GENETIC CHARACTERIZATION AND VIRAL TROPISM OF HIV-1 AMONG PATIENTS AT KENYATTA NATIONAL HOSPITAL

BY

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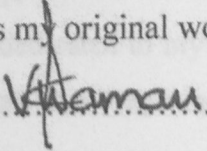
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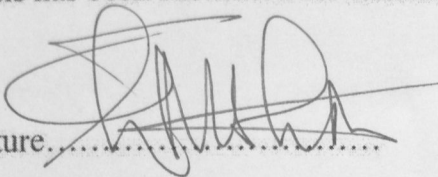
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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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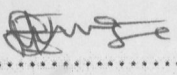
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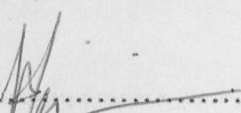
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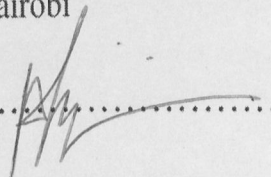
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DEDICATION

This thesis is dedicated to my parents: Dr. Paul K. Ndung'u and Mrs. Maryanne W. Kamau for their support, encouragement and faith in me throughout the course of this thesis.

ABSTRACT

CCR5 antagonists have clinically been approved for prevention or treatment of HIV/AIDS. Countries in Sub-Saharan Africa with the highest burden of HIV/AIDS are yet to adopt these regimens. However, HIV can also use CXCR4 as a co-receptor. There is hence a need to map out cellular tropism of Kenya's circulating HIV strains to guide the impending use of CCR5 antagonists. The study aimed to determine the prevalence of CCR5- and CXCR4-tropic HIV-1 strains among patients attending Kenyatta National Hospital. Blood samples were obtained from HIV infected patients attending the comprehensive care centre, Kenyatta National Hospital in years 2008 and 2009. The samples were separated into plasma and peripheral blood mononuclear cells (PBMCs). Proviral DNA was extracted from PBMCs and Polymerase Chain reaction (PCR) done to amplify the HIV env fragment spanning the C2-V3 region. The resultant fragment was directly sequenced on an automated sequencer (ABI, 3100). The HIV-1 env sequences were then entered into a variety of predictive algorithms: amino acids at position 11/25 rule, V3 net charge rule, Geno2pheno [co-receptor] and dsKernel. Phylogenetic relationships were determined using CLUSTALW and Neighbour Joining method. A total of 84 sequences were successfully amplified and sequenced. HIV-1 R5 tropic strains were more prevalent in the study population according to all algorithms: (71.01%, 69.41%, 72.5% and 82.89% for amino acids at positions 11/25 rule, V3 net charge rule, Geno2pheno[co-receptor] and dsKernel respectively). Phylogenetic analysis showed that 75% were subtype A, 13% subtype C and 12% subtype D. There were no significant differences in predicting the tropism using the four predicting tools ($\chi2$ test, p=0.19). The age, sex and CD4 counts of the study participants were not associated with HIV-tropism ($\chi2$ test, p=0.4447, p=1.000 and p=0.26 respectively). There was a tendency of a higher number of X4 tropic viruses being in the treatment experienced group though not statistically significant ($\chi2$ test, p=0.31). However, a strong association was observed between HIV tropism and HIV subtypes ($\chi2$ test, p=0.04), with subtype D harbouring mainly X4-tropic strains. In conclusion, HIV-1 R5 tropic strains were the most prevalent in the study population and HIV infected patients in Kenya may benefit from CCR5 antagonists. However, there is need for caution where subtype D infection is suspected or where antiretroviral salvage therapy is indicated.

ACKNOWLEDGEMENTS

ACRONYMS AND ABBREVIATIONS:

AIDS	Acquired Immune Deficiency Syndrome
HIV	Human Immunodeficiency Virus
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
mRNA	messenger Ribonucleic Acid
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
dNTPs	Deoxyribonucleoside Triphosphates
MgCl$_2$	Magnesium Chloride
CCR5	Cysteine Chemokine Receptor 5
CXCR4	Chemokine Receptor with Adjacent Cysteines Separated by 4 aminoacids
R5	Receptor 5
ARVs	Antiretrovirals
HAART	Highly Active Antiretroviral Therapy
env	Envelope gene
gag	Group Specific Antigen Gene
tat	Transcriptional Transactivator
rev	Regulator of Virion Gene Expression
nef	Negative effector
vif	Viral Infectivity Factor
vpr	Viral Protein R
vpu	Viral Protein U
pol	Polymerase gene

GP120	Glycoprotein 120
GP41	Glycoprotein 41
KNH	Kenyatta National Hospital
KEMRI	Kenya Medical Research Institute
UNITID	University of Nairobi Institute of Tropical Infectious Diseases
Rt	Reverse Transcriptase
LAV	Lymphadenopathy Associated Virus
SI	Syncitia- inducing
NSI	Non-syncitia-inducing
M-tropic	Macrophage Tropic
T-tropic	T-Cell Tropic
P6	Protein 6
P7	Protein 7
P24	Protein 24
CD4	Cluster of differentiation 4
PSSM	Position Specific Scoring Matrices
SVM	Support Vector machine
VL	Viral Load
µl	Micro Litre
FDA	Food and drug administration
V1	Variable 1 region
V2	Variable 2 region
V3	Variable 3 region
ART	Antiretroviral therapy
EDTA	Ethylene diamine tetra-acetic acid

NCBI   National centre for biotechnology information

SPSS   Statistical package for the social sciences

CHAPTER ONE

1.0 INTRODUCTION & LITERATURE REVIEW

1.1 Introduction

Human immunodeficiency virus (HIV) causes a lethal disease called acquired immunodeficiency syndrome (AIDS). Human immunodeficiency virus has currently surpassed malaria as a leading cause of adult infectious disease mortality worldwide (Nelson, 1999). The Kenya AIDS indicator survey (KAIS), reported by the National AIDS/STIS Control Programme (NASCOP) found that 7.1% of adults (aged 15-64) in Kenya are infected with HIV representing an estimated 1,417,000 people.

Human immunodeficiency virus is dependent on a host cell for its replication and requires binding to receptors on the cell surface in order to gain entry. The receptors are also in interaction with glycoprotein120 (gp120) envelope protein of the virus. The first receptor is the cluster of differentiation 4 antigen (CD4 Ag) which is the main receptor and is always the same for each viral particle. There are other two receptors namely: CCR5 and CXCR4 that serve as co-receptors (Berger, 1999). The preferred phenotypic designations are R5 for the non-syncytium inducing CCR5 using HIV and X4 for the syncytium-inducing CXCR4 using HIV (Berger, 1997). The variable three (V3) region of the envelope is highly associated with the co-receptor phenotype with the overall amino acid charge being central to co-receptor usage. De Jong *et al.* (1992) stated that higher positive charges are associated with the utilization of CXCR4 co-receptor while higher negative charges are associated with utilization of CCR5 co-receptor.

Currently, a new class of anti-HIV drugs are being developed that aim to prevent the virus from entering a host cell. One such drug is the maraviroc that acts by blocking the CCR5 co-receptor hence preventing HIV entry. Since HIV can also use CXCR4, an HIV tropism test must be performed to determine if the drug is suitable for a given patient (Biswas *et al.*, 2007).

1.1.1 Human immunodeficiency virus

In biological taxonomy, human immunodeficiency virus belongs to the genus *Lentivirus* within the family *Retroviridae*. Within the genus lentivirus are other closely related viruses that also cause immunodeficiency in hosts such as cats or in primates (Kober *et al.*, 2000).

Human immunodeficiency virus causes AIDS, a condition in which the immune system slowly becomes depleted, leading to life-threatening opportunistic infections. Previous names for HIV include: human T-lymphotropic virus - III (HTLV-III), lymphadenopathy-associated virus (LAV) or AIDs-associated retrovirus (Coffin *et al.*, 1986).

The Congo region has been shown to play a pivotal role in the genesis of HIV (Gottlieb *et al.*, 1981; Masur *et al.*, 2006; Rambaut *et al.*, 2001). According to Gilbert *et al.* (2007) HIV-1 emerged from Africa and spread worldwide. Gao *et al.* (1999) and Nerrienet *et al.* (2005) believe that HIV-1 entered the human population through cross-species transmission from non-human primates in Africa.

1.1.1.1 Human immunodeficiency virus genome

The virus has several major genes coding for structural proteins found in all retroviruses and several non-structural or accessory genes unique to HIV (Hue *et al.*, 2003). Each of these genes play an essential role in the structure and function of HIV (fig 2). The envelope (*env*), group-specific antigen (*gag*) and polymerase (*pol*) genes are collectively called structural genes (Hue *et al.*, 2003). Group-specific antigen gene codes for protein 24 (p24), the viral capsid, proteins' six and seven (p6 and p7), the nucleocapsid proteins and protein 17 (p17), the matrix protein. The *pol* codes for viral enzymes, including; reverse transcriptase, integrase and proteases. The latter cleave the proteins derived from *gag* and *pol* into functional proteins. The *env* gene encodes the glycoprotein 160 (gp160), the precursor to gp 120 and gp 41, these proteins are embedded in the viral envelope that enable the virus to attach and fuse with target cells.

Transcriptional transactivator (tat) and regulator of virion gene expression (rev) are regulatory genes (Emerman and Malim, 1998). Transcriptional transactivator is crucial for activated transcription from the HIV-1 long terminal repeats. Regulator of virion gene expression modulates the transport of viral mRNA from the nucleus to cytoplasm (Frankel and Young, 1998; Freed, 2001; Chinnasammy *et al.*, 2000)

Human immunodeficiency virus also encodes negative effector (*nef*), viral infectivity factor (*vif*), viral protein r (*vpr*) and viral protein u (*vpu*). These are termed accessory or auxiliary proteins to reflect that they are dispensable for HIV replication even though they play an important role in pathogenesis and immune evasion (Frankel and Young, 1998; Freed, 2001; Chinnasammy *et al.*, 2000).

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1.1.1.2 Human immunodeficiency virus structure

Human immunodeficiency virus is roughly spherical with a diameter of about 120 nm, around 60 times smaller than a red blood cell (Mc Govern *et al.*, 2002). It is composed of two copies of positive single stranded RNA that codes for nine genes, and this RNA is enclosed by a conical capsid composed of 2000 copies of the viral protein 24 (p24). This is in turn surrounded by a plasma membrane, commonly referred to as the envelope. The envelope is made up of lipids and various membrane bound proteins (Cullen, 1998). The membrane bound proteins, including glycoprotein 120 (gp120) and glycoprotein 41 (gp41), bind to CD4 antigen and then chemokine co-receptor on the surface of T-cells resulting in the virus becoming physically attached to the host cell (Kwong *et al.*,1998) (fig 1). The envelope is formed each time the capsid buds from the host cell, taking some of the host's cell membrane with it. A matrix composed of an association of the viral protein 17 (p17) surrounds the capsid ensuring the integrity of the virion particle. Enclosed also within the virion particle are *vif, nef, vpr* and p7.

The single-stranded RNA is tightly bound to nucleoside proteins p7 & p6 and enzymes (Reverse transcriptase, integrase and protease). The nucleocapsid proteins associate with the genomic RNA, one molecule per hexamer and protect RNA from digestion by nucleases. The enzymes are needed for the development of the virions (Chystie and Almeda, 1988).

1.1.1.3 Co-receptors for HIV entry

The primary cellular receptor for HIV entry is CD4. However, expression of CD4 on a host cell is not sufficient for HIV entry and infection. Chemokine receptors act as co-factors that allow HIV entry when co-expressed with CD4.The first chemokine receptor to be identified

was CXCR4, which is expressed on T-cells (Feng *et al.*, 1996). Co-expression of CXCR4 and CD4 on a cell, allow T-tropic HIV isolates to fuse with and infect the cell. Human immunodeficiency virus gp120 interacts with both CD4 and CXCR4 to adhere to the cell and to effect conformational changes in the gp120/gp41 complex that allow membrane fusion by gp41. CXCR4 is expressed on many T-cells but usually not on macrophages and does not allow fusion by M-tropic HIV isolates (Feng *et al.*, 1996).

Shortly after identification of CXCR4, CCR5 was identified which is expressed on macrophages and on some populations of T-cells (Deng *et al.*, 1996; Dragic *et al.*, 1996; Alkhatib *et al.*, 1996). Individuals with certain mutations in CCR5 are resistant to HIV infection (Liu *et al.*, 1996; Samson *et al.*, 1996; Dean *et al.*, 1996).

Figure 1: Human immunodeficiency virus structure.
A representation of the HIV virion and its associated proteins. The interaction between the glycoprotein 120 on the virus surface and the CD4 receptor and a chemokine co-receptor on the T lymphocyte surface is also illustrated. Adapted from Abbas and Litchman, (2004).
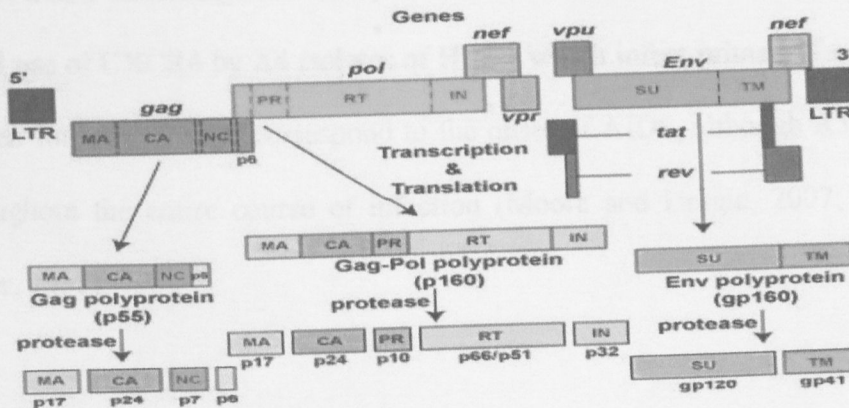
Figure 2: Human immunodeficiency virus genome.
Depiction of the HIV genome showing the organization of the genes and their transcriptional splicing. Adapted from Joshua, (2007).

1.2 Literature review

Chemokine co-receptors are cell-surface molecules that bind peptide ligands called chemokines, thereby inducing migration of the receptor bearing cells toward injured tissues that secrete chemokines into the bloodstream. It is through this mechanism that leukocytes are recruited into sites of inflammation (Murphy, 1996; Premack and Schall, 1996). Based on the positions of two conserved cysteine residues in their N termini, chemokines can be divided into four subfamilies: CC, CXC, CX3C, and C (Wells et al., 1996; Baggiolini et al., 1997).

According to Deng et al. (1996), a number of host cell-surface molecules are used by infectious agents to gain entry into cells. It is now clear that HIV-1 enters cells through an interaction involving chemokine co-receptors. The R5 isolates of HIV-1 use the chemokine co-receptor CCR5 to enter macrophages and primary T cells. These isolates are present early after seroconversion indicating their nearly exclusive role in the initiation of HIV-1 infection. The eventual use of CXCR4 by X4 isolates of HIV-1 which infect primary T cells and T cell lines has been found to closely correspond to the onset of AIDS, although R5 isolates often persist throughout the entire course of infection (Moore and Dragic, 2007; Berger et al., 1998; Berger, 1997).

1.2.1 Cysteine chemokine receptor 5

According to Moyle (2005), it was discovered in 1996 that HIV binds to a second protein on the surface of human cells called CCR5 as part of process of infecting a cell. Cysteine chemokine receptor 5 is the principal co-receptor of a HIV (Easterbrook, 1999). The CCR5

gene has been mapped to the short arm of chromosome 3 along with other genes that encode multiple chemokine receptors (Samson *et al.*, 1996). The HIV strains which use CCR5 are sometimes referred to as macrophage-tropic strain or the non-syncytium inducing (NSI) strain.

CCR5 is a typical G protein-coupled receptor of 352 amino acids, with seven transmembrane domains that presumably adopt an alpha helical structure. It belongs to the subfamily of CC chemokine receptors. The predicted molecular weight of the protein is 40,600 daltons. Like other chemokine receptors, CCR5 has four cysteines within its extracellular domains, involved in the formation of two disulfide bonds. One of these bonds, linking the first and second loops of the receptor, is conserved in most G protein-coupled receptors. The second bond, which is specific to the chemokine receptor family, links the N-terminus to the third extracellular loop. Both bonds are necessary for the chemokine-binding properties and the functional response of the receptor (Blanpain *et al.*, 1999).

Cysteine chemokine receptor 5-tropic strains have been found not to be as virulent as the CXCR4 using strains. Individuals who have a mutation of the CCR5 gene may be partially protected against infection with NSI strains of HIV. However, they are not completely protected against HIV infection.

1.2.2 Cysteine chemokine receptor 5 [delta] 32

Soon after CCR5 was shown to be an HIV co-receptor, the mutant allele CCR5-[Delta] 32 which is characterized by a 32 base-pair deletion in the single coding exon of the gene, was identified in Caucasians (Dean *et al.*, 1996). CCR5-[Delta] 32 causes a truncation that is not

associated with a functional protein, explaining the near-complete protection against HIV-1 infection in individuals homozygous for the allele (Liu *et al.*, 1996). Rare cases of HIV-1 infection in the absence of CCR5 have been reported (Biti *et al.*, 1997; Theodorouo *et al.*, 1997), however, indicating that X4 isolates can sometimes initiate HIV-1 infection. Other homologous chemokine receptors bind an overlapping set of chemokine ligands and may compensate for the absence of CCR5 in individuals homozygous for CCR5-[Delta] 32 (Premack and Schall, 1996). CCR5-[Delta] 32 is also associated with slower progression to AIDS by 2-4 years after HIV-1 seroconversion in individuals heterozygous for the mutation (Dean *et al.*, 1996).

Because the frequency of the CCR5-[Delta] 32/+ genotype is approximately 20% in Caucasians, this genotype has a significant population effect on progression to AIDS. Rather than a simple gene dosage effect, formation of CCR5-[Delta] 32/CCR5 hetero complexes causes CCR5 to be retained in the endoplasmic reticulum resulting in reduced cell surface expression of the wild-type molecule. The CCR5-[Delta] 32/+ genotype is also associated with protection from AIDS-related lymphoma, a non-Hodgkin's B cell malignancy (Dean *et al.*, 1999). Although the mechanism for this protection is not clear, B cells do express CCR5 on their cell surfaces, and RANTES, one of four chemokine ligands of CCR5, is mitogenic for B cells (Dean *et al.*, 1996). Therefore, RANTES may play a role in lymphoma expansion via CCR5 before immune surveillance has a chance to eliminate the malignant cells. If so, then diminished levels of CCR5 in CCR5-[Delta] 32/+ heterozygotes may be advantageous by indirectly controlling B cell expansion.

1.2.3 Chemokine receptor with adjacent cysteines separated by 4 amino acids

The human CXC chemokine receptor 4 (CXCR4) is a receptor for the chemokine stromal cell-derived factor (SDF-1α) and a co-receptor for the entry of specific strains of HIV-1 (Zhou *et al.*, 2001). CXCR4 belongs to the family of seven trans-membrane G protein-coupled receptors that transduce signals via heterotrimeric G-proteins (Murphy, 1994). Recent studies with knockout mice of CXCR4 have demonstrated that this molecule plays an important role in immunomodulation, organogenesis, hematopoiesis, and derailed cerebellar neuron migration (Nagasawa *et al.*, 1998; Zou *et al.*, 1998; Ma *et al.*, 1998). CXCR4 has also been identified as one of co-receptors for HIV-1 (Feng *et al.*, 1996). CXCR4 mediates infection of T cell line tropic HIV-1 strains and has also been found to be used by HIV-2 strains adapted to replication in CD4-negative cell lines (Endres *et al.*, 1996).

While R5 variants are generally detectable over the entire course of HIV-1 infection (Schuitemaker *et al.*, 1991), variants able to utilize CXCR4 emerge in ~40–50% of infected persons over the course of disease (Berger *et al.*, 1999). These "X4 variants" predominantly target naive and resting CD4 cells and display biological properties that differ from those of their R5 counterparts, including increased replication rate, pathogenicity, and syncytium-inducing (SI) capacity in immortalized CD4 cell lines (Schuitemaker *et al.*, 1991; Connor and Ho 1994). In addition, dual-tropic variants capable of using both CXCR4 and CCR5 may also arise over the course of disease (Glushakova *et al.*, 1999). The factors mediating the R5-to-X4 phenotype "switch" over the natural course of HIV-1 infection remain incompletely understood. The emergence of CXCR4-using variants is associated with a rapid decline in CD4 cell counts, accelerated disease progression, and reduced survival time in untreated individuals (Tersmette *et al.*, 1989; Schuitemaker *et al.*, 1992; Koot *et al.*, 1993; Richman

and Bozette 1994), as well as poorer response to treatment in the pre–highly active antiretroviral therapy (HAART) era (Rusconi *et al.*, 1996; Katzenstein *et al.*, 1996; Vidal *et al.*, 1998). However, it is not known whether X4 variants are inherently more pathogenic and are directly responsible for more-rapid disease progression or whether CXCR4-using HIV-1 variants may emerge as a consequence of progressive immune dysfunction (Shaheen and Collman 2004). Regardless of the direction of causation, the association of X4 HIV-1 with poorer prognosis and inferior therapy response remains an important issue in clinical practice and it is important that the prognostic implications of HIV-1 co-receptor use be re-evaluated in the HAART era.

1.2.4 Glycoprotein 120 structure

The *env* gene encodes for the heavily glycosylated surface gp 120 and the non-covalently associated transmembrane subunit gp 41. Glycoprotein 120 and gp 41 are organized on the virion surface as trimeric spikes and mediate viral entry into susceptible cells. The surface gp120 is composed of a core of conserved regions (C1-C5), shielded by variable loop regions (V1-V5) formed by disulphide bonds (except V5) that retain a large degree of flexibility (Rossi *et al.*, 2008).

The hypervariable region 3 is functionally important in viral infectivity (Ivanoff *et al.*, 1992), viral neutralization (Javaherian *et al.*, 1989), replication efficiency and host cell tropism (Shioda and Cheng, 1991). The V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread (Toohey *et al.*, 1995). Lee *et al.* (1995) found that V4 and V5 of gp120 are less flexible regions of the proteins and play roles in CD4 binding and neutralizing antibody responses and is therefore rich in residues involved in CD4 binding (fig

3). The third variable region (V3) of the human immunodeficiency virus type 1 (HIV-1) envelope gp120 subunit participates in determination of viral infection co-receptor tropism and host humoral immune responses (Satoshi *et al.*, 2008) and is therefore rich in residues involved in co-receptor binding (fig 3).
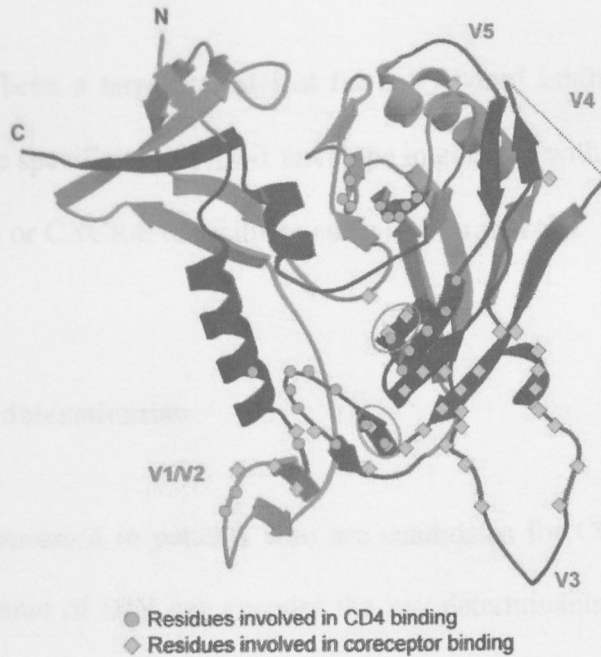
Fig 3: Structure of Glycoprotein 120.
The location of the residues involved in CD4 receptor and co receptor binding are indicated. The locations of the gp120 variable loops (V1-V5) and of the N- & C-termini of the sequence are also indicated. Adapted from Neurath *et al.*, 2004

1.2.4.1 Variable 3 (V3) region of glycoprotein 120

The third hypervariable domain (V3) of gp120 is a cysteine bounded loop structure usually composed of 35 amino acids. Different regions of gp120 V3 have been categorized as the base (residues 1-8 and 25-35), stem (residues 9-14 and 18-24) and turn (residues 15-17) regions (Lynch *et al.*, 2009).

The V3 domain is located between amino acids 296 and 331 of gp120 and has a type 2β-turn conserved secondary structure (Jean *et al.*, 1992). Of the five gp120 hypervariable domains,

V3 has relatively conserved regions and does not exhibit the dramatic insertions, deletions and shifts of glycosylation that are characteristic of other domains, perhaps because V3 participates directly in co-receptor binding and is therefore functionally constrained from displaying further variability (Cardozo *et al.*, 2007; Cormier and Dragic, 2007).

The V3 region has long been a target of interest for entry-based inhibitors because of its critical role in defining the specificity of HIV-1 envelope interaction with cellular co-receptor molecules, usually, CCR5 or CXCR4, to facilitate entry into target cells.

1.2.5 Co-receptor usage determination

HIV-1 tropism must be assessed in patients who are candidates for CCR5 antagonists. As stated above, the V3 domain of HIV *env* encodes the key determinants of viral co-receptor usage (Hwang *et al.*, 1991). Recombinant virus phenotypic entry assays are considered as the gold standard method for determining co-receptor usage (Whitcomb *et al.*, 2007). However, routine use is hampered by technical and cost limitations. Therefore genotypic prediction approaches have great potential, as they could be easier, faster and cheaper than phenotypic assays for routinely assessing HIV-1 tropism.

1.2.5.1 Amino acids at positions 11/25 and V3 net charge rules

A pioneering paper in 1992 by de Jong *et al.* implicated the presence of a basic residue at V3 positions 11 or 25 with the usage of the CXCR4 receptor. The amino acids at positions 11/25 rule states that there are uncharged residues at position 11 of V3 [typically serine (S) or glycine (G)], negatively charged residues at position 25 (typically glutamic (E) or aspartic

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(D) acid), and a net charge of the V3 loop of less than +5 have been reported to predict CCR5 chemokine receptor usage. Conversely, positively charged residues at position 11 or 25 typically arginine (R) or lysine (K) and a net charge of the V3 loop equal and more than +5 have been reported to predict CXCR4 chemokine receptor usage (Lwembe *et al.*, 2009).

A study was designed to determine the minimal number of naturally occurring amino acid changes within the V3 region required for production of a syncytium-inducing phenotype starting from two naturally occurring V3 regions derived from non-syncytium-inducing isolates from two patients in whom a switch to syncytium-inducing variants occurred. Using a HXB-2 background, a cassette system was developed; the viruses constituting the cassette differed in the V3 region only. Regions of the virus other than V3 that influence syncytium-forming capacity and replication rate (Shioda *et al.*, 1991) were not affected. Insertion into this cassette of V3 sequences derived from non-syncytium inducing and syncytium-inducing isolates from the same patient, with mutations at a positions 11 and 25, resulted in hybrid viruses with marked differences in syncytium inducing capacity corresponding to the phenotype of the original isolates (de Jong *et al.*, 1992; Kuiken *et al.*, 1992). Comparison of a large number of V3 sequences from field isolates revealed a strong correlation between positively charged amino acid residues at two of these sites and the syncytium inducing phenotype (Fouchier *et al.*, 1992). To date, the charge rule is the most accepted method of sequence-based prediction. However, prediction based on this rule does not always align with experimental determination of co-receptor usage (McDonald *et al.*, 2001). The inaccuracy of the charge rule is most likely due to the comparatively sparse and unreliable data that were available at the time of its creation. Since then, the number of sequences with known phenotype has increased substantially, and the laboratory-based assays used to generate the data have improved. Another possible candidate for a deficiency in this predictive scheme is the consideration of only 2 of the 35 available amino acid positions in the V3 loop.

1.2.5.2 Trofile assay

Trofile® assay is the current gold standard phenotypic method for the determination of co-receptor tropism for the replicating viral population, although other in-house or commercial tests are available (Braun and Weismann, 2007; Coakley *et al.*, 2009). The drawbacks of any phenotypic test include high costs, long turn-around time, and reduced efficiency in patients with low viremia. For this reason, there is a demand for a fast and cheap HIV-1 tropism assay to fully exploit CCR5 antagonists as a treatment option in clinical routine (Rose *et al.*, 2009; Soriano *et al.*, 2009). Given that most of the determinants of viral co-receptor tropism are based on polymorphisms of the V3 region of the gp120, an alternative to the phenotypic approach is the usage of machine learning tools based on viral genotypic information. So called *in silico* or virtual phenotype models may be indeed convenient for clinical practice due to the reduction of costs and turn-around time. During the recent years, several prediction models have been studied, from the first simple rule based on the polymorphisms at V3 codons 11 and 25, to the position specific scoring matrices (PSSM), neural networks, support vector machines, random forests and logistic models (Resch *et al.*, 2001; Xu *et al.*, 2007; Lamer *et al.*, 2008; Prosperi *et al.*, 2009). Some of these studies identified additional factors possibly impacting viral tropism, such as viral subtype and CD4 cell counts (Sander *et al.*, 2007; Sing *et al.*, 2007; Prosperi *et al.*, 2009).

1.2.5.3 In silico strategies

In silico approaches to determining HIV tropism are gaining popularity given their simplicity (Neogi *et al.*, 2010). The strategies use computer simulation and bioinformatics to predict the co-receptor usage from *env* sequence information (Skrabal *et al.*, 2007; Proveda *et al.*, 2006). Positions outside the V3 loop may also influence viral tropism (Pastore *et al.*, 2006). Most

current genotypic bioinformatics tools consider the entire V3 sequence (Resch *et al.*, 2001). More importantly, most genotypic bioinformatics predictors have been designed based on the genetic characteristics of HIV-1 clade B (Poveda *et al.*, 2006; Skrabal *et al.*, 2006). Since non-B subtypes show a wide genetic variability in the V3 region and since X4 viruses might be more prevalent in some clades than others (Andreoletti *et al.*, 2007; Laeyendecker *et al.*, 2006; Tscherning *et al.*, 1998; Tscherning *et al.*, 2000), there is an urgent need to know the reliability of genotypic tools for inferring HIV-1 tropism in non-B subtypes, especially in regions where these HIV-1 variants are quite prevalent and may soon have access to CCR5 antagonists. Such tools include among others: Geno2Pheno [co-receptor] that detects and aligns the V3 loop from a given sequence automatically (Lengauer *et al.*, 2007), the distant segments (ds) Kernel which include relative positional information of segments in a string of symbols which detects R5-, X4- and R5X4-tropic strains (Boisvert *et al.*, 2008) and position-specific scoring matrices (PSSM).

1.2.5.3.1 Reliability of genotypic tools to predict co-receptor usage

A study to evaluate the concordance between eight distinct bioinformatics tools to estimate HIV-1 co-receptor usage by taking the results of a phenotypic tropism assay in a relatively large population of patients infected with non-B subtypes as a reference was the first study ever done to assess the performances of various genotypic bioinformatics tools to predict HIV-1 tropism in non-B subtypes using the results obtained with a phenotypic test as a reference (Garrido *et al.*, 2008).

From the results, the overall concordance between the results obtained using the distinct genotypic tools taking the phenotypic results as a reference was always over 76%. Overall,

genotypic tools performed slightly better in testing clade B than non-B viruses (mean concordance of 85.7% versus 80.2%, respectively). For testing of subtype B samples, the most accurate bioinformatics tools were PSSMX4R5 (91.4%), PSSMsinsi (88.6%), and geno2pheno (88.6%), while the best concordance for testing non-B variants was seen with PSSMsinsi (83.8%), which was closely followed by Charge Rule, PART, C4.5 with positions 8 to 12 only, and PSSMsinsi, with a concordance of 82.5% for each. Overall, no significant differences in concordance were found by comparing B and non-B groups, although the most remarkable difference was found using geno2pheno (88.6% versus 71.3%, respectively. SVM, PSSMR5X4, PSSMsinsi, and geno2pheno discordances generally resulted in an overestimation of X4 viruses, while other bioinformatics tools tended to underestimate X4 viruses more frequently. Geno2pheno was the genotypic predictor more prone to overestimation of X4 tropism (up to 22.5% in non-B samples). Thus, estimations of HIV-1 tropism using bioinformatics tools based on V3 sequences are better for testing clade B than non-B viruses.

1.2.6 Cysteine chemokine receptor 5 antagonist

Advances have been made in understanding the molecular mechanisms by which HIV enters CD4-positive cells leading to several potential new targets for antiviral agents. Compounds targeting viral entry have two advantages over those that target HIV-1 reverse transcriptase and protease enzymes: entry inhibitors do not depend on efficient cellular uptake or intracellular activation processes to exert their biological effects and they are highly unlikely to show any cross-resistance with protease inhibitors or reverse transcriptase inhibitors (Westby *et al.*, 2006).

Lalezari *et al.* (2003) and Lazzarin *et al.* (2003) validated viral entry as a clinically effective pathway by the first fusion inhibitor, enfurvirtide. According to De Clercq, (2002) and Pierson *et al.* (2004), other classes of entry inhibitors under development target the initial binding of viral gp120 to CD4 and the interaction of gp120 with CCR5 for HIV entry. The HIV co-receptors represent attractive targets for drug development since they are members of G protein-coupled receptor superfamily, a group of proteins targeted by several commonly used and tolerated drugs (Gurrath, 2001).

Cysteine chemokine receptor 5 is of particular interest since a natural polymorphism exists in humans (CCR5 [Delta]-32) that leads to reduced or absent cell surface expression of CCR5 in heterozygotic or homozygotic genotypes respectively (Dean *et al.*, 1996). Maraviroc is the first CCR5 antagonist to be approved by the Food and Drug Administration (FDA) for clinical use.

1.2.6.1 Maraviroc

Maraviroc blocks the cysteine chemokine receptor 5 (CCR5) which HIV uses as a co-receptor to enter a human macrophage (Jay, 2009). Human immunodeficiency virus can use another co-receptor, CXCR4. Therefore, a HIV tropism test must be performed to determine if the drug will be effective (Biswas *et al.*, 2007). Originally designated UK-427857, it was developed by the drug company Pfizer in its United Kingdom (UK) laboratory located in Sandwich. In April 24, 2007 the United States Food and Drug Administration advisory panel reviewing maraviroc's new drug application unanimously recommended approval for the new drug and the drug received full FDA approval in August 6, 2007 for use in treatment experienced patients.

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Two phase II studies have demonstrated that 10 days of maraviroc monotherapy, at doses from 100 mg-300 mg once daily or twice daily, decreased viral load by >1.0 $\log_{10}$ copies/ml in HIV-1 infected patients (Fatkenheuer *et al.*, 2005)

1.2.6.1.1 Discovery and development of maraviroc

The trigger for the discovery of the CCR5 antagonists was the observation that a small percentage of high risk populations showed either resistance or delayed development of the disease. The population was identified in Caucasians who had a mutant allele CCR5-[Delta] 32, characterized according to Dean *et al.* (1996), by a 32 base pair deletion in the single coding exon of the gene. Scientists then discovered the key role of the cell surface receptors CCR5 and CXCR4 in successful viral fusion and infection. Samson *et al.* (2005) and Dragic *et al.* (1996) demonstrated that CCR5 serves as a co-receptor for the most commonly transmitted HIV-1 strain, R5, which is predominant during early stages of infection and remains dominant in over 50% of late stage HIV-1 patients. According to De Clerq *et al.* (2007), R5 HIV-1 strains can eventually evolve to X4 as the disease progresses.

1.2.6.1.2 Maraviroc mechanism of action

Viral entry to the CD4 positive T-cell begins with attachment of the R5 HIV-1 gp120 and allows it to bind to CCR5, thereby triggering gp41 mediated fusion of the viral envelope with the cell membrane and the nucleocapsid enters the host cell (Ray, 2006; Westby, 2005).

According to Britz and Proveda (2006), CCR5 co-receptor antagonists prevent HIV-1 from entering and infecting immune cells by blocking CCR5 cell-surface receptor. Small molecule

antagonists of CCR5 bind to a hydrophobic pocket formed by the trans-membrane helices of the CCR5 receptor (Murga *et al.*, 2006). According to Watson *et al.* (2005), CCR5 antagonists are thought to interact with the receptor in an allosteric manner locking the receptor in a conformation that prohibits its co-receptor function.

1.3 PROBLEM STATEMENT

The current interest in antiretroviral therapy is on CCR5 antagonists. While it has been proven that other HIV medications are very effective, there is growing concern that as time goes by, more and more people are becoming resistant to the drugs currently in the market. The problem with resistance is that often, resistance to one medication means resistance to an entire class of medications. Maraviroc is the first drug from the new class of HIV medications called CCR5 entry inhibitors. Maraviroc being in the new class means people living with HIV will not have resistance to the drug because they have not been exposed to any drugs from this class. Human Immunodeficiency Virus can also use CXCR4 co-receptor for fusion and entry into a host cell. Therefore HIV tropism assays are crucial before administering maraviroc to a patient.

1.4 JUSTIFICATION

Cysteine chemokine receptor 5 antagonists have arrived at the final stage of the drug development pipeline. Before considering treatment with a CCR5 antagonist, finding out about the co-receptor usage of the viral population in the host will reduce the emergence of resistance to CCR5 antagonists as well as save the patient money used in purchasing the wrong drug. The findings will also be of great benefit to the Ministry of Health which purchase the antiretrovirals for hospitals in Kenya. The Ministry can use the statistics on R5 HIV prevalence to approve the drug in Kenya or provide an alternative fusion inhibitor in Kenyan hospitals.

1.5 HYPOTHESIS

1.5.1 Null Hypothesis

Cysteine chemokine receptor 5 tropic HIV-1 strains are not prevalent among patients attending the Comprehensive Care Centre (CCC) at the Kenyatta National Hospital (KNH).

1.6 OBJECTIVES

1.6.1 General Objective

To characterize the HIV-1 strains based on co-receptor tropism among HIV patients attending the CCC at KNH.

1.6.2 Specific Objectives

1) To determine the prevalent HIV-1 tropic strain among HIV-1 patients attending the CCC at the KNH.

2) To evaluate the concordance between the four co-receptor usage prediction tools used in the study.

3) To evaluate the correlation between HIV-1 tropism and HIV-1 sub-types.

4) To evaluate the correlation between HIV-1 tropism and current ART strategies.

5) To evaluate the correlation between HIV-1 tropism and the demographic characteristics of the study population.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study site

Between February 2008 and February 2009 blood samples were collected from patients attending the Comprehensive Care Centre (CCC) at the Kenyatta National Hospital (KNH). KNH based in Nairobi is the oldest hospital in Kenya with a capacity of 1800 beds and over 6000 staff members. The hospital plays a major role in health care delivery system in the country, East Africa and the whole of Africa as provided for in its mandate. The hospital has an efficient and effective referral system and receives referral cases for specialized healthcare from other health institutions within and outside the country. KNH is the hospital of choice to the majority of the population in the capital city, Nairobi, and its environs due to its affordability and quality healthcare. KNH facilitates medical training for students of the College of Health Sciences of the University of Nairobi, The Kenya Medical Training College and other higher learning institutions. This is in addition to facilitating research either directly or through collaboration with other health institutions. The hospital also participates in national health policy planning. KNH has the Comprehensive Care Centre that handles HIV/AIDS patients, dispensing medicine, monitoring viral loads and counselling. Currently, 6,294 patients are on follow up at CCC, KNH: 4,980 are on antiretroviral therapy and 1,314 are treatment-naive patients.

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2.2 Study population

The subjects were part of a prospective study on antiretroviral resistant genotypes among treatment-experienced patients attending the CCC at KNH.

2.2.1 Inclusion criteria

i. Consenting HIV-positive adults (above 16 years of age) on antiretroviral therapy.

ii. Consenting HIV-positive adults (above 16 years of age) not yet on antiretroviral therapy (treatment- naïve).

2.2.2. Exclusion criteria

i. Non-consenting HIV-positive adults (above 16 years of age) who were either treatment naïve or on antiretroviral therapy.

ii. HIV-positive minors (below 16 years of age) who were either treatment naïve or on antiretroviral therapy.

2.3 Sample size

The sample size was calculated using the formula given by Lemesshow *et al.* (1986), used to calculate the adequacy of sample size in health studies.

$N = Z^2 P (1 - P)/d^2$,

Where N = minimum sample size

Z = Standard normal deviation value corresponding to 95% confidence interval (= 1.96)

P = Estimated prevalence of HIV (in Kenya it is 6.3%)

D = Degree of precision (set = 5%)

Therefore $N = 1.92^2 \times 0.063(1- 0.063)/0.05^2 = 87$ samples

The study adjusted the sample size to 96 in order to complement for any error during

sequencing.

2.4 Ethical considerations

Relevant information regarding purpose and procedures of the project were explained in detail and clearly to the study subjects'. The study subjects' names were not asked for to ensure confidentiality but codes were given to show their centre of origin. An informed consent form for demographic survey had also been previously obtained from each study participant before collecting the blood sample. The study was approved by the Kenya Medical Research Institute's National Ethical Review Committee, under SSC1252.

2.5 LABORATORY PROCEDURES

2.5.1. Proviral HIV DNA Extraction

To the peripheral blood mononuclear cells (PBMCs) pellet, 500 μl of DNAzol genomic DNA extraction reagent (Gibco BRL®) was added and dissolved completely by pipetting the reagent-pellet mixture up and down with a pipette. Two volumes (1000 μl) of chilled (cooled

to 4° C) absolute ethanol was added to the dissolved pellet and mixed gently. This mixture was spun at 11269 g in a microcentrifuge at 4° C for 15 minutes and the supernatant discarded. To the pellet, 1000 µl of 70% ethanol was added and vortexed thoroughly. Spinning was done again at 11269 g in a microcentrifuge at 4° C for 15 minutes and supernatant discarded. The pellet was dried at room temperature in a biosafety cabinet. DNAse/RNase free water (100 µl) was used to dissolve the pellet and stored at -30° C till further use.

2.5.2. Polymerase Chain Reaction (PCR)

A master mix was made containing 2.8 µl of 25 mM $MgCl_2$, 2.0 µl of 10 mM dNTPs, 0.2 µl of 0.5 units Taq polymerase, 2.0 µl of 10x PCR Buffer, 10.2 µl of RNase free water and 0.4 µl of 10 µM of each primer. Master mix (18 µl) was added to 2 µl (10 ng/ µl) of each DNA template in PCR tubes for the first PCR. The thermalcycler conditions were: 10 min at 95°C, 35 cycles of 30s at 95 °C, 30s at 55°C and 1min at 72 °C followed by a final extension of 10 min at 72 °C. The first PCR products were used as template for the second PCR using the same thermalcycler conditions.

Table 1: Nested polymerase chain reaction primers used for amplification of viral env gp120 (C2V3 Region)

	Primer Name	Sequence
1st PCR	M5 F1: forward 1	CCCCTATTCCTTTTCCCCTTCTTTTAAAA
	M10 R1:reverse 1	CCAATTCCCATACATTATTGTGCCCCAGCTGG
2nd PCR	M3 F2: forward 2	GTCAGCAACAGTACAATGACACATGG
	M8 R2 :reverse 2	TCCTTCCATGGGAGGGGCATACATTGC

2.5.3. Gel Electrophoresis

Once the PCR products were obtained, they were electrophoresed at a voltage of 100 volts on a 1.5% agarose gel for 20 minutes. The 1.5% agarose gel was prepared by mixing 0.75g of agarose in 50 ml 0f 1xTBE (Tris Base, Boric acid and NaEDTA) buffer and heating till the agarose dissolved. The mixture was then poured into the casting plate containing the casting comb to form well-defined wells. Staining was done with ethidium bromide (10 mg/ml) solution (18 µl of EtBr was dissolved in 50 ml 1xTBE) for 20 minutes and a U.V transilluminator used to visualize the bands of the amplified DNA.

2.5.4. Sequencing PCR

Sequencing PCR was done using a reaction mixture containing 3.5 µl of 5X Sequencing buffer, 1.0 µl BigDye, 1.5 µl Primers (Sense/Antisense), 13µl RNase free ddH$_2$O and 1.0 µl

PCR product making up a total reaction volume of 20 µl. The thermalcycler conditions were: 5 min at 96°C, 25 cycles of 10s at 96 °C, 5s at 50 °C and 4min at 55 °C. The sense primer was M5 F1 while the antisense primer was M10 R1 used during the first round of the nested PCR

2.5.5 Purification of sequencing PCR product

To each 1.5 ml microtube, the following were added: 20 µl of sample, 2 µl 125 mM of EDTA, 2 µl of 3M NaOAc and 50 µl of 100% EtOH. Vortexing was then done and left at room temperature for 15 minutes followed by centrifugation at 15,344g for 20 minutes. The supernatants were then discarded and 70 µl of EtOH added and mixing done gently. Centrifugation was done at 15,344g–for 10 minutes and supernatants discarded. The pellet was finally dried at room temperature for 10 minutes before re-suspending in 25 µl of formamide with gentle vortexing and the solution spun down. Incubation at 95 °C for 2 min was done and immediately placed on ice followed by a brief spin to collect the sample at the bottom of the tube and then transferred to sequencing tubes.

2.6 DATA ANALYSIS

Editing of the nucleotide sequences was done using Sequencher 4.10.1. (Gene Codes corporation, U.S.A). A blast was then done for the edited sequences using the HIV-1 Los Almos database to confirm that the sequences coded for the HIV envelope functional protein.

http://www.hiv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html

The C2V3 nucleotide sequences were translated into the corresponding 35 amino acids of the V3 loop using Genetic Information Processing Software (Gentyx-Win) Version 9.0 (Gentyx, Tokyo, Japan).

After a successful translation, co-receptor prediction was done using two genotypic rules: Amino acids at positions 11/25 rule and the V3 net charge rule. Two Bioinformatic tools were applied: Geno2pheno [coreceptor] and dsKernel. Geno2pheno is available at http://coreceptor.bioinf.mpi-inf.mpg.de/cgi-bin/coreceptor.pl. dsKernel is available at http://genome.ulaval.ca/hiv-dsKernel/

Phylogenetic relationships of the newly derived viral sequences were estimated from comparisons with those of previously reported HIV-1 group M from the Los Almos sequences database using the CLUSTAL W (http://align.genome.jp/) profile alignment option. The phylogenetic tree was constructed by the neighbour joining method with its reliability being estimated by 1000 bootstraps replications. The profile of the tree was visualized with Tree View PPC version 1.6.5. (Taxonomy and Systematics, Glasgow, UK). To improve the accuracy of subtyping, two tools were used: REGA subtyping tools v2.0 (http://wwwbioafrica.net/rega-genotype/html/subtyinghiv.html) and NCBI Viral Genotyping tools (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

2.7 STATISTICAL ANALYSIS

The correlation of gender, HIV-1 subtype and ARV regimen to viral tropism were performed by chi square test. These were done using SPSS version 17 software (IBM Company, New York) and a P value less than 0.05 was considered statistically significant. SAS 9.1 software

was used to summarise the percentages of the outcome of the four test protocols using proc genmod. Outcomes in form of frequencies of the four protocols were analysed by chi square test in proc freq. P value less than 0.05 was considered statistically significant.

CHAPTER THREE

3.0 RESULTS

3.1 Characteristics of the study population

From an initial sample size of 96, only 84 samples were successfully amplified and sequenced. Clinical and general characteristics with respect to sex, age and CD4+T count was determined as presented in table (2) below. The results indicated that 54 HIV-1 strains were from the ART experiencing group. Their mean age was 41(16-65), 33(61%) were women, 21(39%) male. The mean CD4 count was 253(12-917) per μl of whole blood. 30 HIV-1 strains were from the treatment naive group. Their mean age was 34(17-49), 21(70%) women, 9(30%) male. The mean CD4 count was 384 (54-820).

Table 2: Clinical and General Characterization of the Study Population

	Treatment Naive group N=30	ART group N=54
Sex		
Female	21(70%)	33(61%)
Male	9(30%)	21(39%)
Age (yrs)	34(17-49)	41(16-65)
CD4+T count	384(54-820)	253(12-917)

3.2 Co-receptor prediction

The amino acid sequences deduced from sequencing the variable 3 region of the gp120 protein were used to determine co-receptor types. Four co-receptor prediction tools were used and results summarized in figure 4. The bars in figure 4 represent the percentages of R5- and X4- tropic strains predicted by each of the four predicting tools (amino acids at position 11/25 charge rule, V3 net charge rule, Geno2pheno[co-receptor] and ds Kernel). Numbers above the bars are the actual populations of strains predicted by each test protocol. There was a high magnitude of R5-tropism in the *env* sequences predicted using the four testing protocols. The testing protocols are not significantly different (χ^2 test, p value = 0.19).

Figure 4: HIV-1 viral tropism analysis using different co-receptor predicting tools

tpA = 11/25 Charge rule

tpB = V3 Net Charge rule

tpC = Geno2pheno [co-receptor]

tpD = ds Kernel
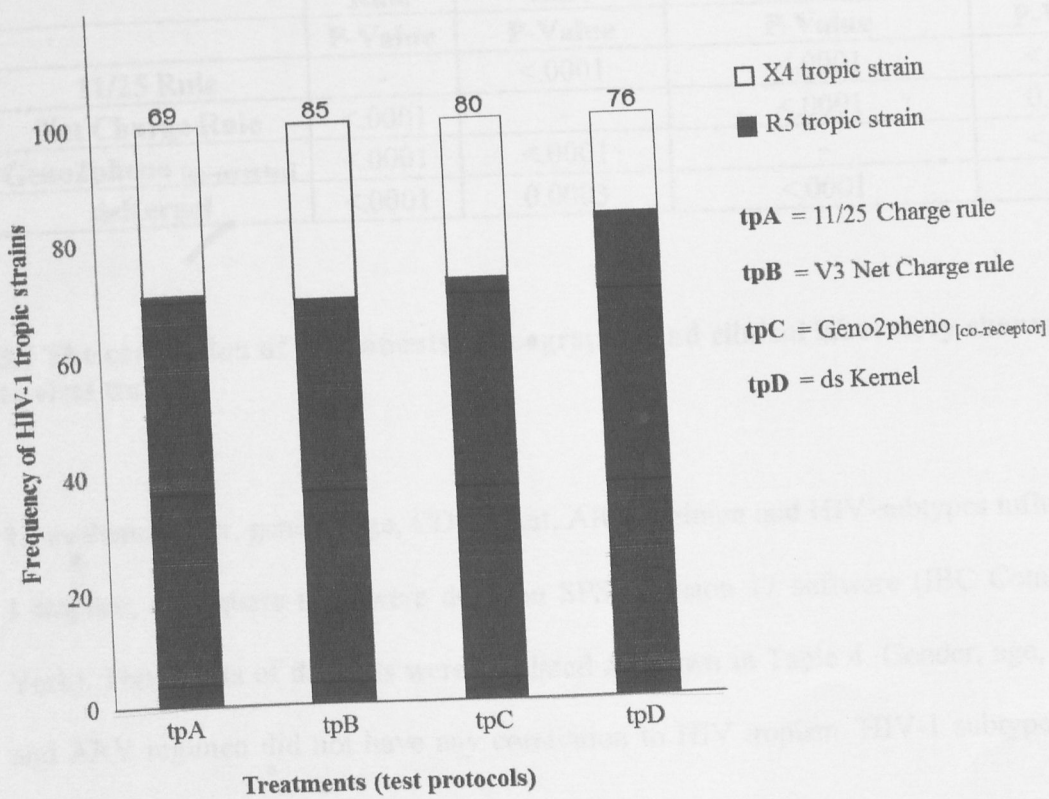
3.3 Correlation of the co-receptor prediction tools used in the study

To find out how the four prediction tools correlated, a chi square test on SPSS version 17 software (IBC Company, New York) was done. The results of the tests were tabulated as shown below (Table 3).The co-receptor predicting tools correlate strongly (p< 0.0001). However, V3 net charge rule and dsKernel do not correlate as strongly (p=0.0003).

Table 3: The correlation between the four co-receptor predicting tools used in the study

	11/25 Rule	Net Charge Rule	Geno2pheno [co-receptor]	dsKernel
	P-Value	P-Value	P-Value	P-Value
11/25 Rule	-	<.0001	<.0001	<.0001
Net Charge Rule	<.0001	-	<.0001	0.0003
Geno2pheno [co-receptor]	<.0001	<.0001	-	<.0001
dsKernel	<.0001	0.0003	<.0001	-

3.4 The correlation of the patients' demographic and clinical laboratory characteristics to viral tropism

To evaluate if sex, gender, age, CD4 count, ARV regimen and HIV-subtypes influence HIV-1 tropism, chi square tests were done on SPSS version 17 software (IBC Company, New York). The results of the tests were tabulated as shown in Table 4. Gender, age, CD4 count and ARV regimen did not have any correlation to HIV tropism. HIV-1 subtypes and HIV-tropism however, correlated strongly.

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Table 4: The correlation of the patient demographic and clinical laboratory characteristics to viral tropism

Characteristic	P-value
Gender	1.00
Age	0.45
CD4 Count	0.26
ARV Regimen	0.31
HIV-1 Sub-type	0.04

3.5 HIV-1 Sub-types of Patients in the Study

To determine the HIV-1 subtypes of the individuals in the study, two subtyping tools were used: REGA subtyping tool v2.0 and NCBI viral genotyping. The pie-chart below (figure 5) represents the percentages of the HIV-1 subtypes of the individuals in the study. The prevalent HIV-1 sub-type was A1 which reported 75%. HIV-1 sub-type C reported 13% and HIV-1 sub-type D reported 12% among the study population.

Figure 5: Pie-chart showing the distribution of HIV-1 sub-types in the study population
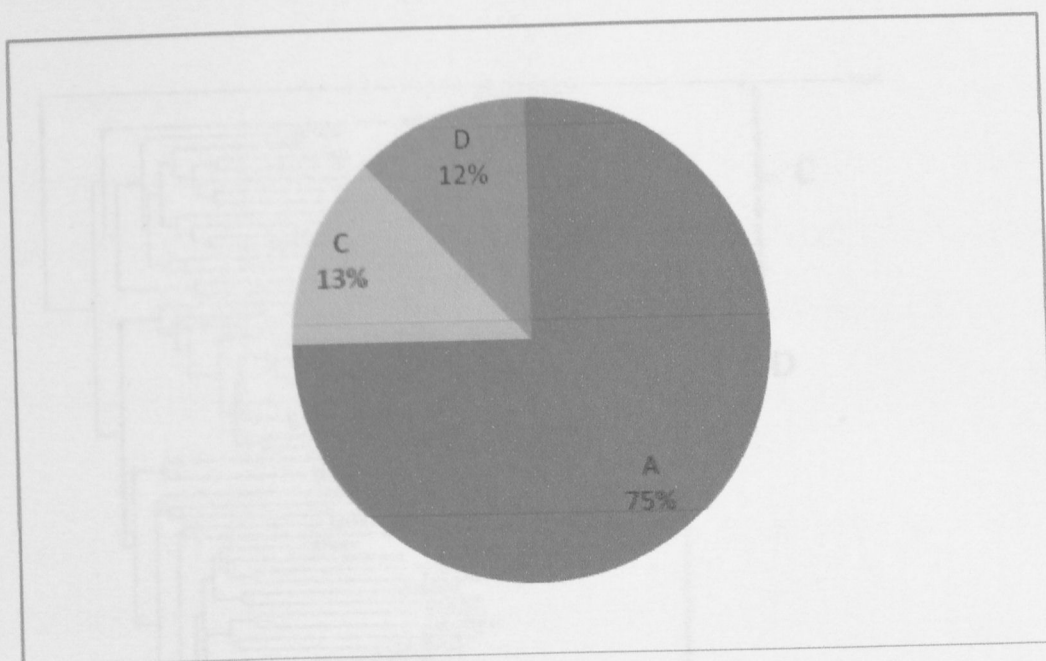
3.6 Phylogenetic relationships of the viral sequences of the study population

C2V3 region sequences of the study population were aligned and compared with reference sequences obtained from the Los Alamos HIV database. Phylogenetic relationships were constructed by neighbour-joining method and rooted with X52154. The bootstrap values (of 1000 replicates) above 70% are indicated next to the node. Brackets on the right indicate the subtype clusters (figure 6).
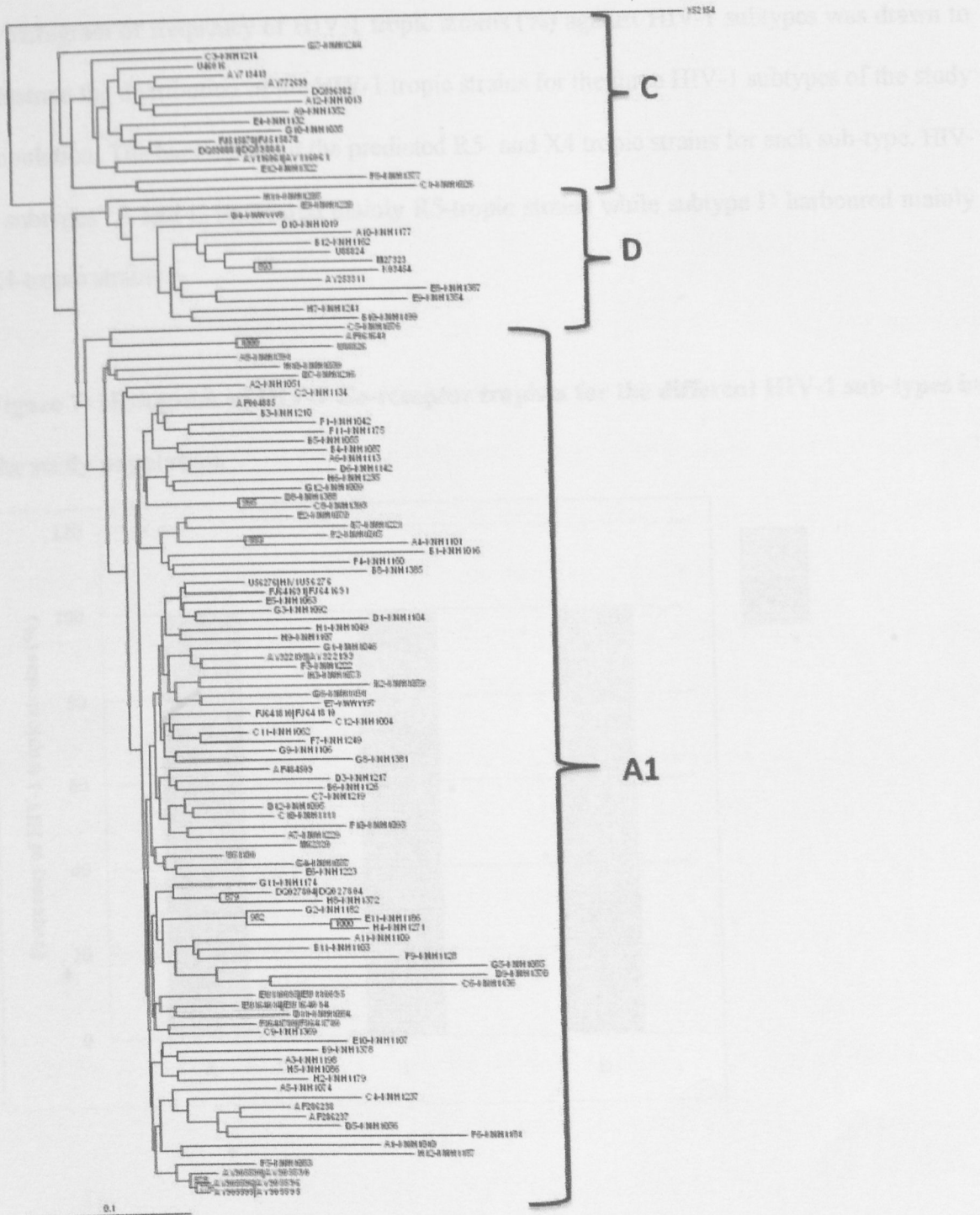
3.7 HIV-1 tropism distribution among the HIV-subtypes in the study population

A histogram of frequency of HIV-1 tropic strains (%) against HIV-1 subtypes was drawn to illustrate the distribution of the HIV-1 tropic strains for the three HIV-1 subtypes of the study population. The bars represent the predicted R5- and X4 tropic strains for each sub-type. HIV-1 subtypes' A and C harboured mainly R5-tropic strains while subtype D harboured mainly X4-tropic strains.

Figure 7: Histogram of HIV-1 Co-receptor tropism for the different HIV-1 sub-types in the study population.
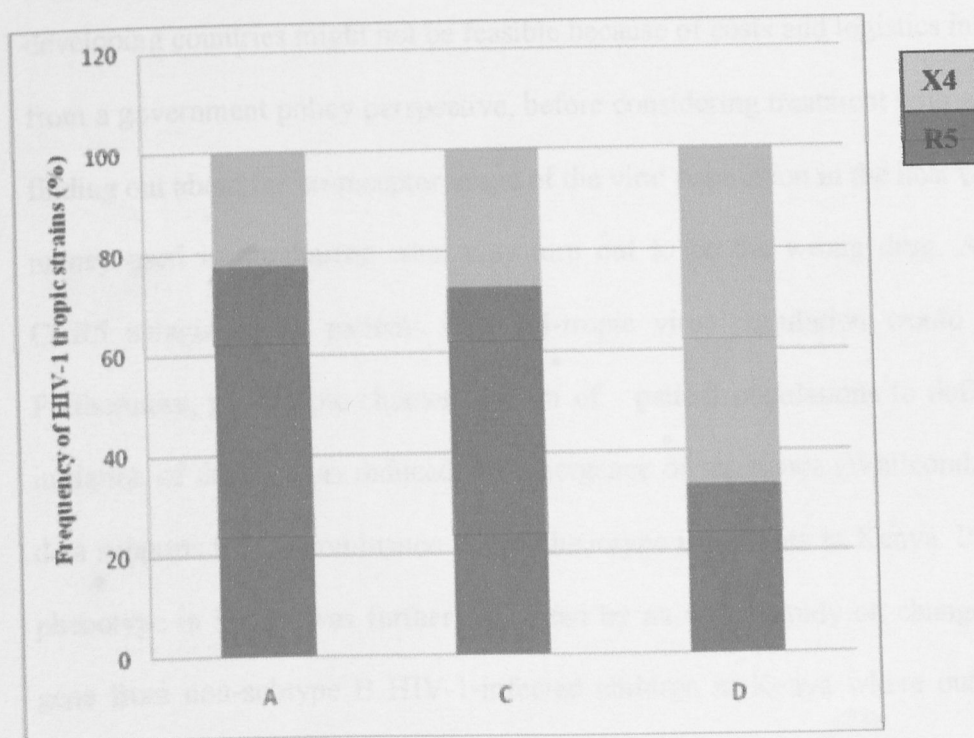
CHAPTER 4

4.0 DISCUSSION, CONCLUSIONS & RECOMMENDATIONS

4.1 Discussion

4.1.1 HIV-1 Co-receptor usage in Kenya

There is little information about co-receptor usage in Kenya despite the impending introduction of CCR5 antagonists. HIV tropism assays are crucial before administering CCR5 antagonists to a patient. Monitoring co-receptor usage in large scale treatment programs in developing countries might not be feasible because of costs and logistics involved. Therefore, from a government policy perspective, before considering treatment with a CCR5 antagonist, finding out about the co-receptor usage of the viral population in the host will save the patient money used in purchasing what may turn out to be the wrong drug. Also, administering CCR5 antagonists to patients with X4-tropic virus population would be of no benefit. Furthermore, phenotypic characterization of patient populations to define R5 only before initiation of therapy has reduced the emergence of resistance (Whitcomb *et al.*, 2007). The data supports the predominance of R5 phenotype in patients in Kenya. Predominance of R5 phenotype in Kenya was further supported by an earlier study on change in the HIV-1 env gene from non-subtype B HIV-1-infected children in Kenya where out of 41 children on ART, 35(85.4%) had HIV-1 using CCR5 as a co-receptor at baseline (Lwembe *et al.*, 2009). In another study out of 19 samples checked for co-receptor usage, 14 (73.7%) were chemokine co-receptor 5 (CCR5) variants while 3(15.8%) were CXCR4 variants (Lihana *et al.*, 2009). This CCR5 co-receptor usage utilization predominance could be due to the fact that CCR5 serves as a co-receptor for the most commonly transmitted HIV-1 strain, R5,

which is predominant during early stages of infection and remains dominant in over 50% of late stage HIV-1 patients (Dragic *et al.*, 1996). Variants able to utilize CXCR4 emerge in ~40%–50% of infected persons over the course of disease (Berger *et al.*, 1999). A similar study done to predict co-receptor tropism in India also revealed the predominance of R5 strains where 96.8% of a total of 1045 HIV-1 subtype C Indian sequences were R5-tropic (Neogi *et al.*, 2010).

4.1.2 Concordance of the Co-receptor prediction tools.

The co-receptor prediction tools used in the study correlated strongly. However, prediction of tropism for some patients was missed or a discrepancy was noted mostly for X4 tropism by the bioinformatics tools especially dskernel. It has been shown previously that genotypic approaches based on V3 perform well for predicting co-receptor usage of HIV-1 sub-type B (Raymond *et al.*, 2006; Delobel *et al.*, 2007). However, the genotypic algorithms built from sub-type B virus data sets could be inadequate for predicting the tropism of non-B viruses (Garrido *et al.*, 2008). It was found that geno2pheno resulted in overestimation of X4 viruses while dsKernel resulted in underestimation of X4 viruses. This was also observed for a study in India where dskernel underestimated X4 viruses (Neogi *et al.*, 2010). Currently Trofile assay (Monogram Biosciences, CA) is the only clinically validated tropism test. However, the use of this type of phenotypic assays has several limitations: (i) the need to perform this assays in a centralized lab; (ii) the limited amplification success of gp120, and (iii) the relatively long turn-around times, high cost and requirement for large fresh specimen. The search for alternatives led to relying on the amplification of the V3 domain of gp120, the major determinant for viral tropism (Sander *et al.*, 2007). Therefore, prediction of co-receptor

usage based on V3 sequences using bioinformatics tools are a good alternative for phenotypic tropism testing in routine clinical practice (Resch *et al.*, 2001).

4.1.3 Correlation between HIV-1 sub-type and HIV-1 viral tropism.

Majority of HIV-1 subtype D population constituted mainly X4 tropic strains (6/10) 60%, p=0.015. HIV-1 subtype D infection is associated with faster disease progression than subtype A (Baeten *et al.*, 2007). A study conducted among Kenyan female sex workers cohort found that HIV-1 clade D infection is associated with higher viral loads and faster disease progression than clade A1. Compared to clade D-infected subjects, those with clade A1 infection progressed to a CD4 count of less than 350 slower than those infected by clade D (mean 5.0 vs 3.4 years from the time of cohort entry). The study also compared available viral load between subjects identified as clade A1 and D and found that infection by HIV-1 clade A1 was associated with a lower VL compared to clade D. While the median VL for clade A1 infected subjects was 28,644 copies/ml, the median VL for clade D infected subjects was approximately 3.5 fold higher (100,033 copies/ml, p=0.048). HIV-1 X4-tropic strains are known to be associated with faster disease progression compared to HIV-1 R5-tropic strains (Kaleebu *et al.*, 2007). It may be hypothesized that those with subtype D switch to X4 earlier thus the strong correlation between CXCR4-tropism and HIV-1 sub-type D. There are 2 other studies indicating that subtype D is associated more with SI (X4 or R5X4) phenotype. V3 loop heterogeneity, length polymorphism, and a large number of positively charged amino acid substitutions associated with SI phenotype have been found more frequently among subtype D variants than among subtype B, C, and E variants (De Wolf *et al.*, 1994). The other study conducted among HIV-infected children (Casper *et al.*, 2002) found that the child with the greatest number of X4 viruses over the greatest time span carried

HIV-1 subtype D. Results from this study imply that CCR5 antagonists may be less effective in individuals infected with HIV-1 subtype D.

4.1.4 Correlation between ARV regimen and HIV-1 viral tropism.

The results showed that the HIV-1 CXCR4 utilization among antiretroviral therapy HIV-1-infected patients was higher than in the treatment-naive population; 73.3% of X4 tropic strains in the ART experiencing population versus 26.7% in the treatment naive population. This was in agreement with a study done in San Francisco in treatment-naive and treated HIV-infected participants with detectable viremia sampled from 2 clinic-based cohorts. Of 182 treated participants, 75 (41%) harboured dual/mixed or X4-tropic viruses, compared with 178 (18%) of the 976 treatment-naive participants (Hunt *et al.*, 2006). The mechanism responsible for the emergence of CXCR4-using viruses on HAART remains unclear. Because CCR5 inhibitors are likely to be initially used in treated patients with resistance to currently available antiretroviral medications, it is important to assess the prevalence of CXCR4 tropism in this population. There are several reasons to believe that antiretroviral therapy might alter the prevalence of X4-tropic viruses. First, partially suppressive therapy may lead to an increase in HIV-specific T cell responses (Deeks *et al.*, 2004). Because X4-tropic variants may be more susceptible to cytotoxic T cell responses than R5-tropic viruses (Harouse *et al.*, 2003), increases in HIV-specific T cell responses during partial treatment-mediated viral suppression might select against X4-tropic viruses. Second, antiretroviral therapy reduces CCR5 expression on T cells, presumably as a consequence of reductions in T cell activation (Anderson *et al.*, 1998), potentially selecting for X4-tropic viruses (Brumme *et al.*, 2005). Last, certain antiretroviral drugs may preferentially select for one virus population, either because of enhanced activity against X4 viruses as has been suggested for enfuvirtide

(Yuan *et al.*, 2004) or because of suboptimal drug metabolism in the cellular reservoirs for X4 viruses as has been suggested for zidovudine (Boucher *et al.*, 1992).

4.2 CONCLUSION

HIV-R5 tropic strains were the most prevalent in the study population, therefore, HIV patients in Kenya may benefit from CCR5 antagonists. However, there is need for caution where subtype D infection is suspected or where antiretroviral salvage therapy is indicated.

4.3 RECOMMENDATION

The incorporation of a phenotypic assay to confirm the V3 co-receptor prediction determined using genotypic algorithms to solve any discrepancies among the genotypic algorithms. The methodology did not also take into effect the possibility of presence of dual tropic strains that have been found to be prevalent in other antiretroviral experienced populations; future studies should therefore incorporate assays to assess dual tropism.

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APPENDIXES

1.0 Patient demographic details and ARV drugs

Patient ID	Age	Sex	CD4 Count	ARV Drugs
KNH1010	30	F	243	Naive
KNH1051	31	F	270	AZT-3TC-EFV
KNH1198	31	F	188	Naive
KNH1074	46	F	820	Naive
KNH1229	46	F	351	D4T-3TC-EFV
KNH1391	24	F	497	Naive
KNH1352	45	M	279	TDF-3TC-LPV/V
KNH1109	42	F	373	TDF-ABC-LPV/V
KNH1013	43	M	79	TDF-ABC-LPV/V
KNH1059	36	F	54	Naive
KNH1087	35	F	405	Naive
KNH1055	36	F	202	d4T(30gm)-3TC-NVP
KNH1126	41	F	242	d4T(30gm)-3TC-NVP
KNH1227	46	M	236	AZT-3TC-NVP
KNH1385	54	M	350	AZT-3TC-EFV
KNH1378	45	F	224	TDF-3TC-LPV/V
KNH1103	40	F	107	TDF-ABC-LPV/V
KNH1162	25	M	471	Naive
KNH1026	32	F	456	Naive
KNH1134	42	F	326	Naive
KNH1214	27	F	167	Naive
KNH1237	49	F	530	Naive
KNH1076	48	F	265	d4T(40gm)-3TC-NVP
KNH1219	44	F	25	TDF-ABC-LPV/V
KNH1369	22	M	917	3TC-AZT-LPV/V
KNH1111	44	M	79	AZT-DDI-LPV/V
KNH1062	41	F	111	ABC-TDF-LPV/V
KNH1004	50	F	210	TDF-3TC-LPV/V
KNH1101	36	F	188	TDF-DDI-LPV/V
KNH1065	36	F	592	Naive
KNH1217	49	M	361	Naive
KNH1118	30	F	121	Naive
KNH1056	30	F	461	d4T(30gm)-3TC-NVP
KNH1142	41	M	401	d4T(40gm)-3TC-NVP
KNH1216	34	F	173	d4T(30gm)-3TC-NVP
KNH1388	50	F	538	3TC-AZT-ALUVIA
KNH1095	44	M	342	TDF-DDI-LPV/V

KNH1220	26	F	661	Naive
KNH1132	44	M	512	Naive
KNH1063	40	F	518	d4T(30gm)-3TC-NVP
KNH1223	49	F	151	d4T(40gm)-3TC-NVP
KNH1197	42	M	190	d4T(30gm)-3TC-NVP
KNH1354	38	M	171	AZT-3TC-LPV/V
KNH1107	52	F	493	TDF-ABC-LPV/V
KNH1186	42	M	137	ABC-DDI-LPV/V
KNH1322	16	M	64	ABC-DDI-LPV/V
KNH1015	31	F	460	Naive
KNH1222	65	F	288	Naive
KNH1160	31	M	141	Naive
KNH1083	53	M	178	d4T(30gm)-3TC-EFV
KNH1181	44	M	98	d4T(30gm)-3TC-EFV
KNH1249	40	F	341	TDF-3TC-EFV
KNH1377	46	F	12	Naive
KNH1128	29	F	58	TDF-3TC-LPV/V
KNH1001	30	F	258	TDF-ABC-LPV/V
KNH1046	47	M	460	Naive
KNH1182	48	F	319	Naive
KNH1092	27	F	510	Naive
KNH1037	35	M	409	Naive
KNH1381	17	M	429	Naive
KNH1106	35	F	418	TDF-3TC-EFV
KNH1035	39	M	119	d4T(30gm)-3TC-EFV
KNH1009	33	F	198	ABC-TDF-LPV/V
KNH1049	40	F	168	Naive
KNH1179	22	F	284	Naive
KNH1073	42	F	654	Naive
KNH1271	31	M	374	Naive
KNH1235	36	M	124	d4T(30gm)-3TC-NVP
KNH1241	53	F	202	TDF-3TC-NVP
KNH1372	29	M	233	d4T(40gm)-3TC-EFV
KNH1039	56	M	239	d4T(40gm)-3TC-EFV
KNH1105	29	F	590	d4T(30gm)-3TC-NVP
KNH1177	41	F	442	TDF-3TC-LPV/V
KNH1016	33	M	252	Naive
KNH1210	37	M	533	Naive
KNH1070	36	F	249	Naive
KNH1387	56	M	342	d4T(30gm)-3TC-NVP
KNH1093	43	M	167	TDF-ABC-LPV/V
KNH1085	42	F	231	Naive

KNH1101	36	F	188	TDF-ABC-LPV/V
KNH1370	24	F	282	ABC-DDI-LPV/V
KNH1019	40	M	170	TDF-ABC-LPV/V
KNH1084	37	F	23	d4T(30gm)-3TC-NVP
KNH1244	40	F	89	d4T(30gm)-3TC-NVP

1.1 V3 loop amino acid sequences

Patient ID	V3 loop Amino acid sequence
KNH1010	CTRPNNNTRTGIHLGPGRTFFATGDIIGDIRKAHC
KNH1051	CTRPANNTRTSIRIGPGQTFFATGAITGNIRQAHC
KNH1198	CTRPDNNTRKSISIGPGQAFYARGEIIGNIRKAHC
KNH1074	RTRPNNNTRRGIHIGPGQAFYAADKIIGNIRQAHC
KNH1229	CIRPNNNTRKGIHIGPGQAFYGT-DIIGDIRQAHC
KNH1391	RTRPNNNTRTSIRIGPGQAFYATGGIIGDIRQAHC
KNH1352	CVRPGNITRRSIRIGPGRAFYTTGPILGDIRKAYC
KNH1109	CTRPGTTTRRSVRIGPGRVFYATGDIIRDIKKAYC
KNH1013	CVRPNNNTRKSVRIGPGQTFYATGDIIGDIRQAYC
KNH1059	CIRPNNNTRTSVRIGPGQMFYATGEIIGNIRQAHC
KNH1087	RSRPNNNTRKSIHIGPGQAFYAT-DIIGDIRQAHC
KNH1055	CSRPNNNTRKSIHIGPGQAFYATGEIIGNIRKASC
KNH1126	RTRPSNNTRKGIHIGPGQAFYTTGDIIGDIRQAHC
KNH1227	RTRPNNNTRKSVRIGPGQAFYATG-IIGDIIKPHC
KNH1385	CIRPGNNTRKSVRIGPGQAFYTT-DVIGDIIQAHC
KNH1378	CTRPNNNTRTSVRIGPGQTFYATGTIIGDIRQAHC
KNH1103	RTRPGNIQGQRIHIGPGRAFSATRAILGDIRKAYC
KNH1162	CVRPYNNTRQGTHIGPGQALYTT-KIIGDIRQAHC
KNH1026	CTRPNNNTRKSIRIGPGQTYYATGDIIGNIRLAYC
KNH1134	CTRPNNNTRKDIGIGPGQTFYATGAIIGNIREAYC
KNH1214	CMRPGNNTRQSIRIGPGQTFYATGDITGDIRQAHC
KNH1237	CTRPYKNTRQRTPIGPGQALFTT-RIVGDIRQAYC
KNH1076	RTRPYNSTKKSIRLGPGKVVYTR-RIIGHIRQAHC
KNH1219	RIRPNNNTRKSVRIGPGQAFYATGDIIGNIRQAYC
KNH1369	RTRLNNNTRKSIHIGPGQAFYATGDIIGNIRQAHC
KNH1111	CIRPNNNTRKRVNIGPGRAFYATDRIIGDIRQAHC
KNH1062	RTRPSNNTRTSVRIGPGQAFYATDDIIGDIRRAHC
KNH1004	CIRPGNNTRKSVRIGPGQAFYATGDIIGDIRQAHC
KNH1101	CVRPNNNTRIRVPIGPGQTWYAT-DIIGDIIQPYC
KNH1065	CMRPNNNTRTSMRIGPGESFYTTGDIIGDIRQAHC
KNH1217	CVRPNNNTRKSIRIGPGQTFYATGDIIGDIRQAHC
KNH1118	RTRPNNNTRTSIRIGPGQTFYATGDVIGDIRQAHC
KNH1056	CTRPNNNTRKYIWFGPGQAFRTHNNIIGDIRKAHC
KNH1142	RIRPNNNTRKGIHMGPGQAFYATGDIIGNIRQAHC
KNH1216	CIRPNNNTRQSTHIGPGRTFYSTGDIIGDIRQAHC
KNH1388	RTRPSNNTRKSITIGPGRAFFATGEIIGDIRAAHC
KNH1095	RIRPNNNTRKSVRIGPGQAFYATGDIIGNIRKAHC
KNH1220	CTRPGNNTRKGIHIGPGQALYAMSKVIGDIRQAHC
KNH1132	CTRPGNNTRQSIRIGPGQTFYATGDIIGDIRQAHC
KNH1063	CTRPNNNTRTSVRIGPGQTFYATGDIIGNTRQAHC

KNH1223	RIRPNNNTRRGVHIGPGQAFFTT-DIIGDIRQAHC
KNH1197	CIRPNNNTRQSVRIGPGQTFLCTRRHNRGYKTSTF
KNH1354	CTRPYKKITRKITIGPGRADF-TSKIEGDIRQAYC
KNH1107	CTRPSNNTRTSIRIGPGQTFHATGEIIGDIRRAHC
KNH1186	CSRPDNKQKIQRVIGRGQSLY-TNNIREDIRKAYC
KNH1322	RMRPGNNTKKYIRIGPGYAFPATDRIIGDIRQAHC
KNH1015	CIRPNNNTRQSIRIGPGQTFY-ASNIIGNIIQAHC
KNH1222	CTRPNNNTRKSVRIGPGQTFYATGDIIGNIRQAHC
KNH1160	RTRPNNNTRKSVRIGPGQTFYGTGDIIGNIRQAHC
KNH1083	CTRPSNNTRTSVRIGPGQAFYATGDIIGDIRQAHC
KNH1181	CTRTRPQQKKSTHTGPGRAFY-TNDIGGNIKKAYC
KNH1249	RTRPSDNKRTSVRIGPGQTFYATGDIIGDIRRAHC
KNH1377	CTRPGNNTRQSIRIGPGQTFYARGDIIGDIRQAHC
KNH1128	CTRPSKNKRHSVRIRPGRAFFKMGNIYGDIKKSQC
KNH1001	GIRPNNNTRIRVGIGPGQAFRGT-DIIGDIRQAHC
KNH1046	CTRPNNNTRKSVHIGPGQAFYARGDIIGNIRQAHC
KNH1182	RFRPNNNTRQSVHIGPGQSLY-TNNIIGDIRKAYC
KNH1092	RIRPNNNTRRSVPIGPGQAFYATGDIIGNIRQAHC
KNH1037	RTRPNNNTRKSIHIGPGQAFYATGDIIGNIRAAHC
KNH1381	CTRPNNNTRQSVHIGPGQALYATGAIIGDIRKAHC
KNH1106	CTRPNNNTRKGIRIGPGQVFYATGKIIGNIRQAHC
KNH1035	CTRPNNNTRESIRIGPGQTFFATGDIIRDIRQAHC
KNH1009	RIRPGNNTRRSIHIGPGKAFYAPGDIIGDIRQAHC
KNH1049	CIRPNNNTRRSVHLGPGQAFYATGDIIGDIRQAHC
KNH1179	CSRPNNNTRKSVRIGPGQTFYATGDIIGDIRQAHC
KNH1073	CIRPNNNTRKNVRIGRGHTFYATGAIIGDIRRAHC
KNH1271	CSRPDNKQQIQRVIGRGQSLY-TNNIRGDIRKAYC
KNH1235	RIRPGNNTRKFIRPGPGHAFY-THDIIGDIRKAHC
KNH1241	RTRPNNNTRLSTHMGPGQALFTGTVIGDIRQAHC
KNH1372	CFRPNNNTRKSVRIGPGQTFYATGGIIGNIRQAHC
KNH1039	CTRPNNNTRESIRIGPGQAFYATGDIIGDIRQAHC
KNH1105	RTRPNNNTSKGIRIGPGRAFYATERIKGDVRAAHC
KNH1177	CTRPYTNIRRRTSIGLGQALY-TSKITGYAGKAYC
KNH1016	CIRPNNNTRKSIRIGPGQSYYATGDIIGDIRKAHC
KNH1210	RTRPNNNTRKSVRIGPGQSFFATGDVIGDIRKAHC
KNH1070	RTRPNNNTRKSIRIGPGQTFYATGDIIGDIRAAHC
KNH1387	CTRPYNNTRRGVPIGPGSAFYASNNIIGDIRQAHR
KNH1093	RTRPNNNTRKSVSIGPGQAFFATGDIIGDIRQAHC
KNH1085	RSRPNNYRRRSVRIGPGPGFPTDANVGDREKAQC
KNH1101	CIRPNNNTRQSIHIGPGQTFYATGKIRKNIQPPCC
KNH1370	CMRPGNNTIKRVRIGPGPGLYFYSDRIRDREKAHG
KNH1019	CTRPYSNTRRRIHIGPGRAFYTHTNIKGDIRQAHC
KNH1084	CTRLNNNTRKSFRIGPGLKYYTRGDIVGNIRQAHC
KNH1244	CTRPNNNAKKRIRIGPGRTLYATRDIRRDIRQHCC

1.2 Patient HIV-1 subtype & predicted HIV-1 co-receptor tropism

Patient ID	Subtype	Position 11/25 rule			V3 Net charge rule		Geno2pheno	dsKernel
		11	25	Strain	Charge	Strain		
KNH1010	A1	G	D	R5	5	R5	R5	R5
KNH1051	A1	S	A	R5	5	R5	R5	R5
KNH1198	A1	S	E	R5	5	R5	R5	R5
KNH1074	A1	G	K	X4	7	X4	X4	R5
KNH1229	A1	G	K	X4	4	R5	X4	X4
KNH1391	A1	S	G	R5	5	R5	R5	R5
KNH1352	C	S	P	-	6	X4	-	-
KNH1109	D	S	D	R5	5	R5	R5	R5
KNH1013	C	S	D	R5	3	R5	R5	R5
KNH1059	A	S	E	R5	4	R5	R5	R5
KNH1087	A1	S	D	R5	5	R5	R5	R5
KNH1055	A1	S	E	R5	5	R5	R5	R5
KNH1126	A	G	D	R5	5	R5	R5	R5
KNH1227	A	S	-	-	6	X4	-	-
KNH1385	A1	S	D	R5	4	R5	R5	R5
KNH1378	A	S	T	R5	4	R5	R5	R5
KNH1103	A	R	A	X4	7	X4	X4	X4
KNH1162	D	S	K	X4	6	X4	X4	X4
KNH1026	C	S	D	R5	4	R5	R5	R5
KNH1134	A	D	A	-	2	R5	-	R5
KNH1214	C	S	D	R5	4	R5	R5	R5
KNH1237	AD	R	R	X4	6	X4	X4	X4
KNH1076	D	S	R	X4	11	X4	X4	X4
KNH1219	A1	S	D	R5	5	R5	R5	R5
KNH1369	A1	S	D	R5	6	X4	R5	R5
KNH1111	A1	R	R	X4	6	X4	X4	R5
KNH1062	A1	S	D	R5	4	R5	R5	R5
KNH1004	A	S	D	R5	5	R5	R5	R5
KNH1101	A1	R	D	X4	3	R5	X4	X4
KNH1065	C	S	D	R5	3	R5	R5	R5
KNH1217	A	S	D	R5	4	R5	R5	R5
KNH1118	CD	S	D	R5	4	R5	R5	R5
KNH1056	A1	Y	N	R5	7	X4	R5	R5
KNH1142	A	G	D	R5	5	R5	R5	R5
KNH1216	A	S	D	R5	4	R5	R5	R5

KNH1388	A1	S	E	R5	5	R5	R5	R5
KNH1095	A1	S	D	R5	7	X4	R5	R5
KNH1220	D	G	K	X4	6	X4	X4	R5
KNH1132	BC	S	D	R5	4	R5	R5	R5
KNH1063	A1	S	D	R5	4	R5	R5	R5
KNH1223	B	G	D	R5	5	R5	R5	R5
KNH1197	A1	S	R	X4	7	X4	X4	R5
KNH1354	D	K	K	X4	4	R5	X4	X4
KNH1107	A1	S	E	R5	5	R5	R5	R5
KNH1186	A1	Q	NULL	-	5	R5	X4	R5
KNH1322	BC	Y	R	X4	6	X4	X4	X4
KNH1015	A1	S	N	-	4	R5	R5	-
KNH1222	A1	S	D	R5	5	R5	R5	R5
KNH1160	A	S	D	R5	6	X4	R5	R5
KNH1083	A	S	D	R5	3	R5	R5	R5
KNH1181	A2	S	D	R5	7	X4	R5	R5
KNH1249	A	S	D	R5	5	R5	R5	R5
KNH1377	BC	S	D	R5	4	R5	R5	-
KNH1128	A1	S	N	-	8	R5	R5	R5
KNH1001	C	R	D	X4	4	R5	R5	R5
KNH1046	A1	S	D	R5	6	X4	R5	-
KNH1182	A1	S	N	-	5	R5	R5	R5
KNH1092	A	S	D	R5	5	R5	R5	R5
KNH1037	A1	S	D	R5	6	X4	R5	R5
KNH1381	A1	S	A	R5	5	R5	R5	R5
KNH1106	A1	G	K	X4	6	X4	X4	R5
KNH1035	C	S	D	R5	2	R5	R5	R5
KNH1009	A1	S	D	R5	5	R5	R5	R5
KNH1049	A1	S	D	R5	4	R5	R5	R5
KNH1179	A	S	D	R5	4	R5	R5	R5
KNH1073	A1D	A	N	X4	8	X4	X4	R5
KNH1271	A1D	Q	N	-	5	R5	X4	-
KNH1235	A1	F	D	-	7	X4	-	-
KNH1241	D	S	T	R5	5	R5	R5	R5
KNH1372	A1	S	G	R5	4	R5	R5	R5
KNH1039	C	S	D	R5	2	R5	R5	R5
KNH1105	A1D	G	R	X4	6	X4	X4	R5
KNH1177	D	R	K	X4	6	X4	X4	X4
KNH1016	A1	S	D	R5	5	R5	R5	R5
KNH1210	A1	S	D	R5	5	R5	R5	R5
KNH1070	A1	S	D	R5	5	R5	R5	R5
KNH1387	D	G	N	R5	5	R5	R5	R5

KNH1093	A1	S	D	R5	4	R5	R5	R5
KNH1085	A1	S	A	-	5	R5	-	-
KNH1101	A	S	K	X4	6	X4	X4	X4
KNH1370	A	R	D	X4	3	R5	X4	X4
KNH1019	D	R	N	X4	9	X4	X4	X4
KNH1084	A1	S	D	R5	5	R5	R5	R5
KNH1244	C	R	D	X4	9	X4	X4	X4

EVALUATION OF THE KENYA ARV SCALE UP PROGRAM:

SURVEILLANCE FOR EMERGENCE OF ANTIRETROVIRAL

RESISTANT GENOTYPES AMONG TREATMENT EXPERIENCED

PATIENTS

a) Description/Purpose of the study

The use of antiretroviral drugs has greatly prolonged lives of people infected by Human Immunodeficiency virus. In Kenya more than 120,000 people are on treatment with antiretroviral drugs. Though these drugs are effective, they may fail when the virus develops resistance towards them. The reasons why resistance develops in other people and not in others is unknown. We are intending to carry out an investigation study to determine the extent of HIV drug resistance in Kenya and possible reasons for the resistance. This study is very important, for it will help doctors treating HIV/AIDS patients to know the alternative type of drugs to give when patients fail treatment. It will also help us to test newer drugs that may be more effective. As you are about to begin ARV treatment or has been on treatment, we are asking for your participation in this study.

If you agree, we will use the blood sample that you have given out for CD4+ analysis and other blood work also for this study. We will use the blood sample to culture the virus, amplify the virus and compare it with other viruses that have been known to have defeated antiretroviral drugs. We will also use the same sample to determine if there are other genetic factors within your body that may help HIV viruses to easily evade some types of antiretroviral drugs.

Use of DNA Material

1.3 Consent form

CONSENT INFORMATION

EVALUATION OF THE KENYA ARV SCALE-UP PROGRAM: SURVEILLANCE FOR EMERGENCE OF ANTIRETROVIRAL RESISTANT GENOTYPES AMONG TREATMENT EXPERIENCED PATIENTS

a) Description/Purpose of the study

The use of antiretroviral drugs has greatly prolonged lives of people infected by Human Immunodeficiency virus. In Kenya more than 120,000 people are on treatment with antiretroviral drugs. Though these drugs are effective, they may fail when the virus develops resistance towards them. The reasons why resistance develops in other people and not in others is unknown. We are intending to carry out an investigative study to determine the extent of HIV drug resistance in Kenya and possible reasons for the resistance. This study is very important, for it will help doctors treating HIV/AIDS patients to know the alternative type of drugs to give when patients fail treatment. It will also help us to test newer drugs that may be more effective. As you are about to begin ARV treatment or has been on treatment, we are asking for your participation in this study.

If you agree, we will use the blood sample that you have given out for CD4+ analysis and other blood tests also for this study. We will use the blood sample to culture the virus, classify the virus and compare it with other viruses that have been known to have defeated antiretroviral drugs. We will also use the same sample to determine if there are other genetic factors within your body that may help HIV viruses to easily evade some types of antiretroviral drugs.

Use of DNA Material

The DNA fragment obtained from your sample shall be sequenced to determine if you are at risk of developing antiretroviral treatment failure. The DNA material will not be modified or engineered in any way.

Laboratory Sites.

Sequencing will be done at the University of Nairobi Institute of Tropical Medicine laboratories. This is a WHO accredited lab for viral sequencing. The Public Health Agency of Canada laboratories (PHAC) in Winnipeg act as a site for external quality assurance for the UoN labs. As a quality assurance procedure, representative sequenced samples will be picked randomly and sent to PHAC for confirmation. PHAC lab has been involved in genetic studies for the past 10 years. The high number of publications in peer review journals on genetic studies from PHAC is proof of the facility to handle such studies.

a) Research team

You are being asked to participate in a research study called EVALUATION OF THE KENYA ARV SCALE UP: SURVEILLANCE FOR EMERGENCE OF ANTRETROVIRAL RESISTANT GENOTYPES AMONG TREATMENT EXPERIENCED PATIENTS which is being conducted by two Principal Investigators: Dr. Martim Songok, PhD and Dr. Sobha Vakil, MD. Dr. Songok is a Senior Research Officer at Centre for Virus Research, Kenya Medical Research Institute (KEMRI) and Dr. Vakil is the Medical Officer of the antiretroviral treatment program at the national AIDS control Program (NASCOP) of the Ministry of Health.

The Co-Investigators in this study are Dr. Fredrick Okoth, MD, Dr. Solomon Mpoke PhD, Dr. John Vulule PhD, and Dr. Michael Kiptoo PhD, all of Kenya Medical Research Institute. The other co investigators from the University of Nairobi and Moi University are Prof. Benson Estambale, MD, Prof. James Ochanda, PhD, Dr. David Mburu PhD and Dr. Ernest Emonyi, PhD. Dr. Jack Nyamongo, MD is the head of National Public Health Laboratories of the Ministry of Health. External co-investigators are Dr. Rami Kantor, MD of Bown University, USA, Dr. Blake Ball PhD of Univesity of Manitoba Canada and Dr. Paul Sandstrom, PhD of Public Health agency of Canada in Ottawa.

b) Benefits of Participation in the Study

You will receive no personal benefit from your participation in this study. The information generated from this study will however be provided to the Ministry of Health to help them make decisions on the type of antiretroviral drugs to use for people who fail first line of treatment. To you in particular, it will help you directly when you develop resistance to the type of drugs that you will using. Based on our observations, we will provide information to your doctor to use to decide on the possible effective drug regimen to change to as an alternative.

c) Archiving of specimens

Your sample will be stored at Kenya Medical Research Institute (CVR and CGHR), University of Nairobi (UNITID) or Moi University Eldoret (AMPATH labs) as a dried blood spot on a filter paper. The AIDS virus is in constant evolution. The type that is in circulation now will likely be different from the one that will be prevalent in 5, 10, 15 or 25 years time. We will wish to compare the current virus and the type that will be in circulation then. In addition newer and better technologies for analyzing the AIDS virus keep emerging every year; we will need to test your sample with these new methods. In this regard we will store your sample (about 5-10ug) for a period of 25 years after the end of the study. In the event of our need to do future comparative studies using your sample, we will apply again to the Director, Kenya Medical Research Institute through the Ethical Review Board for approval. No information which may reveal your identity will be attached to the sample. We will protect the confidentiality of the samples by assigning them a specific code. Your DNA sample will not be specifically identified but a code will link you to the sample. Decoding can only be performed by the Principal Investigators (Dr. Songok and Dr.Vakil) or an individual authorized by them.

Similarly, as we have to compare our study methods with those of others abroad, or these newer technologies may appear earlier in developed countries, your sample may be transported to Canada (Public Health Agency of Canada) or/ and USA (Brown University) for training, quality control and confirmation purposes. At end of the storage and study period, the DNA material shall be incinerated and disposed of as per the prevailing regulations of disposal of genetic materials of Public Health Agency of Canada.

Sharing of samples

Your samples will not be shared with any member outside the investigating team and their students. However, any member of the team may use the sample for other genetic studies.

The use of the material for other studies will however require a re-approval from the Director Kenya Medical Research Institute through the Ethical Review Board.

Risks of participation

Since this research is being performed with samples that have already been taken for other purposes you will not be exposed to any physical risks associated with the taking of a DNA sample.

There are risks of discrimination against persons who have a genetic medical disorder or at risk of a medical disorder or condition in their family. Discrimination may include barrier to obtaining life or health insurance and employment. All efforts shall be made to protect our research subjects from prejudice or use of this information that may adversely affect them. Specifically clinical and research information specific to this study will be maintained in a separate location from your hospital medical records and will not be shared or placed in your medical file in the hospitals that you attend.

Confidentiality

All information obtained about you and the results of the research will be treated confidentially. This information will be coded and kept under a password protected database. The study files will be kept electronically at Centre for Virus Research, KEMRI under the responsibility of Dr. E. Songok. Your participation and your genetic results of the research will not appear in your medical record nor will it be shared with other medical personnel with your identifying information. The results of this study maybe published, deposited on a public database or communicated in other ways but it will be impossible to identify you.

You may also choose not to know your genetic results. In this regard, we will not return the results to you.

Disclosure of potential economic gain

The analysis of your sample may contribute to creation of commercial products from which you will receive no financial benefit.

Basis of participation

You are free to consent or refuse to give consent for your participation in this study. You are also free to withdraw your consent to participate in the study at any given point in time. Your choice to consent or not consent to this study will in no way affect your relationship with KEMRI, Ministry of Health or the Universities involved in this project.

Obtaining additional Information

You are free to seek clarity or ask any questions at any point in time in the course of the study. If you desire to get more information concerning the study, feel free to call or sms Dr. Elijah Songok at +254711870333, or Dr. S.N. Vakil at +254724301518 or Dr. Fredrick Okoth at +254733731121 or Dr. Solomon Mpoke at +254722393067. You may also call Secretary, KEMRI/national Ethical Review Board at Tel. No.2542027222541.

CONSENT

EVALUATION OF THE KENYA ARV SCALE-UP PROGRAM: SURVEILLANCE FOR EMERGENCE OF ANTIRETROVIRAL RESISTANT GENOTYPES AMONG TREATMENT EXPERIENCED PATIENTS

I have read the information stated above and have had the opportunity to ask questions regarding the study. I therefore consent to:

- My sample to be used in this study
- My sample to be stored for periods upto 25 years after end of the study
- My sample to be analyzed abroad whenever the need arises
- My sample to be used by students for training purposes
- My sample to be used for other studies approved by the KEMRI Ethical Review Board

Name........................Signature....................Date........................

I, the undersigned, have fully explained the relevant details of this study to the patient.

Name..Signature...........................Date...........................

Witness...........................Signature:.......................Date............................