HIV-1 PROTEASE INHIBITOR DRUG RESISTANCE AND THEIR SOCIO-DEMOGRAPHIC AND CLINICAL DETERMINANTS IN ANTIRETROVIRAL TREATMENT-NAIVE AND -EXPERIENCED INJECTION DRUG USERS FROM MOMBASA COUNTY, KENYA

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other university or institution for a degree or any other award.

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DEDICATION

This thesis is dedicated to my family members especially, for the days of silence while working on this research.

ABSTRACT

The burden of Human Immunodeficiency Virus (HIV) is greatly attributed to treatment failure and high transmission rates among HIV risk groups such as injection drug users (IDUs). In Kenya and other sub-Saharan African countries, protease inhibitors (PIs) especially Ritonavir boosted Lopinavir (LPV/r) or Atazanavir (ATV/r) are main substitute drugs following first line antiretroviral treatment (ART) failure. However, the circulating protease inhibitor drug resistance mutations (PI-DRMs) are yet to be established among Kenyan IDUs who have been shown to posses high drug resistance (13.8%) to first line ART than the general population. In addition, the social demographic and clinical characteristics associated with development of PI-DRMs among Kenyan IDUs are unknown. This study aimed at determining the frequencies of HIV-1 major and minor PI-DRMs and their socio-demographic and clinical determinants among ART-naive and -experienced IDUs from Mombasa County, Kenya. Mombasa County has the highest IDU related HIV incidence in Kenya who also serve as an epidemiological link of HIV-1 infections and drug resistance to the general Kenyan population. This comparative cross-sectional study targeted HIV positive IDUs from Mombasa County (ART-naive=37; ART-experienced=55). Consenting IDUs were recruited and social-demographic information obtained using questionnaires while body mass index (BMI) determined using weight and height ratios. From the three millilitres of venous blood obtained, CD4+ T-cell counts were determined using BD FACSCalibur and an additional 50µL used to prepare dried blood spots (DBS). HIV-1 viral loads were determined from plasma obtained from centrifugation of the remnant venous blood using Abbott *m*2000. To determine PI-DRMs, HIV-1 proviral DNA was extracted from DBS and the entire protease gene amplified using gene-specific primers. The amplicons were sequenced using BigDye® chemistries and assembled sequences interpreted using Stanford University HIV drug resistance database (HIVDB). Statistical analysis based on the 76 successfully sequenced samples (ART-naive; n =31 and –experienced; n=45) was conducted using the Chi-square test for comparing proportions between the groups, and Mann-Whitney U test to establish associations between continuous variables. An overall prevalence was 5.3% following detection of three major PI-DRMs [D30N (n=1), D30N+M46I (n=2) and L90M (n=1)] in one (3.2%) ART-naive and three (6.7%) ART-experienced IDUs (p=0.459) which confer resistance to LPV/r and ATV/r. Similarly, nine (29.0%) ART-naive and eight (17.8%) ART-experienced IDUs had minor PI-DRMs (p=0.190) comprising of G48E (n=2), G48R (n=1), K20I (n=2), L10I (n=6), L10V (n=1) and T74S (n=1). Additionally, major PI-DRMs coexisted with minor PI-DRMs and were relatively more in males (75%; p=0.182) and associated with high viral loads (median=175,606, IQR, 38,803-270,810 copies/ml, p=0.04) among ART-experienced IDUs indicating that major PI-DRMs are significant contributors to treatment failure. However, no significant differences were observed in BMI, $CD4^+$ T-cell counts and viral loads among ART-naive and –experienced IDUs with minor PI-DRMs. Collectively, this findings demonstrate that both ART-naive and –experienced IDUs are likely reservoirs of PI-DRMs associated with high viral loads. There is need for PI-DRMs testing prior to regimen switching as well as re-adjust the currently used protease inhibitors in Kenya with more effective PIs.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Treatment
ARV	Antiretroviral
BMI	Body mass index
CA	HIV capsid protein
Copies/ml	Copies per millilitre
DBS	Dried blood spots
DNA	Deoxyribonucleic Acid
DRMs	Drug Resistance Mutations
env	envelope
FASTA	Fast-All sequence format
FSW	Female Sex Workers
gag	group associated antigens
HIV-1	Human Immunodeficiency Virus type 1
IDU	Injection Drug Use
IDUs	Injection Drug Users
IUPAC	International Union of Pure and Applied Chemistry

MA	HIV matrix protein
MARPs	Most-At-Risk-populations
MOH	Ministry of Health, Kenya
MoPHS	Ministry of Public Health and Sanitation
MSM	Men having Sex with Men
NACC	National AIDS Control Council
NASCOP	National AIDS and STI Control Programme
NC	HIV nucleocapsid protein
NNRTIs	Nonnucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside / Nucleotide Reverse Transcriptase Inhibitors
PCR	Polymerase chain reaction
PI-DRMs	Protease inhibitor drug resistance mutations
PIs	Protease Inhibitor(s)
pol	polymerase
RNA	Ribonucleic Acid
SU	surface proteins or gp120
TM	transmembrane proteins
U.S. FDA	United States' food and drug adminstration

UNAIDS	Joint United Nations Programme on HIV/AIDS
UNODC	United Nations Office on Drugs and Crime
WHO	World Health Organization

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CHAPTER ONE

1.0. **BACKGROUND**

HIV/AIDS is a significant cause of morbidity and mortality. Significantly, injection drug users (IDUs) have the greatest risk of HIV infection than other risk populations and act as an epidemiological link to the spread of HIV and drug resistance to other populations (UNAIDS, 2013, UNODC, 2014). Globally, 13.1% of the 12.7 million IDUs are living with HIV (UNAIDS, 2012, UNODC, 2014). It is estimated that the HIV prevalence among IDUs is 36-43% which is six times above the national prevalence and accounts for 17% of new HIV infections in Mombasa County alone (Nieburg and Carty, 2011, NASCOP and MOH, 2012). To curb HIV-1 transmission and morbidity, antiretroviral treatment (ART) has been upscaled both globally and in Kenya (Gilks *et al.*, 2006, UNAIDS, 2013). This has seen the use of nucleoside (or nucleotide) or thymidine analogues together with non-nucleosides as first line regimens in Kenya and other sub-Saharan African countries while protease inhibitors as the second line in cases of treatment failure to first line treatment (NASCOP, 2012). However, the emergence of drug resistance impedes these benefits. For instance, IDUs from Mombasa County have been shown to possess 13.8% resistance to first line ART. It therefore implies that there is need to substitute their current regimen with second line ART which is composed of PIs (Osman *et al.*, 2013). Further, studies among non-IDUs from Mombasa County revealed that 4.4% of the general population of Mombasa County have resistance to protease inhibitors implying that IDUs might have a relatively higher resistance rates to PIs (Sigaloff *et al.*, 2012). Challenges faced by IDUs such as poor health seeking behaviour among male IDUs, limited access to ART, poor ART adherence, homelessness and poor nutrition collectively augment viral resistance (Mathers *et al.*, 2010, Palepu *et al.*, 2011). Thus, timely and effective interventions for IDUs are vital in order to curtail rapid spread of HIV-1 drug

resistance. As such, targeting and screening of drug resistance mutations (DRMs) among HIV-1 infected IDUs is important before ART start or regimen switch. This will curb emergence of drug resistance.

There are six currently approved antiretroviral (ARV) drugs by the United States' food and drug administration (U.S. FDA), (Johnson *et al.*, 2013, U.S. FDA, 2014). However, only three classes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) are used in Kenya and other sub-Saharan African countries (MOH and NASCOP, 2014, WHO, 2014b). The current first line therapy in Kenya consists of two NRTIs, plus one NNRTI. Protease inhibitors (PIs), especially ritonavir boosted lopinavir (LPV/r), and atazanavir (ATV/r) are recommended as the main second line substitutes following resistance to first line ART (NASCOP, 2012, UNAIDS, 2013). However, the efficacy of these second-line regimens may be reduced among patients who developed protease inhibitor drug resistance mutations (PI-DRMs) before initiation to PIs (ART-naive) or in the course of treatment (Nijhuis *et al.*, 1999). Drug resistance mutations to PIs can be termed as major or minor. Major PI-DRMs are often associated with treatment failure to PIs (Rhee *et al.*, 2010, Wensing *et al.*, 2014). This is because, they occur near or on the active site of protease enzyme thereby reducing its susceptibility to protease inhibitors (Rhee *et al.*, 2010). They include D30N, G48V, I50V, I54L/M/V, I84A/V, L90M, M46I/L, N88S, V32I, and V82A/F/S/T which are the known seventeen major PI-DRMs that cause high resistance to either one or more PIs (Shafer and Schapiro, 2008, Wensing *et al.*, 2014). For instance, major protease inhibitor mutation D30N has been shown to cause high level resistance to nelfinavir while, M46I/L causes primary resistance to fosamprenavir (FPV), nelfinavir (NFV) atazanavir (ATV), indinavir (IDV), and lopinavir (LPV) treatment (Rhee *et al.*, 2010, Barber *et al.*, 2012). Furthermore, cross-

sectional studies among the general populations have shown that major PI-DRMs may occur in both ART-naive and –experienced individuals (Grossman *et al.*, 2004, Bakhouch *et al.*, 2009). However, the presence of major PI-DRMs in Kenya and especially among the high HIV transmission groups such as IDUs is unknown.

Minor PI-DRMs on the other hand are often considered to be of a lesser significance since they do not necessarily lead to treatment failure on their own (Scherrer *et al.*, 2012). However, they augment the replicative fitness of the virus carrying major mutations thereby increasing its overall drug resistance outcomes caused by major PI-DRMs (Nijhuis *et al.*, 1999, Chang and Torbett, 2011). In addition, the occurrence of a single major or minor mutation may lead to the development of other multiple major or minor mutations either in ART naive or ART-experienced patients. For instance, *in vitro* studies have demonstrated that resistance to indinavir (IDV) develop after acquisition of major PI-DRMs M46I, followed stepwise by minor PI-DRMs; L63P, V82T, and finally I84V (Condra *et al.*, 1995, Barber *et al.*, 2012, Santos *et al.*, 2012). Cross-sectional studies globally and in Kenya have demonstrated that HIV risk groups especially IDUs are of high risk of acquiring or developing HIV drug resistance (Nieburg and Carty, 2011, NASCOP and MOH, 2012, Osman *et al.*, 2013, UNODC, 2014). More importantly, IDUs from Mombasa County have been shown to have 13.8% resistance to first line ART which is higher (1.1%) than the general populations from the same region (Lihana *et al.*, 2009, Osman *et al.*, 2013). ART-naive IDUs may acquire PI-DRMs following infection or multiple re-infection with an already resistant strain while, ART-experienced IDUs mainly develop PI-DRMs due to poor adherence to ART or immunological failure (Wood *et al.*, 2005, Maia Teixeira *et al.*, 2006, Rhee *et al.*, 2010). Therefore, targeting and screening of PI-DRMs among HIV infected IDUs is important before enrolment to ART or regimen switch in order to curb transmission of

DRMs (Shafer *et al.*, 2007, Bertagnolio *et al.*, 2012). This information on minor PI-DRMs among Kenyan IDUs is yet to be established. This study determined minor PI-DRMs in ART-naive and –experienced IDUs from Mombasa County, Kenya.

Upon effective ART, it is expected that plasma viral load is maintained below detectable levels (Ledergerber *et al.*, 2004, Greig *et al.*, 2013). This can be reflected by a rise of $CD4^+$ T-cell count and increase in body mass index (BMI) and absence of DRMs. However, viral loads may rebound in some patients or some may never achieve a fully undetectable level. While in other patients, despite ART, $CD4^+$ T cell counts may remain below 200 cells/mm^3(Meriki *et al.*, 2014). This has been attributed to development of DRMs which is majorly contributed by non-adherence, a characteristic of the most at risk populations (MARPs) (Greig *et al.*, 2013, Meriki *et al.*, 2014). Since drug abuse has been shown to have a detrimental effect on the liver, which in turn reduces cellular immunity (CD4 T-helper cells) as well as impaired pharmacodynamics of antiretrovirals (Were *et al.*, 2014). Drug resistance mutations are therefore more likely to be present even in cases of low or high $CD4^+$ T-cell count, BMI and HIV viral load. However, the effect of PI-DRMs on $CD4^+$ T-cell count, BMI and HIV viral load among this population is unknown.

1.1. Statement of the problem

Injection drug users (IDUs) are severely burdened with HIV-1 infections and drug resistance mutations both globally and in Kenya. Of the 12.7 million people injecting drugs globally, 13.1% are living with HIV. Africa has the second highest prevalence of IDUs infected with HIV (12.1%). In Kenya, 18.3% of IDUs are living with HIV. In addition, IDUs are 22 times more at risk of acquiring and transmitting HIV infection and drug resistance mutations compared to the general population. They account for 17% of the HIV prevalence in Mombasa. Thus, immediate and effective intervention for this group to reduce HIV transmission rate is important. Protease inhibitors are the main substitutes following HIV treatment failure with first line ART. However, achieving quality care is affected by emergence of resistance. Most importantly, IDUs are faced with challenges in accessing ART as well as ART adherence which are significant contributors to the emergence of drug resistance. Major PI-DRMs in a viral strain significantly reduces its susceptibility to protease inhibitor drugs leading to treatment failure. The presence of these mutations in Kenyan IDUs as well as other HIV risk groups is yet to be established. The HIV replication and disease progression are likely to accelerate if patients are given one or more regimens that they are already resistant to. Minor PI-DRMs on the other hand up-regulate the replicative fitness of the virus carrying major mutations thereby increasing its overall drug resistance outcomes. Their presence or absence is also largely unknown among Kenyan IDUs. Similarly, the co-occurrence of specific minor and major PI-DRMs has not been established. Cross-sectional studies have shown a 14% drug resistance to first line ART (NRTIs and NNRTIs) among IDUs in Mombasa County. This therefore necessitates switch to second line therapy that entails PIs. However, there is no information on whether mutations related to PIs already exists in this population or not. In HIV-1 infected patients, the baseline characteristics,

demographic and anthropometric measures, BMI, $CD4^+$ T-cell count and HIV-1 viral load are important measures for defining the acquisition and development of drug resistance mutations. Their association with drug resistance mutations in IDUs remains undefined. In addition, the impact of drug injection on these underlying factors and its burden in occurrence of PI-DRMs among IDUs is unknown.

1.2. Significance of the study

Mombasa hosts the largest number of IDUs in Kenya who contribute to 17% of the HIV-1 infections in the region (Beckerleg *et al.*, 2005, NASCOP, 2014). Targeting IDUs for drug resistance testing was vital in order to reduce morbidity and mortality. This is because PIs in Kenya are the main resort in case of treatment failure to first line antiretroviral drugs (mainly NRTIs and NNRTIs). In areas like Mombasa where high prevalence of primary resistance to first line regimens had been documented among IDUs, regimen switch to PIs is recommended (NASCOP, 2012, Osman *et al.*, 2013). Therefore, continuous monitoring of PI-DRMs before enrolment to treatment (ART-naive) as well as those continuing with treatment (ART-experienced) is of benefit to reduce spread of drug resistance to other IDUs and to the general population. Although, a majority of the Kenyan HIV infected population are on first line ART (NASCOP, 2014), the large numbers of holiday makers and tourists visiting Mombasa and the high HIV-1 risk behaviours among IDUs places them at an increased risk of acquiring PI-DRMs (Deveau *et al.*, 2006, Brodish *et al.*, 2011). Therefore, by determining major PI-DRMs, critical information on effective and non-effective PIs in IDUs harbouring these mutations was generated indicating that IDUs from Mombasa County carried resistance mutations to LPv/r and ATV/r which are the currently recommended PIs. These findings also provide measures on how to prevent the development and further spread of major PI-DRMs. Similarly, data concerning minor PI-DRMs provide useful information

on their contribution to the emergence and virulence of major PI-DRMs. In addition, the association between major and minor PI-DRMs with demographic characteristics, CD4+ T cell count, BMI and viral load provides important information on HIV-1 interventions, care and treatment approaches targeting IDUs. Finally, the general findings of this study reveal 5.3% prevalence of major PI-DRMs which is an important insight in the molecular epidemiology of PI-DRMs aimed at guiding policy makers and health service providers on measures to reduce the spread of drug resistance through effective therapy choice and HIV-1 disease monitoring.

1.3. Objective of the Study

1.3.1. Main objective

To investigate HIV-1 protease inhibitor drug resistance mutations (PI-DRMs) and their socio-demographic and clinical determinants in ART-naive and -experienced injection drug users from Mombasa County, Kenya.

1.3.2. Specific objectives

1. To determine the frequencies of major HIV-1 PI-DRMs in ART-naive and -experienced IDUs from Mombasa County, Kenya.
2. To determine the frequencies of minor HIV-1 PI-DRMs in ART-naive and -experienced IDUs from Mombasa County, Kenya.
3. To determine the associations between major and minor HIV-1 PI-DRMs and the socio-demographic characteristics, CD4+ T cell count, viral load and BMI in ART-naive and -experienced IDUs from Mombasa County, Kenya.

1.3.3. Research questions

1. What are the frequencies of major HIV-1 PI-DRMs in ART-naive and -experienced IDUs from Mombasa County, Kenya?
2. What are the frequencies of minor HIV-1 PI-DRMs in ART-naive and ART-experienced IDUs from Mombasa County, Kenya?
3. What are the associations between major and minor HIV-1 PI-DRMs and the Socio-demographic characteristics, CD4+ T cell count, viral load and BMI in ART-naive and -experienced IDUs from Mombasa County, Kenya?

CHAPTER TWO

2.0. **LITERATURE REVIEW**

2.1. Epidemiology of HIV-1 and injection drug use

HIV and AIDS still stands out as a significant cause of morbidity and mortality globally and in sub-Saharan Africa despite various efforts to curb its transmission. A significant contributor in the transmission of HIV being the most-at-risk population (MARPs), consisting of injection drug users (IDUs) that are often characterized by a high HIV prevalence and transmission rate (UNAIDS, 2013). United Nations office on drugs and crime (UNODC) estimated that 1.6 million IDUs globally and 0.12 million in Africa (11.8%) are living with HIV (UNODC, 2013). In Kenya, Mombasa County has the highest percentage of IDUs who accounted for 6.1% of the new HIV-1 infections in the region (Beckerleg *et al.*, 2005, NACC, 2009). Since injection drug use is a predictor of other HIV risk behaviours, it is estimated that 17% of the HIV-1 infections at the Kenyan Coast are linked to drug injection (NACC, 2009, Musyoki, 2012). Among the other HIV risk behaviours by IDUs, studies in Mombasa and in other places have attributed the greatest risk to "blood flushing" and frequent sharing of unsterile needles or syringes (Musyoki, 2012, NASCOP and MOH, 2012). Since most ART care programs in developing countries such as Kenya encounter challenges targeting IDUs, acquisition of a single drug resistant strain by IDUs may amount to a factorial of drug resistance mutations which can be transmitted later to the general population. Thus, IDUs are a significant group that define HIV drug resistance, transmission and epidemiology in Kenya. However, HIV-1 disease burden in terms of presence of PI-DRMs and disease severity (based on $CD4^+$ T cell counts, BMI and viral load) in IDUs remain unknown. This study therefore sought to investigate the frequencies of HIV-1 major and minor protease inhibitor drug

resistance mutations (PI-DRMs) and their association with socio-demographic and clinical determinants ($CD4^+$ T cell counts, BMI and Viral load) among ART-naive and -experienced injection drug users from Mombasa County, Kenya.

2.2. HIV-1 genome, replication, antiretroviral drugs and protease drug resistance mutations

2.2.1. HIV-1 genome

The HIV-1 genome encodes nine open reading frames of 9749 nucleotides consisting of 15 proteins (**Figure 2.1**) and one RNA strand (Frankel and Young, 1998, Freed, 2001). Among them, are the three major proteins; *gag*-group associated antigens, *pol*-polymerase, and *env*-envelope that are subsequently cleaved into individual polyproteins (Watts *et al.*, 2009). The other six (*vif*-virion infectivity factor, *vpr*- viral protein R, *nef*- negative regulatory factor, *tat*-trans-activator of transcription, *rev*- regulator of virion protein expression, and *vpu*-viral protein U) are accessory proteins (Watts *et al.*, 2009, Sundquist and Krausslich, 2012). Further, *vif, vpr,* and *nef* are incorporated in the virus during replication. The *tat, rev* and *vpu* regulate and assist in viral assembly (Sundquist and Krausslich, 2012). The *gag* is cleaved into four polyproteins; matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins. While the *env* is cleaved into two proteins (**Figure 2.1**); SU (surface or gp120) and TM (transmembrane or gp41), these consist the structural components that make up the core of the virion and outer membrane envelope (Sundquist and Krausslich, 2012). The *pol* (polymerase) gene is the most significant gene of HIV-1 in terms of viral infectivity and antiretroviral drug design. It is cleaved into three enzymes; *protease, reverse transcriptase* and *integrase* (**Figure 2.1**), that participate in viral integration and replication

(Hill *et al.*, 2005, Watts *et al.*, 2009). The *protease* (PR) gene cleaves the non-infectious *pol* and *gag*-encoded polyproteins resulting into a mature infectious virus, while, the *reverse transcriptase* (RT) gene transcribes RNA genome into double stranded DNA (Frankel and Young, 1998, Freed, 2001). The *integrase* (INT) gene on the other hand integrates the double stranded DNA into the hosts' cell chromosome (Hill *et al.*, 2005, Sundquist and Krausslich, 2012).

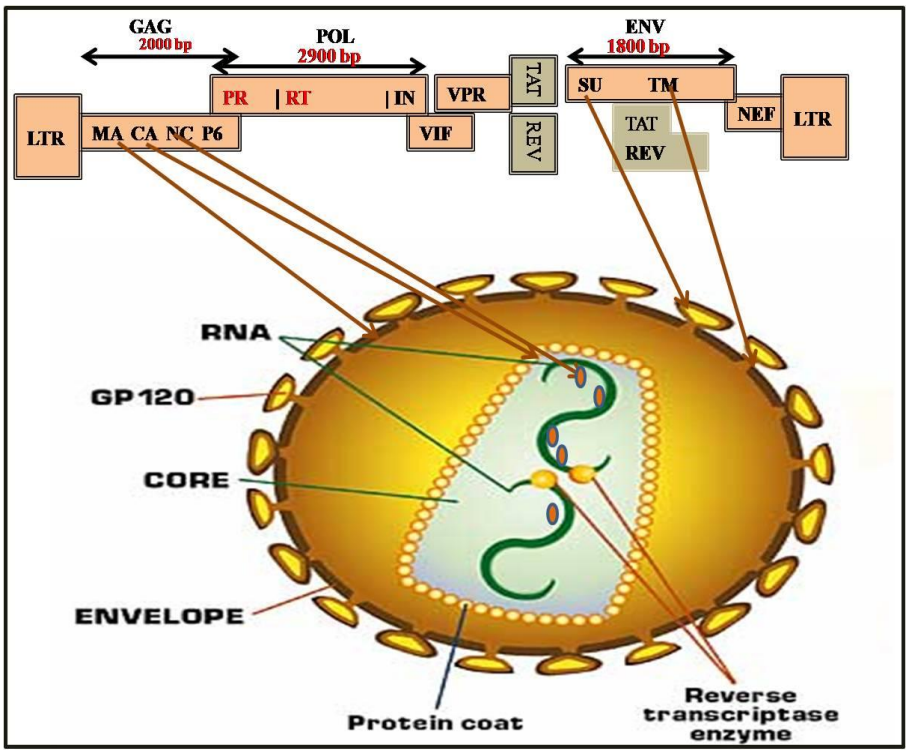

Figure 2.1 HIV-1 genome and structure

Figure legend. Figure 2.1 show the HIV genome and structure with the 15 key proteins at their respective location on the viral particle. Abbreviations used include- gag, group associated antigens; *pol*, polymerase; *env*, envelope; *vif*, virion infectivity factor; *vpr*, viral protein *R*; *nef*, negative regulatory factor, *tat:* trans-activator of transcription, rev: regulator of virion protein expression and *vpu:* viral protein U. MA matrix, CA: capsid, NC: nucleocapsid, SU: surface or gp120 and TM: transmembrane or gp41. Adopted from (Frankel and Young, 1998) with modifications to show corresponding genome structure.

2.2.2. HIV-1 replication and antiretroviral drug targeting

HIV enters host cells through interaction of an envelope glycoprotein (gp120) and the cellular receptor (**Figure 2.2.**), CD4, and other co-receptors such as CCR5 and CXCR4 expressed on T-helper lymphocytes (CD4 lymphocytes), macrophages and dendritic cells, (Bleul *et al.*, 1997, Freed, 2001). This is followed by reverse transcription of the viral RNA into pro-viral DNA mediated by the enzyme *reverse transcriptase* (Freed, 2001, Munir *et al.*, 2013). The *reverse transcriptase* enzyme has no proof-reading function, and therefore the process is error prone and responsible for rapid development of HIV-1 drug resistant mutations (Abram *et al.*, 2014). The pro-viral DNA is then inserted into the host cells' chromosomal DNA by the viral *integrase* enzyme where, the host cell enzymes transcribe the pro-viral DNA into an infectious viral particle (Munir *et al.*, 2013). The viral RNA uses host cell energy and synthetic pathways to make viral proteins. Lastly, viral proteins and RNA aggregate on the cell surface for assembly into a mature viral particle by budding through host cell membrane. More importantly, the *protease* enzyme is responsible for this maturation. (Freed, 2001).

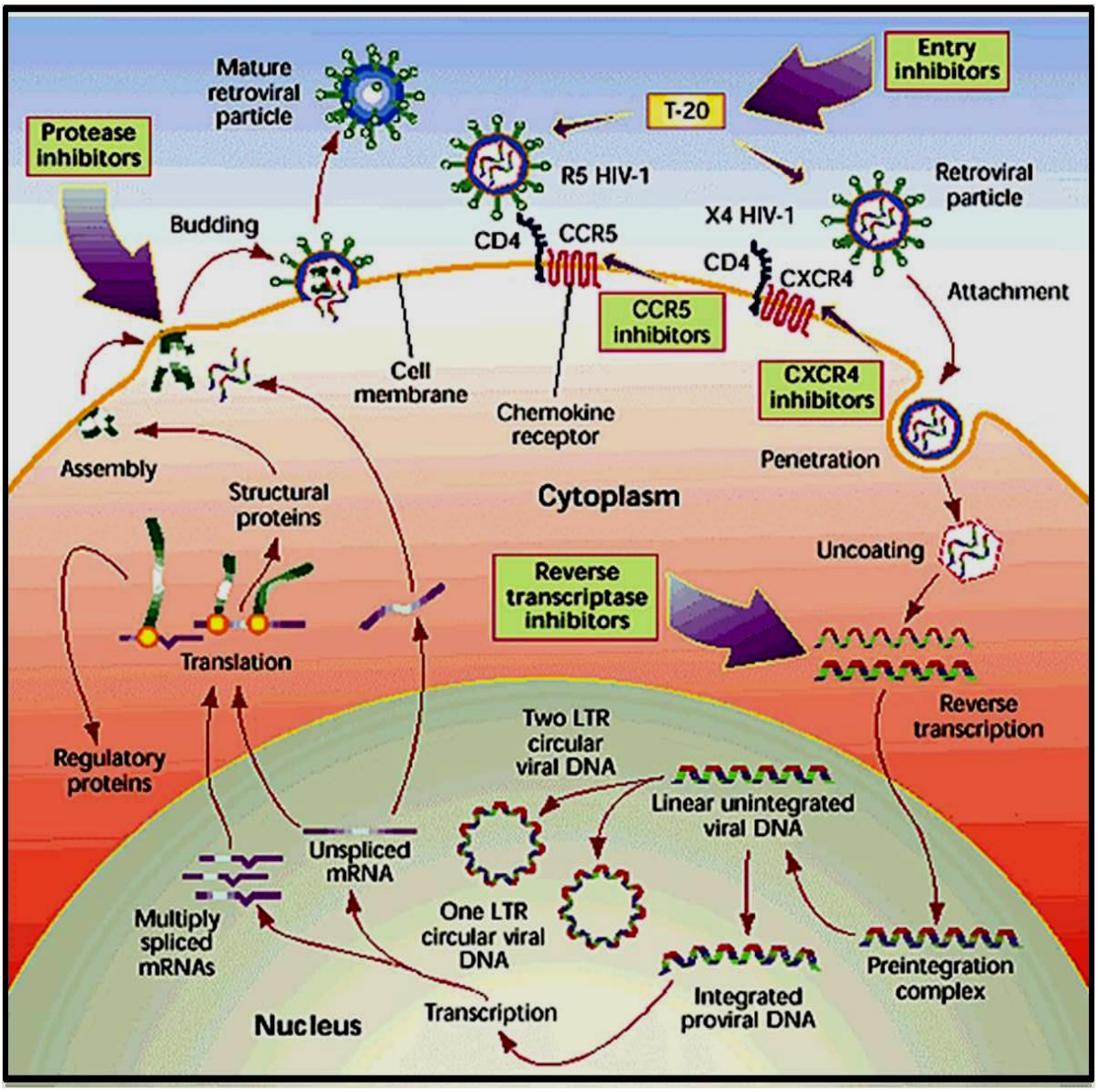

Figure 2.2. HIV-1 replication Cycle and Antiretroviral drug targets

Figure legend. Figure 2.2. show the HIV-1 lifecycle and antiretroviral drug targets. Different antiretroviral drugs are directed against cell penetration and receptor binding (fusion and entry inhibitors), integration of pro-viral DNA into the host cell (integrase inhibitors), reverse transcription (reverse transcriptase inhibitors) or viral assembly (protease inhibitors) (Michael and Moore, 1999).

2.2.3. Antiretroviral treatment and drug resistance

Effective antiretroviral treatment (ART) is pertinent in reduction of HIV-1 disease severity and transmission. However, drug resistance remains a crucial issue since significant proportions of individuals are infected with HIV-1 strains that are already resistant to one or more ARVs (NASCOP, 2012, Johnson *et al.*, 2013). According to the United States food and drugs administration (U.S FDA), there are six main classes of antiretroviral drugs (**Table 2.1)** consisting of; nine nucleoside reverse transcriptase inhibitors (NRTIs), nine protease inhibitors (PIs), four nonnucleoside reverse transcriptase inhibitors (NNRTIs), one fusion inhibitor and one C-C chemokine receptor type 5 (CCR5) inhibitor (U.S. FDA, 2014). Each of these antiretroviral drug bind to respective HIV-1 genes thereby interfering with viral replication and integration into the host cell by inhibiting related replication processes (**Figure 2.2**), (Nielsen *et al.*, 2005). Among the six approved classes, only three classes (NRTIs, NNRTIs and PIs) are widely used in sub-Saharan Africa and Kenya. NRTIs and NNRTIs are often prescribed as first line regimen while PIs substitutions are the only alternatives after failure to first line regimens or intolerable drug effects (NASCOP, 2012). Duration and adherence in the use of antiretrovirals among ART-experienced individuals has been associated with development of drug resistance (Nijhuis *et al.*, 1999, Eshleman *et al.*, 2004, Kowalski *et al.*, 2009). In addition, IDUs have been shown to possess a threefold more PI-DRMs than non-injection drug users thus, they may be transmitting these resistance to ART-naive counterparts (Kowalski *et al.*, 2009). Although high resistance (13.8%) to first line ART has been documented among IDUs from Mombasa County (Osman *et al.*, 2013), neither is drug resistance testing done before regimen switch to second line ART nor, data on PI-DRMs among Kenyan IDUs available. This study therefore determined the association of

major and minor PI-DRMs with exposure and duration of ART in ART-naive and –experienced IDUs from Mombasa, Kenya.

Table 2.2.1: HIV-1 antiretroviral drugs currently approved by United States Food and Drug Administration (U.S FDA)

DRUG CLASS	DRUGS			MODE OF ACTION
	Abbreviation	Generic name	Brand name	
Nucleoside reverse transcriptase inhibitors (NRTIs)	**3TC**	**Lamivudine**	***Epivir***	Terminate DNA chain formation as the enzyme reverse transcriptase copies viral RNA into DNA
	ABC	Abacavir	*Ziagen*	
	AZT/ ZDV	**Zidovudine**	***Retrovir***	
	d4T	Stavudine	*Zerit*	
	ddI	Didanosine	*Videx EC*	
	FTC	Emtritabine	*Emtriva*	
	TDF	**Tenofovir**	***Viread***	
Nonnucleoside reverse transcriptase inhibitors (NNRTIs)	DLV	Delavirdine	*Rescriptor*	Bind to reverse transcriptase enzyme and inhibit transcription of viral RNA to viral DNA
	EFV	**Efavirenz**	***Sustiva/ tocrin***	
	ETR	Etravirine	*Intelence*	
	NVP	**Nevirapine**	***Viramune***	
	-	Rilpivirine	*Edurant*	
Protease inhibitors (PIs)	APV (no longer marketed)	Amprenavir	*Angenerase*	Bind to the viral protease enzyme and block the formation of viral proteins
	FOS-APV	Fosamprenavir	*Lexiva /Telzir*	
	ATV*	Atazanavir	*Reytaz*	
	DRV*	Darunavir	*Prezista*	
	IDV	Indinavir	*Crixivan*	
	LPV/RTV	**lopinariv and ritonavir**	***kaletra/Aluvia***	
	NFV	Nelfinavir	*Viracept*	
	RTV	Ritonavir	*Norvir*	
	SQV	Saquinavir	*Invirase*	
	TPV	Tipranavir	*Aptivus*	
Integrase strand transfer inhibitors (INSTI)	RAL	Raltegravir	*Isentress*	Prevents integration of pro-viral DNA into the host cells' DNA
	DTG	Dolutegravir	*Tivicay*	
Fusion inhibitors	T-20	Enfuvirtide	*Fuzeon*	Prevent entry of the virus into the host cell i.e. CD4
Entry inhibitors/ CCR5 co-receptor antagonist	MVC	Maraviroc	*Celsentri or Selzentry (US)*	Bind to CCR5 receptor and prevents the fusion process involving gp41 from proceeding.

Table legend. Table 2.1 show the clinically-approved HIV-1 antiretrovirals including their mode of action (U.S. FDA, 2014). Drugs in bold are the antiretrovirals currently used in Kenya. Those with asterisk (*) are administered as ritonavir boosted combination (MOH and NASCOP, 2014).

2.2.4. Protease inhibitors drug resistance mutations

HIV has been shown to develop many more mutations to protease inhibitors than any other antiretroviral drugs though with different levels of resistance (Nijhuis *et al.*, 1999, Rhee *et al.*, 2010, Chang and Torbett, 2011). Based on the HIV-1 drug resistance nomenclature by International AIDS Society (IAS-USA), PI mutations are denoted as: the wild type amino acid name, position at which the mutation is occurring on protease gene, followed by the amino acid substitution conferring resistance (Johnson *et al.*, 2013, Wensing *et al.*, 2014). For instance, mutation M46L denotes, the wild type amino acid M (methionine) existing at amino acid position 46 on the protease gene has been substituted with a mutant amino acid L (leucine) in a resistant virus strain (Wensing *et al.*, 2014). These mutations are then classified either as major, minor or accessory based on their interaction with drugs (Rhee *et al.*, 2010, Wensing *et al.*, 2014). Major mutations occur at residues that primarily bind to protease inhibitors (Rhee *et al.*, 2003, Wensing *et al.*, 2014). These mutations may occur at positions 30, 32, 46, 47, 48, 50, 54, 58, 76, 82, 83, 84, 88 and 90 depending on the type of PI regimen. Examples include mutations D30N, G48V, I50V, I54L/M/V, I84A/V, L90M, M46I/L, N88S, V32I, and V82A/F/S/T which are associated with a major decrease in sensitivity to one or more protease inhibitors (Johnson *et al.*, 2013, Wensing *et al.*, 2014).

Minor mutations on the other hand, independently do not have large effects on drug resistance (Scherrer *et al.*, 2012, Wensing *et al.*, 2014). They have been shown to occur at 32 different amino acid loci on the protease gene (Chang and Torbett, 2011). However, presence of multiple minor mutations may up regulate the replicative fitness of the virus carrying major protease mutations and lead to multi-drug protease resistance (Johnson *et al.*, 2013, Rabi *et al.*, 2013). Accessory mutations occur as natural polymorphisms that have not been

shown to have any significant effect to the available PIs and thus considered of less significance (Scherrer *et al.*, 2012).

The presence of drug-resistant virus before starting a new drug regimen is an independent predictor of low virologic response the antiretroviral (DeGruttola *et al.*, 2000, Hanna and D'Aquila, 2001, Haubrich and Demeter, 2001). Thus, surveillance of antiretroviral drug resistance helps map regions with high prevalence of primary resistance. To do this, the resistance testing of ART-naive patients is used to determine resistance to the initial first-line drugs. In sub-Saharan Africa, this is rarely done due to limited resources. Studies have shown that for patients with access to drug resistance data, response to therapy is better (Baxter *et al.*, 2000, EuroGuidelines, 2001, Cohen *et al.*, 2002). Resistance testing is therefore highly recommended as part of treatment of HIV-1-infected patients to guide on the most effective therapy throughout the treatment course (Pillay *et al.*, 2008, Dunn *et al.*, 2011, Parkin *et al.*, 2012). Most HIV-1 drug resistance and genotyping systems target the HIV-1 *pol* gene (Zhou *et al.*, 2011). The use of these genotyping systems facilitates the study of the relationship between mutations and viral resistance to antiretroviral drugs, specifically the protease and reverse transcriptase inhibitors (Hirsch and Richman, 2000, Shafer *et al.*, 2001). Currently, data on resistance to protease inhibitors is yet to be established among injection drug users (IDUs) from Kenya. This study therefore determined both major and minor PI-DRMs in ART-naive and –experienced IDUs from Mombasa County, Kenya.

2.3. Clinical determinants of HIV drug resistance and disease progression

The patient's $CD4^+$ T cell count, Viral RNA copies and BMI are the main laboratory indicators of HIV-1 disease progression to full-blown AIDS (de Wolf *et al.*, 1997, Salomon

et al., 2002). Higher viral loads, low BMI and $CD4^+$ T cell counts are significant indicators of HIV related mortality (Van der Sande *et al.*, 2004). Among HIV infected patients, the interplay of these significant indicators is as described below.

2.3.1. Association of $CD4^+$ T cell count with major and minor PI-DRMs

On average, people with untreated HIV-1 infection show a marked $CD4^+$ T cell count decline of approximately 50-80 cells/µL per year. A persistent $CD4^+$ T cell percentage of less than 14% or an absolute $CD4^+$ T cell count of less than 200 cells/µL is considered as immunosuppression (immunological failure) (Ledergerber *et al.*, 2004). Effective ART is expected to amount to a $CD4^+$ T cell count increase of greater than 50 cells/µL within weeks after viral suppression and increases of 50-100 cells/µL per year thereafter (Sethi *et al.*, 2003, Achhra *et al.*, 2014). However, other factors such as nutrition, drug interactions and co-infections as seen in IDUs may prevent the effectiveness of ART (Addy *et al.*, 2003, Bertagnolio *et al.*, 2012). It is therefore expected that the $CD4^+$ T cell counts in ART-naive and –experienced IDUs are expected to be significantly different within the groups (ART-naive and –experienced) as well as those presenting with major or minor PI-DRMs. Since this vital data on association of $CD4^+$ T cell count with major and minor PI-DRMs in ART-naive and –experienced IDUs in Kenya is unknown, this study went further to establish the association between $CD4^+$ T cell count and major and minor PI-DRMs in ART-naive and –experienced IDUs from Mombasa County, Kenya.

2.3.2. Association of Basal Metabolic Index with major and minor PI-DRMs

In HIV infection, weight loss and wasting as indicated by low basal metabolic index (BMI) are frequent symptoms (Salomon *et al.*, 2002). Weight loss of greater than 10% of body weight due to HIV infection itself is a common condition in HIV infection (Salomon *et al.*,

2002, Van der Sande *et al.*, 2004). This is used as a diagnostic criterion in the classification of HIV disease (Suttmann *et al.*, 1995, NASCOP, 2012). However, weight loss can also be due to a range of underlying conditions such as malnutrition (Salomon *et al.*, 2002). Since many IDUs in developing countries are poor and live in hideouts, they are often overwhelmed with malnutrition and poor health conditions (Beckerleg *et al.*, 2005). Thus, their BMI levels are expected to be different compared with the general population (MOH and NASCOP, 2012). As such, drug users may need to be investigated exclusively. However, this information on BMI in ART-naive and experienced Kenyan IDUs is unestablished. To some extent, there is need to set new cut-offs for body wasting due to HIV infection and clearly differentiated from wasting due to other underlying causes (Achhra *et al.*, 2014). As such, the current study determined the association between BMI and major and minor PI-DRMs in ART-naive and –experienced IDUs from Mombasa County, Kenya.

2.3.3. Association between HIV-1 viral load and major and minor PI-DRMs

Viral load testing is essential for detecting virologic failure (repeated HIV RNA levels greater than 1000 copies per ml of blood) in a timely manner (Moore *et al.*, 2006). After initial infection with HIV, the viral load quickly peaks to very high levels, usually greater than 100,000 copies per millilitre of blood (copies/ml) which then declines and remain in a steady state in 3 to 6 months after primary infection (Kelley *et al.*, 2007, Greig *et al.*, 2013). Higher plasma viral loads are associated with a higher risk of HIV transmission as well as increased risk of progression to symptomatic and full blow Acquired Immunodeficiency syndrome (AIDS) disease (Abu-Raddad *et al.*, 2013). Though antiretroviral therapy is aimed at achieving undetectable viral load (less than 40-150 copies/ml), studies have also reported virologic failure (high viral loads) among HIV patients on ART (Hassan *et al.*, 2014, Meriki *et al.*, 2014). This is majorly attributed to antiretroviral drug resistance, poor adherence to

ART, or reduced drug exposure (Greig *et al.*, 2013). Furthermore, IDUs inject hard drugs such as heroin and cocaine that trigger metabolic derangements in the liver that may in turn interfere with pharmacodynamics of antiretrovirals (Kerr *et al.*, 2004, Ross *et al.*, 2008, Were *et al.*, 2014). Thus, even with full adherence to ART, emergence of drug resistance and virologic failure is expected among IDUs. Kenyan IDUs from Mombasa County presenting with high level resistance to first line ART (reverse transcriptase inhibitors) were found to have high viral loads by various cross-sectional studies in the area (Steegen *et al.*, 2009, Sigaloff *et al.*, 2012, Osman *et al.*, 2013). However, similar data defining HIV viral load among IDUs presenting with major and minor PI-DRMs is currently not available. As such, the current study determined the association between major and minor PI-DRMs and HIV-1 viral load in ART-naive and –experienced IDUs from Mombasa County, Kenya.

2.3.4. Association between antiretroviral treatment and major and minor PI-DRMs among IDUs

The current 90-90-90 HIV treatment and care targets by joint United Nations programme on HIV/AIDS (UNAIDS) has seen the up scaling of ART in Kenya and in most parts of sub-Saharan Africa (UNAIDS, 2014). However, as more people are enrolled on ARVs, emergence and transmission of HIV-1drug resistance may eventually hamper the effectiveness of the current therapeutic regimens (Moore *et al.*, 2006, Bertagnolio *et al.*, 2012). Adherence to ART is a predictor of viral suppression (undetectable viral load) and hence prevention of the development of drug resistance. However, many IDUs stay in hideouts with limited access to health facilities as such, interruptions and poor adherence to ART is common (Beckerleg *et al.*, 2005, Mathers *et al.*, 2010, Palepu *et al.*, 2011). This interrupted ART consequently allows the virus to improve its replicative fitness due to low drug dosage leading to a likelihood of development of acquired DRMs among the IDUs (van

Maarseveen *et al.*, 2006). In addition, IDUs may have multiple re-infections due to their HIV risk behaviours (Beckerleg *et al.*, 2005, Brodish *et al.*, 2011). This may in turn lead to development and transmission of drug resistant strains in case the patient was on ART (Nijhuis *et al.*, 1999, van Maarseveen *et al.*, 2006). An early indicator of drug resistance can be viral load rebound after becoming undetectable. Since there is limited access to viral load measurement in sub-Saharan Africa and more especially in IDUs, such viral load relapses will always go unnoticed and lead to more pronounced DRMs. Studies have shown that, the changes in BMI and $CD4^+$ T-cell count upon initiation of ART predicts the trends in viral replication pattern and hence the treatment outcomes (Moore *et al.*, 2006, Greig *et al.*, 2013). However, the combinatory effect of BMI, $CD4^+$ T cell count and viral load in determination of the types of PI-DRMs remains inconclusive. Since injection drug users have been characterised with malnutrition (low BMI), and impaired immunity (Were *et al.*, 2014), the sum effect of the two poses a good ground for HIV-1 replication fitness and virulence leading to virological failure (high viral loads) (Bertagnolio *et al.*, 2012, Meriki *et al.*, 2014). However, the effect of drug pressure (abused substances) on disease progression and development of PI-DRMs remains unestablished. As such, the current study established the association between major and minor HIV-1 PI-DRMs and the socio-demographic characteristics, $CD4^+$ T cell count, viral load and BMI in ART-naive and -experienced IDUs from Mombasa County, Kenya.

CHAPTER THREE

3.0. **MATERIALS AND METHODS**

3.1. Study site

The study was conducted in Mombasa County and enrolment done at Bomu Hospital. Mombasa is Kenya's second largest city located on the Eastern coastline of Kenya (latitude, longitude; 4° 2' S, 39° 40' E) bordering the Indian Ocean (Figure 3.1), (World Atlas, 2015). As an island town and main East Africa's entry and exit port, Mombasa town attracts many tourists and has also been categorised among the world major drug trafficking routes by United Nations Office for Drugs and Crimes (UNODC, 2013). Heroine has been reported to be the most injected drug among IDUs in this region (Osman *et al.*, 2013, Were *et al.*, 2014). The study participants from different parts of Mombasa County were recruited at Bomu Hospital. Bomu is a private health facility located in Changamwe, 3 kilometres from Mombasa International Airport and 8 kilometres from Mombasa town. The hospital receives an average of 250 outpatients per day and is one of the sites in the region which offers ART. It is strategically placed to enable most-at-risk populations to access antiretrovirals (NACC and Office of the President, 2009). Bomu hospital offers ART care services to HIV patients, rehabilitation services to drug users and other youth friendly services among others. This makes the hospital accessible by many patients especially drug users and HIV positive patients from various parts of Mombasa County.

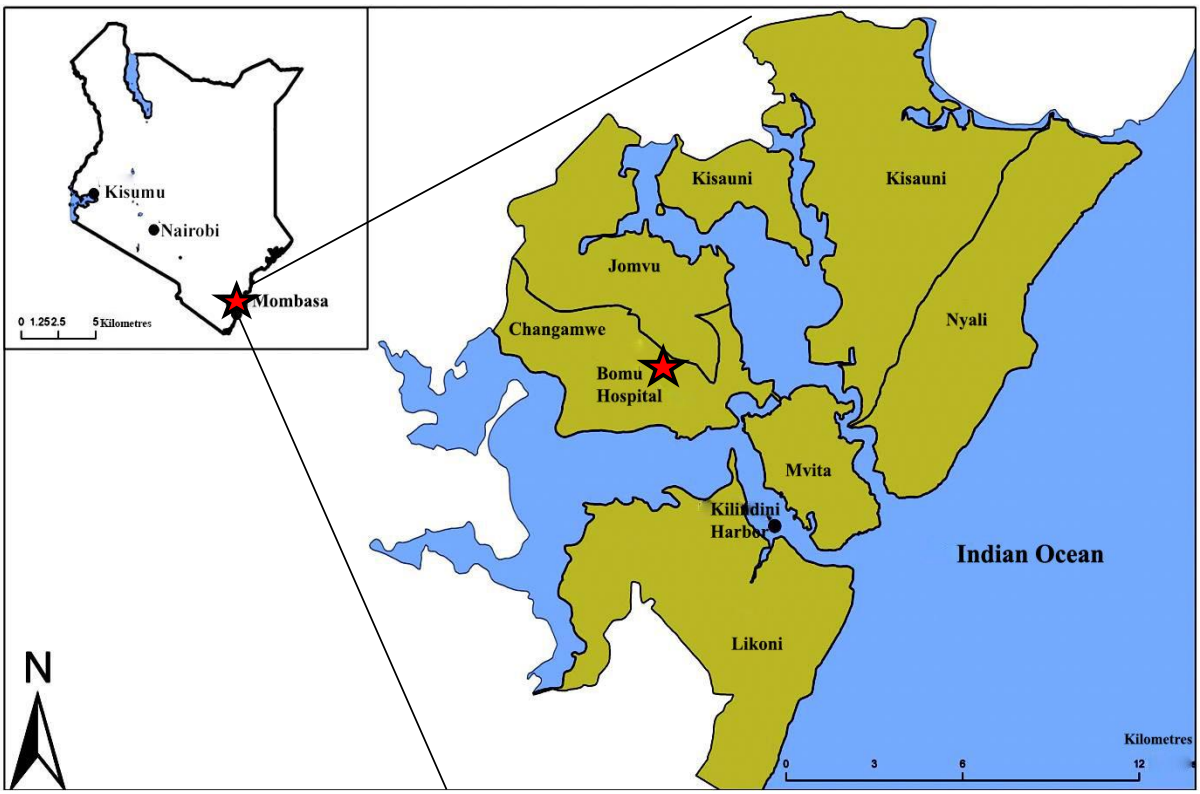

Figure 3.1: Study area -Mombasa County, Kenya

Figure Legend. Map of Kenya showing Mombasa County, and the recruitment site at Bomu Hospital in Changamwe, Mombasa Kenya, modified from the World atlas (World Atlas, 2015).

3.2. Eligibility criteria

Injection drug users (IDUs) were defined as people with a history of injecting illicit drugs and substances. Criteria such as physical examination for needle stick scars and re-confirming the route of drug administration during previous one month were used to confirm drug injection and minimized misclassification.

3.2.1. Inclusion criteria

This study recruited HIV positive injection drug users only. These were adults (over the age of 18 years) of Kenyan citizenship or residents of Mombasa County (foreigners reporting to

have stayed in Mombasa County for at least 6 months during the sampling period). They were also required to be able to provide a written informed consent.

3.2.2. Exclusion criteria

Non-injection drug users and HIV negative drug users were not included in this study. In addition, neither individuals without any physical signs of needle stick scars nor unable to re-confirm their route of drug administration during previous one month excluded from the study.

3.3. Study design, population and recruitment of participants

This was a comparative cross-sectional study that targeted ART-naive and -experienced HIV-1 positive IDUs from Mombasa County, Kenya. ART-experienced IDUs were those IDUs who reported to use antiretroviral drugs before enrolment to the study while, ART-naive participants were those who had never used ART in their lifetime. Recruitment of participants was via snowball and respondent driven methods. Here, a small but diverse group of drug users (heterogeneous in age, gender and geographic location) was selected to initiate the process. After providing informed consent (**Appendix 1**), the study participants were then interviewed with the aid of a questionnaire (**Appendix 2**), educated on how to refer other eligible IDUs without necessarily coaxing them to participate. In addition, they were given three uniquely coded coupons to refer their peers. On each coupon, the title, site, and a brief explanation of the study were printed. This process of continuous interview and training of the newly recruited consenting peers was undertaken for a period of six months.

3.4. Sample size estimation

Sample size was calculated based on methods for estimating sample size for comparative cross-sectional studies by Israel (Cochran, 1977, Israel, 1992).

Total sample size $\mathbf{n}_0$= {($n_1 = \frac{z^2pq}{e^2}$) + ($n_2 = \frac{z^2pq}{e^2}$)}

Where:

n_1 and n_2= required sample size from each arm

Z (Z-score) = at 95% confidence level, Z score = 1.96

P= prevalence among the target population.

q = (1- *p);*

e = allowable margin of error. 10% (***e***= ±0.1) was used in this study;

Thus;

Since previous studies in Mombasa County have shown that the prevalence of drug resistance to first line among ART- experienced IDUs is 13.8% (Osman *et al.*, 2013), while among ART-naive is 7.4% (Sigaloff *et al.*, 2012). *P* =0.138 for ART-experienced and *P*=0.074 for ART-naive was used in sample size calculation.

For ART-naive ($\mathbf{n_1}$); since ***P*** = 0.074; ***q*** = (1-0.074) = 0.926

For ART-experienced ($\mathbf{n_2}$); since ***P*** = 0.138; ***q*** = (1-0.138) = 0.862

Therefore:

ART-naive ($\mathbf{n_1}$) = $\{(1.96)^2 (0.074 \times 0.926)/(0.1)^2\}$ = 36+10% for non-responders =40

ART-experienced (n_2) = $\{(1.96)^2 (0.138 \times 0.862)/ (0.1)^2\}$ = 46 +10% for non-responders =51

Therefore, at least 40 ART-naive and 51 ART-experienced IDUs were required. The study successfully recruited, 37 ART-naive and 55 –experienced IDUs.

3.5. Data collection tools and Laboratory methods

3.5.1. Socio-demographic information

From each consenting participant, structured interview schedules with open- and closed-ended questions were conducted and the information filled in a questionnaire (**Appendix 2**). The information included; Socio-demographic characteristics (gender and age), antiretroviral treatment status (ART-naive or –experienced) and history of injection drug use (frequency and duration of injection, needle and syringe sharing and blood flushing).

3.5.2. Sample collection and processing

Upon consenting to the study, and after undergoing counselling, 3 millilitres (ml) of venous blood was collected using sterile butterfly needles (Becton Dickinson, Sparks, MD) inserted into EDTA tubes (Becton Dickinson, Sparks, MD). This sample was used for subsequent HIV serology testing, CD4 testing and preparation of dried blood spots and plasma.

Dried blood spots (DBS) to be used for protease drug resistance testing were prepared from the EDTA whole blood immediately after HIV serology testing. Briefly, four blood spots were prepared on pre-marked Whatman-FTA® 903 filter paper cards (Schleicher & Schuell, Keene, NH, USA) by carefully pipetting 50µL of whole blood on each circle. The blood spotted cards were left to dry overnight at room temperature (25^0C) in a class-II Biosafety cabinet and then each card was placed in separate glassine envelopes before being packed in air tight, plastic Zip lock bag (Fisher Scientific Company, Pittsburgh, PA, USA) containing three sachets of silica gel desiccant (Mini Pax Sorbent, Multisorb Technologies, Buffalo, NY, USA) to absorb moisture and keep the sample dry thereby maintaining their stability. The Zip lock bags containing DBS cards were then packed in larger Zip lock bags in batches of 20

bags and stored at -20^{0}C temporarily (WHO, 2012). These were later shipped frozen to Kenya Medical Research Institute, on dry ice, and stored at -70^{0}C for until analysis.

Plasma for viral load testing was prepared from the remaining blood sample after CD4 testing at Bomu Hospital in Mombasa County. Here, whole blood was centrifuged at 3000 revolutions per minute (rpm) for 10 minutes at room temperature. The plasma was then aliquoted in 2 ml Nalgene™ cryogenic tubes (Thermo Fisher Scientific, MA, USA) and stored at −70°C until shipment while frozen on dry ice to the Kenya Medical Research Institute, for viral load testing.

3.5.3. HIV Serology

HIV serology testing was performed on whole blood collected as described above in the EDTA tube at Bomu Hospital in the presence of participant after counselling and blood draw. This Serology was based on Kenya's recommended algorithm for HIV rapid testing (MoPHS and NASCOP, 2010). Here, the sample first tested with *Alere* Determine™ HIV-1/2 test (Alere, Australia) then followed by Uni-Gold™ HIV (Trinity Biotech, Ireland) as a confirmatory test if the sample turned positive on *Alere* Determine™ HIV-1/2 test.

Briefly, *Alere* Determine™ HIV-1/2 test was performed by applying 50 µL of the whole blood sample using a calibrated p200 pipette on the absorbent pad of a new test trip marked with participant number followed by addition of one free-falling drop (30 µL) of Chase Buffer (Trinity Biotech, Ireland). The sample was allowed to adsorb on the absorbent pad and results read after 15 minutes (no longer than 60 minutes). Similarly, Uni-Gold™ HIV confirmatory test was performed by pippeting 60µL of the whole blood sample in to the sample port of the Uni-Gold™ test device followed by addition of 60µL of Uni-Gold™ buffer. The sample and buffer were allowed to adsorb in the membrane and results read after

10 minutes (not longer than 20 minutes). For both tests, a sample with two clear red bars in the control and test window was interpreted as positive while the presence of only one band at the control window interpreted as negative. However, absence of non-clear red bar in the control window even if the bar in the test window was clearly visible was interpreted as invalid and the sample repeated.

3.5.4. Estimation of $CD4^+$ T cell counts

In addition to HIV serology testing above, the samples from HIV positive participants were further subjected to $CD4^+$ T cell testing at the recruitment facility (Bomu Hospital) for immunological profiling. Briefly, 50 µL of whole blood was analysed for $CD4^+$ T cell counts 6 hours within collection using a FACSCalibur flow cytometer (Becton-Dickinson, NJ) coupled with fluorochrome-tagged monoclonal antibodies to detect; anti-CD3, anti-CD45, and anti-CD4. The flourochromes were analysed with its automated acquisition and analysis software following the manufacturers' instructions.

3.5.5. Anthropometric measures and estimation of Body Mass index

Height in centimetres was measured at recruitment in a standardized way on a wall scale to the nearest 0.5 cm, without shoes and with the feet together. Weight to the nearest 0.1 kg was measured using standard electronic weighing scale (Rich forth Electronics Co., Fuzhou, China), with individuals dressed without shoes. Body mass index (BMI) was calculated as weight in kilograms divided by height in square meters (kg/m^2). Participants were then grouped in quartiles based on WHO BMI classifications (WHO, 2014a).

3.5.6. Quantitation of HIV-1 viral load

HIV-1 viral loads were determined using the Abbott m2000 System with automated sample extraction, amplification and detection according to the manufacturer's instructions (Abbott

Molecular Inc., Illinois, U.S.A). Briefly, RNA was extracted from plasma using the 0.2ml plasma RNA extraction and master mix addition protocol by the Abbott m2000sp sample preparation system. The master mix containing the viral RNA was then transferred to Abbott m2000rt instrument for amplification and viral load quantitation using the program for 0.2 ml RNA amplification for plasma with a lower limit of detection of 150 copies/ml (2.18 $\log_{10}$).

3.5.7. Extraction of HIV-1 proviral DNA

The entire DBS circle was cut with a pair of sterilised scissors and placed into a sterile 1.5ml eppendorf tube. The HIV-1 proviral DNA was then extracted with QIAamp DNA blood mini kit (QIAGEN, Valencia, CA) spin column protocol using the manufactures instruction (**Appendix 3**). This commercial kit is composed of buffers ATL, AL, AW1 and AW2 as one pack (QIAGEN, Valencia, CA). Briefly, 180 µL of buffer ATL was added to the 1.5 ml eppendorf tube containing cut DBS and incubated at 80 ^{0}C for 10 minutes. This was followed by addition of 20 µL QIAGEN proteinase K enzyme and incubated for one hour at 56 ^{0}C. Further, the blood spot was lysed with addition of 200 µL of lysis Buffer AL and an incubation of 70 ^{0}C for 10 minutes to release the proviral DNA. Following release, the proviral DNA was bound to the QIAamp silica membrane in the spin column and purified by a first and second wash with 500 µL of Buffers AW1 and AW2, respectively. Finally, 50 µL of proviral DNA was eluted in a sterile and nuclease free 1.5 ml eppendorf tube with Buffer AE. Purity and concentration of the extracted DNA was established by measuring its absorbance using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The minimum acceptable absorbance at 260 nm and 280 nm was set at 1.8 for DNA purity. While, DNA concentration of 20 ng/µL was regarded as sufficient for downstream polymerase chain reactions (BioTek, 2010). The sample was then kept frozen at -20 ^{0}C until testing.

3.5.8. Amplification of HIV-1 pro-viral Deoxyribonucleic Acid (DNA)

A 500 base pair (bp) fragment of HIV-1 pro-viral DNA encoding the whole protease gene (330 bp) and partial gag (100bp) and reverse transcriptase (70 bp) genes was amplified by complete nested PCR approach with primers; Nyupol_7 (5'-GGGAATTTTCTTCAGAGCAG-3') HXB2 nucleotide position 2125 to 2144, and Nyupol_8 (5'-TCTTCTGTCAATGGCCATTGT-3') HXB2 nucleotide position 2635 to 2615 in the first round. While, primers; Nyupol_9 (5'-TCCTTAACTTCCCTCAAATCACT-3') HXB2 nucleotide position 2241 to 2264 and Nyupol_10 (5'-CTGGCACGGTTTCAATAGGACT-3') HXB2 nucleotide position 2577 to 2556 were in the second round PCR reaction (Shafer *et al.*, 2001, Konings *et al.*, 2004).

For the first round of PCR, 5 µL of extracted DNA was added in a 20 µL master mix reaction consisting of 1X PCR buffer, 1.5 mM $MgCl_2$, 1U/ µL Taq polymerase (KemTaq®), 200 µM of each dNTP (Invitrogen, Carlsbad, CA), and 0.5 µM of each first round primer. This was then amplified with thermocycling conditions set at an initial denaturation of 94°C for 4 minutes, followed by 40 cycles of 94°C for 15 seconds, 46°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for 5 minutes.

The second round of PCR amplification was performed using similar master mix and thermocycling conditions. Two microliters of the first round PCR product was used as the template and the first round primers replaced by the second round primer set.

The PCR products were electrophoresed in 2% ethidium bromide stained agarose gel, and visualized under ultraviolet light. The presence of a single clear band at the 330 bp mark was indicative of successful amplification (**Appendix 4: Plate 1**).

3.5.9. Purification of Pro-viral DNA amplicons

The PCR amplicons were purified using ExoSAP-IT® enzymatic technique (Affymetrix USB®, Ohio, USA). This technique includes Exonuclease I and Shrimp Alkaline phosphatase enzymes which purify the amplicons upon heat incubation (Bell, 2008). Briefly, 6 µL of ExoSAP-IT® was mixed with 15 µL of PCR product in a PCR tube. The mixture was incubated in a thermocycler at 37°C for 15 minutes, followed by 15 minutes incubation at 80°C to completely inactivate the two active enzymes.

After enzyme purification, the products were then quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) as previously described above and the final concentration of all samples adjusted to 50 ng/µL using molecular grade water (Invitrogen, Carlsbad, CA).

3.5.10. Protease cycle sequencing PCR reaction

Sequencing PCR amplification products was done using the inner primers (Nyupol_9 and Nyupol_10) in two separate sequencing reactions (forward and reverse) with the BigDye® Terminator V3.1 sequencing chemistry (Applied Biosystems, Foster City, CA, USA). In summary, for each primer, 2 µL (initial concentration adjusted to 5 ng/µL) of purified proviral DNA amplicon was added in a 18 µL master mix reaction composed of 3.5 µL of 5X Sequencing buffer, 1.0 µL of BigDye® Terminator v3.1, 2 µL of 1X primer and 11.5 µL of molecular grade water (Invitrogen, Carlsbad, CA). The cycle sequencing PCR was set with initial denaturation of 96°C for 5 minutes, followed by 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes, then a final hold at 4 °C.

3.5.11. Purification of protease cycle sequencing PCR products and sequence analysis

To remove unincorporated dideoxynucleotide triphosphates (ddNTPs) and other PCR remnants, 20 µL of the BigDye-labeled sequencing PCR products was added into an eppendorf tube containing 2 µL of 3M sodium acetate (Applied Biosystems, Foster City, CA, USA) and 50 µL of 200 % proof ethanol (Sigma Aldrich., St. Louse, MO, USA). This was then incubated at room temperature for 15 minutes in the dark followed by centrifugation at room temperature for 20 minutes at 14,000g. Further, 500 ml of 70% ethanol was added and centrifugation performed at 14,000g for 10 minutes and the supernatant discarded. The two steps above were repeated once more and the resulting DNA sediment air-dried for at least 45 minutes. Upon drying, 25 µL of HiDi formamide (Applied Biosystems, Foster City, CA, USA) was added and heated at 95 ^{0}C for 2 minutes. The tubes were chilled on ice immediately for 2 minutes. The contents were transferred into sequencing tubes and loaded into an ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

3.5.12. Sequence data preparation and interpretation of protease resistance mutations

The sequence data from the ABI 3500 genetic analyzer was imported into the Genetyx-Windows computer software version 9.1.0 (Genetyx Corporation, Tokyo, Japan) to assemble the two sequence segments into a single contiguous sequence and saved into a FASTA format.

To determine drug resistance mutations, the generated FASTA sequences encoding the entire protease gene were analyzed and interpreted using the International AIDS Society (IAS) algorithm(Johnson *et al.*, 2013). Briefly, the FASTA sequences were deposited directly to the Stanford HIV drug resistance database (HIVdb) program (http://sierra2.stanford.edu/sierra/servlet/JSierra). This database identifies both major and

minor PI-DRMs by comparing the submitted protease and RT sequences sequences to reference sequences in HIV drug resistances database. The database designates these mutations by comparing a list of five sequences with a mutation at a particular amino acid position and then generates a consensus sequence using at least four sequences to rule out that the position actually has a mutation. Furthermore, the differences in the submitted query sequence to that of the consensus reference sequence were scored as major or minor drug resistance mutations. The overall prevalence of protease drug resistance among IDUs was determined as the proportion of sequences presenting with major or minor PI-DRMs' to the total number of sequences included in the final analysis.

3.6. Data analysis

All the statistical analyses were based on successfully sequenced samples from HIV positive IDUs. Gender, drug use characteristics, and the proportions of major and minor PI-DRMs were compared between ART-naive and experienced IDUs using Chi-square test. Differences in continuous data on age, height, weight, BMI, CD4+ T cell count and HIV viral loads between ART-naive and –experienced IDUs were analysed by Mann-Whitney U test. All analyses were performed using IBM® SPSS® software version 20.0.0 with significance levels set at $p \leq 0.05$.

3.7. Ethical consideration

All participants provided written informed consent (**Appendix 1**) before enrolment into the study. Initial clearance to carry out this study was sought from Maseno University's School of Graduate Studies and ethical approval obtained from Kenyatta University Ethical Review

Committee (ERC number: PKU019/116 of 2012) (**Appendix 5**). Confidentiality was ensured throughout the study by the use of codes and excluding personal identifiers. All study forms were archived in secure cabinets with access limited to authorised investigators. The participants benefited from free health education on the risks of drug injection, prevention of HIV transmission, sexually transmitted infections, good hygiene and nutrition. HIV-positive IDUs who were not on ARV treatment were referred to the nearest comprehensive care centres (CCC) of their choice for treatment, support and care.

CHAPTER FOUR

4.0. **RESULTS**

4.1. Demographics and drug use characteristics of IDUs

Ninety two HIV-1 infected injection drug users were recruited (ART-naive; n =37 and –experienced; n=55). All of them had successful pro-viral DNA amplification for the protease gene. However, only 76 were successfully sequenced for protease drug resistance mutations and used for subsequent analysis. Baseline demographics, laboratory and drug use characteristics of these study participants are summarized in Table 4.1. The 76 successfully sequenced samples comprised of ART-naive (n=31; males, n=14 and females, n=17), and -experienced (n=45; males, n=17 and females, n=28) whose distributions did not vary between the groups (p=0.342). Median age was similar between the ART-naive (median, 30.6; IQR, 27.2-33.7) and -experienced (median, 32.3; IQR, 29.1-36.2) study groups (p=0.168). Likewise, height (p=0.361) and weight (p=0.103) for ART-naive (median, 1.7; IQR, 1.7-1.8 and median, 54.0; IQR, 52.0-60.0 respectively) and, -experienced (median, 1.7; IQR, 1.6-1.7 and median, 53.0; IQR, 50.5-55.5 respectively) were similar between the study groups. However, the ART-experienced group (51.1%) presented with significantly higher rates of malnutrition (p=0.046) compared to the ART-naive group (29.0%).

Heroin was the most frequently reported injection drug both in ART-naive (77.4%) and -experienced (71.1%) groups (p=0.325) followed by cocaine [ART-naive (19.4%) and –experienced (26.7%); (p=0.367)] . However, injection of both heroin and cocaine was equally reported (p=0.653) by 3.2% of the ART-naive and 2.2% of ART-experienced IDUs. In addition, $CD4^+$ T cell counts in ART-naive (median, 542; IQR, 364-760) were similar (p=0.104) to ART-experienced (median, 398; IQR, 218-629) IDUs. Further, the distribution

of HIV-1 viral load (HIV-1 RNA Copies/ml) were comparable (p=0.485) both in ART-naive (median, 4,196; IQR, 150-72793) and -experienced IDUs (median, 4,825; IQR, 150-75115).

Table 4.1.1: Demographics and drug use characteristics of ART-naive and -experienced IDU from Mombasa County, Kenya

Characteristic	ART-naive n=31	ART-experienced n=45	*p*-Value
Gender, n (%)			
Male	14 (45.2)	17 (37.8)	0.342
Female	17 (54.8)	28 (62.2)	
Demographic measures, M(IQR)			
Age, years	30.6 (27.2-33.7)	32.3 (29.1-36.2)	0.168
Height, m	1.7 (1.7-1.8)	1.7 (1.6-1.7)	0.361
Weight, kg	54 (52-60)	53 (50.5-55.5)	0.103
BMI, kg/m^2	18.8 (18.0-20.1)	18.4 (17.7-19.5)	0.204
Nutritional status, n (%)			
Normal (BMI≥18.5<25.0)	22 (71.0)	22 (48.9)	**0.046**
Malnutrition (BMI<18.5)	9 (29.0)	23 (51.1)	
Injection drugs, n (%)			
Heroin	24 (77.4)	32 (71.1)	0.367
Cocaine	6 (19.4)	12 (26.7)	0.325
Cocaine and heroin	1 (3.2)	1 (2.2)	0.653
Clinical disease markers M (IQR)			
CD4+ T cell counts/ml	542 (364-760)	398 (218-629)	0.104
HIV-1 RNA Copies/ml	4,196 (150-72,793)	4,825 (150-75,115)	0.485

Data presented are medians (M) and interquartile range (IQR) or indicated as number (*n*) and proportions (*%*) of subjects. Statistical analysis of data was conducted using the Chi-square test for comparing proportions between the groups, and Mann Whitney U test for comparing differences in median age, height, weight, CD4+ T cell counts, and HIV-1 RNA between the study groups. Cross-tabulations for two-by-two tables in which cells had less than five counts were compared using the Fisher's exact tests. Malnutrition was defined using BMI <18.5 kg/m^2 based on the WHO criteria for assessing the nutritional status in adults (WHO, 2014a). Significant *p*-values are shown in bold. Abbreviations are shown as; HIV-1, human immunodeficiency virus type-1; *n*, number; m, metres; kg, kilograms; ART, antiretroviral treatment; RNA, Ribonucleic Acid.

4.2. Major PI-DRMs in ART-naive and -experienced injection drug users

Following bioinformatics analysis of sequence data, major HIV-1 protease mutations were found in both ART-naive and -experienced injection drug users. Table 4.2. summarizes major protease-inhibitor drug resistance mutations among the IDUs. Three major protease mutations (D30N, M46I and L90M) were detected in four (5.3%) IDUs (**Figure 4.2**). The four IDUs with major-PI-DRMs comprised of one ART-naive (3.2%) and three ART-experienced (6.7%) IDUs, though the proportions major PI-DRMs were comparable between the groups (p=0.459). It is worth noting that, while major mutation D30N only occurred in ART-naive IDUs (3.2%), mutation L90M and M46I in combination with D30N (M46I+D30N) were not found in the naive group (**Figure 4.2**) but only among ART-experienced IDUs (2.2% and 4.4%, respectively; p=0.315). Contrary to other major PI-DRMs detected among these IDUs, major mutation L90M did not co-exist with minor PI-DRMs. Major mutation D30N co-existed with minor mutation T74S but this was only detected in ART-naive IDUs (3.2%) while the duo-combination of major M46I+D30N detected among ART-experienced IDUs co-existed with minor mutation G48E (2.2%; **D30N+M46I**+G48E) or K20I (2.2%; **D30N+M46I**+K20I).

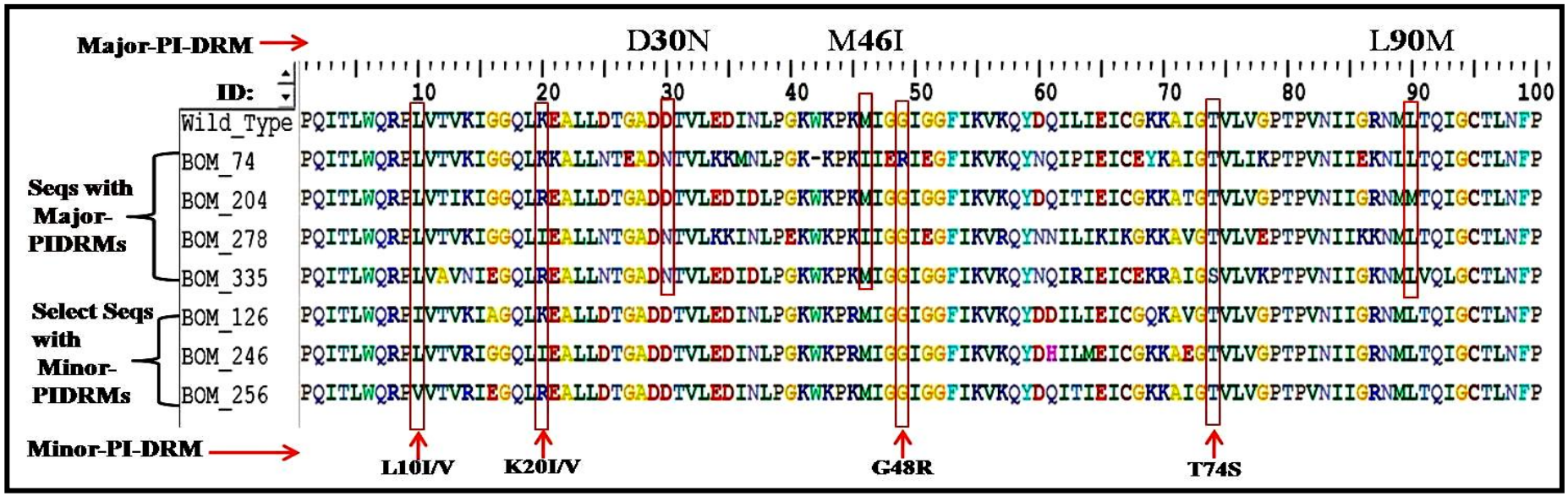

Figure 4.2. Selected sequences with major and minor PI-DRMs from IDUs in Mombasa County

Figure legend. Figure 4.2 show *protease* gene amino acid position for selected ART-naive and -experienced IDUs with major and minor PI-DRMs in relation to the wild type amino acid *protease* gene sequence. Mutations are denoted based on the International AIDS Society criteria as follows: wild-type amino acid, Amino acid position relative to protease gene followed by mutant amino acid substitute. Amino acids are abbreviated as; D, aspartate; E, glutamate; G, glycine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; R, arginine; S, serine; T, threonine; and, V, valine using the IUPAC nomenclature.

Table 4.2: Major PI-DRMs in ART-naive and -experienced Injection Drug Users from Mombasa County, Kenya

Major PI-DRMs	ART-naive n=31	ART-experienced n=45	Totals N=76	*P*-value
PI-DRMs frequencies, n (%)				
Major PI-DRMs present	1 (3.2)	3 (6.7)	4 (5.3)	0.459
Major PI-DRMs absent	30 (96.8)	42 (93.3)	72 (94.7)	
Major PI-DRMs types, n (%)				
D30N	1 (3.2)	None	1 (1.3)	0.315
L90M	None	1 (2.2)	1 (1.3)	
M46I+ D30N	None	2 (4.4)	2 (2.6)	
None	30 (96.8)	42(93.3)	72 (94.7)	
Multiple PI-DRMs, n (%)				
D30N+T74S	1 (3.2)	None	1 (1.3)	0.408
D30N+M46I+G48E	None	1 (2.2)	1 (1.3)	0.592
D30N+M46I+K20I	None	1 (2.2)	1 (1.3)	0.592

Table legend. Data presented are number (n) and proportions (%) of subjects. Chi-square test was used to compare the proportions of mutations between the groups. Cross-tabulations for two-by-two tables in which cells had less than five counts were compared using the Fisher's exact tests. Mutations are denoted based on the International AIDS Society criteria as follows: wild-type amino acid, Amino acid position relative to protease gene followed by mutant amino acid substitute (Wensing *et al.*, 2014). Major PI-DRMs are shown in bold. Amino acids are abbreviated as: D, aspartate; E, glutamate; G, glycine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; S, serine; and T, threonine using the International Union of Pure and Applied Chemistry (IUPAC) nomenclature (IUPAC, 1972). Abbreviations are indicated as; ART, antiretroviral treatment and PI-DRMs, Protease Inhibitor-Drug Resistance Mutations.

4.3. Minor PI-DRMs in ART-naive and -experienced injections drug users

Minor HIV-1 protease mutations were also detected in both ART-naive and -experienced IDUs. This information is summarized in Table 4.3. (see also in figure 4.2). Nine (29.0%) ART-naive and eight (17.8%) ART-experienced had minor PI-DRMs (p=0.190). These comprised of G48E (n=2), G48R (n=1), K20I (n=2), L10I (n=6), L10V (n=1) and T74S (n=1). Although the distribution of individual minor PI-DRMs did not vary greatly between the ART-naive and -experienced IDUs (p>0.05), minor mutations L10V and L10I were the commonly detected mutations both in ART-naive (9.7% each) and -experienced (4.4% and 6.7%, respectively) IDUs. Minor mutation G48E was detected in 3.2% of ART-naive and 2.2% of -experienced IDUs. However, minor mutation K20I was only detected in ART-experienced IDUs (3.2%) while, mutations G48R and T74S only detected in ART-naïve individuals (3.2% each).

Table 4.3: Minor PI-DRMs in ART-naive and -experienced Injection Drug Users from Mombasa County, Kenya

Minor PI-DRMs	ART-naive n=31	ART-experienced n=45	Total N=76	*p*-value
Mutations frequencies, n (%)				
Minor PI-DRMs present	9 (29.0)	8 (17.8)	17 (22.4)	0.190
Minor PI-DRMs absent	22 (71.0)	37 (82.2)	59 (77.6)	
Minor mutations types, n (%)				
L10I	3 (9.7)	3 (6.7)	6 (7.9)	0.473
L10V	3 (9.7)	2 (4.4)	5 (6.6)	0.327
G48E	1 (3.2)	1 (2.2)	2 (2.6)	0.653
K20I	None	2 (4.4)	2 (2.6)	0.347
G48R	1 (3.2)	None	1 (1.3)	0.408
T74S	1 (3.2)	None	1 (1.3)	0.408

Table legend. Data presented are number (n) and proportions (%) of subjects. Statistical analysis to compare proportions of mutations between the groups was conducted using Chi-square test while Fisher's Exact test used for two-by-two cross-tabulations tables in which cells had less than five counts. Mutations are denoted based on the International AIDS Society criteria as: wild-type amino acid, Amino acid position relative to protease gene followed by mutant amino acid substitute (Wensing *et al.*, 2014). Amino acids are abbreviated as: G, glycine; I, isoleucine; K, lysine; L, leucine; R, arginine; S, serine; T, threonine; and, V, valine using the International Union of Pure and Applied Chemistry (IUPAC) nomenclature (IUPAC, 1972). Abbreviations are indicated as; ART, antiretroviral treatment and PI-DRMs, Protease Inhibitor-Drug Resistance Mutations.

4.4. Association between major and minor PI-DRMs and demographics and HIV disease markers in ART-naive and -experienced injection drug users

To establish the associations between major and minor PI-DRMs and injection drug user's demographics, $CD4^+$ T cell count and HIV-1 viral load in ART-naive and –experienced participants, additional statistical analyses were conducted and summarized in the following sub-sections.

4.4.1. Association between major PI-DRMs and demographics and HIV disease markers in ART-naive and -experienced IDUs from Mombasa County, Kenya

The association between participant's demographics and HIV-1 disease markers are summarized in Table 4.4. The four IDUs presenting with major PI-DRMs comprised of one female (25%) and three males (75%) depicting about 3 times burden of major PI-DRMs in male IDUs over female counterparts (p=0.182). Although age, height, and, weight were not associated with the presence or absence of major PI-DRMs (p>0.05), IDUs with major PI-DRMs were slightly older (median=34.0 yrs, IQR, 30.9-36.0 yrs) compared to those without major PI-DRMs (median=30.8 yrs, IQR, 26.4-34.9 yrs) similar to body weight for IDUs with PI-DRMs (median=56.5 kg, IQR, 50.5-63.3 kg) versus those without major mutation (median=53.0 kg, IQR, 50.0-56.6 kg). While body height (median=1.7 meters) was similar between the groups (p=0.398). Consistently, BMI levels did not significantly vary between those presenting with or without major-PIDRMs (p=0.564). There was an equal proportion of IDUs presenting normal BMI and malnutrition (50% each).

Furthermore, factors affecting development of drug resistance such as experience and duration on ART and daily drug injection frequency did not predict the presence or absence of major PI-DRMs. However, none of the IDUs reported to have adhered to ART. Most

importantly, although $CD4^+$ T-cell counts were low (less than 500 counts/ml) in both groups, IDUs with major PI-DRMs were associated with significantly higher viral loads (median=175,606; IQR, 38,803-270,810 copies/ml) as opposed to those without the major PI-DRMs (p=0.004).

Table 4.4: The demographic characteristics and HIV disease markers in ART-naive and -experienced injection drug users with major PI-DRM from Mombasa County, Kenya

Confounding factors	Major PI-DRMs present n=4	Major PI-DRMs Absent n=72	Total	*p*-value
Gender, n (%)				
Male	3 (75.0)	28 (38.9)	31 (40.8)	0.182
Female	1 (25.0)	44 (61.1)	45 (59.2)	
IDU demographics, M(IQR)				
Age, years	34.0 (30.9-36.0)	30.8 (26.4-34.9)		0.303
Height, m	1.7 (1.7-1.8)	1.7 (1.6-1.7)		0.398
Weight, kg	56.5 (50.5-63.3)	53 (50.0-56.6)		0.552
Nutritional status, n (%)				
Normal (BMI≥18.5<25.0)	2 (50.0)	42 (58.3)	44 (57.9)	0.564
Malnutrition (BMI<18.5)	2 (50.0)	30 (41.7)	32 (42.1)	
ART, n (%)				
ART-naive	1 (25.0)	30 (41.7)	31 (40.8)	0.459
ART-Experienced	3 (75.0)	42 (58.3)	45 (59.2)	
Duration on ART, *n (%)*				
Naive	1 (25.0)	30 (41.7)	31 (40.8)	0.754
<1 year	1 (25.0)	18 (25.0)	19 (25.0)	
>= 1Year	2 (50.0)	24 (33.3)	26 (34.2)	
IDU frequency, *n (%)*				
Once daily	None	17 (23.6)	17 (22.4)	0.355
Twice or more daily	4 (100)	55 (76.4)	59 (77.6)	
Adherence to ART, *n (%)*				
Compliant	None	None		
Non-compliant	4 (100)	72 (100)	-	-
Markers of disease, M (IQR)				
CD4+ T cell counts/ml	318 (68-596)	497 (241-677)		0.104
HIV-1 RNA Copies/ml	175,606 (38,803-270,810)	41,904 (150-75,115)		**0.040**

Table legend. Data presented are medians (M) and interquartile range (IQR) or indicated as number (n) and proportions (%) of subjects. Statistical analysis was conducted using Chi-square test for comparing proportions between groups, while Mann Whitney-U test for association of median age, height, weight, CD4+ T cell counts, and HIV-1 RNA between the study groups. Abbreviations: m, metres; kg, kilograms; ≥, greater or equal to; <, less than; HIV-1, human immunodeficiency virus type-1; BMI, body mass index; ART, antiretroviral treatment RNA, ribonucleic acid. Significant p-values are shown in bold.

4.4.2. Association between minor PI-DRMs and demographics and HIV disease markers among ART-naive and -experienced injection drug users

The association between minor PI-DRMs and demographic measures, CD4+ T cell counts, HIV-1 viral load and drug use characteristics in both ART-naive and -experienced IDUs was also evaluated. This information is summarized in Table 4.5. A higher proportion of males 64.7% harboured minor PI-DRMs compared to females 35.3% (p=0.028). However, age (p=0.441) and height (p=0.151) were similar among IDUs with carriage and non-carriage of the minor PI-DRMs. On contrast, IDUs harbouring minor PI-DRMs significantly had a heavy weight (median=54.0; IQR, 53.0-61.0 Kg) relative to those without minor PI-DRMs (median=53.0; IQR, 50.0-56.0 Kg, p=0.028) with majority (76.5%) of them presenting with normal BMI (p=0.067). However, the presence of minor PI-DRMs was not associated with ART experience, duration and daily drug injection frequency (p>0.05) while none of the IDUs reported to have adhered to antiretroviral treatment.

Similarly, neither CD4+ T cell counts (p=0.464) nor HIV-1 RNA viral load (p=0.793) accounted for carriage and non-carriage of minor PI-DRMs (Table 4.5).

Table 4.5: The associations between demographic characteristics and HIV disease markers among ART-naive and -experienced IDUs with minor PI-DRMs from Mombasa county, Kenya

Characteristic	Minor PI-DRMs present, n=17	Minor PI-DRMs Absent, n=59	Total	*p*-value
Gender, n (%)				
Male	11(64.7)	20 (33.9)	31 (40.8)	**0.028**
Female	6 (35.3)	39 (66.1)	45 (59.2)	
IDU demographics, M(IQR)				
Age, years	31.8 (27.7-32.9)	30.6 (27.2-35.9)		0.441
Height, m	1.7 (1.7-1.8)	1.7 (1.6-1.7)		0.151
Weight, kg	54.0 (53.0-61.0)	53.0 (50.0-56.0)		**0.028**
Nutritional status, n (%)				
Normal (BMI≥18.5<25.0)	13 (76.5)	31 (52.5)	44 (57.9)	0.067
Malnutrition (BMI<18.5)	4 (23.5)	28 (47.5)	32 (42.1)	
ART, n (%)				
ART-naive	9 (52.9)	22 (37.3)	31 (40.8)	0.190
ART-Experienced	8 (47.1)	37 (62.7)	45 (59.2)	
Duration on ART, *n (%)*				
Naive	9 (52.9)	22 (37.3)	31 (40.8)	0.084
<1 year	6 (35.3)	13 (22.0)	19 (25.0)	
≥1Year	2 (11.8)	24 (40.7)	26 (34.2)	
IDU frequency, *n (%)*				
Once daily	3 (17.6)	14 (23.7)	17 (22.4)	0.435
Twice or more daily	14 (82.4)	45 (76.3)	59 (77.6)	
Adherence to ART, *n (%)*				
Compliant	None	None		
Non-compliant	4 (100)	72 (100)	-	-
Markers of disease, M (IQR)				
CD4+ T cell counts/ml	522 (225-759)	466 (241-637)		0.464
HIV-1 RNA, Copies/ml	1,427 (150-89,267)	2,974 (150-72,793)		0.793

Table legend. Data presented are medians (M) and interquartile range (IQR) or indicated as number (n) and proportions (%) of subjects. Statistical analysis of data was conducted using the Chi-square test for comparing proportions between the groups, and Mann-Whitney U test for to establish associations the continuous variables. Abbreviations: m, metres; kg, kilograms; ≥, greater or equal to; <, less than; HIV-1, human immunodeficiency virus type-1; BMI, body mass index; ART, antiretroviral treatment RNA, ribonucleic acid.

4.5. Sequence data

All the seventy six HIV-1 protease sequences obtained in this study **(Appendix 6)** were submitted to GenBank with accession numbers: KP791995- KP792070.

CHAPTER FIVE

5.0. **DISCUSSION**

The up-scaling of HIV-1 treatment in sub-Saharan Africa has heavily relied on first line treatment regimens consisting of NRTI and non-NRTIs that has led to significant reductions in HIV-1 related morbidity and mortality (Gilks *et al.*, 2006, Jordan *et al.*, 2008). However, the emergence and spread of resistance to NRTIs and NNRTIs threatens the gains of the up-scaled HIV-1 treatment (Hassan *et al.*, 2014). Protease inhibitors (PIs) are integral drugs for second line HIV-1 treatment regimens that are increasingly becoming available in the treatment of virologic failures and intolerance to first line ART (Clumeck *et al.*, 2014, Paton *et al.*, 2014). Injection drug users are one of the most-at-risk populations with high transmission rates resulting in new HIV-1 infections (Brodish *et al.*, 2011, UNODC, 2013, NASCOP, 2014). Successful treatment of HIV-1 infections in IDUs is hampered by poor adherence and the high rates of transmissions leading to higher rates of development and transmission of drug resistance to antiretroviral (Kerr *et al.*, 2004, Palepu *et al.*, 2011).

Out of the 76 IDUs whose samples were successfully analysed, the overall prevalence of major PI-DRMs was 5.3%. The prevalence of major PI-DRMs among the ART-experienced (6.7%) about two times that of ART-naive 3.2% (p=0.459). The major PI-DRMs among these IDUs were L90M or a combination of both D30N and M46I for the ART-experienced or only D30N for the ART-naive. A similar study among ART-experienced IDUs from Mombasa County detected 13.8% prevalence of resistance to first line ART (NRTIs and NNRTIs) which was higher than the prevalence (1.1%) in ART-naive patients (Hassan *et al.*, 2013, Osman *et al.*, 2013). The findings from this study, summed by the above related studies can therefore suggest that ART-experienced IDUs have a relatively higher burden of major PI-

DRMs than the ART-naive counterparts. Mutation D30N is a nonpolymorphic substrate-cleft mutation that causes high-level resistance upon nelfinavir (NFV) treatment (Rhee *et al.*, 2010). While, mutation L90M reduces susceptibility to each of the PIs except tipranavir (TPV) and darunavir (DRV) and it is primarily selected by indinavir (IDV), lopinavir (LPV), NFV and saquinavir (SQV) (Rhee *et al.*, 2010). When present with other mutations, M46I reduces susceptibility to atazanavir (ATV), fosamprenavir (FPV), indinavir (IDV), lopinavir (LPV), and nelfinavir (NFV) and is known to increase the protease catalytic efficiency(Schapiro *et al.*, 2010, Henderson *et al.*, 2012). Since ATV and LPV (LPV/r) are the main PIs advised as second line substitutions in Kenya and other developing countries (NASCOP, 2012, Paton *et al.*, 2014), the detection of these mutations among IDUs from Mombasa County, especially M46I and L90M may suggest a reduction of efficacy of the currently used PIs in Kenya. A study carried out in Mombasa among ART-experienced non-IDUs also detected major PI-DRMs L90M, I85V and N88D which confirms that indeed PI-DRMs including those detected in this study are circulating among residents of Mombasa County (Sigaloff *et al.*, 2012). It therefore implies that, achieving complete benefits of ART among subjects carrying these major mutations which confer resistance to the recommended PIs (LPV/r and ATV/r) might not be possible thus negating efforts aimed at controlling the emergence and spread of drug resistance.

Although the exact antiretroviral regimens (ARVs) were not evaluated, 6.7% of IDUs presenting with major PI-DRMs were ART-experienced as opposed to 3.2% among the ART-naive (p=0.459). It has been shown that there is poor adherence to ART among ART-experienced IDUs (Vlahov and Celentano, 2006, Graham *et al.*, 2010, Palepu *et al.*, 2011). Therefore, it can be inferred that, sub-optimal levels of ARVs exerts selective pressure on the virus to efficiently develop resistance (Nijhuis *et al.*, 1999, Graham *et al.*, 2010, Ssemwanga

et al., 2015). Immunological studies targeting IDUs have also shown that injection drug use is associated with impaired liver pharmacokinetic functions (Valente *et al.*, 2012, Were *et al.*, 2014). Thus, the cumulative effect of opioid-related hepatotoxicities as well as opiate-ARV interactions could also be suggested as a major contributor of higher frequencies of major PI-DRMs among ART-experienced IDUs.

Six minor PI-DRMs (G48E, G48R, K20I, L10I, L10V and T74S) were observed in 22.4% of the IDUs (29.0% in ART-naive and 17.8% in ART-experienced, p=0.190). Although no high level resistance has been reported to develop in individuals with exclusively minor PI-DRMs, in the presence of major PI-DRMs, minor PI-DRMs have been shown to increase the viral replication fitness and drug resistance to major PIs (Shafer and Schapiro, 2008, Scherrer *et al.*, 2012). For instance, this study established that minor mutation L10I and L10V were the most prevalent in both the ART-naive and -experienced individuals (see Table 4.5). These minor mutations independently or when present with major PI-DRMs reduce the PI efficacy and increase the viral replication (Chang and Torbett, 2011). As such, individuals with minor PI-DRMs should be routinely monitored for a possible emergence of major PI-DRMs (Mitsuya *et al.*, 2006, Zimmer *et al.*, 2008, Rhee *et al.*, 2010).

There were no multiple major PI-DRMs among ART-naive study participants. On the contrary, 66.7% (2/3) of the ART-experienced participants with major PI-DRMs had multiple major PI-DRMs. They all had HIV-1 strains harbouring both D30N and M46I mutations. Interestingly, this combination is not always expected as M46I is mainly known to occur in combination with V32I, I47V, L76V, I84V, and L90M (Varghese *et al.*, 2013). Consistent with other studies, there was no co-occurrence of mutations D30N and L90M - both require mutation N88D that was not found among the study participants (Mitsuya *et al.*, 2006). *In vitro* studies have demonstrated that the co-existence of mutation D30N and M46I in a viral

strain have a complex interplay between PI susceptibility and resistance (Santos and Soares, 2011, Varghese *et al.*, 2013). Although D30N mutation confers high resistance to NFV, this mutation also increases the susceptibility of the virus to SQV and APV as well as neutralizing the resistance conferred by M46I to IDV (Santos and Soares, 2011). This indicates that, individuals infected with HIV-1 strains harbouring mutation D30N alone could be given other boosted PIs such as APV, IDV or SQV because that mutation is only specific to NFV resistance and makes the virus to be very susceptible to other PIs (Santos and Soares, 2011). However, care has to be taken if mutation N88D is also present since it synergistically compensates D30N by increasing the viral resistance to APV and SQV. The N88D mutation will also lead to acquisition of L90M ruling out the other salvage PIs in case of D30N mutation except TPV and DRV (Mitsuya *et al.*, 2006, Rhee *et al.*, 2010, Santos and Soares, 2011).

Though gender was not significantly associated with carriage of major PI-DRMs (p=0.182), a relatively higher number of IDUs with major PI-DRMs were male (75%) than females (25%). Consistent with other studies, males engage more often in drug injection as opposed to females (Deveau *et al.*, 2006, NASCOP and MOH, 2012). Once the virus acquires major drug resistance, both viral replication and depletion of the host immunity are enhanced (DeGruttola *et al.*, 2000). IDUs carrying major PI-DRMs had a significantly higher viral load (median=175,606; IQR, 38,803-270,810 copies/ml) as opposed to those without the major PI-DRMs (p=0.004). Similarly, consisted with high viral loads, the $CD4^+$ T-cell counts were relatively lower (median=318; IQR 68-596) depicting failure of the body's immune response to counter the overwhelming high viral replication. Overall, this suggests that viruses carrying these mutations acquire replicative fitness and thereby overwhelm the body's defence mechanism (O'Brien *et al.*, 1995, de Wolf *et al.*, 1997).

Among the IDUs that harboured minor PI-DRMs, a significantly (p=0.028) proportions were males (64.7%). As pointed out earlier, the burden of injection drug use is usually high in males as opposed to females (UNODC, 2013). It can be speculated that, injection drug use related effects among the HIV-1 infected IDUs might in turn facilitate the virus to develop minor- drug resistant mutations that may trigger the development of major drug resistance mutations. In addition, factors such as homelessness, exclusion from the society, poor nutrition as well as poverty levels in male IDUs can also offer a good ground for emergence of drug resistance (Mathers *et al.*, 2010, Bhunu and Mushayabasa, 2011).

The presence of minor PI-DRMs did not vary with HIV-1 clinical correlates in both ART-naive and -experienced groups. Although body weight was significantly higher (p=0.028) among IDUs with minor PI-DRMs (median=54.0; IQR, 53.0-61.0 Kg), the CD4+ T-cell counts and BMI were equally distributed among IDUs who either lacked, or had minor PI-DRMs regardless of their ART status. These findings are consistent with previous cross-sectional and *in vitro* studies on the effect of minor PI-DRMs showing that minor PI-DRMs do not impact on HIV-1 disease progression but increase viral replication fitness and drug resistance in strains with major PI mutations (Chang and Torbett, 2011, Scherrer *et al.*, 2012). However, further investigations on the effect of injection drug use and viral replication especially among the ART-experienced individuals are required to provide additional insights into the effects of minor PI-DRMs on viral replication. As observed in this study, there was a generally high viral load both in IDUs carrying minor PI-DRM and those without the resistance mutations (median VL>1000 copies/mL). Low viral load is often expected upon antiretroviral treatment (Greig *et al.*, 2013). The high viral load in the ART-naive IDUs could be as a result of poor HIV treatment-seeking behaviour among drug users. More importantly,

many IDUs stay in hideouts and will only seek medications when they are overwhelmed with HIV-1 related opportunistic infections (Broz *et al.*, 2014).

CHAPTER SIX

6.0. SUMMARY OF FINDINGS, CONCLUSION, AND RECOMMENDATIONS

6.1. Summary of findings

A total of 92 HIV-1 positive (ART-naive=37; ART-experienced=55) IDUs were recruited. Among these, samples from 76 IDUs (ART-naive=31; ART-experienced=45) were successfully amplified and sequenced for PI-DRMs. Major PI-DRMs (D30N, M46I and L90M) were detected in four (5.3%) IDUs (ART-naive=3.2% and ART-experienced=6.7% *p*=0.459). Minor PI-DRMs (G48E, G48R, K20I, L10I, L10V and T74S) were detected in 22.4% of the IDUs (29.0% in ART-and 17.8% in ART-experienced; *p*=0.190). Majority of the IDUs presenting with major or minor PI-DRMs were males (75%, *p*=0.182 and 64.7%, *p*=0.028 respectively). Major PI-DRMs were associated with high viral loads *p*=0.04 and relatively low $CD4^+$ T-cell counts (median=318 IQR, 68-596 cells/ml). However, there was no significant difference in viral loads, BMI, and $CD4^+$ T-cell counts between IDUs carrying minor PI-DRMs and those without minor PI-DRMs.

6.2. Conclusion

1. The prevalence of major PI-DRMs was 5.3% among IDUs (ART-naive and –experienced) from Mombasa County, Kenya with ART-experienced IDUs exhibiting twice more major PI-DRMs (6.7%) than ART-naive IDUs (3.2%).
2. The prevalence of minor PI-DRMs was 22.4%. The ART-naive IDUs exhibited relatively higher prevalence (29.0%) compared to the ART-experienced IDUs (17.8%).

3. Major PI-DRMs were significantly associated with high viral loads, and low $CD4^+$ T-cell counts with majority (75%) of the IDUs with major PI-DRMs being male. Similarly, IDUs that harboured minor PI-DRMs presented with high viral loads, low BMI, and $CD4^+$ T-cell counts although, there was no marked differences between the IDUs that exhibited minor PI-DRMs with those that did not.

6.3. Recommendations from the current study

i. Drug resistance testing should be made a requirement before enrolment to therapy in ART-naive subjects and before regimen switching in ART-experienced patients as part of treatment and care services for HIV infected IDUs and other patients in order to improve treatment benefits of ART.

ii. The government and other stakeholders involved in planning HIV-1 treatment guidelines for most-at-risk populations (MARPs) such as IDUs and other HIV patients should supplement the currently used protease inhibitors in Kenya with more effective ones. This is because, the major PI-DRMs reported in this study confer resistance to the currently approved PIs (LPV/r and ATV/r) and are more likely to be transmitted to the general population.

iii. As part of HIV disease monitoring in IDUs and other patients, HIV viral load and CD4 testing should be backed with drug resistance information in order to determine whether treatment failure is as a result of drug resistance, or poor adherence.

6.4. Recommendations for future studies

Information generated from this study shows that HIV-1 strains with major protease drug resistance mutations are in circulation among the IDUs. Data on HIV-1 drug resistance

mutations among IDUs from Mombasa County has now been updated. This information is useful in the strategies for HIV-1 treatment, monitoring, and prevention of the acquisition of drug resistance mutations among the IDUs as well as the general population. These results also form a basis for support of the choice of antiretroviral drugs (especially PIs) by treatment program planners and individual clinicians targeting IDUs and other most-at-risk populations. As such, the study recommends the following future investigations;

i. Additional surveillance and longitudinal studies need to be performed to determine the burden of major and minor PI-DRMs in the general population as well as other HIV risk groups with regard to the underlying drug resistance to first line regimens.

ii. Studies should also be designed to elucidate ART adherence among the Kenyan IDUs and its impact on the development of PI-DRMs.

iii. Programs targeting HIV infected IDUs should come up with strategies to enhance ART adherence since it is a biggest contributor to development of drug resistance.

REFERENCES

Abram, M. E., Ferris, A. L., Das, K., Quinones, O., Shao, W., Tuske, S., Alvord, W. G., Arnold, E. & Hughes, S. H. 2014. Mutations in HIV-1 reverse transcriptase affect the errors made in a single cycle of viral replication. *J Virol,* 88**,** 7589-601.

Abu-Raddad, L. J., Barnabas, R. V., Janes, H., Weiss, H. A., Kublin, J. G., Longini, I. M., Jr. & Wasserheit, J. N. 2013. Have the explosive HIV epidemics in sub-Saharan Africa been driven by higher community viral load? *AIDS,* 27**,** 981-9.

Achhra, A. C., Petoumenos, K. & Law, M. G. 2014. Relationship between CD4 cell count and serious long-term complications among HIV-positive individuals. *Curr Opin HIV AIDS,* 9**,** 63-71.

Addy, C. L., Gavrila, A., Tsiodras, S., Brodovicz, K., Karchmer, A. W. & Mantzoros, C. S. 2003. Hypoadiponectinemia is associated with insulin resistance, hypertriglyceridemia, and fat redistribution in human immunodeficiency virus-infected patients treated with highly active antiretroviral therapy. *J Clin Endocrinol Metab,* 88**,** 627-36.

Bakhouch, K., Oulad-Lahcen, A., Bensghir, R., Blaghen, M., Elfilali, K. M., Ezzikouri, S., Abidi, O., Hassar, M. & Wakrim, L. 2009. The prevalence of resistance-associated mutations to protease and reverse transcriptase inhibitors in treatment-naive (HIV1)-infected individuals in Casablanca, Morocco. *J Infect Dev Ctries,* 3**,** 380-91.

Barber, T. J., Harrison, L., Asboe, D., Williams, I., Kirk, S., Gilson, R., Bansi, L., Pillay, D., Dunn, D., Database, U. H. D. R. & Committees, U. K. C. H. C. S. S. 2012. Frequency and patterns of protease gene resistance mutations in HIV-infected patients treated with lopinavir/ritonavir as their first protease inhibitor. *J Antimicrob Chemother,* 67**,** 995-1000.

Baxter, J. D., Mayers, D. L., Wentworth, D. N., Neaton, J. D., Hoover, M. L., Winters, M. A., Mannheimer, S. B., Thompson, M. A., Abrams, D. I., Brizz, B. J., Ioannidis, J. P. & Merigan, T. C. 2000. A randomized study of antiretroviral management based on plasma genotypic antiretroviral resistance testing in patients failing therapy. CPCRA 046 Study Team for the Terry Beirn Community Programs for Clinical Research on AIDS. *AIDS,* 14**,** F83-93.

Beckerleg, S., Telfer, M. & Hundt, G. L. 2005. The rise of injecting drug use in East Africa: a case study from Kenya. *Harm Reduct J,* 2**,** 12.

Bell, J. 2008. A simple way to treat PCR products prior to sequencing using ExoSAP-IT. *Biotechniques,* 44**,** 834.

Bertagnolio, S., De Luca, A., Vitoria, M., Essajee, S., Penazzato, M., Hong, S. Y., McClure, C., Duncombe, C. & Jordan, M. R. 2012. Determinants of HIV drug resistance and public health implications in low- and middle-income countries. *Antivir Ther,* 17**,** 941-53.

Bhunu, C. P. & Mushayabasa, S. 2011. HIV/AIDS Transmission Dynamics in Male Prisons. *Global View of HIV Infection* [Online]. Available: http://www.intechopen.com/books/global-view-of-hiv-infection/hiv-aids-transmission-dynamics-in-male-prisons [Accessed 25, July, 2013].

BioTek. 2010. Tech Resources – *Nucleic Acid Purity Assessment using A260/280 Ratios*. Available: http://www.biotek.com/resources/articles/nucleic-acid-purity-a260a280.html.

Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A. & Mackay, C. R. 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc Natl Acad Sci U S A,* 94**,** 1925-30.

Brodish, P., Singh, K., Rinyuri, A., Njeru, C., Kingola, N., Mureithi, P., Sambisa, W. & Weir, S. 2011. Evidence of high-risk sexual behaviors among injection drug users in the Kenya PLACE study. *Drug Alcohol Depend,* 119**,** 138-41.

Broz, D., Wejnert, C., Pham, H. T., DiNenno, E., Heffelfinger, J. D., Cribbin, M., Krishna, N., Teshale, E. H., Paz-Bailey, G. & National, H. I. V. B. S. S. S. G. 2014. HIV infection and risk, prevention, and testing behaviors among injecting drug users -- National HIV Behavioral Surveillance System, 20 U.S. cities, 2009. *MMWR Surveill Summ,* 63**,** 1-51.

Chang, M. W. & Torbett, B. E. 2011. Accessory mutations maintain stability in drug-resistant HIV-1 protease. *J Mol Biol,* 410**,** 756-60.

Clumeck, N., Mwamba, C., Kabeya, K., Matanda, S., Vaira, D., Necsoi, C., Kadiebwe, D., Delforge, M., Kasamba, E., Milolo, C., Ilunga, J. & Kapend, L. 2014. First-line antiretroviral therapy with nevirapine versus lopinavir-ritonavir based regimens in a resource-limited setting. *AIDS,* 28**,** 1143-53.

Cochran, W. G. 1977. Sampling Techniques. *John Wiley and Sons, Inc.* [Online]. Available: http://ruangbacafmipa.staff.ub.ac.id/files/2012/02/William_G._Cochran_Sampling_Techniques_Third_EdBookFi.org_.pdf [Accessed 28th, Aug, 2013].

Cohen, C. J., Hunt, S., Sension, M., Farthing, C., Conant, M., Jacobson, S., Nadler, J., Verbiest, W., Hertogs, K., Ames, M., Rinehart, A. R. & Graham, N. M. 2002. A randomized trial assessing the impact of phenotypic resistance testing on antiretroviral therapy. *AIDS,* 16**,** 579-88.

Condra, J. H., Schleif, W. A., Blahy, O. M., Gabryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shivaprakash, M. & et al. 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature,* 374**,** 569-71.

de Wolf, F., Spijkerman, I., Schellekens, P. T., Langendam, M., Kuiken, C., Bakker, M., Roos, M., Coutinho, R., Miedema, F. & Goudsmit, J. 1997. AIDS prognosis based on HIV-1 RNA, CD4+ T-cell count and function: markers with reciprocal predictive value over time after seroconversion. *AIDS,* 11**,** 1799-806.

DeGruttola, V., Dix, L., D'Aquila, R., Holder, D., Phillips, A., Ait-Khaled, M., Baxter, J., Clevenbergh, P., Hammer, S., Harrigan, R., Katzenstein, D., Lanier, R., Miller, M., Para, M., Yerly, S., Zolopa, A., Murray, J., Patick, A., Miller, V., Castillo, S., Pedneault, L. & Mellors, J. 2000. The relation between baseline HIV drug resistance and response to antiretroviral therapy: re-analysis of retrospective and prospective studies using a standardized data analysis plan. *Antivir Ther,* 5**,** 41-8.

Deveau, C., Levine, B. & Beckerleg, S. 2006. Heroin use in Kenya and findings from a community based outreach programme to reduce the spread of HIV/AIDS. *Afr. J Drug & Alcohol Studies,* 5.

Dunn, D. T., Coughlin, K. & Cane, P. A. 2011. Genotypic resistance testing in routine clinical care. *Curr Opin HIV AIDS,* 6**,** 251-7.

Eshleman, S. H., Guay, L. A., Mwatha, A., Brown, E. R., Cunningham, S. P., Musoke, P., Mmiro, F. & Jackson, J. B. 2004. Characterization of nevirapine resistance mutations in women with subtype A vs. D HIV-1 6-8 weeks after single-dose nevirapine (HIVNET 012). *J Acquir Immune Defic Syndr,* 35**,** 126-30.

EuroGuidelines 2001. Clinical and laboratory guidelines for the use of HIV-1 drug resistance testing as part of treatment management: recommendations for the European setting. The EuroGUidelines Group for HIV resistance. *AIDS,* 15**,** 309-20.

Frankel, A. D. & Young, J. A. 1998. HIV-1: fifteen proteins and an RNA. *Annu Rev Biochem,* 67**,** 1-25.

Freed, E. O. 2001. HIV-1 replication. *Somat Cell Mol Genet,* 26**,** 13-33.

Gilks, C. F., Crowley, S., Ekpini, R., Gove, S., Perriens, J., Souteyrand, Y., Sutherland, D., Vitoria, M., Guerma, T. & De Cock, K. 2006. The WHO public-health approach to antiretroviral treatment against HIV in resource-limited settings. *Lancet,* 368**,** 505-10.

Graham, S. M., Masese, L., Gitau, R., Jalalian-Lechak, Z., Richardson, B. A., Peshu, N., Mandaliya, K., Kiarie, J. N., Jaoko, W., Ndinya-Achola, J., Overbaugh, J. & McClelland, R. S. 2010. Antiretroviral adherence and development of drug resistance are the strongest predictors of genital HIV-1 shedding among women initiating treatment. *J Infect Dis,* 202**,** 1538-42.

Greig, J. E., du Cros, P. A., Mills, C., Ugwoeruchukwu, W., Etsetowaghan, A., Grillo, A., Tayo-Adetoro, A., Omiyale, K., Spelman, T. & O'Brien, D. P. 2013. Predictors of raised viral load during antiretroviral therapy in patients with and without prior antiretroviral use: a cross-sectional study. *PLoS One,* 8**,** e71407.

Grossman, Z., Paxinos, E. E., Averbuch, D., Maayan, S., Parkin, N. T., Engelhard, D., Lorber, M., Istomin, V., Shaked, Y., Mendelson, E., Ram, D., Petropoulos, C. J. & Schapiro, J. M. 2004. Mutation D30N is not preferentially selected by human immunodeficiency virus type 1 subtype C in the development of resistance to nelfinavir. *Antimicrob Agents Chemother,* 48**,** 2159-65.

Hanna, G. J. & D'Aquila, R. T. 2001. Clinical use of genotypic and phenotypic drug resistance testing to monitor antiretroviral chemotherapy. *Clin Infect Dis,* 32**,** 774-82.

Hassan, A. S., Mwaringa, S. M., Obonyo, C. A., Nabwera, H. M., Sanders, E. J., Rinke de Wit, T. F., Cane, P. A. & Berkley, J. A. 2013. Low prevalence of transmitted HIV type 1 drug resistance among antiretroviral-naive adults in a rural HIV clinic in Kenya. *AIDS Res Hum Retr,* 29**,** 129-35.

Hassan, A. S., Nabwera, H. M., Mwaringa, S. M., Obonyo, C. A., Sanders, E. J., Rinke de Wit, T. F., Cane, P. A. & Berkley, J. A. 2014. HIV-1 virologic failure and acquired drug resistance among first-line antiretroviral experienced adults at a rural HIV clinic in coastal Kenya: a cross-sectional study. *AIDS Res Ther,* 11**,** 9.

Haubrich, R. & Demeter, L. 2001. International perspectives on antiretroviral resistance. Clinical utility of resistance testing: retrospective and prospective data supporting use and current recommendations. *J Acquir Immune Defic Syndr,* 26 Suppl 1**,** S51-9.

Henderson, G. J., Lee, S. K., Irlbeck, D. M., Harris, J., Kline, M., Pollom, E., Parkin, N. & Swanstrom, R. 2012. Interplay between single resistance-associated mutations in the HIV-1 protease and viral infectivity, protease activity, and inhibitor sensitivity. *Antimicrob Agents Chemother,* 56**,** 623-33.

Hill, M., Tachedjian, G. & Mak, J. 2005. The packaging and maturation of the HIV-1 Pol proteins. *Curr HIV Res,* 3**,** 73-85.

Hirsch, M. S. & Richman, D. D. 2000. The role of genotypic resistance testing in selecting therapy for HIV. *JAMA,* 284**,** 1649-50.

Israel, G. D. 1992. Determining Sample Size. Available: http://www.soc.uoc.gr/socmedia/papageo/metaptyxiakoi/sample_size/samplesize1.pdf [Accessed 27, October, 2013].

IUPAC 1972. IUPAC-IUB Commission on Biochemical Nomenclature. Symbols for amino-acid derivatives and peptides. Recommendations (1971). *J Biol Chem,* 247**,** 977-83.

Johnson, V. A., Calvez, V., Gunthard, H. F., Paredes, R., Pillay, D., Shafer, R. W., Wensing, A. M. & Richman, D. D. 2013. Update of the drug resistance mutations in HIV-1: March 2013. *Top Antivir Med,* 21**,** 6-14.

Jordan, M. R., Bennett, D. E., Bertagnolio, S., Gilks, C. F. & Sutherland, D. 2008. World Health Organization surveys to monitor HIV drug resistance prevention and associated factors in sentinel antiretroviral treatment sites. *Antivir Ther,* 13 Suppl 2**,** 15-23.

Kelley, C. F., Barbour, J. D. & Hecht, F. M. 2007. The relation between symptoms, viral load, and viral load set point in primary HIV infection. *J Acquir Immune Defic Syndr,* 45**,** 445-8.

Kerr, T., Palepu, A., Barness, G., Walsh, J., Hogg, R., Montaner, J., Tyndall, M. & Wood, E. 2004. Psychosocial determinants of adherence to highly active antiretroviral therapy among injection drug users in Vancouver. *Antivir Ther,* 9**,** 407-14.

Konings, F. A., Zhong, P., Agwara, M., Agyingi, L., Zekeng, L., Achkar, J. M., Ewane, L., Saa, Afane Ze, E., Kinge, T. & Nyambi, P. N. 2004. Protease mutations in HIV-1 non-B strains infecting drug-naive villagers in Cameroon. *AIDS Res Hum Retr,* 20**,** 105-9.

Kowalski, J., Gange, S. J., Schneider, M. F., Tsai, H. L., Templeton, A., Shao, Q., Zhang, G. W., Yeh, M. F., Young, M. & Markham, R. B. 2009. Relationship of injection drug use, antiretroviral therapy resistance, and genetic diversity in the HIV-1 pol gene. *J Acquir Immune Defic Syndr,* 50**,** 381-9.

Ledergerber, B., Lundgren, J. D., Walker, A. S., Sabin, C., Justice, A., Reiss, P., Mussini, C., Wit, F., d'Arminio Monforte, A., Weber, R., Fusco, G., Staszewski, S., Law, M., Hogg, R., Lampe, F., Gill, M. J., Castelli, F. & Phillips, A. N. 2004. Predictors of trend in CD4-positive T-cell count and mortality among HIV-1-infected individuals with virological failure to all three antiretroviral-drug classes. *Lancet,* 364**,** 51-62.

Lihana, R. W., Khamadi, S. A., Lubano, K., Lwembe, R., Kiptoo, M. K., Lagat, N., Kinyua, J. G., Okoth, F. A., Songok, E. M., Makokha, E. P. & Ichimura, H. 2009. HIV type 1 subtype diversity and drug resistance among HIV type 1-infected Kenyan patients initiating antiretroviral therapy. *AIDS Res Hum Retr,* 25**,** 1211-7.

Maia Teixeira, S. L., Bastos, F. I., Hacker, M. A., Guimaraes, M. L. & Morgado, M. G. 2006. Trends in drug resistance mutations in antiretroviral-naive intravenous drug users of Rio de Janeiro. *J Med Virol,* 78**,** 764-9.

Mathers, B. M., Degenhardt, L., Ali, H., Wiessing, L., Hickman, M., Mattick, R. P., Myers, B., Ambekar, A. & Strathdee, S. A. 2010. HIV prevention, treatment, and care services for people who inject drugs: a systematic review of global, regional, and national coverage. *Lancet,* 375**,** 1014-28.

Meriki, H. D., Tufon, K. A., Afegenwi, M. H., Nyindem, B. A., Atanga, P. N., Anong, D. N., Cho-Ngwa, F. & Nkuo-Akenji, T. 2014. Immuno-haematologic and virologic responses and predictors of virologic failure in HIV-1 infected adults on first-line antiretroviral therapy in Cameroon. *Infect Dis Poverty,* 3**,** 5.

Michael, N. L. & Moore, J. P. 1999. HIV-1 entry inhibitors: evading the issue. *Nat Med,* 5**,** 740-2.

Mitsuya, Y., Winters, M. A., Fessel, W. J., Rhee, S. Y., Hurley, L., Horberg, M., Schiffer, C. A., Zolopa, A. R. & Shafer, R. W. 2006. N88D facilitates the co-occurrence of D30N and L90M and the development of multidrug resistance in HIV type 1 protease following nelfinavir treatment failure. *AIDS Res Hum Retr,* 22**,** 1300-5.

MOH & NASCOP. 2012. Most-At-Risk-Populations: Unveiling new evidence for accelerated programing. Available: http://nascop.or.ke/library/Marps/MARPs%20BOOK%20REPORT%20.pdf [Accessed 24th, July, 2013].

MOH & NASCOP. 2014. Guidelines on Use of Antiretroviral Drugs for Treating and Preventing HIV Infection: A rapid advice. Available: http://www.faces-kenya.org/wp-content/uploads/2014/04/Rapid-Advice-Booklet-2014-24-June-12-noon.pdf [Accessed 23, July, 2014].

Moore, D. M., Mermin, J., Awor, A., Yip, B., Hogg, R. S. & Montaner, J. S. 2006. Performance of immunologic responses in predicting viral load suppression: implications for monitoring patients in resource-limited settings. *J Acquir Immune Defic Syndr,* 43**,** 436-9.

MoPHS & NASCOP. 2010. National Guidelines for HIV Testing and Counselling in Kenya. Available: http://nascop.or.ke/library/HTC/National%20Guidelines%20for%20HTC%20in%20Kenya%202010.pdf [Accessed 25, July, 2013].

Munir, S., Thierry, S., Subra, F., Deprez, E. & Delelis, O. 2013. Quantitative analysis of the time-course of viral DNA forms during the HIV-1 life cycle. *Retrovirology,* 10**,** 87.

Musyoki, H. 2012. Overview of programs for people who inject drugs. *MARPs and Vulnerable Groups Program* [Online]. Available: http://www.uonbi.ac.ke/sites/default/files/chs/chs/An%20overview%20of%20programs%20for%20people%20who%20inject%20drugs.pdf.

NACC. 2009. KENYA HIV Prevention Response and Modes of Transmission Analysis. Available: http://www.nacc.or.ke/images/stories/Documents/KenyaMOT22March09Final.pdf [Accessed 25, July, 2013].

NACC & Office of the President. 2009. Kenya national HIV/AIDS strategic plan 2009/10 – 2012/13. Available: http://www.nacc.or.ke/nacc%20downloads/knasp_iii.pdf [Accessed 22nd, October, 2013].

NASCOP. 2012. Guidelines for antiretroviral therapy in Kenya 2011. Available: http://healthservices.uonbi.ac.ke/sites/default/files/centraladmin/healthservices/Kenya%20Treatment%20Guidelines%202011.pdf [Accessed 25, July, 2013].

NASCOP. 2014. Kenya AIDS Indicator Survey 2012: Final Report. Available: http://www.nacc.or.ke/attachments/article/403/KAIS_II_2014_Final_Report.pdf [Accessed 5th, August, 2014].

NASCOP & MOH. 2012. MARPs SURVEILLANCE REPORT 2012. Available: http://nascop.or.ke/library/Marps/MARPs%20BOOK%20REPORT%20.pdf [Accessed 25, July, 2013].

Nieburg, P. & Carty, L. 2011. HIV Prevention among injection drug users in Kenya and Tanzania: New opportunities for progress. Available: http://csis.org/files/publication/110428_Nieburg_HIV_Web.pdf [Accessed 27th,October,2013].

Nielsen, M. H., Pedersen, F. S. & Kjems, J. 2005. Molecular strategies to inhibit HIV-1 replication. *Retrovirology,* 2**,** 10.

Nijhuis, M., Schuurman, R., de Jong, D., Erickson, J., Gustchina, E., Albert, J., Schipper, P., Gulnik, S. & Boucher, C. A. 1999. Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. *AIDS,* 13**,** 2349-59.

O'Brien, T. R., Hoover, D. R., Rosenberg, P. S., Chen, B., Detels, R., Kingsley, L. A., Phair, J. & Saah, A. J. 1995. Evaluation of secular trends in CD4+ lymphocyte loss among human immunodeficiency virus type 1 (HIV-1)-infected men with known dates of seroconversion. *Am J Epidemiol,* 142**,** 636-42.

Osman, S., Lihana, R. W., Kibaya, R. M., Ishizaki, A., Bi, X., Okoth, F. A., Ichimura, H. & Lwembe, R. M. 2013. Diversity of HIV type 1 and drug resistance mutations among injecting drug users in Kenya. *AIDS Res Hum Retr,* 29**,** 187-90.

Palepu, A., Milloy, M. J., Kerr, T., Zhang, R. & Wood, E. 2011. Homelessness and adherence to antiretroviral therapy among a cohort of HIV-infected injection drug users. *J Urban Health,* 88**,** 545-55.

Parkin, N., Bremer, J. & Bertagnolio, S. 2012. Genotyping external quality assurance in the World Health Organization HIV drug resistance laboratory network during 2007-2010. *Clin Infect Dis,* 54 Suppl 4**,** S266-72.

Paton, N. I., Kityo, C., Hoppe, A., Reid, A., Kambugu, A., Lugemwa, A., van Oosterhout, J. J., Kiconco, M., Siika, A., Mwebaze, R., Abwola, M., Abongomera, G., Mweemba, A., Alima, H., Atwongyeire, D., Nyirenda, R., Boles, J., Thompson, J., Tumukunde, D., Chidziva, E., Mambule, I., Arribas, J. R., Easterbrook, P. J., Hakim, J., Walker, A.

S. & Mugyenyi, P. 2014. Assessment of second-line antiretroviral regimens for HIV therapy in Africa. *N Engl J Med,* 371**,** 234-47.

Pillay, V., Ledwaba, J., Hunt, G., Rakgotho, M., Singh, B., Makubalo, L., Bennett, D. E., Puren, A. & Morris, L. 2008. Antiretroviral drug resistance surveillance among drug-naive HIV-1-infected individuals in Gauteng Province, South Africa in 2002 and 2004. *Antivir Ther,* 13 Suppl 2**,** 101-7.

Rabi, S. A., Laird, G. M., Durand, C. M., Laskey, S., Shan, L., Bailey, J. R., Chioma, S., Moore, R. D. & Siliciano, R. F. 2013. Multi-step inhibition explains HIV-1 protease inhibitor pharmacodynamics and resistance. *J Clin Invest,* 123**,** 3848-60.

Rhee, S. Y., Gonzales, M. J., Kantor, R., Betts, B. J., Ravela, J. & Shafer, R. W. 2003. Human immunodeficiency virus reverse transcriptase and protease sequence database. *Nucleic Acids Res,* 31**,** 298-303.

Rhee, S. Y., Taylor, J., Fessel, W. J., Kaufman, D., Towner, W., Troia, P., Ruane, P., Hellinger, J., Shirvani, V., Zolopa, A. & Shafer, R. W. 2010. HIV-1 protease mutations and protease inhibitor cross-resistance. *Antimicrob Agents Chemother,* 54**,** 4253-61.

Ross, M. W., McCurdy, S. A., Kilonzo, G. P., Williams, M. L. & Leshabari, M. T. 2008. Drug use careers and blood-borne pathogen risk behavior in male and female Tanzanian heroin injectors. *Am J Trop Med Hyg,* 79**,** 338-43.

Salomon, J., de Truchis, P. & Melchior, J. C. 2002. Body composition and nutritional parameters in HIV and AIDS patients. *Clin Chem Lab Med,* 40**,** 1329-33.

Santos, A. F. & Soares, M. A. 2011. The impact of the nelfinavir resistance-conferring mutation D30N on the susceptibility of HIV-1 subtype B to other protease inhibitors. *Mem Inst Oswaldo Cruz,* 106**,** 177-81.

Santos, J. R., Llibre, J. M., Imaz, A., Domingo, P., Iribarren, J. A., Marino, A., Miralles, C., Galindo, M. J., Ornelas, A., Moreno, S., Schapiro, J. M., Clotet, B. & Call Conference, G. 2012. Mutations in the protease gene associated with virological failure to lopinavir/ritonavir-containing regimens. *J Antimicrob Chemother,* 67**,** 1462-9.

Schapiro, J. M., Scherer, J., Boucher, C. A., Baxter, J. D., Tilke, C., Perno, C. F., Maggiolo, F., Santoro, M. M. & Hall, D. B. 2010. Improving the prediction of virological response to tipranavir: the development and validation of a tipranavir-weighted mutation score. *Antivir Ther,* 15**,** 1011-9.

Scherrer, A. U., Ledergerber, B., von Wyl, V., Boni, J., Yerly, S., Klimkait, T., Cellerai, C., Furrer, H., Calmy, A., Cavassini, M., Elzi, L., Vernazza, P. L., Bernasconi, E. & Gunthard, H. F. 2012. Minor protease inhibitor mutations at baseline do not increase the risk for a virological failure in HIV-1 subtype B infected patients. *PLoS One,* 7**,** e37983.

Sethi, A. K., Celentano, D. D., Gange, S. J., Moore, R. D. & Gallant, J. E. 2003. Association between adherence to antiretroviral therapy and human immunodeficiency virus drug resistance. *Clin Infect Dis,* 37**,** 1112-8.

Shafer, R. W., Dupnik, K., Winters, M. A. & Eshleman, S. H. 2001. A Guide to HIV-1 Reverse Transcriptase and Protease Sequencing for Drug Resistance Studies. *HIV Seq Compend,* 2001**,** 1-51.

Shafer, R. W., Rhee, S. Y., Pillay, D., Miller, V., Sandstrom, P., Schapiro, J. M., Kuritzkes, D. R. & Bennett, D. 2007. HIV-1 protease and reverse transcriptase mutations for drug resistance surveillance. *AIDS,* 21**,** 215-23.

Shafer, R. W. & Schapiro, J. M. 2008. HIV-1 drug resistance mutations: an updated framework for the second decade of HAART. *AIDS Rev,* 10**,** 67-84.

Sigaloff, K. C., Mandaliya, K., Hamers, R. L., Otieno, F., Jao, I. M., Lyagoba, F., Magambo, B., Kapaata, A., Ndembi, N. & Rinke de Wit, T. F. 2012. Short communication: High prevalence of transmitted antiretroviral drug resistance among newly HIV type 1 diagnosed adults in Mombasa, Kenya. *AIDS Res Hum Retr,* 28**,** 1033-7.

Ssemwanga, D., Lihana, R. W., Ugoji, C., Abimiku, A., Nkengasong, J., Dakum, P. & Ndembi, N. 2015. Update on HIV-1 acquired and transmitted drug resistance in Africa. *AIDS Rev,* 17**,** 3-20.

Steegen, K., Luchters, S., Dauwe, K., Reynaerts, J., Mandaliya, K., Jaoko, W., Plum, J., Temmerman, M. & Verhofstede, C. 2009. Effectiveness of antiretroviral therapy and development of drug resistance in HIV-1 infected patients in Mombasa, Kenya. *AIDS Res Ther,* 6**,** 12.

Sundquist, W. I. & Krausslich, H. G. 2012. HIV-1 assembly, budding, and maturation. *Cold Spring Harb Perspect Med,* 2**,** a006924.

Suttmann, U., Ockenga, J., Selberg, O., Hoogestraat, L., Deicher, H. & Muller, M. J. 1995. Incidence and prognostic value of malnutrition and wasting in human immunodeficiency virus-infected outpatients. *J Acquir Immune Defic Syndr Hum Retr,* 8**,** 239-46.

U.S. FDA. 2014 *Antiretroviral drugs used in the treatment of HIV infection* [Online]. New Hampshire Avenue. Available: http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm [Accessed 22nd, Aug. 2014.

UNAIDS. 2012. Global report: UNAIDS report on the global AIDS epidemic 2012. Available: http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2012/gr2012/20121120_UNAIDS_Global_Report_2012_with_annexes_en.pdf [Accessed 25, July, 2013].

UNAIDS. 2013. Global report: UNAIDS report on the global AIDS epidemic 2013. Available: http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/UNAIDS_Global_Report_2013_en.pdf [Accessed 5/12/2013].

UNAIDS. 2014. 90-90-90 An ambitious treatment target to help end the AIDS epidemic. Available: http://www.unaids.org/sites/default/files/media_asset/90-90-90_en_0.pdf.

UNODC. 2013. World Drug Report 2013. Available: http://www.unodc.org/unodc/secured/wdr/wdr2013/World_Drug_Report_2013.pdf [Accessed 25, July, 2013].

UNODC. 2014. World Drug Report 2014. Available: https://www.unodc.org/documents/wdr2014/World_Drug_Report_2014_web.pdf [Accessed 6th, Oct. 2014].

Valente, M. J., Carvalho, F., Bastos, M., de Pinho, P. G. & Carvalho, M. 2012. Contribution of oxidative metabolism to cocaine-induced liver and kidney damage. *Curr Med Chem,* 19**,** 5601-6.

Van der Sande, M. A., Schim van der Loeff, M. F., Aveika, A. A., Sabally, S., Togun, T., Sarge-Njie, R., Alabi, A. S., Jaye, A., Corrah, T. & Whittle, H. C. 2004. Body mass index at time of HIV diagnosis: a strong and independent predictor of survival. *J Acquir Immune Defic Syndr,* 37**,** 1288-94.

van Maarseveen, N. M., de Jong, D., Boucher, C. A. & Nijhuis, M. 2006. An increase in viral replicative capacity drives the evolution of protease inhibitor-resistant human immunodeficiency virus type 1 in the absence of drugs. *J Acquir Immune Defic Syndr,* 42**,** 162-8.

Varghese, V., Mitsuya, Y., Fessel, W. J., Liu, T. F., Melikian, G. L., Katzenstein, D. A., Schiffer, C. A., Holmes, S. P. & Shafer, R. W. 2013. Prototypical Recombinant Multi-Protease Inhibitor Resistant Infectious Molecular Clones of Human Immunodeficiency Virus Type-1. *Antimicrob Agents Chemother*.

Vlahov, D. & Celentano, D. D. 2006. Access to highly active antiretroviral therapy for injection drug users: adherence, resistance, and death. *Cad Saude Publica,* 22**,** 705-18.

Watts, J. M., Dang, K. K., Gorelick, R. J., Leonard, C. W., Bess, J. W., Jr., Swanstrom, R., Burch, C. L. & Weeks, K. M. 2009. Architecture and secondary structure of an entire HIV-1 RNA genome. *Nature,* 460**,** 711-6.

Wensing, A. M., Calvez, V., Gunthard, H. F., Johnson, V. A., Paredes, R., Pillay, D., Shafer, R. W. & Richman, D. D. 2014. 2014 update of the drug resistance mutations in HIV-1. *Top Antivir Med,* 22**,** 642-50.

Were, T., Wesongah, J. O., Munde, E., Ouma, C., Kahiga, T. M., Ongecha-Owuor, F., Kiarie, J. N., Ahmed, A. A., Makokha, E. P. & Budambula, V. 2014. Clinical chemistry profiles in injection heroin users from Coastal Region, Kenya. *BMC Clin Pathol,* 14**,** 32.

WHO. 2012. WHO manual for HIV drug resistance testing using dried blood spot specimens. WHO/HIV/2012.30. Available: http://apps.who.int/iris/bitstream/10665/75829/1/WHO_HIV_2012.30_eng.pdf?ua=1 [Accessed July, 2013].

WHO. 2014a. *Global database on body mass index* [Online]. Geneva, Switzerland. Available: http://apps.who.int/bmi/index.jsp?introPage=intro_3.html [Accessed 21, 9 2014].

WHO 2014b. March 2014 supplement to the 2013 consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection. Recommendations for a public health approach.

Wood, E., Hogg, R. S., Yip, B., Dong, W. W., Wynhoven, B., Mo, T., Brumme, C. J., Montaner, J. S. & Harrigan, P. R. 2005. Rates of antiretroviral resistance among HIV-infected patients with and without a history of injection drug use. *AIDS,* 19**,** 1189-95.

World Atlas. 2015. *Where is Mombasa?* [Online]. Available: http://www.worldatlas.com/af/ke/28/where-is-mombasa.html.

Zhou, Z., Wagar, N., DeVos, J. R., Rottinghaus, E., Diallo, K., Nguyen, D. B., Bassey, O., Ugbena, R., Wadonda-Kabondo, N., McConnell, M. S., Zulu, I., Chilima, B., Nkengasong, J. & Yang, C. 2011. Optimization of a low cost and broadly sensitive genotyping assay for HIV-1 drug resistance surveillance and monitoring in resource-limited settings. *PLoS One,* 6**,** e28184.

Zimmer, J. M., Roman, F., Lambert, C., Jonckheer, A., Vazquez, A., Plesseria, J. M., Servais, J. Y., Covens, K., Weber, J., Van Laethem, K., Schmit, J. C., Vandamme, A. M., Quinones-Mateu, M. E. & De Maeyer, M. 2008. Impact on replicative fitness of the G48E substitution in the protease of HIV-1: an in vitro and in silico evaluation. *J Acquir Immune Defic Syndr,* 48**,** 255-62.

APPENDICES

APPENDIX 1: INFORMED CONSENT FORM

2(a): Informed consent in English

MASENO UNIVERSITY

SCHOOL OF PUBLIC HEALTH AND COMMUNITY DEVELOPMENT

Title of the study: HIV-1 protease inhibitor drug resistance mutations (PI-DRMs) and their socio-demographic and clinical determinants among ART-naive and -experienced injection drug users from Mombasa County, Kenya

A. Introduction

Human Immunodeficiency Virus (HIV) is a virus that causes the disease called AIDS (Acquired Immunodeficiency Syndrome). HIV is mainly spread through having unprotected sex, sharing needles or receiving blood and/or blood products or other tissues that are infected with HIV. Injection drug users have the greatest risk of being infected and transmitting HIV. As part of this research, we intend to determine your HIV status and thereafter know your history concerning the use of addictive drugs and substances. It is important for you to know the risks and benefits before you are tested and enrolled to this study. In addition, you may decide not to take part to this study for whatever reason.

This information form therefore seeks your informed consent for your participation in this study that seeks to determine HIV-1 major and minor protease inhibitor drug resistance in antiretroviral treatment-naive and -experienced injection drug users from Mombasa County, Kenya.

Please read this consent form with care so that you can make an informed choice about participating in this study.

B. Purpose of the study

The objective of this study is:

> *To the frequencies of HIV-1 major and minor protease inhibitor drug resistance mutations (PI-DRMs) and their socio-demographic and clinical determinants among ART-naive and -experienced injection drug users from Mombasa County, Kenya*

Procedure

If you agree to participate in this study, you will receive an identification number. A trained interviewer will ask you several questions on your HIV status, substance abuse and the risk factors associated with use of injection drugs. The questions will be about your socio-demographic characteristics, drug and substance abuse, HIV status and whether you are attending HIV Comprehensive Care Centres (CCC).

We will draw about 250 µL of blood from a finger prick to test you for HIV. If your test is positive, it means you have or may have the HIV virus and that you can pass it on to others. We will then draw 5-8mls of blood from your arm for further testing (CD4+ T-cell count, viral load and HIV drug resistance mutations). If you are not currently enrolled to any HIV comprehensive care centres (CCC), we will enrol you at Bomu hospital or refer you to a CCC centre nearest to you or that of your choice.

C. Risks/ Discomforts

This study does not have adverse physical risks. However, drawing of blood for this test may cause pain or bruising where the needle enters the vein. could be a minor invasion to your

privacy when sensitive questions are being asked. Being tested and learning your HIV test results may cause you and any partner(s) stress and anxiety. If the test is negative, you might be tempted to have unsafe sex which would increase your risk of becoming infected with HIV. If others find out your test is positive it might result in discrimination.

D. Benefits

By participating in this study, you will benefit from free health education on the risks of drug injection, prevention of HIV transmission, sexually transmitted infections, good hygiene and nutrition. You will also be enrolled to a comprehensive care centre as well as attend free rehabilitation training for those willing to quit taking drugs.

E. Confidentiality

Information that identifies you will be kept secure and strictly confidential. We will do our best to protect all the information we collect from you and your medical history. Your name and other identifiers will not be used if information from this research is published or presented at scientific meetings. When this research is complete, all information which identifies you will be destroyed.

F. Voluntariness

Your participation in this study is voluntary. You will be required to answer the interview questions in the provided form and later provide 3 ml of venous blood. You are free to choose whether to participate in this study or not. You are also free to withdraw from the study at any time you wish to do so.

G. Who to contact

You are encouraged to ask any questions to clarify any issues at any time during your participation in the study. If you need more information on the study, below are the contacts of persons coordinating the study.

H. Declaration

I have read and understood the study information. I have been given the opportunity to ask questions about the study. I understand that my taking part is voluntary; I can withdraw from the study at any time and I will not be asked any questions about why I no longer want to take part. I understand my personal details will be kept private. I hereby consent to participate in the study as explained and as I have understood.

Participants' signature: _____ Date: ____/____/_____

Researcher's name: _____

Researcher's signature: _____ Date: ____/____/_____

Note: For any clarifications or inquiry, feel free to contact
Principle investigator: Dr. Tom Were, PhD (0720326127)
KU-ERC kuerc.chairman@ku.ac.ke and kuerc.secretary@ku.ac.ke

2(b): INFORMED CONSENT IN SWAHILI/ FOMU YA KUOMBA RIDHAA

CHUO KIKUU CHA MASENO

IDARA YA AFYA YA UMMA NA MAENDELEO YA JAMII

Jina la utafiti: HIV-1 major and minor protease inhibitor drug resistance mutations (PI-DRMs) and their socio-demographic and clinical determinants among ART-naive and -experienced injection drug users from Mombasa County, Kenya

A. Utangulizi

Virusi vya HIV husababisha ugonjwa wa UKIMWI. Virusi hivi vya HIV vinaweza kusambazwa kupitia kufanya ngono bila kinga, kutumia sindano moja au kupokea damu, bidhaa damu au tishu nyingine na watu walioathirika na UKIMWI. Watumiaji wa madawa ya kujidunga wamo katika hatari kubwa ya kuambukizwa na pia kusambaza virusi vya HIV. Kama sehemu ya utafiti huu, tutakupima ilitujua ikiwa una virusi ya HIV na pia,tutataka kufahamu historia yako kuhusu matumizi ya madawa ya kulevya. Yakupasa kujua hatari na faida kabla ya wewe kupimwa na kushiriki katika utafiti huu. Aidha, unaweza kuamua kutoshiriki katika utafiti huu kwa sababu yoyote ile.

Fomu hii ina habari inayoomba ridhaa yako ili ushiriki kwenye utafiti utakaofanywa ilikubaini vipimo vya chembe chembe zinazosababisha madawa ya UKIMWI kutofanya kazi katika watu wanaotumia madawa ya kulevya kwa kujidunga au kutojidunga na walio au wasiokua kwenye matibabu ya ART wanoishi kata ya Mombasa Kenya.

Tafadhali soma fomu hii kwa makini ya idhini kwa makini ili uweze kufanya uchaguzi sahihi kuhusu kushiriki katika utafiti huu.

B. Madhumuni

Utafiti huu utafanywa ili:

> *Kubaini chembe chembe kuu na mbadala zinazosababisha madawa ya UKIMWI kutofanya kazi pamoja na uhusiano wake na dalili za kiafya za gonjwa la UKIMWI katika watu wanaotumia madawa ya kulevya kwa kujidunga ambao wanapata au kutopata matibabu ya ART wanoishi kata ya Mombasa Kenya.*

C. Utaratibu

Ukikubali kushiriki katika utafiti huu, utapewa nambari ya utambulisho. Mhojaji aliyehitimu atakuuliza maswali kuhusu ya hali yako ya UKIMWI, utumizi wa madawa ya kulevya na mambo ya hatari yanayotokana na kujidunga dawa za kulevya. Maswali haya haswa yatahusu tabia na hali zako za kimaisha, madawa ya kulevya na jinsi unavyoyatumia, hali yako ya UKIMWI na ikiwa wewe unahudhuria vituo vinavyo toa huduma kwa waasiriwa wa UKIMWI (CCC).

Ilikupimwa virusi vya HIV, utatolewa microlita 250 ya damu kwenya kidole. Kipimo hiki hakibaini virusi vya HIV bali kinabaini ikiwa mwili wako unaonyesha athari za virusi vya HIV. Ikibainika kuwa wewe ni muathirika, ina maana kuwa wewe una virusi vya HIV na unaweza kuwaambukiza watu wengine. Hivyo basi, tutakutoa mililita kati ya 5 au 8 ya damu kutoka kwko wa kwa vipimo zaidi vya UKIMWI (seli za CD4, kiwango cha virusi, na chembe chembe zinazosababisha madawa ya UKIMWI kutofanya kazi). Ikiwa wewe haujajiandikisha katika kituo cha kutoa huduma kwa waasiriwa wa UKIMWI (CCC), tutakuandikisha katika hospitali ya Bomu au tukuelekeze katika kituo cha CCC kilichokaribu nawe au kile kituo utakachokipenda.

D. Madhara au changamoto

Utafiti huu hauna hatari kubwa za kimwili. Hata hivyo, utoaji wa damu unaweza kusababisha uchungu mdogo au kugwarushwa katika sehemu ambayo sindano itaingia katika mshipa wa damu. Baadhi ya maswali nyeti yatakayo ulizwa yanaweza kusababisha uvamizi madogo ya faragha yako. Pia, kupimwa na kujua matokeo yako ya HIV inaweza kusababisha wewe au unayeshiriki naye tendo la ngono dhiki au wasiwasi. Istoshe, kwa wale ambao si waathiriwa, wanaweza kuwa walegevu na kuanza kufanya ngono bila kinga. Tendo hili litawatia katika hatari ya kuambukizwa virusi vya HIV. Kwa upande mwinginw, ikiwa mtu atagunduliwa kuwa na virusi ya HIV, inaweza sababisha kubaguliwa na wenzake.

E. Manufaa

Kwa kushiriki katika utafiti huu, utapewa mafunzo ya bure kuhusu afya, madhara yanayotokana na kujidunga madawa ya kulevya, kuzuia kuenea kwa virusi vya HIV, magonjwa ya zinaa, usafi na afya njema. Licha ya hayo, utaandikishwa katika kituo cha kutoa huduma kwa waasiriwa wa UKIMWI (CCC) pamoja na kupewa huduma za bure katika kituo cha kurekebisha tabia kwa wale watakao taka kuwacha kutumia madawa ya kulevya.

F. Njia mbadala za kushiriki

Ukiamua kutoshiriki utafiti huu hakuna mtu yeyote ambaye atakulazimisha kufanya hivyo, kwa hivyo utakuwa huru kufanya uamuzi wako mwenyewe na pia utajichagulia kushiriki kwenye utafiti mwingine wa siku zijazo.

G. Usiri

Habari zinazoweza kutuiwa kukutambua zitawekwa salama na kwa siri . Tutafanya juhudi zote zile kulinda habari zote tutakazokusanya kutoka kwako pamoja na historia yako ya matibabu. Jina lako na vitambulisho vingine havitatumika ikiwa taarifa kutokana na utafiti

huu itachapishwa au kuwasilishwa katika mikutano ya kisayansi. Utafiti huu utakapokamilika, taarifa zote zinakuhusu zitaharibiwa.

H. Hiari

Ushirikiano wako kwenye utafiti huu ambao utakuwa kwa njia ya mahojiano ya moja kwa moja pamoja na kutolewa mililita tatu za damu ni kwa kujitolea na kwa hiari yako. Utakuwa huru kuchagua kama utashiriki au kutoshiriki utafiti huu pia, utakuwa huru kujiondoa kwenye utafiti huu wakati wowote utakaotaka.

Nani wa kuwasiliana nao

Unashauriwa kuuliza maswali yoyote ilikubaini maswala yote yanayoibuka wakati wa kushiriki kwenye utafiti. Kama utahitaji habari au mambo mengine kuhusu utafiti huu haya ndiyo majina ya wale ambao watahakikisha utafiti huu utafanyika bila tashwishi.

I. Mkataba au tangazo

Nimesoma na nimeelewa habari inayohusiana na utafiti huu. Nimepatiwa nafasi kuuliza maswali yanayohusiana na utafiti huu. Nimeelewa kwamba kushiriki kwangu ni wa kujitolea kwa hiari na ninaweza kujiondoa kwenye utafiti wakati wowote na sitaulizwa maswali kama vile mbona haushiriki tena kwenye utafiti. Ninaelewa kuwa mambo yanayonihusu yatawekwa kwa siri kikamilifu kwa hivyo nimekubali kushiriki kwenye utafiti huu kama vile nimeelezwa na kuelewa.

Sahihi ya mshiriki: _____ Tarehe: ____/___/___

Jina la mtafiti: _____

Sahihi ya mtafiti: _____Tarehe:___/____/___

Note: Ukihitaji maelezo zaidi usisite kuwasiliana na:
Mtafiti Mkuu: Dr. Tom Were, PhD (0720326127)
KU-ERC kuerc.chairman@ku.ac.ke na kuerc.secretary@ku.ac.ke

APPENDIX 2: QUESTIONNAIRE

Interview to determine HIV-1 major and minor protease inhibitor drug resistance mutations (PI-DRMs) and their socio-demographic and clinical determinants among ART-naive and -experienced injection drug users from Mombasa County, Kenya

Good morning/afternoon, my name is ……from Maseno University. I am currently conducting a study to determine HIV-1 major and minor protease inhibitor drug resistance mutations in antiretroviral treatment-naive and -experienced injection drug users from Mombasa County, Kenya. Kindly allow me ask you a few questions to make this study successful. The information you give will strictly be kept anonymous and confidential and will be used solely for the purposes of the study.

A. IDENTIFICATION

Participant code _____

Date of interview _____/_____/_____ (date/month/year)

Time of interview (Start) _____ (Finish) _____

Language of interview _____

B. SOCIAL DEMOGRAPHIC CHARACTERISTICS

i. Age _____ (Years) Birth date: _____/_____/_____

ii. Gender: ☐Male ☐Female

C. SUBSTANCE AND DRUG ABUSE

i. Have you ever taken any addictive drugs? ☐Yes ☐No

If yes,

Which drugs have you ever used?

I. …… IV. ……

II. …….. V. ……..

III. …….. VI. ……..

ii. For how long have you been using these drugs? ……(Weeks/ Months/ Years)

How often do you use these drugs?

☐ Once [Daily, weekly, Monthly, Yearly]

☐ Twice [Daily, weekly, Monthly, Yearly]

☐ Thrice [Daily, weekly, Monthly, Yearly]

☐ More than Thrice [Daily, weekly, Monthly, Yearly]

iii. Have you ever injected yourself any drug? ☐ Yes ☐ No

If yes;

For how long have you been injecting drugs? …… (Weeks / Months/ Years)

How often do you inject drugs?

☐ Once [Daily, weekly, Monthly, Yearly]

☐ Twice [Daily, weekly, Monthly, Yearly]

☐ Thrice [Daily, weekly, Monthly, Yearly]

☐ More than Thrice [Daily, weekly, Monthly, Yearly]

iv. Have you ever shared a needle/syringe with any one? ☐ Yes ☐ No

If yes;

For how long have you been sharing needles/syringes?……(Weeks/ Months/ Years)

How many people have you shared with?

☐ Only one person: …….. ☐ Once, ☐ Twice, ☐ More than Twice

☐ Two people: …….. ☐ Once, ☐ Twice, ☐ More than Twice

☐ More than three different people ….. ☐ Once, ☐ Twice, ☐ More than Twice

How often do you share?

☐ Once [Daily, weekly, Monthly, Yearly]

☐ Twice [Daily, weekly, Monthly, Yearly]

☐ Thrice [Daily, weekly, Monthly, Yearly]

☐ More than Thrice [Daily, weekly, Monthly, Yearly]

v. Have you ever "flushed blood" in order to get 'high'? ☐ Yes ☐ No

If yes;

For how long have you been "flushing blood"? …… (Weeks / Months/ Years)

How many people have you "flushed blood" from?

☐ Only one person: …….. ☐ Once, ☐ Twice, ☐ More than Twice

☐ Two people: …….. ☐ Once, ☐ Twice, ☐ More than Twice

☐ More than three different people ….. ☐ Once, ☐ Twice, ☐ More than Twice

How often do you "flush blood"?

☐ Once [Daily, weekly, Monthly, Yearly]

☐ Twice [Daily, weekly, Monthly, Yearly]

☐ Thrice [Daily, weekly, Monthly, Yearly]

☐ More than Thrice [Daily, weekly, Monthly, Yearly]

D. ANTIRETROVIRAL HISTORY

i. Are you currently enrolled to a comprehensive care centre? ☐ Yes ☐ No

If yes;

Are you taking ARVs? ☐ Yes ☐ No

For how long have you been taking ARVs? …… (Weeks / Months/ Years)

ii. Have you ever skipped taking your ARVs? ☐ Yes ☐ No

If yes;

How often do you skip?

☐ Once [Daily, weekly, Monthly, Yearly]

☐ Twice [Daily, weekly, Monthly, Yearly]

☐ Thrice [Daily, weekly, Monthly, Yearly]

☐ More than Thrice [Daily, weekly, Monthly, Yearly]

APPENDIX 3: QIAamp DNA EXTRACTION PROTOCOL FROM DRIED BLOOD SPOTS

A. Introduction

QIAamp DNA Mini and QIAamp DNA Blood Mini Kits provide fast and easy methods for purification of total DNA for reliable PCR and Southern blotting. Total DNA (e.g., genomic, viral, mitochondrial) can be purified from whole blood, plasma, serum, buffy coat, bone marrow, other body fluids, lymphocytes, cultured cells, tissue, and forensic specimens.

The simple QIAamp spin and vacuum procedures, which are ideal for simultaneous processing of multiple samples, yield pure DNA ready for direct amplification in just 20 minutes. The QIAamp spin procedures can be fully automated on the QIAcube® for increased standardization and ease of use. The QIAamp procedure is suitable for use with fresh or frozen whole blood and blood which has been treated with citrate, heparin, or EDTA. Prior separation of leukocytes is not necessary.

Purification requires no phenol/chloroform extraction or alcohol precipitation, and involves very little handling. DNA is eluted in Buffer AE or water, ready for direct addition to PCR or other enzymatic reactions. Alternatively, it can be safely stored at -20°C for later use. The purified DNA is free of protein, nucleases, and other contaminants or inhibitors.

DNA purified using QIAamp Kits is up to 50 kb in size, with fragments of approximately 20-30 kb predominating. DNA of this length denatures completely during thermal cycling and can be amplified with high efficiency.

For purification of genomic DNA from blood for in vitro diagnostics in Europe, the QIAamp DSP DNA Blood Mini Kit is CE-IVD-marked, compliant with EU Directive 98/79/EC. This kit is not available in the USA and Canada.

B. Purification of viral RNA and DNA

For purification of viral RNA, the QIAamp Viral RNA Mini Kit is recommended. All buffers and components are guaranteed to be RNase-free. Alternatively, for simultaneous purification of viral DNA and RNA, we recommend using the QIAamp MinElute® Virus Vacuum Kit or the QIAamp MinElute Virus Spin Kit. These kits provide viral nucleic acid purification with minimal elution volumes for higher sensitivity in downstream applications. All buffers and components of these kits are guaranteed to be RNase-free. Viral nucleic acid purification using the QIAamp MinElute Virus Spin Kit or the QIAamp Viral RNA Mini Kit can be fully automated on the QIAcube for increased standardization and ease of use. Purification of viral DNA is possible with QIAamp DNA Mini or QIAamp DNA Blood Mini Kits. Since cellular DNA copurifies with viral DNA, cell-free samples (e.g., plasma, serum) are necessary to obtain pure viral DNA.

For purification of viral nucleic acids for in vitro diagnostics in Europe, the QIAamp DSP Virus Kit is CE-IVD-marked, compliant with EU Directive 98/79/EC. This kit is not available in the USA and Canada.

C. Principle and procedure

QIAamp DNA Mini and QIAamp DNA Blood Mini Kits are designed for rapid purification of an average of 6 µg of total DNA (e.g., genomic, viral, mitochondrial) from 200 µL of whole human blood, and up to 50 µg of DNA from 200 µL of buffy coat, 5 x10^6 lymphocytes, or cultured cells that have a normal set of chromosomes. The procedure is suitable for use with whole blood treated with citrate, heparin, or EDTA;* buffy coat; lymphocytes; plasma; serum; and body fluids. Samples may be either fresh or frozen. For

larger volumes of whole blood or cultured cells, we recommend using QIAamp DNA Blood Midi or Maxi Kits.

The QIAamp DNA Mini Kit performs all the functions of the QIAamp DNA Blood Mini Kit, and also allows rapid purification of DNA from solid tissue. On average, up to 30 µg of DNA can be purified from 25 mg of various human tissues.

D. Lysis with QIAGEN Protease or proteinase K

QIAamp DNA Blood Mini Kits contain QIAGEN Protease. Intensive research has shown that QIAGEN Protease is the optimal enzyme for use with the lysis buffer provided in the QIAamp DNA Blood Mini Kit. QIAGEN Protease is completely free of DNAse and RNAse activity.

When using the QIAamp DNA Blood Mini Kit for a sample that requires a modified protocol, please contact our Technical Service Department for advice about whether your lysis conditions are compatible with QIAGEN Protease. When >8 mM EDTA is used in conjunction with >0.5% SDS,* QIAGEN Protease activity decreases. For samples that require an SDS-containing lysis buffer or that contain high levels of EDTA, the QIAamp DNA Mini Kit is recommended. The QIAamp DNA Mini Kit contains proteinase K, which is the enzyme of choice for SDS-containing lysis buffers used in the Tissue Protocol, but which performs equally well in the Blood and Body Fluid Protocol.

The activity of the proteinase K solution is 600 mAU/ml solution (or 40 mAU/mg protein). This activity provides optimal results in QIAamp protocols.

E. Purification on QIAamp Mini spin columns

The QIAamp DNA purification procedure comprises four steps and is carried out using QIAamp Mini spin columns in a standard microcentrifuge, on a vacuum manifold, or fully

automated on the QIAcube. The procedures are designed to ensure that there is no sample-to-sample cross-contamination and allow safe handling of potentially infectious samples.

QIAamp Mini spin columns fit into most standard microcentrifuge tubes. In the spin protocol, due to the volume of filtrate, 2 ml collection tubes (provided) are required to support the QIAamp Mini spin column during loading and wash steps. Eluted DNA can be collected in standard 1.5 ml microcentrifuge tubes (not provided).

F. Adsorption to the QIAamp membrane

The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the QIAamp membrane before the sample is loaded onto the QIAamp Mini spin column. DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation or vacuum step. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane. If the initial sample volume is larger than 200 µL, it will be necessary to load the lysate onto the QIAamp Mini spin column in several steps. If larger sample volumes are required, we suggest using QIAamp DNA Blood Midi or Maxi Kits (Midi: 1–2 ml; Maxi: 5–10 ml starting material).

G. Removal of residual contaminants

DNA bound to the QIAamp membrane is washed in 2 centrifugation or vacuum steps. The use of 2 different wash buffers, Buffer AW1 and Buffer AW2, significantly improves the purity of the eluted DNA. Wash conditions ensure complete removal of any residual contaminants without affecting DNA binding.

H. Elution of pure nucleic acids

Purified DNA is eluted from the QIAamp Mini spin column in a concentrated form in either Buffer AE or water. Elution buffer should be equilibrated to room temperature (15–25°C) before it is applied to the column. Yields will be increased if the QIAamp Mini spin column is incubated with the elution buffer at room temperature for 5 minutes before centrifugation. The eluted genomic DNA is up to 50 kb in length (predominantly 20–30 kb) and is suitable for direct use in PCR or Southern-blotting applications.

If the purified DNA is to be stored, elution in Buffer AE (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0) and storage at –20°C is recommended. If high pH or EDTA affects sensitive downstream applications, use water for elution. However, ensure that the pH of the water is at least 7.0 (deionized water from certain sources can be acidic). DNA stored in water is subject to degradation by acid hydrolysis.

I. Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Ethanol (96–100%)*
- 1.5 ml microcentrifuge tubes
- Pipet tips with aerosol barrier
- Microcentrifuge (with rotor for 2 ml tubes)
- Vortexer
- Water bath or heating block at 56°C
- Phosphate-buffered saline (PBS) may be required for some samples

J. Preparation of reagents

QIAGEN Protease stock solution (store at 2–8°C or –20°C)

When using the QIAamp DNA Blood Mini Kit (50), pipette 1.2 ml protease solvent* into the vial containing lyophilized QIAGEN Protease, as indicated on the label. When using the QIAamp DNA Blood Mini Kit (250), pipette 5.5 ml protease solvent into the vial containing lyophilized QIAGEN Protease, as indicated on the label.

Dissolved QIAGEN Protease is stable for up to 2 months when stored at 2–8°C. Storage at –20°C is recommended to prolong the life of QIAGEN Protease, but repeated freezing and thawing should be avoided. For this reason, storage of aliquots of QIAGEN Protease is recommended.

Note: If you also use QIAamp MinElute Virus Kits, be aware that the QIAGEN Protease supplied with these kits is reconstituted in protease resuspension buffer or Buffer AVE and is not compatible with the QIAamp DNA Blood Mini Kit. After reconstituting a vial of QIAGEN Protease, label the resuspended QIAGEN Protease to indicate which buffer was used for resuspension.

Buffer AL† (store at room temperature, 15–25°C)

Mix Buffer AL thoroughly by shaking before use. Buffer AL is stable for 1 year when stored at room temperature.

Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

Buffer AW1† (store at room temperature, 15–25°C)

Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle.

Buffer AW1 is stable for 1 year when stored closed at room temperature.

Buffer AW2* (store at room temperature, 15–25°C)

Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle.

Buffer AW2 is stable for 1 year when stored closed at room temperature.

Protocol: DNA Purification from Dried Blood Spots **(QIAamp DNA Mini Kit)**

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from blood, both untreated and treated with anticoagulants, which has been spotted and dried on filter paper (Schleicher and Schuell 903).

Important point before starting

All centrifugation steps are carried out at room temperature (15–25°C).

Things to do before starting

Prepare an 85°C water bath for use in step 2, a 56°C water bath for use in step 3, and a 70°C water bath for use in step 4.

Equilibrate Buffer AE or distilled water to room temperature for elution in step 10.

Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on page 17.

If a precipitate has formed in Buffer AL or Buffer ATL, dissolve by incubating at 56°C.

K. Procedure

1. **Place 3 punched-out circles from a dried blood spot into a 1.5 ml microcentrifuge tube and add 180µl of Buffer ATL.**

Cut 3 mm (1/8 inch) diameter punches from a dried blood spot with a single-hole paper puncher.

2. **Incubate at 85°C for 10 minutes. Briefly centrifuge to remove drops from inside the lid.**

3. **Add 20µl proteinase K stock solution. Mix by vortexing, and incubate at 56°C for 1hour. Briefly centrifuge to remove drops from inside the lid.**

Note: The addition of proteinase K is essential.

4. **Add 200µL Buffer AL to the sample. Mix thoroughly by vortexing, and incubate at 70°C for 10 minutes. Briefly centrifuge to remove drops from inside the lid.**

In order to ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly.

Note: Do not add proteinase K directly to Buffer AL. A white precipitate may form when Buffer AL is added to the sample. In most cases, the precipitate will dissolve during incubation. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5. **Add 200µL ethanol (96–100%) to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove drops from inside the lid.**

It is essential that the sample and ethanol are mixed thoroughly.

6. **Carefully apply the mixture from step 5 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.**

Close each QIAamp Mini spin column in order to avoid aerosol formation during centrifugation.

7. **Carefully open the QIAamp Mini spin column and add 500µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 Xg (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.**
8. **Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.**
9. **Recommended: Place the QIAamp Mini spin column in a new 2ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

This step helps to eliminate the chance of possible Buffer AW2 carryover.

10. **Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 150 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 xg (8000 rpm) for 1 min.**

Three punched-out circles (3 mm diameter) typically yield 150 ng and 75 ng of DNA from anticoagulated and untreated blood, respectively. If the yield from untreated blood is not sufficient, use 6 circles per prep instead of 3.

The volume of the DNA eluate used in a PCR assay should not exceed 10%; for example, for a 50 µl PCR reaction, add no more than 5µl of the eluate.

APPENDIX 4: STUDY PLATES

Plate 1: Protease gene PCR Gel Image

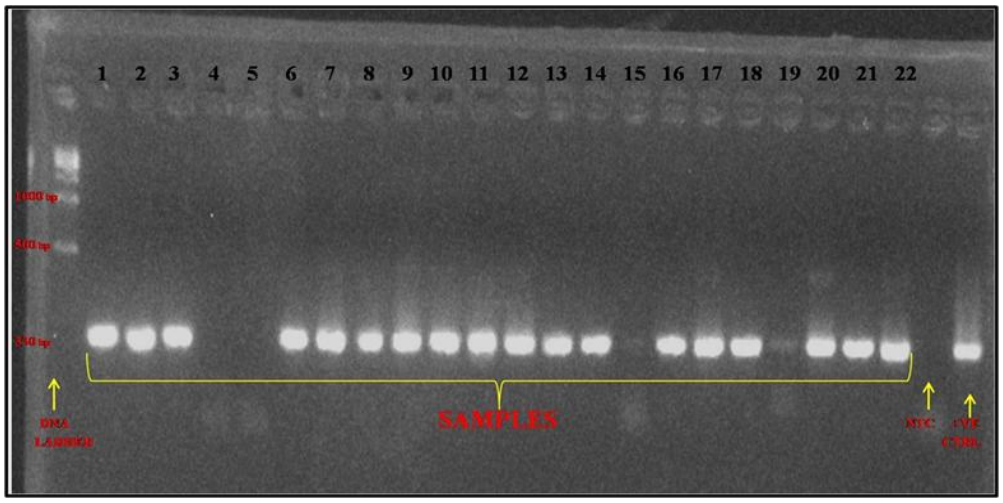

Plate showing a gel image of HIV-1 *protease* gene amplification from selected samples. Abbreviations and legend: NTC, Known negative control; +VE CTRL, Known positive control; DNA ladder, molecular weight marker; 1-22, test samples

APPENDIX 5: ETHICAL APPROVAL

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Fax: 8711242/8711575
Email: kuerc.chairman@ku.ac.ke
kuerc.secretary@ku.ac.ke
Website: www.ku.ac.ke

P. O. Box 43844,
Nairobi, 00100
Tel: 8710901/12

Our Ref: KU/R/COMM/51/32-4

Date: June 6th, 2012

Valentine Budambula
School of Public Health,
Kenyatta University
P.O. Box 43844, Nairobi.

Dear Ms. Valentine

APPLICATION NUMBER PKU019/I16 of 2012 - **'HIV/Pulmonary TB co-infection amongst intravenous drug users in Mombasa, Kenya. Version 4.**

1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research topic 'HIV/Pulmonary TB co-infection amongst intravenous drug users in Mombasa, Kenya', Version 4. Dated 19th May, 2012.

2. APPLICANT

Valentine Budambula
School of Public Health,
Kenyatta University
P.O. Box 43844, Nairobi.

3. SITE

Mombasa County, Kenya.

4. DECISION REACHED.

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines, and is of the view that against the following elements of review,

i. Scientific design and conduct of study,
ii. Recruitment of research participant,
iii. Care and protection of research participants,
iv. Protection of research participant's confidentiality,
v. Informed consent process,
vi. Community considerations.

AND APPROVED that the research may proceed for a period of ONE year from 6th June, 2012.

APPENDIX 6: HIV-1 PROTEASE SEQUENCES OBTAINED FROM IDUs FROM MOMBASA COUNTY

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