gov) must be (1) accessible to the public at no charge, (2) open to all prospective registrants, and (3) managed by a not-for-profit organization. There must be a mechanism to ensure the validity of the registration data, and the registry should be electronically searchable. An acceptable registry must include at minimum the data elements available at the ICMJE website listed above. The title page of a manuscript describing the results of a clinical trial must contain the name of the clinical trial registry and registration number of the trial. Any report of a clinical trial not containing such information will be returned to the corresponding author without review.

  Reports of randomized, controlled trials should follow the recommendations of the Consolidated Standards of Reporting Trials (CONSORT) statement. See for the current CONSORT guidelines and checklist

  http://www.consort-statement.org/statement/revisedstatement.htm.

Special Issues

• Contributions that are part of a Special Issue must include the following footnote on the title page:
"This article is published as part of the Special Issue on [title of the Special Issue]"

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Contributions of Authors statement

It is required to specify the contribution/responsibility of each author in the work in a statement at the end of the manuscript. State the contribution detailed, with relevance to the international guidelines of co-authorship by ICMJE and for each author separately.
The statement should be placed after the Acknowledgments and before the reference list.

Title Page

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide a structured abstract of 150 to 250 words which should be divided into the following sections:

- Purpose (stating the main purposes and research question)
- Methods
- Results
- Conclusions

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Text Formatting

Manuscripts should be submitted in Word.

- The text of a research paper should be divided into Introduction, Materials and Methods, Results, Discussion, Acknowledgements, Conflict of Interest, and References.
- Materials and Methods must include statement of Human and Animal Rights.
- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.

- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

Specific remarks

- Introduction

  This section can be brief and should state the relevant background for and the main purposes of the study reported. Avoid review type introductions.

- **Terminology**

  Generic names of drugs and pesticides are preferred. If trade names are used, the generic name should be given at first mention. The proprietary name, chemical composition, and manufacturer should be stated in full in Materials and Methods. If a generic name has not been created or otherwise is not available, the chemical name should be given. Use of an industry code name alone is not sufficient.

- **SI units**

  Please always use internationally accepted signs and symbols for units. SI units.

- **Statistics**

  Sample size consideration must be given for any clinical study and power calculations are needed for negative results of pivotal variables. This can be done post-hoc if insufficient information was available a priori. Bioequivalence/bioavailability and drug-drug interaction studies should include tests/reference ratios and the respective 90% or 95% confidence intervals.

- **Analytical methods**

  Any method used to quantify drug or metabolite concentrations in body fluids should be characterised at least by the following information:

  - Range of quantification (defined by an acceptable accuracy/precision and not by a factor above baseline noise),

  - accuracy and precision over the entire range of quantification,

  - recovery (if applicable) and stability information for the period of measurement.

  This information is needed either in the manuscript or must be available in a reference the author provides. Normally the methods should be described in such a detailed way that other researchers will be able to repeat it.

References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 20 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

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For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

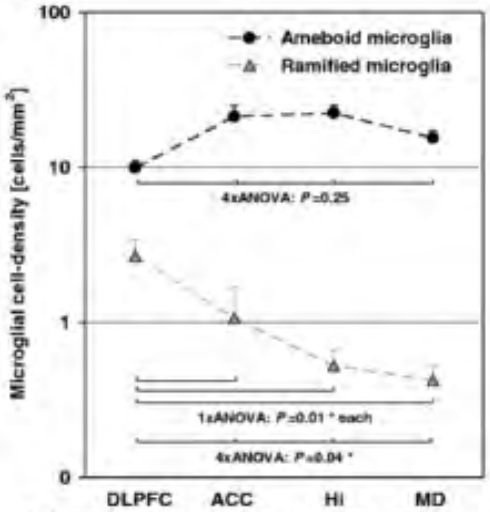
ARTWORK AND ILLUSTRATIONS GUIDELINES

For the best quality final product, it is highly recommended that you submit all of your artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the quality of the artwork provided.

Electronic Figure Submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art



- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

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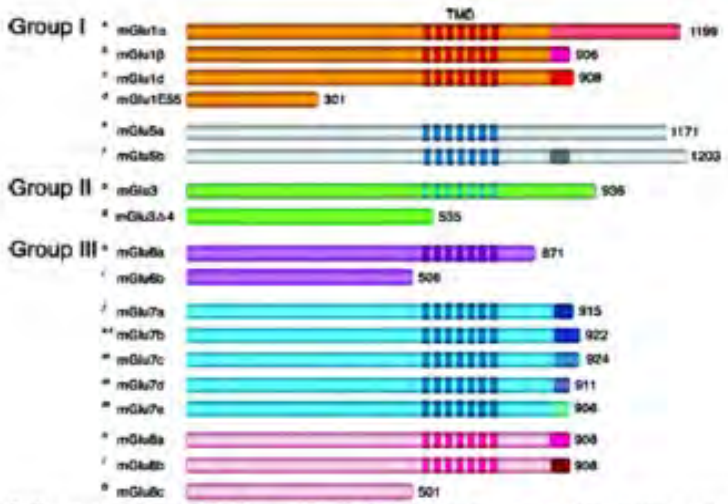
Combination Art



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- Color art is free of charge for online publication.
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Figure Numbering

- All figures are to be numbered using Arabic numerals.
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- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures.
  "A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

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- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
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These statements should be added in a separate section before the reference list. If these statements are not applicable, authors should state: The manuscript does not contain clinical studies or patient data.
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Addendum C	Study Protocol and GLP Laboratory Protocol

RESEARCH PROPOSAL

A PILOT INVESTIGATION ON PLASMA TENOFOVIR LEVELS AND POSSIBLE SIDE EFFECTS IN HIV-INFECTED WOMEN

Student Name: Mwila Mulubwa

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MSc Pharmacology

Promoter:

Dr. Michelle Viljoen, PhD (Pharmacology)

Co-promoter:

Dr. Malie Rheeders, PhD (Pharmacology)

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1.0 INTRODUCTION:

Globally, 34.0 million people were living with human immunodeficiency virus (HIV) at the end of 2011. Sub-Saharan Africa remains most severely affected, with nearly 1 in every 20 adults (4.9%) living with HIV and accounting for 69% of the people living with HIV worldwide **[1]**.

Standard combination antiretroviral therapy (ART) consists of the use of at least three antiretroviral (ARV) drugs to maximally suppress HIV and stop the progression of HIV disease. Treatment-naïve patients in South Africa are initiated on triple therapy, which consists of one non-nucleoside reverse transcriptase inhibitor (NNRTI) (e.g. nevirapine or efavirenz) and two nucleoside reverse transcriptase inhibitors (NRTI) (e.g. tenofovir disoproxil fumarate (TDF), lamivudine or emtricitabine) **[2]**.

Safe and effective management of HIV infection requires an understanding of the adverse effects associated with antiretroviral therapy (ART). Drug-related symptoms decrease adherence, which negatively impacts on virological suppression and immunologic recovery in a disease that requires lifelong therapy. Special considerations in drug-related toxicity have been observed in the years since the beginning of the ART "roll-out" in resource-limited settings **[3]**.

2.0 LITERATURE BACKGROUND:

It is known that the prevalence of chronic kidney disease and the risk factors associated with the development of the renal disease are ageing, diabetes mellitus, hypertension and HIV infection **[4]**.

2.1 Adverse effects of antiretroviral drugs

The NRTIs are associated with renal toxicity, proteinuria, diabetes insipidus,decreased bone mineral density, lipodystrophy, peripheral neuropathy pancreatitis, anaemia, myopathy, hyperpigmentation and lactic acidosis of which symptoms include; fatigue, nausea, diarrhoea and hepatic steatosis. NNRTIs are associated with rash, lipid disorders, severe hepatotoxicity and central nervous system adverse reactions. Protease inhibitors (PIs) are known for gastrointestinal effects, lipodystrophy and glucose intolerance **[5-7]**.

2.2 Tenofovir disoproxil fumarate (TDF)

Tenofovir disoproxil fumarate (TDF) is the prodrug which is converted to tenofovir diphosphate (nucleotide reverse transcriptase inhibitor) that has potent activity against the human immunodeficiency virus type 1 (HIV-1) and relatively low toxicity. However, some patients develop nephrotoxicity, which can lead to discontinuation in 1–2%. It is mainly excreted by the kidneys via a combination of glomerular filtration and active tubular secretion. Intracellular accumulation of tenofovir is responsible for damage to tubular cells and thus nephrotoxicity is usually presented as tubular dysfunction, which can, lead to Fanconi syndrome **[8]**. Treatment with tenofovir has been associated with significant decrease in spine and hip bone mineral density (BMD) **[9]**.

2.3 Tenofovir associated renal failure.

In a retrospective observational study of 183 (96% Caucasian, 85% male) patients in Spain aged between 40-50 years who discontinued TDF-based regimens due to renal failure after average exposure of 22 months, 31% had no improvement in any renal parameter **[8]**.

A cohort study in Brazil of 213 (81.7% euro-descendants, 51.3% men) HIV patients aged between 18-70 years, established tenofovir as the only antiretroviral drug that was independently associated with chronic kidney disease (CKD) defined as estimated glomerular filtration rate of less or equal to 60ml/min/1.73m^2 **[10]**. Furthermore, a cohort study in Switzerland of 1078 (30% female, 81.9% Caucasian) therapy naïve and therapy experienced patients aged between 32-47 years starting TDF showed reduction in glomerular filtration rate (Cockroft-Gault formular) between baseline and 6,12 and 24 months post TDF initiation. Median changes in eGFR from baseline at month 24 were -5.50ml/min and -4.11ml/min in therapy naïve patients, respectively **[11]**, dosing interval adjustment is required when creatinine clearance is less than 50 ml/min and avoidance if less than 30 ml/min **[12]**.
A cohort in 890 South African HIV patients (96.3% blacks,73.5% female) with a median age of 37.1 years on TDF based regimen, reported that 271 (30.4%) patients had mild and 46 (5.2%) had moderate renal dysfunction at TDF initiation. After 48 months post TDF initiation, 21 (2.4%) patients experienced nephrotoxicity **[13]**.

A prospective randomized controlled study of 51 (90% Asian or non-Hispanic white, 84% Men) HIV patients in USA showed that long term therapy with TDF or adefovir cause development of renal tubular dysfunction in 15% of patients. This was associated with proximal renal tubular dysfunction resulting in significant hypophosphatemia which could result in bone mineralization defects, renal insufficiency and osteomalacia **[14]**.
In a case report of a 37 year old male HIV patient in Germany, creatinine steadily increased to a maximum of 172 µmol/L after two days post initiation of TDF containing ART regimen. Upon discontinuation of TDF renal parameters restored within 36 hours to baseline values (GFR-MDRD 104.5ml/min) **[15]**. Furthermore, a case of suspected Fanconi's syndrome of a 38 year old male patient in USA on TDF regimen showed improved renal function during hospitalization after discontinuation of TDF **[16]**.

2.4 TDF and tenofovir pharmacokinetics.

Absorption

TDF is the water soluble prodrug (greater bioavailability and cellular penetration) which is converted to the active ingredient tenofovir diphospate. The oral bioavailability of TDF from fasted subjects is approximately 25%. A single oral dose of 300 mg TDF achieves maximum serum tenofovir concentrations (Cmax) in 1.0 ± 0.4 hrs with Cmax tenofovir and area under the curve (AUC) values of 0.30 ± 0.09 µg/mL and 2.29 ± 0.69 µg•hr/mL, respectively in adults**[17]**. Bioavailability is enhanced by a high fat-meal **[18]**. The absorption rate constant is 1.03h^{-1}**[19]**.

Distribution

In vitro binding of tenofovir to human plasma or serum proteins is less than 0.7 and 7.2%, respectively, over the tenofovir concentration range 0.01 to 25 µg/mL **[17]**. In adults, tenofovir

has a volume of distribution of 0.813 L/kg [20] and best described by a two compartment model [19].

Metabolism and Elimination

In vitro studies indicated that neither TDF nor tenofovir are substrates of cytochrome P-450 (CYP) enzymes. Following a single oral dose, terminal elimination half-life of tenofovir is approximately 17 hours. It has a plasma elimination half-life ($t_{½}$) of 12.0 to 14.4 hours. Tenofovir is eliminated by a combination of glomerular filtration and active tubular secretion and is mainly excreted unchanged (70%–80%) in urine [17, 20]. Apparent and intercompartment clearance is 42 L/h and 181 L/h respectively [19].

2.5 Plasma therapeutic range of tenofovir.

A single centre, pilot cross sectional study followed by a longitudinal prospective study of 27 HIV-infected Caucasians in Spain on TDF-based regimen (26% women, mean age 43.8 years) reported tenofovir C_{max} range of 252.1-349.5 ng/mL and C_{trough} range of 54.4-96.7 ng/mL [21]. Plasma concentration (EC_{50}) producing 50% of maximal intracellular tenofovir concentration was 60.6 ng/mL [22] and 24-hour trough concentration at steady state was 41 µg/L (5^{th} percentile 12 µg/L, 95^{th} percentile 91 µg/L) [18].

2.6 Creatinine clearance and tenofovir plasma concentration.

In a 24–week open-label multicentre trial of 53 patients in France (96% male) with median age of 41 (29-62) years on TDF-based regimen associated with atazanavir/ritonavir showed no difference in creatinine clearance observed at week 24 between patients with average tenofovir trough levels ≤ 58 µg/L and those with levels > 58 µg/L (p= 0.48). No difference in creatintine clearance was observed at 24 weeks in patients with peak levels ≤ 217 µg/L [23].

In light of the above there is a need to establish the average plasma tenofovir concentration that is associated with estimated glomerular filtration rate (eGFR) of < 50ml/min so as to promptly consider dose adjustment or TDF replacement in the management of HIV/AIDS.

TDF was introduced in 2010 and phased in as part of first line adult ART regimen in South Africa and research aimed at assessing the impact TDF on the South African population is highly called for, hence this study proposal.

3.0 PROBLEM STATEMENT:

Published information confirms that TDF is linked to renal failure [10, 14-16] and reduction in bone mineral density [9, 17]. The plasma concentration of tenofovir associated with a decreased renal function as defined by a glomerular filtration rate (eGFR) of less than 50 mL/min is not yet established.

4.0 MAIN OBJECTIVE:

To establish correlation between tenofovir plasma concentration and renal function and bone metabolism in HIV-infected women on TDF-based ART regimen.

4.1 Specific objectives

Primary objectives

- To develop and validate a high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method to determine TFV concentrations in human plasma.
- To determine plasma TFV concentrations in HIV-infected female participants on a TDF-based ART regimen in the PURE-SA sub-study.
- To investigate the correlation between plasma TFV concentrations and renal function markers [estimated glomerular filtration rate (eGFR), CrCl, albuminuria, maximum renal tubular reabsorption of phosphate ($TmPO_4$/GFR), serum urea, serum creatinine (SCr), serum uric acid, glucosuria or urine sodium (UNa)] in HIV-infected female participants.
- To establish possible correlations between TFV concentration and bone turnover markers [C-terminal telopeptide (CTx), alkaline phosphatase (ALP), parathyroid hormone (PTH), serum phosphate (SrP), serum calcium (SrCa) or total vitamin D (VitD)] in HIV-infected female participants.
- To compare renal function and bone turnover markers (BTM) between HIV-infected female participants on a TDF-based ART regimen and matched female HIV-uninfected controls within the PURE-SA sub-study.

Secondary objectives

- To track immunological recovery/status by comparing CD4+ cell count values between baseline and follow-up in HIV-infected female participants on a TDF-based ART regimen.
- To evaluate renal function status by comparing CrCl and eGFR between baseline and follow-up in HIV-infected female participants on a TDF-based ART regimen.

5.0 STUDY DESIGN AND METHODS:

This will be retrospective and prospective cross sectional study, which will form part of the already approved sub study (PURE: Bone density project, NWU-00016-10-A1, 23/02/2010) within the multinational longitudinal Prospective Urban and Rural Epidemiology (PURE) study taking place at the Tlokwe and Ganyesa study sites which commenced during November 2012 and will continue in April 2013 until end of August 2013. Since the study is observational, no form of intervention will be made in terms of provision of drugs. The HIV positive female participants will be their own control with regard to their creatinine clearance prior to TDF-based therapy and in addition a comparative HIV negative female group will also be identified within this PURE-SA sub study.

All other parameters such as serum phosphate, serum creatinine, urine creatinine and albumin, bone density measurements, weight, height and BMI already form part of the approved tests within the approved sub study (PURE: Bone density project).

5.1 Collection and storage of blood samples for tenofovir plasma determination and CD4-cell count.

In addition to the already approved blood sampling as per the sub study (PURE: Bone density project) protocol, 3.5 mL of whole blood in a sodium citrate tube and 1.0 mL in an ethylenediaminetetra acetic acid (EDTA) microtainer will be collected. All the blood drawing

procedure will take place at the same time in one action and no additional needle pricking would be required at any stage. The additional 4.5 mL extra whole blood would be used for the tenofovir plasma (3.5 mL) analysis and the CD4-cell count (1.0 mL). The sodium citrate collected blood will be centrifuged for 10 minutes at 4000 x g and the plasma will be frozen at -20 °C until further drug analysis. **[25]**. The 0.5 mL microtainer will be sent to the Lancet Laboratories for the CD4-cell count test using their standard method on a flow cytometer.
The standard adult dose for TDF is 300 mg taken once a day **[17]** and the current practise in the local primary health care (PHC) clinics is to establish standardised dosage directions with all patients in the North West province and TDF is directed to be taken at night to ensure the best patient compliance. Recording the medication use history of all the study participants will also include when was the last dose of TDF taken the previous night and or day. It is anticipated that the tenofovir plasma samples will correspond to a typical 10-16 hours post last dose which will correspond to a typical mid-dose interval range for these study participants.

5.2 Analytical sample preparation and analysis of tenofovir in plasma

A simple solid phase extraction method will be employed for extraction of tenofovir and internal standard from human plasma before injecting the sample into the HPLC-MS/MS apparatus for the analyte sample detection and quantification as described in a previously published method with some modification **[26]**.
Validation procedures will be performed by incorporating calibration curves constructed by plotting peak height ratios (tenofovir/internal standard) as a function of tenofovir plasma concentration. Linearity will be tested using analysis of variance. Significance of the slope and the validity of the linear calibration curves will be tested using F-test. Extraction recovery of tenofovir will be determined at different concentrations **[25]**.

5.3 Study sample size

This is a pilot study and the sample size will include all of the HIV-positive women who are on TDF-based ART regimen at the time of the recordings and observations (April 2013 – August 2013) of the PURE-SA sub study. The longitudinal PURE-SA study follow up that were performed in 2010 included 137 HIV-infected participants of whom only 66 (54 women) were on ART (mainly consisting of stavudine, lamivudine and efavirenz) and 71 were treatment naïve. An estimate based on this 2010 data would include at least 48 female patients on TDF-based treatment during the April 2013 – August 2013 sub study follow-up.

5.4 Inclusion and exclusion criteria

Inclusion criteria

- All the HIV positive women on TDF-based ART regimen that are being followed up during the PURE-SA sub study duration.
- Baseline serum creatinine values prior to TDF treatment should be available from the participant's clinic or hospital records, provided that the participants give consent to access their health information/records from the North West Department of Health (NW DoH).
- An equal number of HIV negative women of similar age, weight, height, BMI will also be identified retrospectively to act as a control group for the HIV-positive women.

- Concomitant medication use for hypertension, diabetes, asthma, epilepsy, tuberculosis (e.g rifampicin, metformin, aminophyllin, enalapril, hydrochlorothiazide, nifedipine, isoniazid, phenobarbitone) will be acceptable but the specific medication history must be available and recorded.
- Signed informed consent.

Exclusion criteria

- Amputation
- Known renal disease
- Drugs (streptomycin, lithium, sulfadiazine, phenytoin, allopurinol, amphotericin B deoxycholate, methotrexate, statins, mesalazine), due to known renal damage that they can cause.

6.0 DATA ANALYSIS:

The pharmacokinetic results of the tenofovir plasma concentration will be described by descriptive statistics such as mean (standard deviation) and median (interquartile range). Other pharmacokinetic parameters to be reported on would include clearance (CL). Correlation and linear regression analysis will be applied to obtain statistical relationship between TDF plasma concentrations and eGFR below 50 mL/min. eGFR will be calculated using MDRD and Cockroft –Gault formulae with parameters accessed from PURE data base. T-test will be used to determine significance in eGFR in TDF exposed and TDF non exposed participant. F-test will be used to determine significance in variation.

7.0 ETHICS:

Ethics approval for the sub study (PURE: Bone density project: NWU-00016-10A1) was already approved by the ethics committee of the North-West University (refer to attachment) in 2010. This protocol will include the same female population and the same observational period. The additional request for 4.5 mL whole blood in sodium citrate (3.5 mL) and EDTA microtainer (1.0 mL) tubes will all be drawn at the same time when all the other already approved bloods will be drawn. This protocol will also be assessed for ethical suitability and to determine if it could be incorporated into the confines of the already approved sub study (PURE: Bone density project).
Written signed informed consent will be sought from participants and they will have the right to withdraw the consent at any time. Numeric codes shall be used to identify participants during data collection and all study participants information will be confidential.
The study will be conducted in accordance with the Declaration of Helsinki 2013 and according to National Guidelines of Good Clinical Practice, Department of Health.

8.0 COLLABORATORS:

Dr CMT Fourie from Department of Physiology (HART) will be advising and conceptualising some aspects of the renal function degeneration within this study population and will also be contributing as an author on proposed manuscripts for publication.

Prof HS Kruger will also be consulted with regard to the body mass index (BMI) and other relevant anthropometric measurements.

The possible effect or no effect of long term TDF-based ART on bone density would be investigated as part of the primary aims, in collaboration with Prof A Kruger, Drs L Kruger and H Wright. Scientific contributions to a manuscript by these collaborators would be welcomed and encouraged should the study numbers and power of the study be statistical significant.

9.0 TIME TABLE

	JAN$_{13}$	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN$_{14}$	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV
Protocol development																							
Protocol presentation																							
Ethics approval																							
Data collection: Feb-wet April-May																							
HPLC method development and validation																							
Data collection: Gauteng July-Aug																							
Analysis of samples																							
Data analysis																							
Report and dissertation writing																							
Presentation at Pharmacology Dept.																							
Presentation World Pharmacology congress																							
Final dissertation & publication																							

10.0 BUDGET

SN	ITEM DESCRIPTION	QUANTITY	UNIT PRICE (R)	TOTAL (R)
1	EDTA tubes	600		600
2	HPLC method validation and analysis	R120 /hr		10 000
3	Smac analysis (phosphate, creatinine)	600		10 000
4	Urine analysis (creatinine, phosphate, albumin)	600		20 000
5	CD4 counts (Lancet)	60	@R180	10 800
6	Statistical consultations			2 000
7	Stationery			600
8	Chemical reagents			1 000
9	Tenofovir analytical grade (99%)	50 mg	@R120	6 000
10	Laboratory consumables			5 000
11	Accommodation and food in Ganyesa	20 days/nights	@R500	10 000
12	PURE study contribution for Clinic use/fieldworkers/fuel combi's/Participant reimbursement			3 200
13	Incidentals			5 000
	TOTAL			**R84 200**

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PCDDP	GLP Study Protocol		
Title	Development and validation of high performance liquid chromatography-mass spectrometry method for determination of tenofovir in human plasma.		
Study no	GLP Plan-Dr.M.Viljoen-2014-001	**Issue no**	Draft 2
Study initiation date:		**Page no**	Page 1 of 9

1. COMPILATION AND AUTHORISATION

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Ethical Approval no:	NWU-00016-10-A1

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Facility Head / co-ordinator	L Webster		
Sponsor	Dr M Viljoen		

3. DOCUMENT HISTORY

Date	Issue no	Reason for revision
	Draft 1	First issue of draft based on initial process
	Draft 2	Developed a better procedure

PCDDP	GLP Study Protocol		
Title	Development and validation of high performance liquid chromatography-mass spectrometry method for determination of tenofovir in human plasma.		
Study no	GLP Plan-Dr.M.Viljoen-2014-001	**Issue no**	Draft 2
Study initiation date:		**Page no**	Page 2 of 9

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5. **INTRODUCTION and OBJECTIVES:**

Tenofovir is a nucleotide reverse transcriptase inhibitor that has potent activity against HIV-1 and forms part of first line ART regimen in South Africa. It has been associated with renal tubular dysfunction, nephrotoxicity and reduced eGFR. Plasma tenofovir levels associated with decreased renal function have not yet been established. Thus the objective will be to develop and validate HPLC/MS/MS method to determine tenofovir plasma concentrations and to further determine tenofovir concentrations in HIV-infected plasma samples.

6. **SCOPE:**

Preparation of standard solutions, plasma sample processing, extraction and analysis of tenofovir and internal standard (cimetidine). Complete validation and system suitability data of the specific HPLC/MS/MS method

7. **REFERENCE DOCUMENTS:**

a) Bi, Hui-chang, et al. "Determination of adefovir in human plasma by liquid chromatography/tandem mass spectrometry: application to a pharmacokinetic study." Rapid communications in mass spectrometry 19.20 (2005): 2911-2917.

b) Xiong, Zhili, et al. "Hydrophilic interaction liquid chromatography–tandem mass spectrometry for the determination of adefovir in human plasma and its application to a pharmacokinetic study." Journal of Chromatography B 878.23 (2010): 2111-2116.

c) Xie, Hai-Tang, et al. "A New LC–MS–MS Method for Quantitative Analysis of Adefovir, and Its Use for Pharmacokinetic Studies in Healthy Chinese Volunteers." Chromatographia 71.7-8 (2010): 587-593.

d) Sentenac, S., et al. "Sensitive determination of tenofovir in human plasma samples using reversed-phase liquid chromatography." Journal of Chromatography B 793.2 (2003): 317-324.

e) Yadav, Manish, et al. "Rapid and specific liquid chromatographic tandem mass spectrometric determination of tenofovir in human plasma and its fragmentation study."

Journal of Chromatographic Science 47.2 (2009): 140-148.

f) Bennetto-Hood, Chantelle, Mary C. Long, and Edward P. Acosta. "Development of a sensitive and specific liquid chromatography/mass spectrometry method for the determination of tenofovir in human plasma." Rapid communications in mass spectrometry 21.13 (2007): 2087-2094.

g) Barkil, Mima El, Marie-Claude Gagnieu, and Jérôme Guitton. "Relevance of a combined UV and single mass spectrometry detection for the determination of tenofovir in human plasma by HPLC in therapeutic drug monitoring." Journal of Chromatography B 854.1 (2007): 192-197.

h) Hirt, Déborah, et al. "Plasma and intracellular tenofovir pharmacokinetics in the neonate (ANRS 12109 trial, step 2)." Antimicrobial agents and chemotherapy 55.6 (2011): 2961-2967.

i) Kandagal, P. B., et al. "RP-HPLC Method for the Determination of Tenofovir in Pharmaceutical Formulations and Spiked Human Plasma." Analytical letters 41.4 (2008): 561-570.

j) Shah, Syeda AB, et al. "Simultaneous analysis of antiretroviral drugs abacavir and tenofovir in human hair by liquid chromatography tandem mass spectrometry." Journal of pharmaceutical and biomedical analysis (2012).

k) Xiong, Zhili, et al. "Hydrophilic interaction liquid chromatography–tandem mass spectrometry for the determination of adefovir in human plasma and its application to a pharmacokinetic study." Journal of Chromatography B 878.23 (2010): 2111-2116.

l) European Medicines Agency, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf: 19/01/2014

m) U.S. Food And Drug Administration: Centre For Drug Evaluation And Research, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidelines/UMC070107.pdf: 19/01/2014

8. ABBREVIATIONS AND/OR DEFINITIONS:

Abbreviation	Description
DST	Department of Science and Technology
GLP	Good Laboratory Practice
NWU	North West University
OECD	Organisation for Economic Co-operation and Development
PCDDP	Preclinical Drug Development Platform
QA	Quality Assurance
SOP	Standard Operating Procedure
HIV-1	Human immunodeficiency virus, type 1
ART	Anti-retroviral therapy
eGFR	Estimated glomerular filtration rate
HPLC/MS/MS	High performance liquid chromatography/mass spectrometry

9. RESPONSIBILITIES:

Reflects the persons and facilities that are responsible for or involved in the execution of the study.

Study director: Prof Anne Grobler
QA Manager: Liezl-Marie Scholz
Sponsor: Dr Michelle Viljoen
The Facility Heads and Co-ordinators: Linzie Webster
Study Personnel: Mr Mwila Mulubwa; Dr Michelle Viljoen; Dr Malie Rheeders

10. MATERIALS AND EQUIPMENT:

Tenofovir 99.9% (analytical grade)
Blank human plasma
Acetonitrile (MS grade)

Methanol (MS grade)
Formic acid (MS grade)
Water (HPLC grade)
Centrifuge
Mixer
HPLC/MS/MS (Agilent 1290/ AB SCIEX 4000 QTRAP®)

11. SAFETY:

All biological material will be handled with the required GLP requirements. Biological waste will be removed as per the prescribed SOP.

All required safety steps and precautions will be adhered to in the laboratory working with the various chemicals, apparatus and biological samples according to the relevant SOPs.

HIV infected plasma will be heated at 57°C to inactivate the virus. In an event where samples accidentally come in contact with body fluids, post exposure prophylaxis will be sought from University Health Centre immediately.

12. JUSTIFICATION:

12.1. Test system elected and on the number of test subjects used.
12.2. Method of administration
12.3. Dose levels and /or concentration
12.4. Frequency and durations of administration / application
12.5. Statistical methods:

Regression analysis will be used to determine linearity of the calibration curves. best model that will describe the linear regression of the ratio of the areas of plasma tenofovir and internal standard peaks versus tenofovir concentration will be fitted with a weighting factor of either $1/x$, $1/x^2$ or unweighted.

13. PROCEDURE: PLASMA TENOFOVIR EXTRACTION

13.1. Methods:

Materials: (Protein precipitation)

Tenofovir 99.9% (analytical grade)
Acetonitrile (
Methanol
Blank human plasma
Water (HPLC grade)

1. Preparation of standard and quality control stock solutions:

- Dissolve 8.5 mg of TFV reference standard in 50 mL of Milli-Q water to make 170 µg/mL.
- Prepared stock quality control solution (QC) by dissolving 9.1 mg of TFV reference standard in 50 mL of Milli-Q water to make 182 µg/mL.
- Dilute (QC) with Milli-Q water to make 170 µg/mL.
- Dissolve 10.08 mg of cimetidine (internal standard) in 25 mL of methanol: water (40:60, v/v) to make 403.2 µg/mL.
- Dilute internal standard solution with Milli-Q water to 10 ng/ml.

2. Preparation of calibration standards and QC samples:

- Spike 4235 µL of drug free plasma with 15 µL of TFV stock solution.
- Dilute spiked plasma with drug free plasma and make nine calibration standard concentrations at 600, 500, 400, 300, 200, 100, 50, 25 and 12.5 ng/mL.
- Prepare QC samples at 450,150 and 30 ng/mL (HQC, MQC, LQC) by spiking 4235 µL of drug free plasma with 15 µL of stock QC solution and dilute with drug free plasma.

3. Extraction:

- Heat nine calibration standards and QC plasma samples using water bath at 57°C for 30 minutes.
- Leave samples to equilibrate with room temperature.
- Pipette 25 µL (10 ng/mL) of internal standard into Eppendorf tube.
- Add 10 µL of the plasma sample to internal standard solution.
- Add 100 µL of methanol: acetonitrile (50:50 v/v) was added to the sample.
- Vortex for 10 seconds, left in a freezer at -23°C for 10 minutes.

- Vortex for a second time and centrifuge for 4 minutes at 6000 rpm.
- Transfer 50 µL of the supernatant into a clean auto sampler vial.
- Add 120 µL of Milli-Q water and vortex.
- Inject 20 µL of this solution onto the column for analysis.
- Repeat the same procedure for all human plasma samples (Heating of plasma samples in a water bath ensured inactivation of HIV).

3. **Method validation**

- As described in the European Medicines Agency and US Food and Drug Administration guidelines for bio analytical method validation.

13.4. Conditions

Chromatographic conditions

Run time (min)	Flow rate (µL/min)	A (%)	B (%)
0.00	250	95.0	5.0
0.10	250	95.0	5.0
0.60	250	5.0	95.0
1.30	250	5.0	95.0
1.40	250	90.0	10.0
2.00	250	90.0	10.0

Mass spectrometric conditions

Drug	Precursor ion (m/z)	Product ion (m/z)	DP (v)	CE (v)	CXP (v)	Dwell time (ms)
Tenofovir	288.072	176.038	106	35	8	200
Cimetidine	253.13	158.987	61	21	12	200

13.5. Analysis and measurements

- Measurements will be in Nano grams per millilitre.
- The measurement will be done on a AB Sciex QTRAP 4000

13. **ADDENDUMS and RECORDS**

Addendum D1	Ethics clearance letters

health
Department of
Health
North West Province
REPUBLIC OF SOUTH AFRICA

2ND Floor Tirelo Building
Dr. Albert Luthuli Drive
Mafikeng, 2745
Private Bag X2068
MMABATHO, 2735

Tel: (018) 387 1766
Fax: 018 392 6710
kshogwe@nwpg.gov.za
www.nwhealth.gov.za

POLICY, PLANNING, RESEARCH, MONITORING AND EVALUATION

To : **Mr M Mulubwa and Dr M Viljoen**

From : **Policy, Planning, Research, Monitoring & Evaluation**

Subject : **Approval Letter- A pilot study investigating on plasma tenofovir levels and possible side effects in HIV positive women: Sub study within the PURE-SA study**

Purpose

To inform the researcher that permission to undertake the above mentioned study has been granted by the North West Department of Health. The researcher is expected to arrange in advance with the chosen districts or facilities, and issue this letter as prove that permission has been granted by the provincial office.

Upon completion, the department expects to receive a final research report from the researcher.

Kindest regards

Acting Director: PPRM&E
Mr. L Moaisi

07/08/2013
Date

DEPARTMENT OF HEALTH
PRIVATE BAG X2068
2013 -08- 08
SUPERINTENDENT GENERAL

Healthy Living for All

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: +2718 299-1111/2222
Web: http://www.nwu.ac.za

Faculty of Health Sciences
Tel: +2718 2992095
Fax: +2718 2992088
Email: Annamarie.Kruger@nwu.ac.za

To whom it may concern

12 April 2013

Dear Dr. Viljoen

Additional request: NWU-00016-10-A1

Your request to include the study "A pilot investigation on plasma tenofovir levels and possible side effects in HIV positive women: substudy within the PURE-SA study" within the PURE SA study has been approved.

Yours sincerely

Prof. A. Kruger
Ethics Sub-Committee Chair

Original details: Prof. A. Kruger(10062416) C:\Users\13210572\Documents\ETIEK\2010 ETHICS\NWU-00016-10-A1b Additional Request.docm
12 April 2013

File reference: NWU-00016-10-A1 Add. Request

Addendum D2	Informed consent form: original PURE study at recruitment (2005)

YUNIBESITI YA BOKONE-BOPHIRIMA
NORTH WEST UNIVERSITY
NOORDWES UNIVERSITEIT

POTCHEFSTROOM CAMPUS

PURE-SA Project
INFORMED CONSENT FORM

Title of the project: PURE-Project (Prospective Urban and Rural Epidemiology)

INFORMED CONSENT

I, the undersigned ..... (full names) read/listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project

I indemnify the University, also any employee or student of the University, of any liability against myself, which may arise during the course of the project.

I will not submit any claims against the University regarding personal detrimental effects due to the project, due to negligence by the University, its employees or students, or any other subjects.

I agree to be tested for HIV : YES NO

I want to know my HIV-status YES NO

(Signature of the subject)

Signed at ..... on .....

Witnesses

1. .....

2. .....

Signed at ..... on .....

YUNIBESITI YA BOKONE-BOPHIRIMA
NORTH WEST UNIVERSITY
NOORDWES UNIVERSITEIT

CONFIDENTIAL

PART 1

1. School/Institute:
   School for Physiology, Nutrition and Consumer Sciences

2. Title of project/trial:
   PURE: Prospective Urban and Rural Epidemiology

3. Full names, surname and qualifications of project leader:
   Annamarie Kruger, Ph.D.(Nutrition)

4. Rank/position of project leader:
   Research Manager

5. Aim of this project
   PURE's aim is that understanding the different lifestyle and health transitions of individuals in response to societal changes will elucidate societal and individual adaptive strategies that could diminish the adverse health effects of industrialization and urbanization on health, while retaining its benefits.

8. Explanation of the nature of all procedures, including identification of new procedures:
   Each participant will have to fill in a number of questionnaires (Adult questionnaire, Physical activity questionnaire, Food frequency questionnaire, Health questionnaire) with the help of field workers. A blood and urine sample will be taken. Physical measures will be performed, including anthropometric measures (such as weight, height, and waist circumference), blood pressure, lung capacity and lung volume and an ECG will be performed.

9. Description of the nature of discomfort or hazards of probable permanent consequences for the subjects which may be associated with the project:
   (Including possible side-effects of and interactions between drugs or radio-active isotopes which may be used.)
   It will take each participant quite a while (about an hour) to complete the questionnaires and discomfort may be experienced with the taking of blood samples. No measures will have permanent damage or consequences for the participants.

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NORTH WEST UNIVERSITY
NOORDWES UNIVERSITEIT

10. Precautions taken to protect the subjects:

The research nurse will be present at all times, and will be responsible for the blood sampling. She is very experienced and has performed these procedures numerous times in previous studies.

11. Description of the benefits which may be expected from this project:

When measures with immediate results are taken, such as blood glucose levels or blood pressure, the information will be communicated to the individual to seek professional help.

Since this study is a longitudinal study, subjects that are high at risk will be identified from the dataset and personal feedback will be given.

12. Alternative procedures which may be beneficial to the subjects:

There will be tested for HIV/AIDS, therefore pre-test counselling will be given. If the subject wants to know his/her status and he/her tests positive, post counselling will also be given.

PART 2

To the subject signing the consent:

You are invited to participate in a research project. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. Participation in this project is voluntary.
2. It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.
3. You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.
4. The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.

YUNIBESITI YA BOKONE-BOPHIRIMA
NORTH WEST UNIVERSITY
NOORDWES UNIVERSITEIT

5. We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.
6. The University staff will use standardised procedures and take all possible precaution to protect the subject from risks. We require that you indemnify the University from any liability due to detrimental effects of treatment by University staff or students or other subjects to yourself or anybody else. We also require indemnity from liability of the University regarding any treatment to yourself or another person due to participation in this project, as explained in Part 1. Lastly it is required to abandon any claim against the University regarding treatment of yourself or another person due to participation in this project as described in Part 1.
8. If you are married, it is required that your spouse abandon any claims that he/she could have against the University regarding treatment or death of yourself due to the project explained in Part 1.
9. All information will be kept CONFIDENTIAL.

Signature: electronically signed on Date: 25 March 2005
Project leader

Contact details: **082 7715778 / 018 2994037 (W) / 018 2907024 (H)**

YUNIBESITI YA BOKONE-BOPHIRIMA
NORTH-WEST UNIVERSITY
NOORDWES-UNIVERSITEIT

Potchefstroom Campus

The PURE project
Information to communities

Dear Participant

Thank you for being willing to help us in this very important project. We are sure that the project will contribute to improve health of all the people of the North West Province.

The aim of the project is to get enough information regarding the development of chronic diseases like Diabetes, Stroke, Lung disease and heart disease with urbanisation to plan appropriate health and nutrition intervention strategies.

For this study we need 2 000 subjects whom we can follow for 12 years. The baseline survey will be done from April 2005 to November 2005. The subjects must be from rural as well as urban communities. Therefore, 500 subjects from 4 different levels of urbanisation will be needed. Ganyesa and Tlakgameng were chosen for the rural and semi-rural areas because they are still under tribal law with a good infra structure and stability. We also spoke to Chief M. Letlhogile and the mayor Mr E. Tladinyane and both gentlemen gave us permission to do the research in these two communities. Ikageng and the informal Ikageng were chosen as it is convenient and near the University. Cllr GG Megalanyane and Cllr Mahesh Roopa are informed about the study.

You are one of the 2000 people (250 men and 250 women from all four sites (Ganyesa, Tlakgameng, Ikageng, and the Informal Ikageng) that are selected from the previous questionnaires to be asked to proceed with the study. You should be

- Older than 35 years
- Healthy – which means that they must not be aware of any disease and do not take any chronic medication

You will be asked to fill out the adult questionnaire, the food frequency questionnaire, the health questionnaire and the physical activity questionnaire. We will also make an appointment with you to take some measurements such as weight, height, skinfold thicknesses, ECG (test for heart abnormalities), lung functions, blood pressure, blood glucose, blood samples and a urine sample. You should understand that participation is voluntarily.

YUNIBESITI YA BOKONE-BOPHIRIMA
NORTH-WEST UNIVERSITY
NOORDWES-UNIVERSITEIT

It is very important that we gather quality data and knowledge. Because HIV/AIDS is such a devastating illness and affects almost all aspects of health, it is necessary to know if HIV is absent before we analyse the data. Therefore we will ask questions about your HIV status which you are allow not to answer.

It is also very important to us that you feel free to participate in this study and that you understand what the study is all about. The fieldworker will ask you to sign this form after you have read and understood it.

Kind regards

Dr ANNAMARIE KRUGER
Contact details: 082 7715778 / 018 2994037(W) / 018 2907024(H)

Addendum D3	**Informed consent form: bone sub-study (2010 – 2013)**

NORTH-WEST UNIVERSITY
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NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

PURE-SA PROJECT (Prospective Rural and Urban Epidemiology) and Bone Health Study

INFORMED CONSENT

I, the undersigned ..... (full names) read/listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project.

I indemnify the University, also any employee or student of the University, of any liability against myself, which may arise during the course of the project.

I will not submit any claims against the University regarding personal detrimental effects due to the project, due to negligence by the University, its employees or students, or any other subjects.

I agree to be tested for HIV:	YES	NO
I want to know my HIV-status	YES	NO

I give consent that the outcome of results can be disclosed with members of the PURE-SA research team for effective management and compliance to the project. The test results will be treated with the highest confidentiality.

_____
(Signature of the subject)

Signed at _____ on _____

Witnesses

1. _____ 2. _____

Signed at _____ on _____

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

CONFIDENTIALITY
PART 1

1. *School/Institute:*
   Africa Unit for Transdisciplinary Health Research (AUTHeR)

2. *Title of project/trial:*
   PURE: Prospective Urban and Rural Epidemiology and Bone Health Study

3. *Full names, surname and qualifications of project leader:*
   Annamarie Kruger, Ph.D. (Nutrition)
   Lanthé Kruger, Ph.D. (Physiology)

4. *Rank/position of project leader:*
   Research Manager

5. *Aim of this project*
   PURE's aim is that understanding the different lifestyle and health transitions of individuals in response to societal changes will elucidate societal and individual adaptive strategies that could diminish the adverse health effects of industrialization and urbanization on health, while retaining its benefits.

   The aims of the **Bone Health study** is to investigate and improve bone health in women. It is very important to build strong and healthy bones in the childhood and teen years to avoid osteoporosis and other bone problems later in life. Osteoporosis is a condition in which bones are fragile, making them fracture or break much easier. The spine, wrist and hips are particularly vulnerable to fracture.

6. *Evaluation/measurement points:*
   Each participant will undergo multiple evaluations at different stations. These include:

   - HIV counselling and testing
   - Blood sample (18.5 ml)

- Anthropometrics (height, weight, skinfold thickness, circumference of arm, leg and waist)
- Urine sample (spot urine sample)
- Bone density measurement - Osteometer
- Blood pressure measurement
- Carotid IMT scan (Intimal-medial thickness)
- Electrocardiogram
- Physical strength test
  - Forearm and hand strength test by squeezing an object
  - Getting up from a chair and walking on straight line for 6 meters
  - Stepping on and off a step
- Questionnaires
  - Bone Health
  - Attitudes towards HIV/AIDS
  - Physical Activity
  - Socio-Demographic
  - Medicine use

7. *Description of the nature of discomfort or hazards of probable permanent consequences for the subjects which may be associated with the project:*
(Including possible side-effects of and interactions between drugs or radio-active isotopes which may be used.)
It will take each participant quite a while (about an hour) to complete the questionnaires and discomfort may be experienced with the taking of blood samples. No measures will have permanent damage or consequences for the participants. If participant is unable to perform a test (especially physical strength test) they will not be forced to take the test.

8. *Precautions taken to protect the subjects:*
The research nurse will be present at all times, and will be responsible for the blood sampling. She is very experienced and has performed these procedures numerous times in previous studies.

9. *Description of the benefits which may be expected from this project:*
When measures with immediate results are taken, such as blood pressure, the information will be communicated to the individual to seek professional help.
Since this study is a longitudinal study, subjects that are high at risk will be identified from the dataset and personal feedback will be given.

10. *Alternative procedures which may be beneficial to the subjects:*
There will be tested for HIV/AIDS, therefore pre-test counselling will be given. If the subject wants to know his/her status and he/her tests positive, post counselling will also be given.

PART 2

To the subject signing the consent:

You are invited to participate in a research project. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. Participation in this project is voluntary.

2. It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.

3. You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.

4. The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.

5. We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.

6. The University staff will use standardised procedures and take all possible precaution to protect the subject from risks. We require that you indemnify the University from any liability due to detrimental effects of treatment by University staff or students or other subjects to yourself or anybody else. We also require indemnity from liability of the University regarding any treatment to yourself or another person due to participation in this project, as explained in Part 1. Lastly it is required to abandon any claim against the University regarding treatment of yourself or another person due to participation in this project as described in Part 1.

8. If you are married, it is required that your spouse abandon any claims that he/she could have against the University regarding treatment or death of yourself due to the project explained in Part 1.

9. All information will be kept CONFIDENTIAL.

_____ _____

Signature of project leader Date

Addendum D4	**Informed consent form: access to patients' records and TDF data (2012 – 2013)**

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

PURE-SA PROJECT (Prospective Rural and Urban Epidemiology) and Bone Health Study
INFORMED CONSENT

I, the undersigned ……(full names) read/listened to the information on the project regarding medicine use and/or other related clinical tests. I declare that I understand the information. I had the opportunity to discuss aspects of the project with the data collector and I declare that I participate in the project as a volunteer.

I indemnify the University, also any employee or student of the University, of any liability against myself, which may arise during the course of the project.

I will not submit any claims against the University regarding personal detrimental effects due to the project, due to negligence by the University, its employees or students, or any other subjects.
I agree that any additional information regarding my medicine use and/or other related clinical tests may be gathered from my clinic or hospital file if needed on a later date.
I give consent that the outcome of results can be disclosed to members of the PURE-SA research team for effective management and compliance to the project. The information on my medicine use and/or other related clinical tests will be treated with the highest confidentiality and anonymously.

_____

(Signature of the subject)

Signed at _____ on _____

Witnesses

1. _____ 2. _____

Signed at _____ on _____

Addendum D5	Medication questionnaire

Pure Study: Medicine Usage Questionnaire

1. Full Patient Names: _____

2. PURE Study number: _____ Patient nr. _____Patient consent signed: Yes / No

3. Date of birth:

Y	Y	Y	Y	M	M	D	D

4. Contact numbers:_____(Cellphone/Home)

5. Tick the sites the patient visits (All sites)

Public Clinic	1
Public Hospital	2
Private medical doctor	3
Pharmacies	4
Other:Traditional healers Homeopath(specify)	5

6. Public Clinic Name: _____

7. Gender

M	1
F	2

8. Race

African (Blacks)	1
Coloured	2
Indian	3
White	4
Other, Specify	5

9. A Language (Mother tongue / Huistaal)

Tswana	1
Afrikaans	2
English	3
Other ,Specify	4

9. B Second language

Tswana	1
Afrikaans	2
English	3
Other ,Specify	4

10. Does the patient use any medication ? (If answer is yes fill in Question 11 and 12)	Yes=1	No=2
11. Did the patient bring their clinic book ?	Yes=1	No=2
12. Did the patient bring their medicines ?	Yes=1	No=2

MEDICATION LIST

(Ask about any other medication in use, like Grandpa, KPP powders, etc.)

Note: Acute medication = medicines prescribed within the last 14 days + Chronic medication = medicine prescribed monthly

Prescription date	Trade name	Active component	Dose	Acute (A)/ Chronic (C)	ATC classification/ Pharmacological classification	Pure Study coding (0-29)	NOTES / COMMENTS (Specific disease)
							According to doctor / clinic book:
							According to patient :

Addendum E1	Summary statistics of all variables

Variable	HIV-infected						HIV-uninfected						*P*-value[a]	Adjusted *P*-value[c]
	N	Mean (SD)	Median (IQR)	Max	Min	95% CI	N	Mean (SD)	Median (IQR)	Max	Min	95% CI		
Age (years)	30	53 ±9.3	52 (49-59)	67	37	51-56	30	60 ±11	57 (54-63)	88	45	56-64	**0.008**	
BMI (kg/m^2)	30	24.2 ±9.5	20.5 (18-29)	43.1	15.7	21.2-27	30	26.2 ±8.2	24.4 (19.4-33)	42.5	14.1	23.5-29	0.333	
Weight (kg)	30	58 ±22.5	52.5 (44-69)	101	37.4	51-65	30	64.7 ±20.5	60.4 (49-84)	101	34.4	54.4-72	0.179	
SCr (µmol/L)	30	50 ±20	43 (38-58)	131	31	48-58	30	54 ±12.1	53.3 (47-61)	100	35.5	50-59	**0.028**[b]	0.441
SrP (mmol/L)	30	1.05 ±0.2	1.06 (0.1-1.16)	1.62	0.75	0.97-1.13	30	0.99 ±0.16	0.99 (0.87-1.11)	1.29	0.65	0.93-1.05	0.204	0.165[d]
Serum urea (mmol/L)	30	3.3 ±1.7	3.13 (1.9-4.50	7.0	0.96	2.62-3.91	30	3.5 ±1.32	3.21 (2.5-4.5)	6.97	1.54	3.04-4	0.507	
Serum uric acid (µmol/L)	30	278.7 ±122	251 (186-323)	649	121	233-324	30	311 ±99.6	313 (238-380)	563	149	274-348	0.265	
Glucosuria (mmol/L)	30	0.12 ±0.09	1 (0.04-1.9)	0.35	0.01	0.09-0.15	30	0.19 ±0.27	0.1 (0.06-0.21)	1.32	0.0	0.09-0.29	0.59[b]	
UCr (mmol/L)	30	4.3 ±3.1	3.5 (1.6-5.8)	13.5	0.53	3.07-5.43	30	6.0 ±5.8	4.18 (2.9-6.7)	31	1.4	3.88-8.2	0.145	
UP (mmol/L)	30	5.4 ±5.3	4.15 (1.3-8)	27.4	0.45	3.4-7.4	30	7.5 ±9.4	5.2 (1.8-9)	46.5	0.18	4-11	0.524[b]	
UNa (mmol/L)	30	75.5 ±55.7	49 (28-17)	191	20	-12-48	30	101.7 ±42.3	103 (76-127)	210	22	85-117	**0.048**	0.119
Albuminuria (mg/mmol)	30	18.0 ±78	2.4 (1.2-4.2)	425	0.41	1.09-1.27	30	8.8 ±27.9	1.11 (0.65-3.0)	131	0.44	-1.6-19	**0.028**[b]	**0.048**
$TmPO_4$/GFR	30	1.18 ±0.24	1.18 (0.96-1.3)	1.7	0.78	111-141	30	1.15 ±0.27	1.18 (0.91-1.4)	1.61	0.6	1.05-1.25	0.645	
eGFR (mL/min/1.73 m^2)	30	125.8 ±39.6	134 (92-153)	199	37	97-128	30	105 ±25.5	101 (89-120)	163	49	96-115	**0.02**	**0.047**
CrCl (mL/min)	30	112.5 ±40.5	112 (84-137)	229	34	97-128	30	104.9 ±43	101 (67-138)	200	42	89-121	0486	**0.048**
Urine albumin (mg/L)	30	33.9 ±131	5.6 (4.5-12)	713	2.9	-16-84	30	35.5 ±112.4	4.52 (3.4-13)	566	2.8	-6.4-78	0.126[b]	
SrCa (mmol/L)	30	2.08 ±0.15	2.1 (2-2.1)	2.4	1.7	2.03-2.14	30	2.12 ±0.18	2.1 (2-2.2)	2.5	1.6	2.05-2.19	0.406	0.835[d]
CTx (ng/mL)	30	0.68 ±0.38	0.68 (0.39-0.89)	1.61	0.036	0.54-0.82	30	0.56 ±0.35	0.49 (0.31-0.76)	1.38	0.052	0.43-0.68	0.181	**0.027**[d]
ALP (U/L)	30	112 ±27.7	114 (95-136)	159	56	102-123	30	78.7 ±25	75 (60-93)	156	45	69-88	**< 0.001**	**< 0.001**[d]
VitD (ng/mL)	25	38 ±10	40 (26-39)	50	3	34-42	24	37.6 ±14	41.5 (25-46)	64	11	32-44	0.935	0.676[d]
BMD (g/cm^2)	18	0.39 ±0.15	0.36 (0.26-0.44)	0.76	0.24	0.31-0.46	28	0.42 ±0.1	0.41 (0.35-0.48)	0.67	0.215	0.38-0.46	0.366	0.822[d]
T-score	18	1.49± 17.7	-2.2 (-6-0.2)	70.4	-6.8	-7-10	28	15.9 ±34.2	-0.45 (-0.3-4.7)	93	-7.4	2.6-29	**0.049**[b]	0.168[d]
PTH (pg/mL)	30	56.30 ±32	47 (36-79)	126	12	44-68	30	46.3 ±18	43 (34-54)	97	23	40-53	0.383[b]	**0.050**[d]

[a] *P*-value calculated from unpaired t-test. [b] *P*-value calculated from Mann-Whitney U test. [c] *P*-value calculated using ANCOVA adjusted for weight.
[d] *P*-value calculated using ANCOVA adjusted for smoking status and alcohol use

Addendum E2 — Self reported alcohol consumption and smoking status

HIV infected			HIV uninfected		
Participant's number	**Smoking status**	**Alcohol consumption**	**Participant's number**	**Smoking status**	**Alcohol consumption**
8	0	1	111	0	0
19	-	0	205	0	0
23	0	0	171	1	0
46	0	0	297	0	0
56	0	0	308	0	0
76	1	1	40	1	1
106	0	1	5	0	0
113	1	0	396	0	0
120	0	1	137	0	0
134	0	1	123	1	0
158	0	0	459	1	1
162	0	0	232	0	0
166	0	0	394	1	-
174	1	0	236	1	1
176	1	1	313	0	-
181	-	-	69	1	1
183	-	1	310	1	0
186	-	1	410	0	0
202	1	1	212	0	0
221	1	-	369	-	0
241	-	1	328	0	0
258	1	0	29	-	-
314	-	0	362	0	-
343	0	0	289	1	1
368	0	1	71	0	0
380	0	0	14	-	1
398	1	1	408	0	0
413	0	-	227	-	0
449	1	1	345	-	0
462	1	0	415	-	0

Key	
0	No
1	Yes
-	Did not respond

Addendum E3

Participants' plasma TFV concentrations

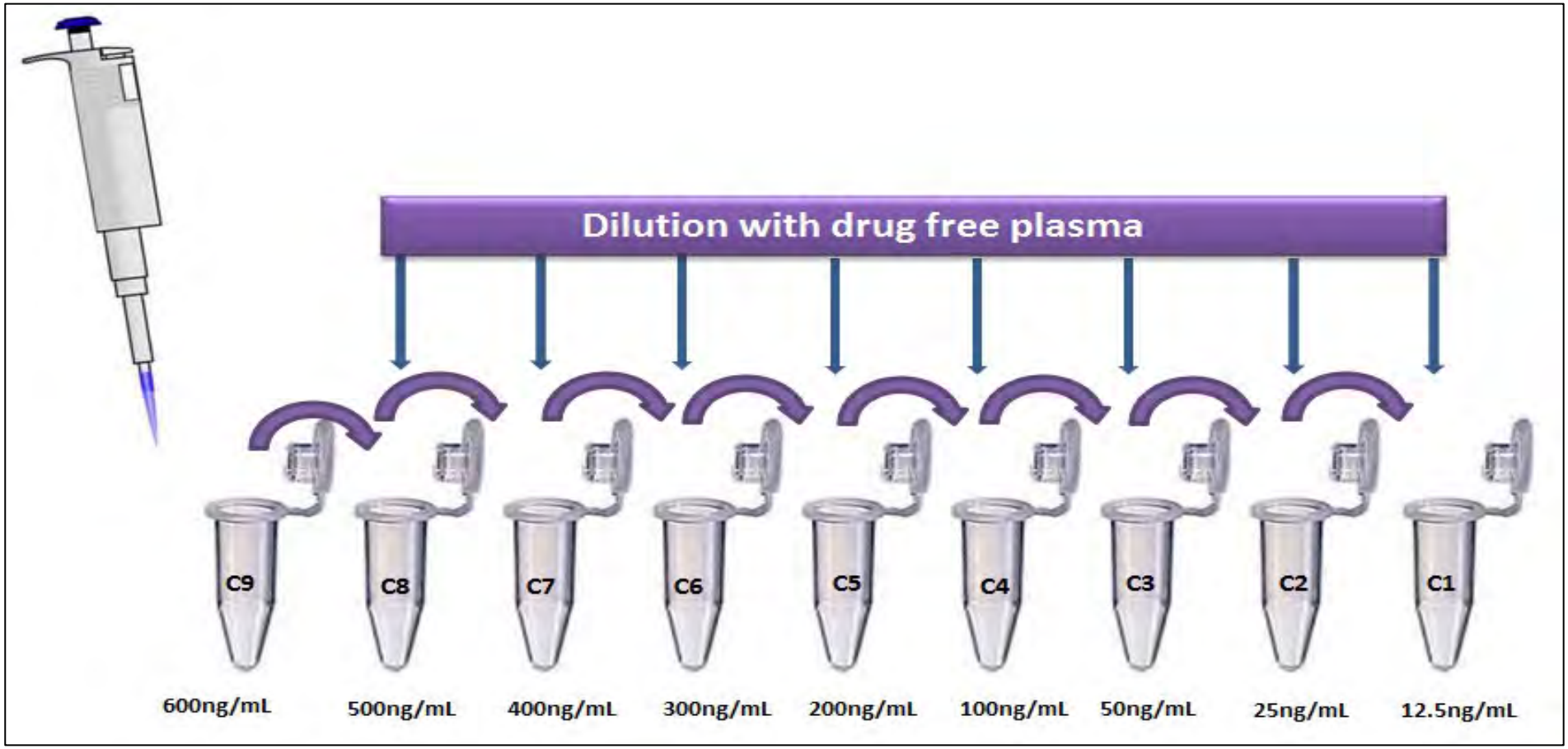
Participant's number	Plasma TFV concentrations (ng/mL)
1	150.08
2	30.82
3	124.16
4	83.56
5	-
6	177.52
7	70.80
8	158.42
9	-
10	-
11	117.00
12	138.29
13	69.93
14	122.83
15	-
16	77.81
17	17.22
18	140.55
19	112.98
20	-
21	434.21
22	92.97
23	39.50
24	128.75
25	151.61
26	124.00
27	83.56
28	82.98
29	44.20
30	77.08

-Means no TFV was detected in plasma

Descriptive statistics of TFV plasma concentrations

N	Mean (SD)	Median (IQR)	Max	Min	95%CI
25	114 ±78.9	113 (74-139)	434.2	17.2	81.2-146.6

Addendum E4	Plasma dilutions



Non-serial dilution of calibration standards.

Addendum E5 — Correlations: TDF treatment exposure and VitD status

	Variable	N	*r*	*p*-value
VitD deficiency (<30 ng/mL)	-	3	-	-
VitD sufficiency (> 30 ng/mL)	ALP:CTx	19	0.465	0.045
	SrCa:T-score	19	-0.675	0.046
	PTH:VitD	19	-0.52	0.023

TDF exposure	Variable	N	*r*	*P*-value
> 12 months	PTH:VitD	14	0.626	0.017
	CTx:SrP	16	0.562	0.024
< 12 months	CTx:BMD	4	0.999	0.001
	CTx:T-Sore	4	0.999	0.001
> 24 months	-	4	-	-
< 24 months	ALP:CTx	19	0.512	0.025

P-value and r were calculated using SPSS version 22 software