IDENTIFICATION OF THE CX3CR1 GENE POLYMORPHISM T280M IN A SAMPLED POPULATION OF HIV INFECTED PERSONS FROM SELECTED PROVINCES IN KENYA

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DECLARATION

I, LIKHAKO FELIX LIYAYI declare that the work presented herein is my original work and has not been presented for degree award in any other university or for any other award.

Signature.....Date..25/02/2012.....

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DEDICATION

I dedicate this thesis to my beloved wife Adeline Terry, to my mum Veronica, dad Andrew, my brothers and sisters.

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ABBREVIATIONS AND ACRONYMS

AIDS	.....	Acquired Immunodeficiency Syndrome
ALT	.....	Asymptomatic Long-term Progression
ART	.....	Antiretroviral Therapy
ASHM	.....	Australasian Society for HIV Medicine
BCA	.....	B cell-attractant
BSMBI	.....	*Bacillus stearothermophilus B61.*
CCR	.....	Cystein-Cystein linked Chemokine Receptor
CD	.....	Cluster of Differentiation
CD 4+	.....	T- Helper cells
CDC	.....	Centre for Disease Control
CSF	.....	Colony Stimulating Factor
CTL	.....	Cytotoxic T-Lymphocytes
CXCR	.....	Cystein-X-Cystein linked chemokine receptor
CX3CR1	.....	Cystein-X3-Cystein linked chemokine receptor 1
DAG	.....	Diacylglycerol
DNA	.....	Deoxyribonucleic acid
dNTP	.....	Deoxyribonucleotide Triphosphate
EDTA	.....	Ethylene diamine tetra-acetic acid
ELR	.....	Glutamic acid-leucine-arginine motif
ENV	.....	Gene encoding viral envelope
ERB	.....	Ethical Review Board of KEMRI

Gag	.....	Group-specific antigen- a viral gene.
GDP	.....	Guanosine 5' Diphosphate
GTP	.....	Guanosine 5' triphosphate
Gp41	.....	Glycoprotein 41 of the viral coat.
GP120	.....	Glycoprotein 120 of the viral coat
HAART	.....	Highly Active Antiretroviral Therapy
HIV	.....	Human Immunodeficiency Virus
HLA	.....	Human Leucocytes Antigen.
IL	.....	Interleukin
IP	.....	Interferon Inducible Protein
KEMRI	.....	Kenya Medical Research Institute
KDHS	.....	Kenya Demographic and Health Survey
LAV	.....	Lymphadenopathy Associated Virus
LTR	.....	Long Terminal Repeat
MAP	.....	Mitogen Activated Protein
MCP	.....	Monocyte Chemoattractant Protein
MDC	.....	Macrophage Derived Chemokine
$MgCl_2$	.....	Magnesium chloride
MHC	.....	Major Histocombatibility Complex
Mig	.....	Monokine induced by interferon gamma
MIP	.....	Macrophage Inflammatory Protein
MOH	.....	Ministry of Health – Kenya
NACC	.....	National AIDS Control Council

NASCOP	.....	National AIDS and STI Control Programme
Nef	.....	Negative Regulatory Factor
NK cells	.....	Natural Killer Cells
P	.....	Protein
PBMC	.....	Peripheral blood mononuclear cells
PCR	.....	Polymerase Chain Reaction
PEP	.....	Post Exposure Chemoprophylaxis
PF	.....	Platelet factor
PIP2	.....	Phosphatidylinositol (4,5) biphosphate
PK	.....	Protein Kinase
PL	.....	Phospholipase
Pol	.....	Gene encoding HIV polymerase.
PTK	.....	Protein tyrosine kinase
RANTES	.....	Regulated upon Activation T-cell Expressed and secreted
Rev	.....	Regulator of Virion
RFLP	.....	Restriction fragment length polymorphism
RNA	.....	Ribonucleic acid
rpm	.....	Revolutions per minute
RT	.....	Reverse transcriptase
SDF-1	.....	Stromal derived factor-1
SIV	.....	Simian Immunodeficiency Virus
SSC	.....	Scientific Steering Committee
STD	.....	Sexually Transmitted Diseases

Tac	.....	T-cell alpha chemoattractant
TACE	.....	Tumor Necrosi Factor α Converting Enzyme
Taq	.....	*Thermus aquaticus*
Tat	.....	Trans-Activator of Transcription
TBE	.....	Tris Borate EDTA
Teck	.....	Thymus-expressed chemokine
TH	.....	T-helper
TNF	.....	Tumor Necrosi Factor
T280M	.....	Threonine to Methione substitution at point 280
UNAIDS	.....	United Nations programme on HIV disease and AIDS
UNDP	.....	United Nations Development Programme
UTR	.....	Untranslated region
UV	.....	Ultra violet
V3	.....	Third Variable Domain
Vif	.....	Viral infectivity factor
Vpr	.....	Viral protein regulatory
Vpu	.....	Viral protein unknown
Vpx	.....	Viral protein X
WHO	.....	World Health Organization

ABSTRACT

The Acquired Immunodeficiency Syndrome (AIDS) caused by the Human Immunodeficiency Virus (HIV), is one of the leading cause of morbidity and mortality in sub-Saharan Africa where about 22.9 million people accounting for over 68% of the infections worldwide are living with AIDS. It kills young economically productive individuals, causing hardship to families, increased expenditure on health care and adversely affects the economic growth in the region. There is still neither a cure nor a vaccine for HIV infection while the therapies used only delay the onset of AIDS. The T-cell recognition molecules CD4 act as receptors, the chemokine receptors CCR5 and CXCR4 as co-receptors for viral entry into target cells, whereas chemokine receptors CCR2, CCR3, CCR8, D6, RDC1 and CX3CR1 act as secondary coreceptors. Polymorphisms in chemokines and their receptors have been shown to influence the rate of infection, disease progression and viral suppression in individuals receiving antiretroviral therapy. Moreover, an allele mutation namely CCR5-Δ32 confers resistance against HIV-1 infection. This mutation results in a truncated protein devoid of cell surface receptor. The chemokine receptor CX3CR1 gene polymorphism T280M has been shown to increase susceptibility to HIV infection and progression to AIDS in French Caucasian studies. However, the existence of the polymorphism among Africans is still not fully understood. The aim of this study was to determine the prevalence and geographical distribution of T280M polymorphism in various regions in Kenya. Samples were collected from five provinces of Kenya, two with a high prevalence of HIV-1 (Western and Nyanza), two with a low prevalence (Central and North Eastern) and one cosmopolitan population (Nairobi) to determine whether this mutation exists. Whole blood samples from adults in these provinces were collected, genomic DNA extracted and CX3CR1 gene amplified using gene specific primers. BSMBI restriction enzyme was used to determine the existence of the T280M mutation in the sampled population. The results of this study show that T280M polymorphism exists in Kenya. The wild type phenotype had the highest frequency of 70% then heterozygous with a frequency of 28.5% while homozygous mutant were 1.5% in the population. Homozygous mutant was only found in Nyanza and Nairobi provinces. There is no significant difference in the geographical distribution of CX3CR1-280M polymorphism in the sampled population $p=0.382$. Pair wise analysis showed no significant difference in the prevalence of the polymorphism with province $P>0.05$. However, there is a trend towards high prevalence of T280M polymorphism in Nyanza and Western provinces compared to other regions. It was concluded that other factors therefore other than T280M polymorphisms are responsible for variation in the prevalence of HIV in the five provinces studied. The results of this study provide information for future studies on the prevalence and geographical distribution of T280M polymorphism in different provinces of Kenya.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Acquired immune-deficiency syndrome (AIDS) is caused by the human immune-deficiency virus (HIV). The scourge has claimed over 30 million lives and 34 million people are currently living with HIV virus throughout the world (UNAIDS, 2010). Sub-Saharan Africa is more heavily affected than any other region of the world (UNAIDS, 2010). An estimated 22.9 million people are currently living with the virus in the region, accounting for 68% of the global total (UNAIDS, 2010). First diagnosed in Kenya in 1984, AIDS is currently responsible for about 85 000 deaths per year and the number of persons in the country infected with the HIV virus countrywide is estimated to be 1.3million to 1.6 million (NACC, 2010). Over 336 000 Kenyans are on antiretroviral therapy (ART) while the number of HIV and AIDS orphans and vulnerable children is estimated to be 2.4 million (USAID, 2010). This has led to a rise in the number of destitute children who are disinherited of their property and who are denied care, support and access to social amenities and services such as education and health care.

The HIV virus is a member of retrovirus family, subfamily *Lentivirus* which usually display long periods of latent infection prior to causing immunological and neurological diseases (Butler *et al.*, 2007). It is enclosed in a lipid bilayer coat from which 72 knob-like projections that are composed of two viral glycoproteins namely, gp120 and gp41 and CD4 molecules found at the surface of helper T lymphocytes and macrophages (ASHM, 2004). Like all retroviruses, the RNA genome of the virus particle contains three genes; *gag*, *pol* and *env* which encode structural and nonstructural polypeptides (Butler *et al.,* 2007). The HIV genome contains seven additional genes including the essential regulatory genes, *tat* and *rev* which greatly increase its flexibility.

To initiate infection, the HIV external envelop glycoprotein, gp120, interacts with two receptors namely, CD4 and a chemokine receptor CCR5 or CXCR4 (Gabuzda and Wang, 2000). This two-stage receptor interaction strategy allows gp120 to maintain a highly conserved coreceptor binding site in a cryptic conformation protected from neutralizing antibodies (Lusso, 2006). Upon entry into the cytoplasm, the virus is uncoated and the viral enzyme reverse transcriptase converts the viral genomic RNA into double stranded DNA (Butler *et al.,* 2007). Some viral DNA molecules enter the nucleus and are integrated into host cell DNA as provirus (figure 1).

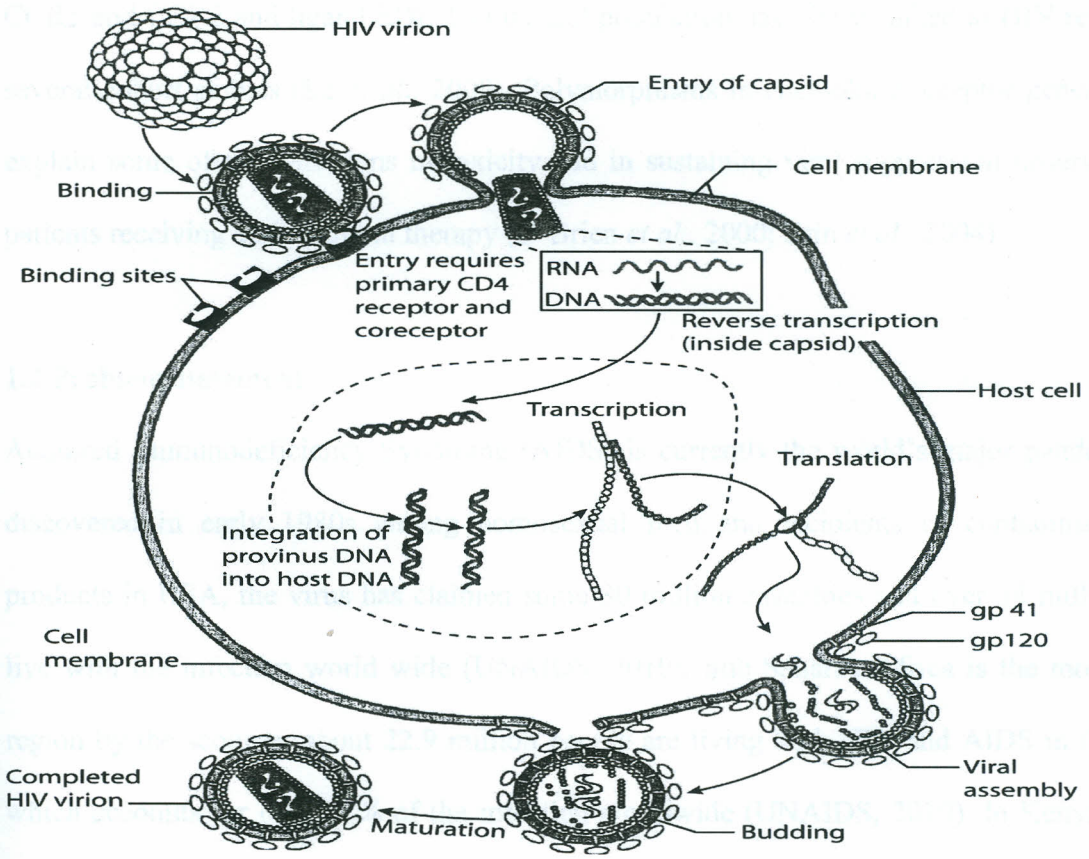

Figure 1 HIV life cycle {from Nelson and Williams (2007)}.

There is variation in the rate of HIV disease progression among individuals living with HIV and AIDS (Suresh *et al.,* 2006). Although majority of patients develop AIDS within 10 years of infection, 5 percent of long term infected individuals remain clinically and immunologically stable for more than a decade (Easterbrook *et al.,* 1999). These individuals are known as non-progressors. The cause of this variation is not clear but virological, genetic and immunological factors have been implicated (Easterbrook *et al.,* 1999). Polymorphisms have been identified in genes encoding some chemokine receptors used by HIV for entry into cells that have an impact on the rate of HIV disease progression. Mutations in the genes encoding chemokine receptors CCR5 and CCR2 and ligand SDF-1 in natural population have been linked to HIV resistance in several cohort studies (Su *et al.*, 2000). Polymorphisms in chemokine receptor genes may also explain some of the variations in toxicity and in sustaining viral suppression observed among patients receiving antiretroviral therapy (O'Brien *et al.,* 2000; Erin *et al.,* 2004).

1.2 Problem statement

Acquired Immunodeficiency Syndrome (AIDS) is currently the world's major pandemic. First discovered in early 1980s among homosexual men and recipients of contaminated blood products in USA, the virus has claimed some 30 million casualties and over 34 million people live with the infection world wide (UNAIDS, 2010). Sub-Saharan Africa is the most affected region by the scourge; about 22.9 million people are living with HIV and AIDS in this region, which accounts for over 68% of the infection worldwide (UNAIDS, 2010). In Kenya about 85 000 people die annually from the disease while around 1.3 million to 1.6 million people have so far been infected (NACC, 2010).

AIDS is a major public health problem with negative impact on economy. It causes loss of skilled man power, absenteeism from work and high expenditure on treatment and care of the infected individuals (IPAR, 2004). Kenya has a severe, generalized HIV and AIDS epidemic that has a devastating impact on all sectors of the society. In 1999, Kenya declared AIDS a national disaster and public health emergency (IPAR, 2004). The country currently has an estimated HIV prevalence rate of 6.3%, with an estimated 1.3 million to 1.6 million people living with HIV and AIDS (NACC, 2010). Kenya is currently spending a total of US$ 34 million per year over the five years 2009 to 2013 to implement various HIV and AIDS interventions (USAIDS, 2010).

There are significant regional variations in the prevalence of AIDS (Appendix 1). For example, the levels of prevalence in various provinces are as follows; Nairobi, 7%; Nyanza, 13.7%; Coast, 4.2%; Western, 6.6%; Central, 4.6%; Rift Valley, 4.7%; Eastern, 3.5% and North Eastern, 0.9% (KDHS, 2009). There are cases of couple discordance among Kenyan couples. Couple discordance refers to a condition where one of the couples is HIV negative while the other spouse is positive. In Kenya about 7.5% of married couples are discordant for HIV (MOH, 2007). There is also variation in toxicity of antiretrovirals among individuals receiving such treatment. Although the cause of these variations is not fully understood studies have suggested that genetic factors maybe one of the most likely cause (Erin *et al.*, 2004).

1.3 Justification

HIV virus binds to a cell surface marker namely, CD4 in order to infect the cells, a chemokine receptor CCR5 or CXCR4 acts as a coreceptor during the process. Chemokine receptor CX3CR1 is a minor coreceptor for HIV infection (Hendel *et al.*, 2001). There are two non synonymous

single nucleotide polymorphisms which have been identified for the CX3CR1 gene. These polymorphisms are V249I and T280M. These two polymorphisms are as a result of a base guanine to adenine base substitution resulting in the change of the cordon for valine to that for isoleucine at codon 249 and the base cytocine to thymine substitution resulting to substitution of threonine for methionine at codon 280 (Garin *et al.,* 2003). Several investigators have reported association between these CX3CR1 polymorphisms and HIV progression and arteriosclesis susceptibility (Faure *et al.,* 2000 and McDermott *et al.,* 2000, 2003). The allele T280M is associated with increased risk of HIV infection and rapid progression to AIDS (Suresh *et al.*, 2006). Individuals homozygous for T280M polymorphism progress to AIDS more rapidly than other genotypes at the locus. No studies have been carried out to investigate the prevalence and geographical distribution of this allele in Kenyan population. The results of such a study will improve our knowledge of the molecular mechanism involved in the propagation of HIV which will be useful in designing better control strategies and shed some light on why some areas of Kenya have a higher HIV and AIDS prevalence compared to others.

1.4 Research questions

a) What is the prevalence of T280M gene polymorphism in HIV infected persons in Kenya?

b) What is the distribution of T280M polymorphism in HIV infected persons in Kenya?

1.5 Null hypotheses

i. There is no CX3CR1 gene polymorphism T280M in the sampled population of HIV infected persons in Kenya.

ii. There is no difference in the geographical distribution of CX3CR1 gene polymorphism T280M in the country.

1.6 Objectives

1.6.1 General objective

The overall objective of this study is to determine the prevalence and geographical distribution of T280M gene polymorphism in HIV infected population in Kenya.

1.6.2 Specific objective

i. To determine the prevalence of T280M polymorphism in a sampled population of HIV infected people from different parts of Kenya.

ii. To assess the distribution of T280M polymorphism among a sampled population of HIV infected people from different parts of the country.

1.7 Significance and anticipated output

The CX3CR1 chemokine receptor is a secondary co receptor for HIV infection and entry into target cells (Devries *et al.,* 2003). It interacts with limited number of commonly tested HIV envelops and fractalkine, its ligand, can efficiently block the HIV co-receptor activity of CX3CR1 (Faure *et al.*, 2000). The CX3CR1 gene polymorphism T280M has been shown to increase susceptibility to HIV infection among French Caucasians (Suresh *et al.*, 2006). Individuals homozygous for this polymorphism progress to AIDS faster than those who are wild type for the polymorphism (Faure *et al.,* 2003). Very little has so far been done to determine the prevalence and distribution of this polymorphism in Kenya. The findings of this study will improve our knowledge on the molecular mechanisms involved in the propagation of HIV and the prevalence of T280M gene polymorphism in the various provinces of Kenya which will be useful in designing better control strategies. The results of the study will also elucidate the basis of high HIV and AIDS prevalence in some areas compared to others in Kenya.

CHAPTER TWO: LITERATURE REVIEW

2.1 Medical and economic importance of HIV and AIDS

Acquired Immunodeficiency Syndrome is currently the world's worst pandemic. Since its discovery, the virus has claimed more than 30 million casualties and over 34 million people live with the infection world wide (UNAIDS, 2010). In Kenya HIV and AIDS epidemic is severe and generalized having devastating impact on all sectors of the society. The country has an HIV prevalence rate of 6.3%, with 1.3 million to 1.6 million people infected with the virus. The country requires over US$ 2000 million in the period 2010 to 2014 to implement the various HIV and AIDS programs in the country (USAID, 2010). The impact of HIV and AIDS on the economic growth and development is significant. It causes loss of manpower for agricultural productivity; education services suffer from loss of teachers due to AIDS and high drop out rate due to loss of parents and fall in household income. Health sector is also affected by loss of staff and increasing burden of treating HIV related infections. There are direct costs and social problems associated with caring for the increasing number of orphans which places heavy burden on families.

2.2 Structure and organization of HIV

The HIV virus is classified in the family Retroviridae, subfamily Lentivirinae, and genus Lentivirus (Coffin *et al.,* 1986). It is the causative agent of HIV and AIDS (Coffin *et al.,* 1986). The structure of HIV comprises of a single-stranded, ribonucleic acid (RNA) genome of about 9.7 kilobases (Butler *et al.,* 2007). There are two strands of HIV RNA and each strand has a copy of the virus's nine genes. The RNA is surrounded by a cone-shaped capsid which consists of the *p24* viral protein (Butler *et al.,* 2007). Surrounding the capsid is the viral envelope which is

composed of a lipid bilayer membrane, formed from the cellular membrane of the host cell (Wang *et al.,* 2000). Host-cell proteins, such as the major histocompatibility complex (MHC) antigens and actin, remain within the viral envelope, along with the viral envelope protein. Each envelope subunit consists of two non-covalently linked membrane proteins: glycoprotein 120 (gp120) and glycoprotein 41 (gp41) that anchors the glycoprotein complex to the surface of the virion (Dragic, 2001). The envelope protein is the most variable component of HIV, although gp120 itself is structurally divided into highly variable (V) and more constant (C) regions (ASHM, 2004). The variability of V regions may be a product of envelope functionality, as shown in V3, where amino acid changes alter co-receptor use. The variability of the HIV envelope also confers a complex antigenic diversity which helps the virus to escape the effects of the immune system (ASHM, 2004). The structure of a virion is shown in figure 2 below.

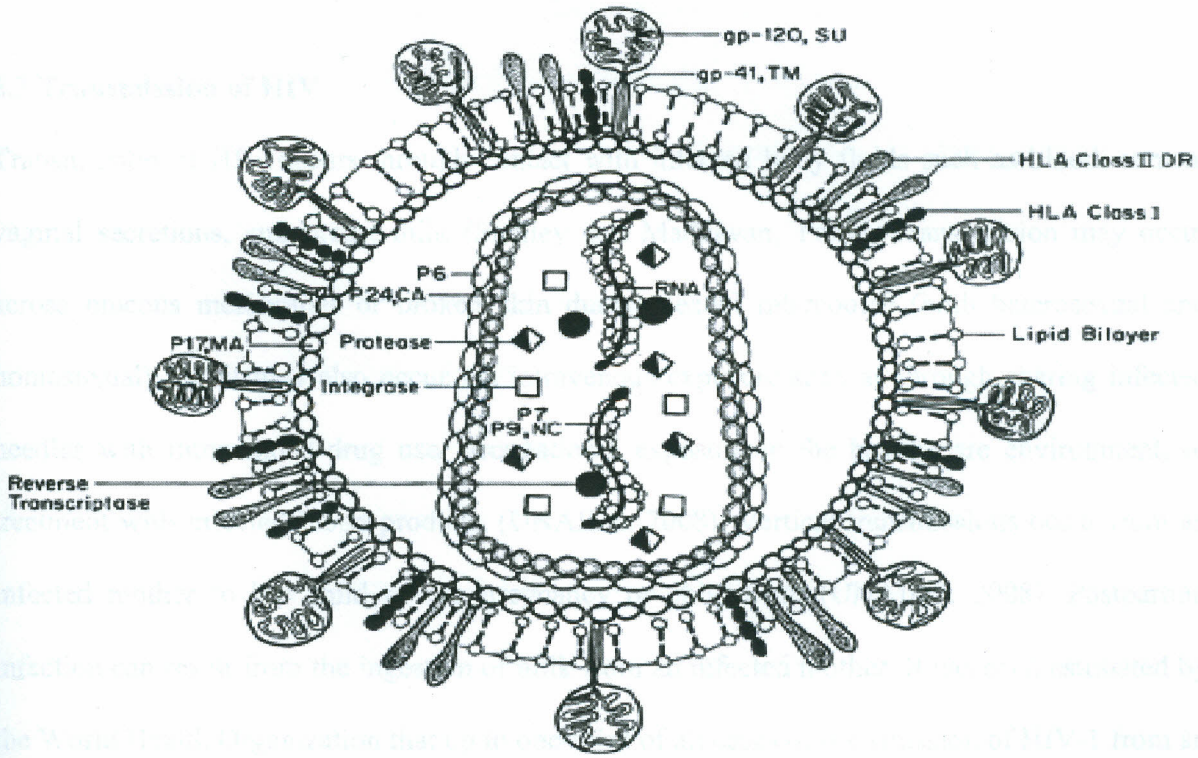

Figure 2 The structure of a virion

From Stanley and Madhvan (1999).

Genes of HIV encode proteins that may be broadly classified into structural, catalytic, regulatory, and accessory classes (ASHM, 2004). The long-terminal repeat (LTR) region has a transcription-promoter function in the integrated deoxyribonucleic acid (DNA) provirus, and contains regions essential for genomic RNA dimerisation, reverse transcription and integration into the host-cell genome (Coffin *et al.,* 1986). There are several major genes which code for structural proteins and several accessory (nonstructural) genes. *Gag* gene (Group-Specific Antigen) codes for *p 24,* the viral caspid, *p 6* and *p 7,* the nucleocaspid proteins and *p 17,* a matrix protein. *Pol* gene codes for viral enzymes such as reverse transciptase, integrase and protease (Levy, 2004). Accessory proteins *tat, vif, rev,nef, vpr* and *vpu* help HIV to enter the cell and enhance replication. Each gene codes for a protein with the same name for example, *tat* gene codes for *tat* proteins (Levy, 2004).

2.3 Transmission of HIV

Transmission of HIV occurs through contact with infected body fluids such as blood, semen, vaginal secretions, and breast milk (Stanley and Madhavan, 1999). Transmission may occur across mucous membranes or broken skin during sexual intercourse (both heterosexual and homosexual), but it may also occur via intravenous exposure such as through sharing infected needles with intravenous drug use, occupational exposure in the health care environment, or treatment with infected blood products (UNAIDS, 2008). Vertical transmissions occur from an infected mother to her child during pregnancy or child birth (UNAIDS, 2008). Postpartum infection can result from the ingestion of milk from an infected mother. It has been estimated by the World Health Organization that up to one-third of all cases of transmission of HIV-1 from an infected mother to her child may occur via breast milk (UNAIDS, 2008).

2.4 Chemokines

Chemokines are small, structurally-related molecules involved in chemotaxis of cells of the immune system to the sites of infection. Chemokines play an important role in many disease processes, including inflammation, autoimmune disease, infectious diseases such as HIV and AIDS, and cancer (Zlotnik *et al.,* 2006). They cause chemotaxis through interaction with receptors on the surface of lymphocytes (Schuitemaker and Van Rij, 2004). These receptors contain a G-protein for signal transduction. There are about 46 different chemokines that have been described (Zlotnik *et al.,* 2006).

Chemokines are classified into various groups namely, C, CC, CXC and CX3C. The C-chemokines have two conserved cysteines instead of four which is a characteristic of most chemokines. The CC-chemokines have four cysteines with the first two cysteines being adjacent to one another. The CXC-chemokines have four cysteines and the first two being separated by a single amino acid while CX3C-chemokines have four cysteines, with the first two being separated by three amino acids. The genes for the CXC chemokines have been localized on the long arm of human chromosome 4 (Mahalingam and Karupia, 1999). The subfamily CXC, is further divided into two; glutamic acid, leucine and arginine (ELR) containing chemokines and non-ELR chemokines. The ELR-CXC chemokines are predominantly neutrophil attractants and activators, while the non-ELR chemokines are mainly chemotactic for T lymphocytes (Mahalingam and Karupia, 1999, Zlotnik *et al.,* 2006). The genes for CC chemokines are localized to human chromosome 17. The CC chemokines are mainly chemotactic for monocytes and T lymphocytes, although they are also known to chemoattract basophils, eosinophils and NK cells.

Chemokine C and CX3C were characterized most recently. Only one chemokine has been identified for each group that is lymphotactin for C–chemokines and fractalkine for CX3C-chemokine. Lymphotactin has been found to be mainly chemotactic for CD8+ T lymphocytes. Fractalkine is a chemokine encoded as a membrane-bound molecule, with the chemokine domain attached to a long mucin-like stalk. The gene for this chemokine is localized on chromosome 16 (Mahalingam and Karupia, 1999). This 'chemokine on a stick' strategy allows the chemokine domain of fractalkine/neurotactin to be presented on the surface of a cell to act on another cell bearing the appropriate receptor. Fractalkine has potent chemoattractant activity for T lymphocytes, NK cells and monocytes (Zlotnik *et al.,* 2006).

Chemokines have two main sites of interaction with their receptors, one in the N-terminal protein region and the other within an exposed loop of the backbone that extends between the second and third cysteine. The N-terminal binding site is essential for triggering of the receptor. It is believed that the receptor recognizes its ligand and that this interaction is necessary for the correct presentation of the triggering domain. Chemokines are produced in relatively large amounts by the cells that produce them. This may be due to the way they function, by establishing concentration gradients along which the responding cells migrate (Zlotnik *et al.,* 2006).

2.5 Chemokine receptors

Chemokine receptors belong to the seven transmembrane spanning families of G-protein-coupled receptors that bind to chemokines (McNicholl *et al.,* 1997). There are 18 known chemokine receptors, many of which exhibit multiple ligand specificity (Zlotnik *et al.*, 2006). Chemokine receptors are grouped into families on the basis of the chemokine ligands they bind: CC, CXC, or

both. Some receptors are selective, while others are not in terms of ligand binding. The receptors are widely distributed on hematopoietic and other cells, but the Duffy antigen of erythrocytes (DARC) is the only member expressed on cells of erythroid lineage (McNicholl *et al.,* 1997).

Receptors for interlukin-8, CXCR1 and CXCR2 are expressed on neutrophils. The receptor CXCR2 has a high affinity for IL-8 and all other ELR-containing CXC chemokines that attract neutrophils, while CXCR1 has a high affinity for IL-8 and GCP-2. Receptors CXCR1 and CXCR2 are also found on monocytes, basophils and eosinophils, but these cells display a weak response to IL-8 (Mahalingam and Karupia, 1999). CXCR3 recognises the CXC chemokines IP-10 and Mig, while interferon-inducible T cell alpha chemoattractant (I-TAC) has been shown to have potent activity on activated T lymphocytes, through high affinity binding to CXCR3. The receptor CXCR3 is highly expressed in IL-2-activated T lymphocytes and NK cells (Mahalingam and Karupia, 1999).

Stromal cell-derived factor (SDF)-1 is a ligand for CXCR4 which is expressed on CD4+ T lymphocytes. CXCR5, which is expressed on B lymphocytes, was identified as the receptor for B cell-attracting chemokine-1 (BCA-1). The CC chemokine receptors CCR1 and CCR2 recognize macrophage inflammatory protein (MIP)-1/regulated upon activation, normal T cell expressed and secreted (RANTES) receptor and monocyte chemoattractant protein (MCP)-1 receptor, respectively (Zlotnik *et al.,* 2006). They also recognize MCP-2, MCP-3 and possibly MCP-4. CCR3 is expressed in eosinophils and T-helper 2 cells and is believed to be the principal chemokine receptor for the recruitment of these cells in allergy. The CCR4 receptor is selectively expressed on T lymphocytes and is the specific receptor for thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC). The CCR5 receptor binds

mainly RANTES and MIP-1, and is expressed in lymphoid organs, such as thymus and spleen, as well as in peripheral blood leucocytes, including macrophages and T lymphocytes (Zlotnik *et al.,* 2006).

Receptor CCR6, the only known receptor for MIP-3 is a CC chemokine chemotactic for lymphocytes and dendritic cells. This receptor is expressed only on memory T lymphocytes, including most 47 memory cells and cutaneous lymphocyte-associated Ag-expressing cells, and on B lymphocytes. The receptor for ELC/MIP-3 is CCR7 and this receptor is expressed on activated T and B lymphocytes (Zlotnik *et al.,* 2006). The receptor CCR8 is expressed constitutively in monocytes and thymus and binds I-309 with high affinity. Thymus-expressed chemokine (TECK) is a specific agonist for a human orphan receptor called CCR9. Orphan receptors are those whose ligands have not been identified.

The CCR9 receptor is expressed in the thymus, T lymphocytes and low concentration in lymph nodes and spleen. The CCR10 receptor is expressed primarily in placenta and foetal liver and binds MCP-1 and MCP-3 with high affinity (Devries *et al.,* 2003). The receptor for lymphotactin is CXCR1. This receptor is expressed strongly in placenta and weakly in spleen and thymus, which correlates with the expression of lymphotactin in these tissues (Zlotnik *et al.,* 2006). The identities of cells expressing CCR10 or XCR1 are not yet known. The CX3CR1 receptor has been found to be expressed predominantly on NK cells and binds fractalkine with high affinity (Hendel *et al.,* 2001).

2.5.1 Structure of chemokine receptors

Chemokine receptors are G protein-coupled receptors containing 7 transmembrane domains that are found predominantly on the surface of leukocytes (Lusso, 2006). They are G- protein because they have three distinct subunits namely, α, β and γ subunits attached to it which gives it a G- appearance. Approximately 18 different chemokine receptors have been characterized to date, sharing many common structural features. For example, they are composed of about 350 amino acids that are divided into a short and acidic N-terminal end, seven helical transmembrane domains with three intracellular and three extracellular hydrophilic loops, and an intracellular C-terminus containing serine and threonine residues that act as phosphorylation sites during receptor regulation (Mahalingam and Karupia, 1999).

The first two extracellular loops of chemokine receptors are linked together by disulphide bridges between two conserved cysteine residues. The N-terminal end of the chemokine receptor binds to chemokines and is important for ligand specificity (Mahalingam and Karupia, 1999). G-proteins couple to the C-terminal end, which is important for receptor signaling following ligand binding (Figure 3). Although chemokine receptors share high amino acid identity in their primary sequences, they typically bind a limited number of ligands (Lusso, 2006).

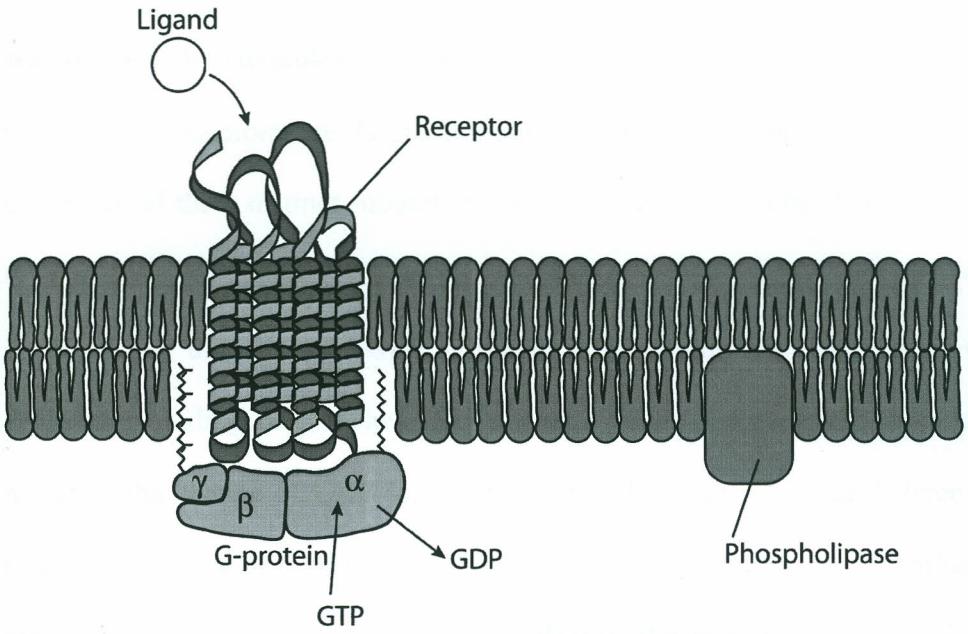

Figure 3 The structure of a G-protein coupled receptor.

Garret and Grisham (1999)

2.5.2 Functional domains of the coreceptor

The N- terminal domain of the coreceptor plays a critical role in the HIV-coreceptor function of both CCR5 and CXCR4 since the gp 120 of the virus recognizes and binds to this end of the coreceptor (Doranz *et al.,* 1997). This domain is modified by the addition of sulfate moieties on tyrosine residues, which facilitates electrostatic interactions with positively charged amino acids in the 'bridging sheet' and the V3 base (Doranz *et al.*, 1997). The second extracellular loop is believed to interact with the tip of V3 (Anjali *et al.,* 1999). The gp120 binding surface of the coreceptor is complex and varies according to the viral envelop examined, with critical residues dispersed throughout the extracellular domain. The signalling function of the coreceptors is not required for viral entry (Cocchi *et al.,* 1996).

2.5.3 The reception and transmission of extracellular information through signal transduction by chemokine receptors

Chemokine receptors are G-protein coupled receptors, the G-protein being a heterotrimer composed of three distinct subunits namely, α, β and γ subunits. The α-subunit binds to GDP or GTP and has an intrinsic GTPase activity which hydrolyses GTP to GDP (Garret and Grisham, 1998). When the molecule GDP is bound to α-subunit, the G-protein is in an inactive state. Following binding of the chemokine ligand, chemokine receptors associate with G-proteins, allowing the change of GDP for GTP, and the dissociation of the different G protein subunits (Toshio and Osamu, 2000). The subunit called Gβ activates phospholipase C (PLC) that is associated with the cell membrane. PLC cleaves phosphatidylinositol (4,5)-bisphosphate (PIP2) to form two second messenger molecules namely, inositol triphosphate (IP3) and diacylglycerol (DAG); DAG activates protein kinase C (PKC), and IP3 triggers the release of calcium from intracellular stores (Toshio and Osamu, 2000).

These events promote many signaling cascades, causing a cellular response (Figure 4). For example, when CXCL8 (IL-8) binds to its specific receptors, CXCR1 or CXCR2, a rise in intracellular calcium activates phospholipase D (PLD) that goes on to initiate an intracellular signaling cascade namely, the MAP kinase pathway. At the same time the G-protein subunit Gα directly activates protein tyrosine kinase (PTK), which phosphorylates serine and threonine residues in the tail of the chemokine receptor causing its desensitisation or inactivation. The initiated MAP kinase pathway activates specific cellular mechanisms involved in chemotaxis, degranulation, release of superoxide anions and changes in the avidity of cell adhesion molecules called integrins (Toshio and Osamu, 2000).

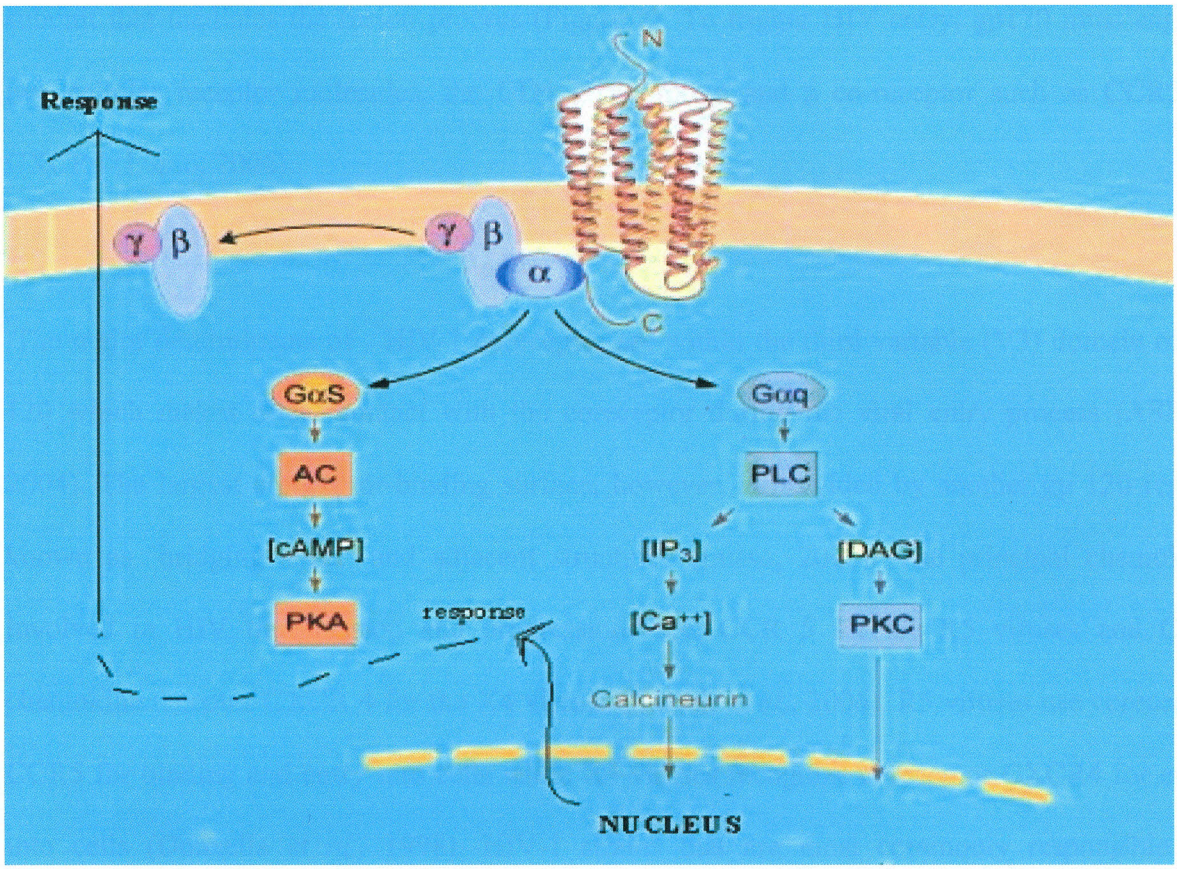

Figure 4 Signal transduction cascade.

Goodman (2008)

2.6 The role of genetic factors in HIV and AIDS

The risk of HIV infection and the rate of HIV disease progression are highly variable in a population, but factors responsible for this remain poorly defined. Studies have shown that host genetic factors significantly influence the risk of infection upon exposure to HIV, the rate of disease progression once infected, and the strength and diversity of the immune response (Brumme and Harrigan, 2006). Mutations in genes for HIV co-receptors and their natural chemokine ligands have been shown to modify HIV transmission and disease progression. Chemokines and their receptors play a critical role in binding and entry of HIV-1 into cells (Suresh *et al.,* 2006). The viral envelope is arranged in a spike-like structure formed by gp120,

the external subunit that mediates virion attachment and gp 41, the transmembrane subunit that mediates the fusion process (Figure 2). To trigger HIV entry, gp120 must engage two cellular receptor molecules, the CD4 glycoprotein and a co-receptor such as CCR5 or CXCR4 (Lusso, 2006).

The major determinants of HIV-1 coreceptor are within the third variable (V3) domain of gp 120 which makes direct contact with the coreceptor during the viral entry process (ASHM, 2004). The largest coreceptor-binding surface, however, is provided by another gp 120 region known as `bridging sheet' and adjacent structures (Lusso, 2006). The principal chemokine involved in HIV transmission and progressions are: CCR 5 for the R5 viruses and CXC chemokine receptor CXCR 4 for the X4 viruses (Suresh *et al.*, 2006). R5 viruses use coreceptor CCR5 for binding and entry into cells while X4 viruses recognize coreceptor CXCR4 for entry into cells (Donzella *et al.,* 1998). HIV-1 strains that are most commonly responsible for transmission and predominate during the long asymptomatic phase are the R5 viruses. X4 variant emerge later in the course of infection (Lusso, 2006). This strain also utilizes a series of minor coreceptors like CCR2b, CCR3, CCR8, CX3CR1, CXCR6, D6 and RDC1 (Lusso, 2006). Polymorphism in the genes encoding these chemokines and chemokine receptors are associated with resistance against acquiring HIV infection and alters the rate of disease progression in infected individuals (Suresh *et al.,* 2006).

2.6.1 The role of cysteine–cysteine linked chemokine receptor-5 (CCR5) on the rate of HIV and AIDS infection and disease progression

CCR5 serves as a co receptor along with T-cell recognition molecule CD4, as entry portals for HIV infection. Over 90% of primary HIV infections involve R5-tropic strains of HIV, which replicate efficiently in CD4+/CCR5+ bearing cell types: macrophages, monocytes and T-cells of

lymph nodes particularly in the intestines and colon. A polymorphism has been identified in the coding region of the CCR5 gene which involves a 32 base pair deletion (CCR5-Δ32) that creates a truncated protein. Homozygosity for CCR5-Δ32 confers near absolute protection against infection in the individuals (Dean *et al.*, 1996).

It has been observed that HIV-1 infected CCR5-Δ32 heterozygotes progress to AIDS less rapidly than patients who are wild type at the locus (O'Brien *et al.*, 2000). The heterozygotes have reduced level of CCR5 receptors on their cell surface, notably rather greater than the expected 50% reduction due to gene dosage effect (O'Brien and Moore, 2003). The nascent CCR5-Δ32 polypeptides dimerize with their wild type CCR5 counterparts in the endoplasmic reticulum, retarding the transport of CCR5 to the cell surface (Li *et al.*, 2005). The heterozygotes display a reduction in the viral load due to fewer available CCR5 receptors on the cell surface.

The frequency of CCR5-Δ32 allele rose rapidly in the Caucasian European population in a very short time, indicating a strong selection pressure which favored the carriers of the allele. The cause of the pressure is not known, although Black Death (bubonic plague) of the 14th century has been suggested as epidemic candidate. The deletion mutant was found primarily in the population of European decent (~10%) but no mutant alleles were reported in indigenous non-European population (Martinson *et al.*, 1997).

2.6.2 The role of cysteine-cysteine linked chemokine receptor-2 (CCR2) on the rate of HIV and AIDS infection and disease progression

The gene for CCR2 is located at the chromosome band 3P21 and contains 3 exons distributed over 7kb of the genomic sequence (Mariani *et al.*, 1999). It is CCR5's closest genomic relative

based on chromosomal proximity and DNA sequence homology (O'Brien and Moore, 2003). A guanine to adenine transition at position 190 of the CCR2 gene was found to produce a substitution of valine to isoleucine in amino acid 641 of the protein. The presence of the CCR2-641 genotype consistently confers two to three years postponement in the median time to both AIDS and death (Smith *et al.,* 1997).

It is not clear how CCR2-641 causes the delay of AIDS onset but unconfirmed reports indicate that the CCR2-641 protein product can dimerize with the CXCR4 polypeptide and sequester it in the endoplasmic reticulum (Francois *et al.,* 2002). This reduces the rate of disease progression by limiting the number of available CCR5 or CXCR4 co-receptors and hence the rate of viral replication.

This mutation CCR2-641 is common in the Asian population with a frequency of 0.250, least common in Europeans with a frequency of 0.098 and of intermediate frequency in African populations of 0.151 (Acosta *et al.*, 2003). In another study, the frequency of CCR2-641 among native Africans from Nairobi was 0.23 twice as high as in American Caucasians which has a frequency of 0.10 and delay in AIDS progression was twice as great. This increase in protective allele frequency and strength of genetic protection may be related in part to the absence of CCR5-Δ32 from the population (Francois *et al.,* 2002).

2.6.3 The role of stromal derived factor-1 (SDF-1) on the rate of HIV and AIDS infection and disease progression

Stromal derived factor-1 is the only known CXCR4 ligand. A single nucleotide polymorphism at position 801 (counting from the AUG codon) in the 3' untranslated region (3'UTR) of a splicing

variant transcript for SDF-1B has been discovered (O'Brien and Moore 2003). A guanine to adenine transition (G/A) in the 3' untranslated region of stromal cell derived factor-1 inhibits transmission of T- cell line tropic HIV strain and is associated with delayed progression to AIDS in homozygous individuals and particularly in the late stage of the disease (Su *et al.,* 1999). The mechanism could be related to up regulation of SDF-1 protein, which competes with HIV to bind the CXCR4 receptor. The protective effects of CCR5-Δ32 and CCR2-641 were shown to be dominant whereas the protective effects of SDF1-3'A is recessive (Su *et al.*, 1999).

2.6.4 The role of cysteine-X3-cysteine linked chemokine receptor-1 (CX3CR1) on the rate of HIV and AIDS infection and disease progression

The chemokine receptor CX3CR1 was originally identified as a receptor without a ligand and was named V28. It has a high sequence similarity to chemokine receptors such as CCR1 and CCR2 (Toshio and Osamu, 2000). Later, a ligand namely, fractalkine, a novel membrane molecule with an N terminal CX3C chemokine domain was found to bind to V28 with high affinity (Toshio and Osamu, 2000). CX3CR1 is expressed in monocytes and NK cells as well as CD8+ cells, glial cells and astrocytes (McDermott *et al.*, 2003).

The chemokine fractalkine exists in both membrane bound and a soluble form (Devries *et al.,* 2003). The membrane bound fractalkine is cleaved by TNF –α- converting enzyme (TACE) and released from the cell to form the soluble form (McDermott *et al.*, 2003). It is the only known ligand for CX3CR1 receptor. It has a mucin-like stalk, a transmembrane domain and a cytoplasmic tail which allows the molecule to be attached to the cells that express it (Steffen *et al.,* 2000). Fractalkine has been shown to function in inflammatory responses and in particular appear to be linked to TH1 adaptive immune responses (Devries *et al.*, 2003). The soluble form

of CX3CL1 recruits lymphocytes and monocytes whereas membrane bound CX3CL1 directly mediate the capture and firm adhesion of CX3CR1 expressing leucocytes (Garin *et al.*, 2003). The rat homolog of CX3CR1 has also been described (Mc Dermott *et al.*, 2003). A comparison of the chemokine domain of fractalkine with sequences of other chemokines shows that it is more related to the CC-chemokines and C-chemokines than the CXC-chemokines (Toshio and Osamu, 2000).

Cells expressing CX3CR1 adhered to the membrane-associated fractalkine without involving integrins (Toshio and Osamu, 2000). Furthermore, this adhesion appeared to occur in the absence of G protein activation (Toshio and Osamu, 2000). In contrast, these cells respond to the soluble form of fractalkine in both chemotaxis and calcium flux assays through a pertussis toxin-sensitive signaling pathway (Toshio and Osamu, 2000). Expression of CX3CR1 mRNA is strongly up regulated in both CD4 and CD8 T cells by IL-2.

CX3CR1 is secondary co receptor for HIV and in fact, HIV entry leads to a dramatic increase in the expression of CX3CR1 in lymphoid tissues and CD4 (Devries *et al.,* 2003). CX3CR1 interacts with a limited number of commonly tested HIV envelops and fractalkine can efficiently block the HIV co-receptor activity of CX3CR1 (Faure *et al.*, 2000). There are two polymorphisms that have been characterized in the CX3CR1 receptor gene that has an effect on HIV transmission and progression. There is a valine to isoleucine substitution at position 249, referred to as V249I polymorphism and a threonine to methionine substitution at position 280 referred to as T280M polymorphism. These two polymorphisms are at linkage disequilibrium such that polymorphism 280M has been shown to occur only when there is 249I, but 249I occurs even in absence of 280M (Qian *et al.*, 2008). This results into only 3 haplotypes commonly

identified in different cohort studies namely, 249V280T, 249I280T and 249I280M. The mutation 280M therefore occurs mostly when there is isoleucine at position 249. Polymorphism leading to the substitution of threonine to methionine at position 280 has been shown to increase susceptibility to HIV among the French caucasians (Faure *et al.*, 2000). It has also been associated with low risk of cardio-vascular diseases in several cohort studies (McDermott *et al.*, 2003).

In a study of 565 individuals from three HIV infected cohorts {patients with intermediate progression (IMMUNOCO), patients with asymptomatic long-term progression (ALT cohort) and patients with a known date of seroconversion (SEROCO cohort)} Faure *et al.*,2000 reported a trend towards a higher frequency of T280M polymorphism in IMMUNOCO and SEROCO cohorts compared to the ALT cohort. Kaplan-Meier survival analysis of seroconverters stratified by genotypes revealed faster progression to clinical AIDS for T280M homozygotes compared with wild type, with relative risk factor of 2.13 which was much higher than those previously described in the chemokine system. They also observed that the binding capacity of fractalkine is much reduced in these individuals than in other haplotypes which suggests that T280M polymorphism is associated with accelerated HIV disease due to reduced expression of CX3CR1 receptors on the cell surface and fractalkine binding capacity. Moreover, the proportion of homozygotes for the 280M mutation among infected subjects was much higher than expected from Hardy-Weinberg equilibrium, suggesting an effect on infection.

Subsequent studies (Suresh *et al.,* 2006, Hendel *et al.*, 2001, Kwa *et al.,* 2003 and Franceisc *et al.,* 2005) have, however, failed to observe such associations between T280M polymorphism and HIV disease progression (Singh *et al.,* 2005). This discrepancy has been attributed to the

deleterious effect of the allele which may have led to premature death of some patients before recruitment in some cohorts. A study of 169 individuals also reported improved CD4 responses after one year of HAART among individuals with the homozygous CX3CR1 280M genotype (Puissant *et al.,* 2006). The expression of CX3CR1 and its ligand, fractalkine, is increased during HIV-1 infection and is reduced on treatment with highly active antiretroviral therapy (HAART), suggesting that they play an important role in directing the immune response against HIV-1. Fractalkine expression has been reported to be up-regulated in the brains of patients with AIDS, and fractalkine produced during interactions between monocytes and HIV-1infected macrophages is thought to play a role in HIV-1 associated dementia (Singh *et al.,* 2005). Little has been done to investigate the prevalence and geographical distribution of this allele in different parts of Kenya. In this study, the prevalence and geographical distribution of T280M polymorphism in a sampled population of HIV infected individuals from different parts of Kenya was determined. Samples from different provinces were analyzed using molecular techniques. The results of this study will be useful in designing better control strategies and shed some light why some areas of Kenya have a high HIV and AIDS prevalence compared to others.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study design

3.1.1 Study population and study site

The study was carried out at KEMRI- HIV laboratory Nairobi. The aim of the study was to determine the prevalence and distribution of T280M gene polymorphism in Kenyan population. Experiments were conducted on samples collected from HIV screening centers, district and provincial hospitals and blood transfusion centers from 5 provinces in Kenya and brought to KEMRI for further analysis. For each province, an average of 40 samples was randomly collected to make a total of 200 samples used in this study.

3.1.2 Inclusion criteria

Samples from HIV positive Kenyans aged between 18-47 years irrespective of their sex were considered for inclusion in the study. This was the criteria used for samples in the larger study carried out at KEMRI for which this study was part.

3.1.3 Exclusion criteria

Samples from HIV negative individuals and individuals below 18 years or above 47 years were excluded from the study.

3.1.4 Ethical considerations

The study did not involve the enrollment of new volunteers but was based on the use of samples already collected from the provinces described. The ethical committee of the Kenya Medical Research Institute approved all the studies in which these samples were collected (Appendix 2 and 3).

3.1.5 Sample size determination

A simple random sampling technique was used to select the samples within the provinces. Using the expected prevalence of 14% (Francesc *et al.*, 2005), the sample size was determined by the formula used by Fisher *et al.* (1998), as shown below.

$$N = \frac{Z^2 PQD}{d^2}$$

Where, N = sample size

Z =Standard error ($1.96 \approx 2$)

P = proportion of target population. (0.14)

Q = 1- p

d = α = Absolute precision (0.05)

D = design effect

Therefore the sample size was

$$N = \frac{1.96^2 \, x \, 0.14 x 0.86 x 1}{0.05}$$

$$= \frac{0.4625}{0.0025}$$

$$= 185 \approx 200$$

200 samples were used to cater for attrition.

3.2 Experimental procedures

3.2.1 Blood collection and separation

Venus blood (3ml) was drawn directly into vacutainer tubes containing EDTA as anticoagulant. The samples were then transported at room temperature to KEMRI/ HIV Laboratory for subsequent procedures. Peripheral blood mononuclear cells were obtained by using 0.84% ammonium chloride. The blood (5ml) was mixed with 10 ml of 0.84% ammonium chloride then incubated for 5 minutes at 37°C. The mixture was then centrifuged for 10 minutes at 1500 rpm and the supernatant discarded. Ammonium chloride (10ml of 0.84% concentration) was then added to the pellet, this was incubated for 5 minutes at 37^0c. The mixture was again centrifuged for 10 minutes at 1500rpm and supernatant discarded. This was repeated until the pellet appeared white in color. The pellets were then washed twice with PBS and stored at -80^0c.

3.2.2 Extraction of DNA from Peripheral Blood Mononuclear Cells

DNA for amplification was obtained from the Peripheral Blood Mononuclear Cells. The DNA was extracted using DNAzol reagent as described by Khamadi *et al.* (2005). DNAzol (500µl) was added to the lymphocytes pellet and the cells lysed by gentle pipeting. Two volumes (1000µl) of chilled (cooled to 4°c) absolute ethanol was added to the dissolved pellet and mixed gently. This was centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and 1000µl of 70% ethanol added to the pellet and then it was vortexed. Centrifugation was repeated at 3000 rpm at 4°C for 10 minutes, and the supernatant discarded. The pellets were then dried in biosafety cabinet at room temperature, prior to dissolution in 100 µl of DNAse and RNAse free water. The DNA samples were then used for amplification.

3.2.3 Polymerase chain reaction and restriction fragment length polymorphism

The DNA which had been extracted from peripheral blood mononuclear cells was used as template. A master mix was made containing the following final volume concentration: 2ng of each primer, 0.2MmdNTP mix, 1X PCR buffer, 3µl genomic DNA, 2µl $mgcl_2$ and 0.5 units *Taq* DNA polymerase in a final volume of 25 µl. The amplification was performed in a Gene Amp PCR system 9700 model (Applied Biosystems). The new DNA synthesis of the 588 base pair sequence of CX3CR1 gene involved the following primers (Erofins MWG/operon).

Forwards: 5'- C.....T-3'

Reverse: 5'-T.....C-3'

The amplified DNA products were subjected to restriction enzyme analysis using restriction enzyme *BSMBI.* Restriction enzyme recognizes specific sites of a DNA strand and hydrolyses the DNA at those specific sites. A master mix was prepared for restriction using the following final volume concentration. 1X NEBuffer 2.0µl, *BSMBI* restriction enzyme 0.5µl, DNA template 10µl, and PCR water 7.5µl in a final volume of 20µl. The PCR products were digested for 1 hour at 55^0C. The CX3CR1 is a 588 bp gene therefore; *BSMB1* restriction enzyme recognizes two restriction sites, one at position 216 and the other at position 291. The normal strand (T280) is completely digested into three fragments of 75, 216 and 297bp and in the mutated strand (M280) the second site is disrupted thus displaying only two fragments of 216 and 372bp. In heterozygous subjects, four bands at 75,216,297 and 372bp are present. The restriction enzyme is obtained from an *E.coli* strain that carries the cloned *BSMBI* gene from *Bacillus stearothermophilus* B61. The enzyme *BSMBI* is an isoschizomer of the restriction enzyme *Esp31;* it recognizes the same restriction site as the enzyme *Esp31.* The digested products were run on agarose gel for separation of bands.

3.2.4 Preparation of Agarose gel

The restricted products were analyzed on a 2.0% Agarose gel after electrophoresis. Agarose powder (2.0g) was put in a conical flask, into which 100ml of 1X TBE (Tris Borate EDTA – Dojindo China) was added. This was then heated in a microwave oven for 3 minutes until completely dissolved. Ethidium bromide solution (2µl) of concentration 10mg/ml was added using a micropipette and shaken gently to ensure uniform mixing. This was left to cool to around 30-40 ^{0}C. The gel casting system (Gibco, UK) was arranged ensuring it was in a flat position using a spirit level. The well forming combs were fixed in position. The cool gel was poured gently on the trough ensuring that no air bubbles formed as this could interfere with the DNA separation process. This was left to polymerize. The combs were then gently removed without breaking the wells.

3.2.5 Preparation of DNA loading buffer-Bromophenol blue.

Bromophenol blue dye was used for visualization of bands on the UV transilluminator. It absorbs ultraviolet light at low PH and appears yellow in solution. Sucrose powder, about 40g, was weighed and dissolved in 100 ml of distilled autoclaved water to give a 40% sucrose solution. 0.25g of Bromophenol Blue powder was weighed and dissolved in the 40% sucrose solution to make 0.25% bromophenol blue solution used in loading of DNA preparations on the gels.

3.2.6 Agarose gel electrophoresis

The aim of agarose gel electrophoresis was to separate the restricted bands. The cast gel was placed on the tank with the wells near the cathode and the running buffer just covered the gel. Using a micropipette, 2-3µl of 0.25% bromophenol blue solution (loading buffer) was placed on the surface of a sterile Parafilm, for each of the sample to be analyzed. The restricted products

(5µl) were then taken and mixed with the loading buffer by repeated pipetting on the parafilm. This was then dispensed into each sample well on the gel. The first well of the gel was loaded with 3µl of 100 base pair molecular weight marker while the consecutive wells were loaded with the restricted samples. Loading was done carefully without touching the gel or overflowing the wells. The apparatus was then closed and connected to the power pack with the cathode connected near the samples end and this was run at 100 volts for 30 minutes until the bands were clearly visible. The samples were loaded together with the ladder. The power supply was then turned off and the gel transferred into the UV transilluminator for observation.

3.2.7 UV transilluminator photography.

The gel was placed directly over the illuminator glass filter. The perpex protective was closed over the gel. The UV protective shield or glasses was put on then the illuminator put on. The gel was checked for the presence of the DNA bands. Where the bands appeared and were desirable, a photograph using UV illumination was taken for recording. The photograph was taken using a Polaroid ® camera loaded with polaroid® instant films.

The camera hood was placed directly over the gel with the UV switched off. This was adjusted to ensure that it was centered in the hood. The focusing was then set, the protective perpex cover put in place and shield worn. The UV was then put on but set at lower intensity and photograph taken. The film was then removed from the camera immediately and allowed to develop at room temperature for 2-3 minutes. The developed photo was then peeled off to expose it and analysed for the results. The gel was removed from the illuminator and disposed appropriately.

3.3 Data Management and Statistical Analysis

Data was analyzed using INSTAT package version 2 to establish measures of central tendency (mean, standard deviations and variance) and find the prevalence of the polymorphism in each province. The data generated was then stored both in hard and soft copies. The analysis of variance was used to determine significance difference between the provinces. Pearson correlation was used to establish the correlation of the various types of polymorphism in the sampled provinces and a regression analysis trend done to determine the trend of the polymorphism in the country. Summary scores of i.e. wild type/wild type, wild type/mutant and mutant/mutant were computed as the number of mutations presented in tabular and graphical forms.

CHAPTER FOUR: RESULTS

4.1 PCR Amplification of DNA

The PCR analysis was carried out to amplify the DNA materials using a Gene Amp PCR system 9700 thermocycler. The PCR amplification was carried out as detailed in materials and methods. In this process, a total of 200 HIV positive samples were used. These samples were drawn from 5 provinces in Kenya namely: - Nairobi, Central, Western, Nyanza and North Eastern. Presence of bands showed that amplification had taken place. Representative photographs of amplified products are shown in Figure 5 and Figure 6. The bands showed that amplification had taken place. Some samples did not amplify (Figure 5 lanes 4, 7,8,14 and15) while Lane 9 contained negative control.

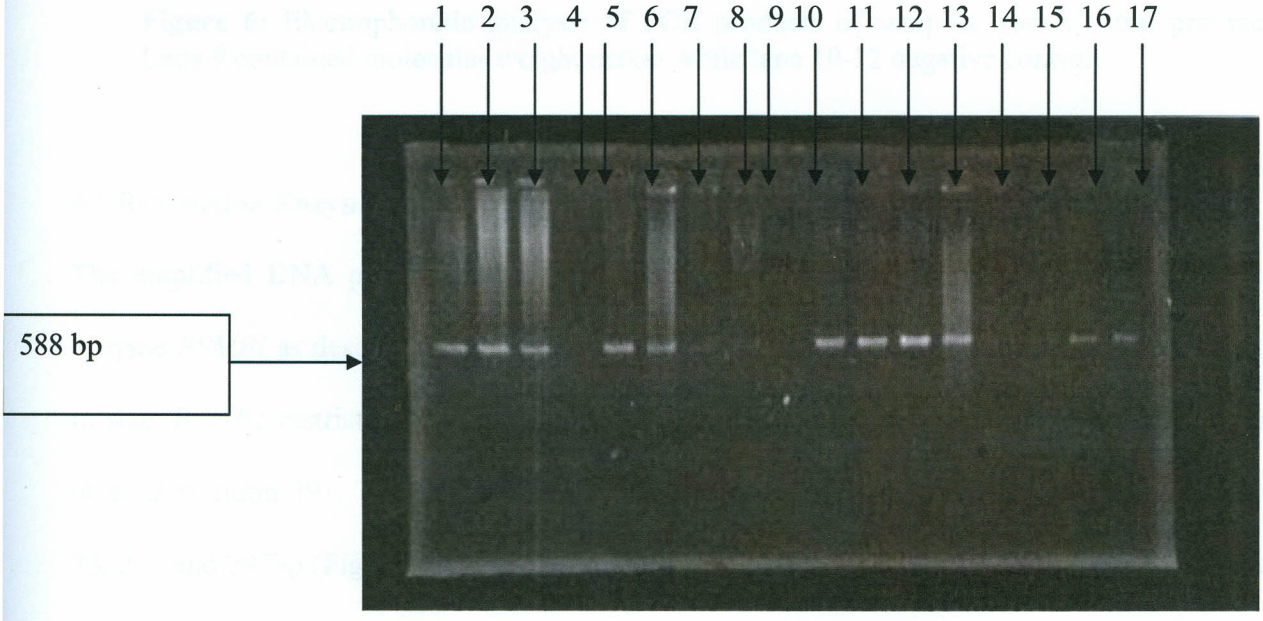

Figure 5: Electrophoretic analysis of PCR products of samples from different provinces: Lane 1 - 3 - Western, lane 5 and 6 - Nyanza, lane 10 – 13 - North Eastern while lane 16 and 17 are from Nairobi province. Lanes 4, 7, 8, 14 and 15 did not amplify, while lane 9 contained negative control.

A representative photograph of amplified samples from Central province is shown below (Figure 6). Lane 9 is the 100bp moleculer weight marker while lanes 10-12 are negative controls.

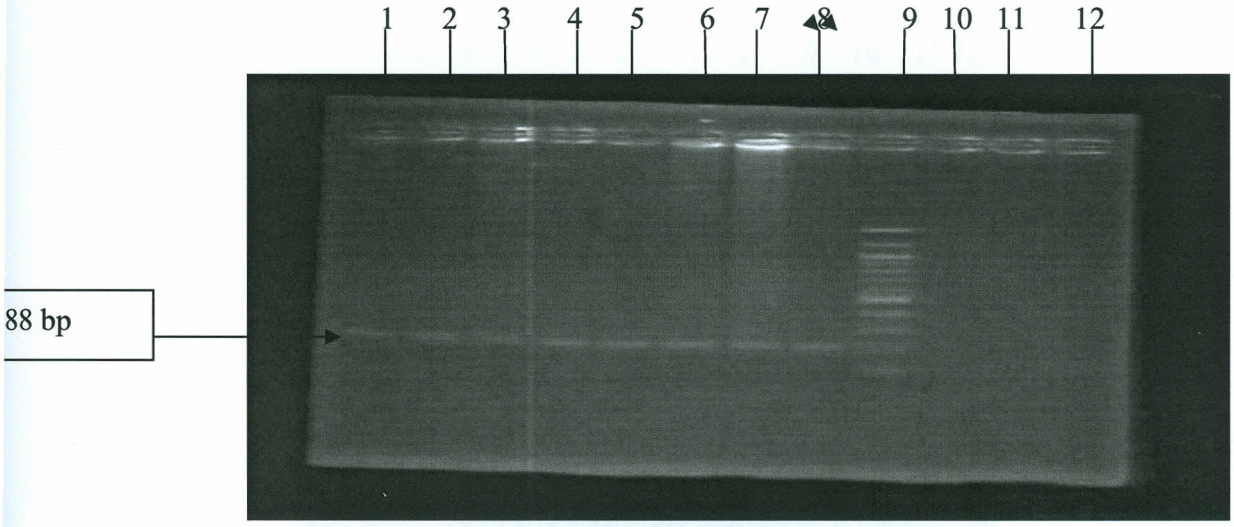

Figure 6: Electrophoretic analysis of PCR products of samples from Central province. Lane 9 contained molecular weight maker while lane 10-12 negative control.

4.2 Restriction Enzyme Analysis

The amplified DNA products were subjected to restriction enzyme analysis using restriction enzyme *BSMBI* as described in materials and methods section. The gene for CX3CR1 is 588 bp in size. *BSMB1* restriction enzyme recognizes two restriction sites, one at position 216 and the other at position 291. The normal strand (T280) is completely digested into three fragments of 75, 216 and 297bp (Figure 8 and 9) and in the mutated strand (M280) the second site is disrupted thus displaying only two fragments of 216 and 372bp. In heterozygous subjects, four bands at 75,216,297 and 372bp are present (Figure 7). Representative photograph for the restriction enzyme analysis is shown in figures 7, 8 and 9. In figure 7 lane 5 contained a sample from an individual who is wild type for the polymorphism. Lane 1and 7 were loaded with moleculer

weight marker while lanes 2, 3, 4, 6, 8, 9, 10, 11 and 12 shows samples that were heterozygous for the polymorphism.

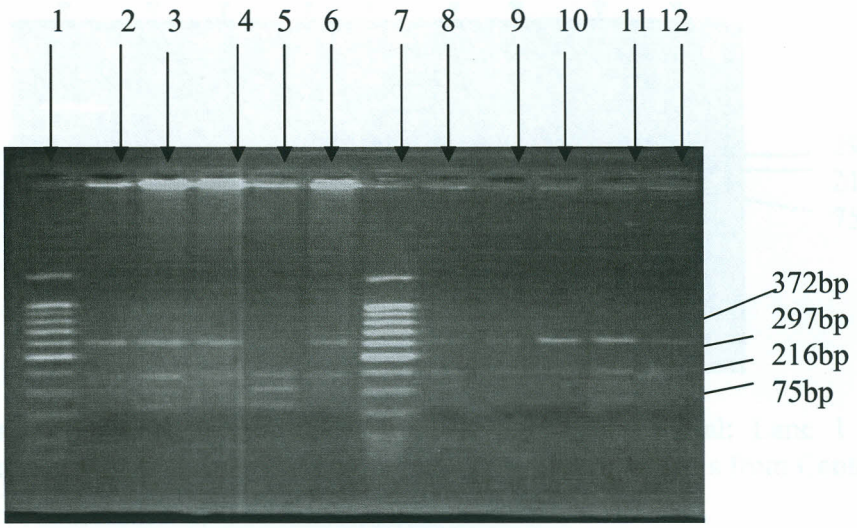

Figure 7: Analysis of restriction products of samples: Lanes 1 and 7 contained molecular weight marker, lanes 2-4 are results of DNA samples from Western, lane 5 and 6, Nyanza, lane 8 and 9, Nairobi while lane 10-12, North Eastern province.

In figure 8 the first lane contained the molecular weight marker. The samples were drawn from Nyanza and Central provinces. The samples used in the photograph were all wild type for the polymorphism. Lane 7 did not amplify probably due to presence of inhibitors or contamination.

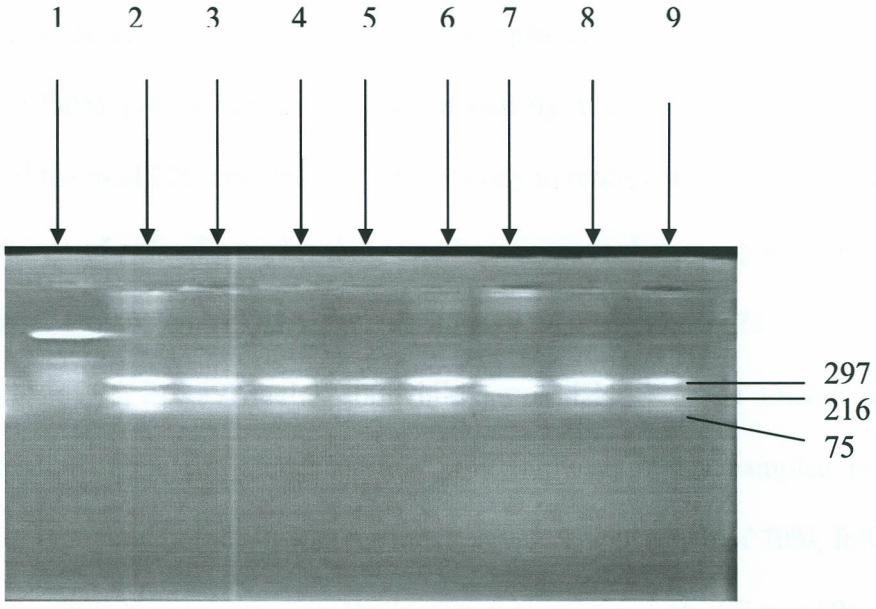

Figure 8: Analysis of restriction products from Nyanza and Central: Lane 1 contained molecular weight marker, Lane 2-5, Nyanza, while lanes 6 - 9 were samples from Central.

Figure 9 contained samples from Nairobi and North Eastern provinces. The first lane was loaded with the moleculer weight marker. All the other samples in the photograph were wild type for the polymorphism.

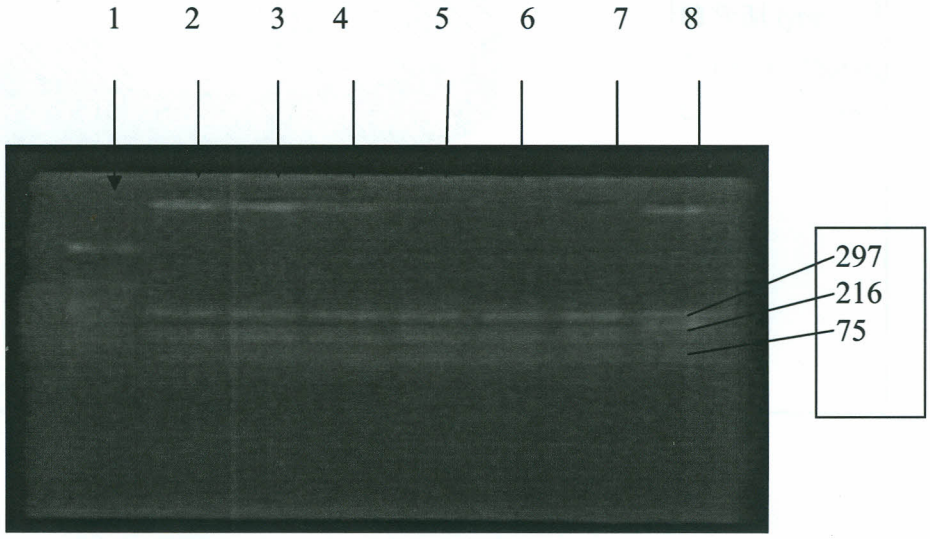

Figure 9: Analysis of restriction products from Nairobi and North Eastern: Lane 1 contained molecular weight marker, in lanes 2-5 were samples from Nairobi while lanes 6-8 contained samples from North Eastern.

4.3. Determination of the prevalence of T280M polymorphism in Kenya

The prevalence of T280M polymorphism was determined by analyzing the prevalence of the polymorphism out of the total 200 samples used in the study to represent the Kenyan population. Total counts were made of gels of samples that were homozygous, heterozygous and wild type for the polymorphism. Homozygous DNA showed 2 bands of 216bp and 372bp, heterozygous DNA showed 4 bands of 75, 216,297 and 372bp while normal DNA showed 3 bands of 75,216 and 297bp. The results of the prevalence of this polymorphism in the sampled population showed that the wild type polymorphism was the most prevalent with a rate of 70%, followed by heterozygous, 28.5%, while homozygous was the least prevalent with 1.5% (Figure 10).

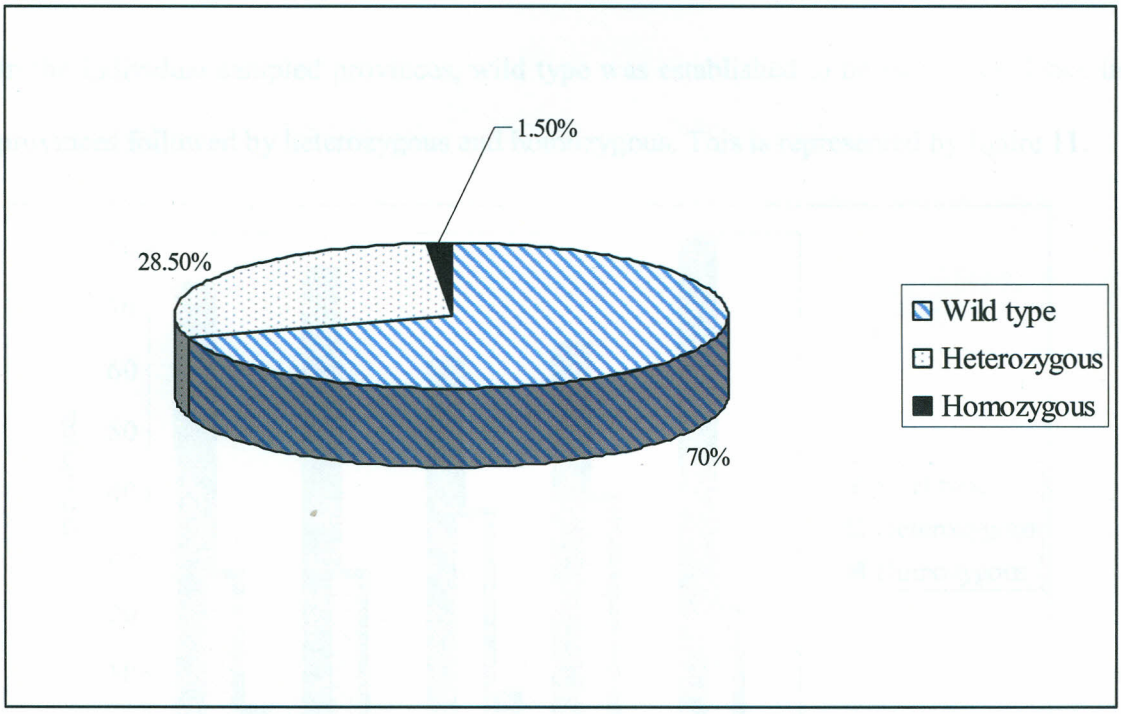

Figure 10: Prevalence of the wild type, heterozygous and homozygous genotypes in Kenya

ANOVA analysis showed a significant difference in the types of T280M polymorphism percentage prevalence in Kenya with $p<0.05$ (table 1). A post ANOVA finding showed that, wild type (mean prevalence (70.0% ± 3.78) is higher than heterozygous (mean 28.5% ± 3.32) and homozygous (mean 1.50% ± 1.0).

Table 1: ANOVA results for the prevalence of T280M in the country

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11905.833	2	5952.917	135.422	.000
Within Groups	527.500	12	43.958		
Total	12433.333	14			

In the individual sampled provinces, wild type was established to be more prevalence in all the provinces followed by heterozygous and homozygous. This is represented by figure 11.

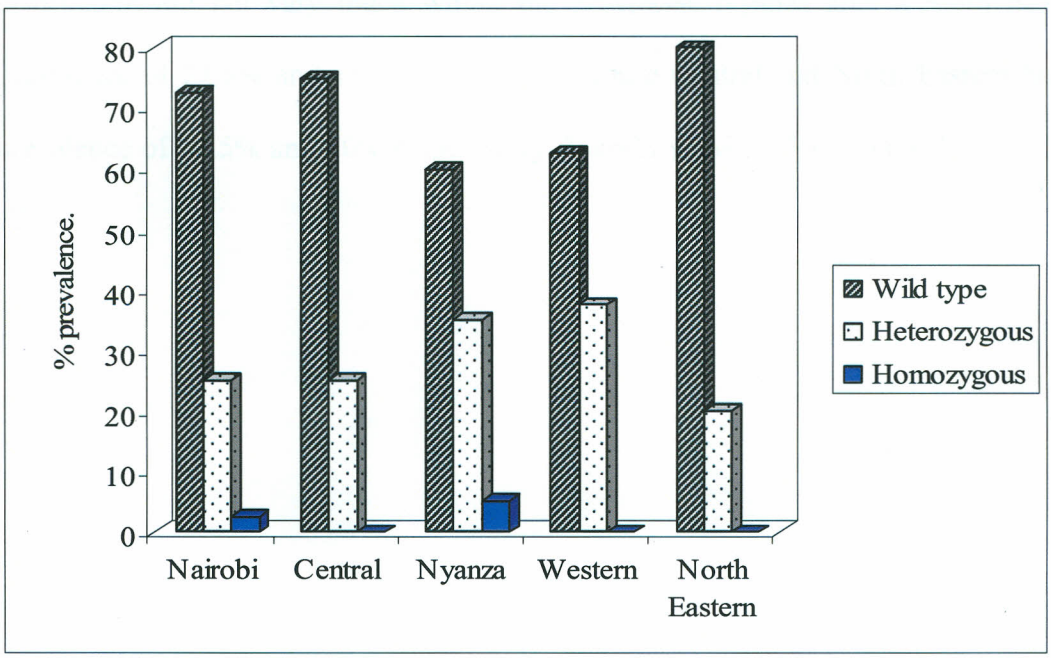

Figure 11: Frequency of the wild type, heterozygous and homozygous genotypes in the provinces

The mutant T-allele was 15% in the total population. Nairobi, North Eastern and Central provinces had the highest percentage of wild type, which was 72.5%, 80% and 75% respectively, while Nyanza had the lowest prevalence of 60%. For heterozygous, the prevalence was high in Western province (37.5%) and low in North Eastern province (20%). Homozygous conditions were observed only in Nairobi (2.5) and Nyanza provinces (5%) accounting for 1.5% of the total.

4.4. Distribution of the T- allele in Kenya

The distribution of the mutated T- allele was determined by counting the total number of homozygous, heterozygous and wild type gels. Homozygous gel has T-allele at both loci; it therefore has two T-alleles. The heterozygous one has only one T-allele at the locus; its allelic pair is C. The normal strand has no T-allele. The prevalence of this allele per province was expressed as a percentage of the possible 80 gene loci available. The study showed that the distribution did not vary much within the provinces. Nyanza and Western had the highest prevalence of 22.5% and 18.8% respectively while Central and North Eastern had the lowest prevalence of 12.5% and 10% respectively. Nairobi province had a prevalence of 14% (Figure 12).

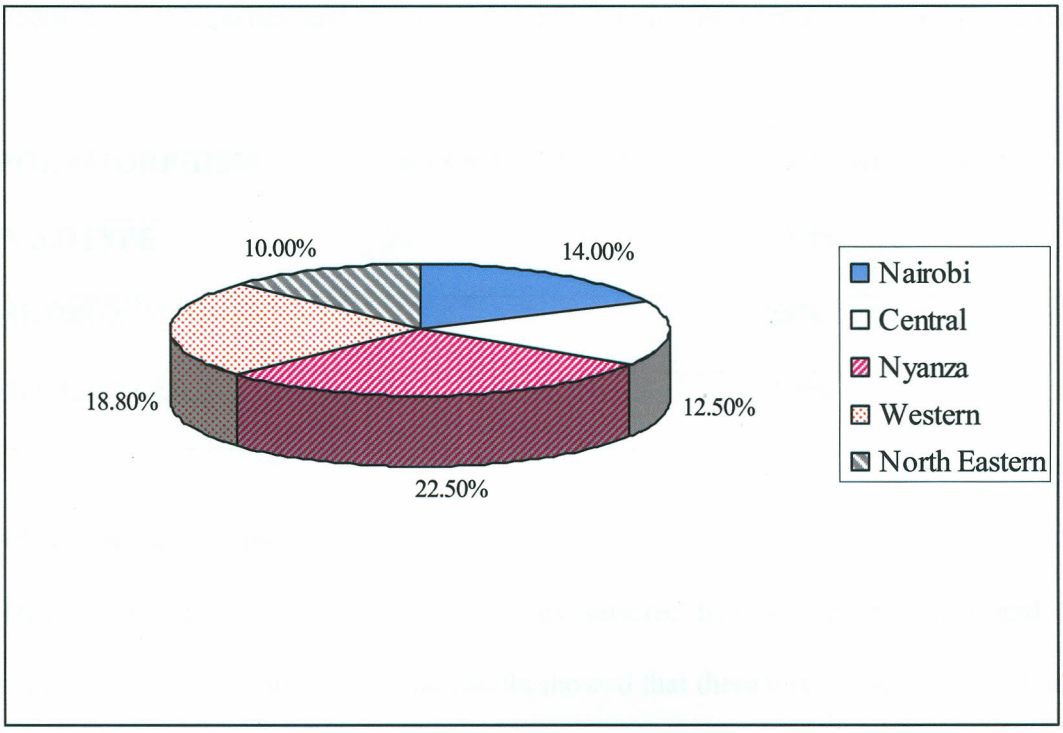

Figure 12: Mutant T-allele prevalence in the provinces

4.5. Prevalence of T280M within individual provinces

A total of 40 samples were selected randomly from each of the 5 provinces and analyzed for presence of the polymorphism. The results are shown below.

4.5.1. Nairobi province

A total of 40 samples were randomly selected for the study from Nairobi province and analysed for the presence of the polymorphism. The results showed that out of the 40 samples, there were 29(72.5%) gels of samples with the wild type CX3CR1 gene, 10(25%) had the heterozygous gene while 1(2.5%) had the homozygous gene for the mutation. This indicated that the wild type was more prevalent in Nairobi province (Table 2).

Table 2: The frequency and prevalence of T280M polymorphism in Nairobi province

POLYMORPHISM	FREQUENCY (40)	PREVALENCE (%)
WILDTYPE	29	72.5%
HETEROZYGOUS	10	25%
HOMOZYGOUS	1	2.5%

4.5.2 Central Province

There were a total of 40 samples randomly selected from Central province and analyzed for presence of the polymorphism. The results showed that there were no samples with the homozygous gene, 10 (25%) had the heterozygous gene while 30 people (75%) had the wild type CX3CR1 gene (Table 3).

Table 3: The frequency and prevalence of T280M polymorphism in Central province

POLYMORPHISM	FREQUENCY (40)	PREVALENCE (%)
WILDTYPE	30	75%
HETEROZYGOUS	10	25%
HOMOZYGOUS	0	0

4.5.3 Nyanza province.

In Nyanza province, there were 24 (60%) samples with wild type gene, 14 samples (35%) had the heterozygous gene while 2 (5%) had the homozygous gene for the mutation out of the 40 samples selected (Table 4).

Table 4: The frequency and prevalence of T280M polymorphism in Nyanza Province

POLYMORPHISM	FREQUENCY (40)	PREVALENCE (%)
WILDTYPE	24	60%
HETEROZYGOUS	14	35%
HOMOZYGOUS	2	5%

4.5.4 Western Province

The results of samples from Western province showed that homozygous gene was absent in the population sampled. However, wild type had the highest prevalence of 62.5% in the province. Heterozygous samples were 15 accounting for 37.5% of the total 40 samples studied (Table 5).

Table 5: The frequency and prevalence of T280M polymorphism in Western Province

POLYMORPHISM	FREQUENCY (40)	PREVALENCE (%)
WILDTYPE	25	62.5%
HETEROZYGOUS	15	37.5%
HOMOZYGOUS	0	0

4.5.5 North Eastern Province

North Eastern province had the highest wild type prevalence than in all the provinces (80%). It was noted that in North Eastern province samples, there was no homozygous type (table 6).

Table 6: The frequency and prevalence of T280M polymorphism in North Eastern Province

POLYMORPHISM	FREQUENCY (40)	PREVALENCE (%)
WILD TYPE	32	80%
HETEROZYGOUS	8	20%
HOMOZYGOUS	0	0%

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4.6 The homozygous genotype

The mutated gene T280M is recessive therefore; homozygous type progress to HIV and AIDS more rapidly than the wild type individual. It was noted that samples with this type of polymorphism were only got from Nairobi (2.5%) and Nyanza (5%) provinces (figure 13).

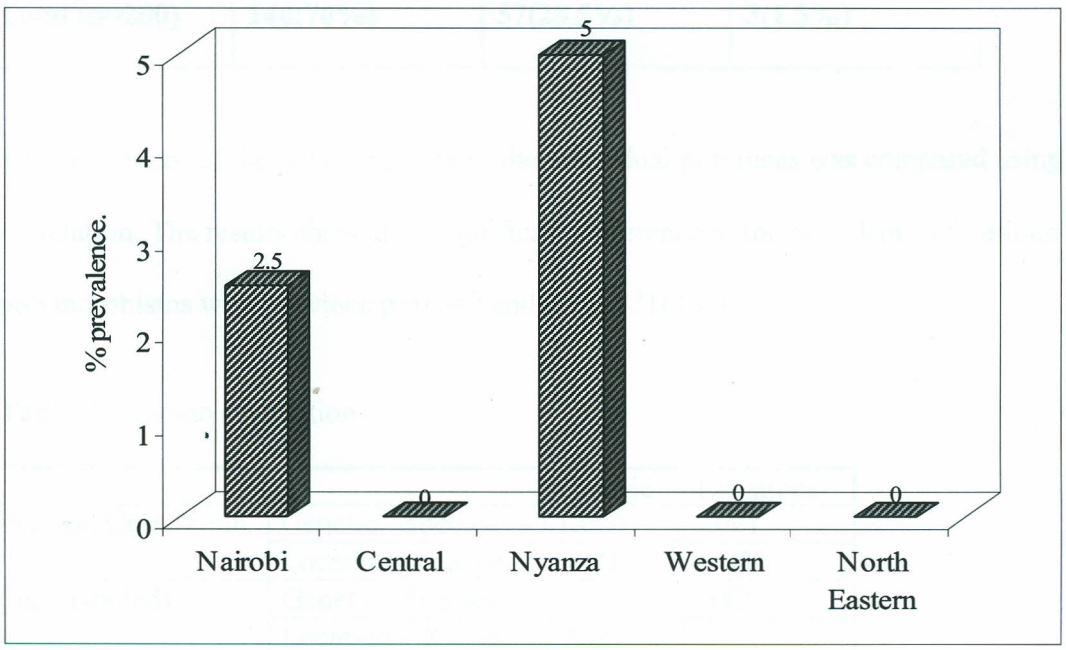


Figure 13: Prevalence of homozygous type of T280M polymorphism

4.7 Distribution of T280M polymorphism in the various provinces in Kenya

The distribution of T280 polymorphism in the various provinces in Kenya was determined by assessing the number of samples with the polymorphism out of the total 200 samples (Table 7).

Table 7: Distribution of T280M in the various provinces in Kenya

	Wild type	Heterozygous	Homozygous
Nairobi	29 (14.5%)	10(5%)	1(0.5%)
Central	30(15%)	10(5%)	0(0%)
Nyanza	24(12%)	14(7%)	2(1%)
Western	25(12.5%)	15(7.5%)	0(0%)
North Eastern	32(16%)	8(4%)	0(0%)
Total (n=200)	**140(70%)**	**57(28.5%)**	**3(1.5%)**

The prevalence of the polymorphism in the individual provinces was compared using Pearson correlation. The results showed no significant difference in the prevalence of various polymorphisms with province p=0.382 and r= -0.021(Table 8).

Table 8: Pearson correlation

		Genetic	Locations-
Pearson Correlation	Genetic Species -	1.000	-.021
	Locations- Kenya	-.021	1.000
Sig. (1-tailed)	Genetic Species -	.	.382
	Locations- Kenya	.382	.
N	Genetic Species -	200	200
	Locations- Kenya	200	200

Bivariate analysis using pearsons correlation showed no significant difference in the prevalence of the polymorphisms (p> 0.05) (Table 9).

Table 9: Pair wise comparison of correlation coefficients.

Rho		Nairobi	Central	Nyanza	Western	North Eastern
Nairobi	Correlation Coefficient	1.000	.023	-.153	-.138	-.132
	Sig. (1-tailed)	.	.445	.174	.198	.208
Central	Correlation Coefficient	.023	1.000	-.023	.030	.000
	Sig. (1-tailed)	.445	.	.443	.428	.500
Nyanza Loc.	Correlation Coefficient	.	.	.	.	.
	Sig. (1-tailed)	.	.	.	.	.
Nyanza	Correlation Coefficient	-.153	-.023	1.000	.068	.126
	Sig. (1-tailed)	.174	.443	.	.339	.220
Western	Correlation Coefficient	.	.	.	.	.
	Sig. (1-tailed)	.	.	.	.	.
Western	Correlation Coefficient	-.138	.030	.068	1.000	.000
	Sig. (1-tailed)	.198	.428	.339	.	.500
North Eastern	Correlation Coefficient	.	.	.	.	.
	Sig. (1-tailed)	.	.	.	.	.
North Eastern	Correlation Coefficient	-.132	.000	.126	.000	1.000
	Sig. (1-tailed)	.208	.500	.220	.500	.

The regression analysis however, showed that the homozygous genotype is evenly distributed in the country. There is a significant negative trend (β_1 = -0.85) in prevalence of heterozygous genotype as one moves from Nyanza to North Eastern provinces with R^2=82.1%. A similar trend (β_1 = -0.2) was noted in homozygous genotype with R^2=50%. In wild type, however, the trend (β_1 = 1.05) in prevalence was significantly positive and R^2=95.9%. Prevalence was higher towards North Eastern province than in Nyanza (Figure 14).

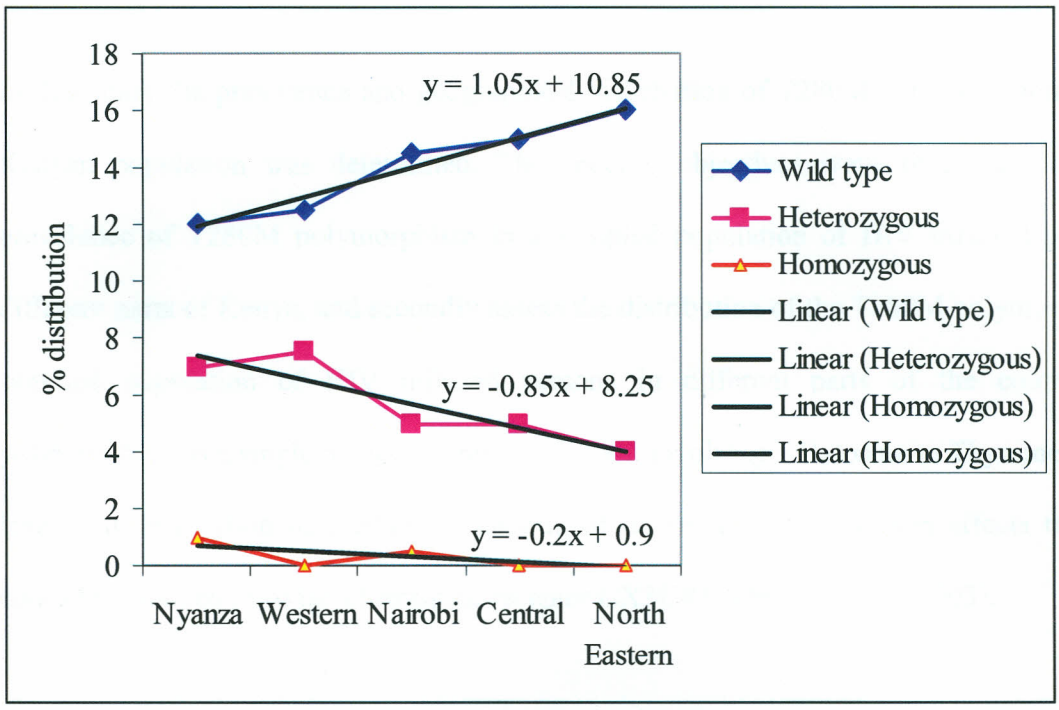


Figure 14: A trend analysis of polymorphism in Kenya

There is a trend towards higher prevalence of wild type genotype as one moves from Nyanza to North Eastern province. Heterozygous and homozygous genotypes, the trend is negative. It is important to note that homozygous samples were very few. Two of the samples were from Nyanza province and one was from Nairobi province.

CHAPTER FIVE: DISCUSSION

In this study the prevalence and geographical distribution of T280M gene polymorphism in the Kenyan population was determined. The specific objectives were firstly to determine the prevalence of T280M polymorphism in a sampled population of HIV infected persons from different parts of Kenya, and secondly assess the distribution of the T280M polymorphism in the sampled population of HIV infected persons in different parts of the country. T280M polymorphism is a single nucleotide polymorphism involving a Cytosine to Thymine substitution leading to translation of methionine instead of threonine. The mutation affects the gene that codes for production of the chemokine receptor CX3CR1 (Devries *et al.,* 2003).

CX3CR1 has been identified as a coreceptor for HIV-1 and a receptor for the chemokine fractalkine. Its role in viral attachment and entry is minor compared to that of the dominant coreceptors, CCR5 and CXCR4. It serves as a minor coreceptor especially for the X4 strain which emerge later in the course of infection (McDermont *et al.,* 2000). Studies have shown that the impact of polymorphisms in CX3CR1 on HIV-1 disease progression is more likely to be through directing inflammatory cells to specific sites of infection to initiate innate adaptive immunity (Toshio and Osamu, 2000). Moreover, it has been shown that HIV-1 infected patients homozygous for the CX3CR1-280M polymorphism progress to AIDS more rapidly than those with other genotypes. Functional analysis has shown that these individuals have less expression of CX3CR1 receptors on the cell surface. The receptors also have a low binding capacity to its ligand - fractalkine (Faure *et al.,* 2000). This study was aimed at determining the prevalence and geographical distribution of this polymorphism. These individuals were also found to have a better response to antiretroviral compared to other genotypes (puissant *et al.,* 2006).

In this study blood samples from HIV positive individuals were collected from HIV screening centers, district and provincial hospitals and blood transfusion centers from 5 provinces in Kenya namely, Nairobi, Central, Nyanza, Western and North Eastern. They were analyzed for presence of T280M gene polymorphism. Western and Nyanza provinces have a high HIV prevalence, Central and North Eastern provinces have low HIV prevalence (MOH, 2005), while Nairobi province is cosmopolitan hence the reason why they were included in the study. The samples consisted of individuals aged 18-47 years since the protective effect of T280M polymorphism has not been shown in young infants below the age of 18 years (Singh *et al.,* 2005). It is also important to note that this was the inclusion criteria for the larger study at KEMRI in which this research was part of. A simple random sampling technique was used to select 40 samples from every province for analysis. The peripheral blood mononuclear cells (PBMCs) were separated from whole blood; genomic DNA extracted from the PBMCs and amplified using DNA specific primers. The amplified DNA was subjected to restriction enzyme analysis using *BSMBI* restriction enzyme for 1 hour and then electrophoresis was done on a 2% agarose gel for separation of bands.

The results of this study show that the prevalence of T280M gene polymorphism is 1.5% for homozygous mutant gene, 28.5% heterozygous gene and 70% for the wild type gene in the total population. These results differ with those of other workers whose results indicated that T280M polymorphism was only present in Caucasians, but absent in Asian and African populations (Faure *et al.,* 2000). The results of this study however, support those of Hendel *et al.* (2001) whose data showed that the polymorphism was present at low frequency in African population. His study was based on samples collected from West African populations. Fancois *et al.* (2002)

also showed that the polymorphism was present in North Africa with prevalence rate of less than 2%. Devries *et al.* (2003) found no significant difference in the inter-ethnic prevalence of T280M polymorphism in Asian, Caucasian and African populations. This study also established that the prevalence of the mutant T-allele in the sampled population was 15% which is similar to the prevalence of T- allele among the Caucasians (Hendel *et al.,* 2001). However, it is much higher than that found among the Chinese 2.1% (Li *et al.,* 2005).

The distribution of various genotypes was significantly different in this study ($F = 135.422$, $P < 0.05$). The wild type prevalence was higher (mean prevalence (70.0% ± 3.78) than heterozygous (mean 28.5% ± 3.32) and homozygous (mean 1.50% ± 1.0). Faure *et al.* (2000) showed that HIV infected patients homozygous for T280M polymorphism progressed to AIDS more rapidly than those with other haplotypes (relative risk (RR) 2.13; p=0.08). Functional CX3CR1 analysis showed that fractalkine binding is reduced among patients homozygous for this particular haplotype. The prevalence of the homozygous gene however, was found to be low in the studied population with a prevalence rate of 1.5% compared to 3.8% among the French Caucasians (Faure *et al.,* 2003). Only 3 samples from the total studied population was homozygous for the polymorphism.

The results of the distribution of T280M polymorphism with provinces showed that there is an even distribution of homozygous genotype in the sampled population ($\beta_1 = -0.2$). The homozygous genotype is important in this study as individuals homozygous for the mutant allele have high HIV and AIDS infection rate and rapid progression to AIDS. This results shows that T280M polymorphism therefore cannot explain the cause of variation in the prevalence of HIV and AIDS in the studied sample population since it is evenly distributed in the studied

population. The study however, established that there is a significant negative trend (β_1 = -0.85) in prevalence of heterozygous genotype as one moves from Nyanza to North Eastern provinces. In the wild type, the trend in prevalence was significantly positive (β_1= 1.05). Prevalence was higher towards North Eastern province than in Nyanza. The correlation in the prevalence of T280M polymorphism with the provinces was positive. The provinces with higher prevalence of the polymorphism include Nyanza, Western and Nairobi compared to North Eastern and Central provinces as stated above however, this correlation is not significant (p=0.382). When two provinces are considered for comparison at a time (pair wise comparison) it showed no significant difference in the prevalence of this polymorphism with province p>0.05. This pair wise comparison further confirms that the T280M polymorphism cannot explain the cause of variation in the prevalence of HIV and AIDS in the studied population.

The prevalence of the T-allele per province was also established. The study showed that; Nyanza and Western had the highest prevalence compared to Central and North Eastern which had the lowest prevalence. There is therefore, a trend towards higher prevalence of the T- allele as one move from Nyanza to North Eastern province though this trend is not significant (p>0.05). It is interesting to note that Nyanza and Western provinces which have high prevalence of HIV and AIDS compared to North Eastern province also have high prevalence of T-allele compared to North Eastern province.

Faure *et al.* (2000) reported that HIV-1 infected patients homozygous for the CX3CR1-280M substitution progress to AIDS more rapidly than those with other genotypes. The protective effect of T280M polymorphism was shown to be recessive since this effect was not observed among the heterozygous individuals. Moreover, Hendel *et al.* (2001) observed a delay in the

median time of progression to AIDS among individuals heterozygous for the mutation. In this study however, only two provinces of Nairobi and Nyanza had samples that were homozygous for the mutation. Two samples from Nyanza province and one sample from Nairobi province were homozygous for the mutation (Figure 10). There is however, no significant difference in the prevalence and distribution of the homozygous genotype within the provinces ($R^2 = 50.0$).

These results shows that the prevalence of T280M polymorphism is very low in the sampled population of HIV infected individuals (1.5%). The results also shows that the distribution of this polymorphism within provinces is not significant ($R^2 = 50.0$) although there is a trend towards higher prevalence in Nyanza and Western provinces compared to North Eastern and Central provinces. It can also be deduced from the results that this polymorphism has no effect on the variation observed in the prevalence of HIV and AIDS in different parts of the country since there is no significant difference in the distribution of the polymorphism ($R^2 = 50.0$). Other factors have also been implicated in the variation in the prevalence of AIDS in different parts of Kenya. These factors include: cultural practices, socio-economic status and religion. Cultural practices such as wife inheritance and widow cleansing which are practiced in Nyanza and Western provinces has been suggested (IPAR, 2004). Lack of circumcision has also been suggested as one of the factors responsible for high prevalence of HIV in Nyanza province (NASCOP, 2006). It has been observed that circumcision provides up to 53% protective effect against HIV and AIDS acquisition (Bailey *et al.,* 2007). Poverty in Western and Nyanza provinces is very high compared to Central province (IPAR, 2004). Poverty pre-disposes one to casual sex and promiscuity, which in turn enhances the spread of the scourge. Nyanza is also major overland trade route. The main truck stops on the interstate highway feeding Uganda,

Rwanda, Burundi and Congo are in Kisumu city (Nyanza province) and Busia town in Western province (IPAR, 2004).

Nairobi is a cosmopolitan province. It has a high population compared to other provinces and it is a business center. These factors have led to the high prevalence of HIV in the province while Islamic religion which is dominant in North Eastern province has contributed to low HIV prevalence in the area (USAID, 2007). The prevalence of mutations that influence AIDS pathology and progression is relevant for the establishment of strategies for the prevention and treatment of AIDS, and, as shown for the T280M mutation, it should be investigated In different populations, taking into account their ethnic background and genetic diversity.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study confirms that T280M polymorphism exists in Kenyan population, wild type genotype is the most common with a prevalence of 70%, and heterozygous gene has a prevalence of 28.5% while homozygous gene has a prevalence of 1.5%. There is no significant difference in the distribution of T280M polymorphism in Nairobi, Nyanza, Central, Western and North Eastern provinces of Kenya. This polymorphism therefore cannot account for variation in the prevalence of HIV/AIDS in these provinces. Other factors other than T280M polymorphism are responsible for this variation. There is, however, a trend towards high prevalence of the polymorphism in Nyanza and Nairobi than in Central and North Eastern but this difference is not significant. This is a baseline study therefore studies involving large sample size need to be done in order to determine the effect of this polymorphism on the rate of HIV and AIDS infection and disease progression.

6.2 Recommendations

- This survey indicated that genetic polymorphism in T280M in Kenya have varied distribution across the provinces, a large cohort of Kenyan population infected with HIV-1 will be necessary to fully describe the distribution of this polymorphism.

- There is need for further studies to determine the influence of T280M polymorphism to HIV-1 infection and disease progression.

- There is need for further studies on the role of chemokine receptors in HIV infection and how information on these receptors can be used to reduce the spread and impact of HIV/AIDS in Kenya.

REFERENCE

Acosta A.X., Sampair R.G., Spinola J.L. and Galvão-Castro B. (2003) Distribution of the CCR2-641 allele in three Brazilian ethnic groups. *Genetics and Molecular Biology*, 26: 241-243

Anjali s., Gideon B., Azina M. and Ronald G.C. (1999) Patterns of chemokine Receptor Fusion Cofactor Utilization by Human Immunodeficiency Virus Type 1 Variants from the Lungs and Blood. *Journal of Virology*, 73:6680-6690

Australasian Society for HIV Medicine (2004). HIV Management in Australasia- a guide for clinical care. LMB 5057 Darlinghurst NSW 1300 Australia

Bailey. R., Moses, S., Parker,C., Agot,K., Maclean, I., Krieger, J.,Carolyn F M Williams, C., Campbell ,R. and Ndinya-Achola, J. (2007) Male circumcision for HIV prevention in young men in Kisumu, Kenya: a randomised controlled trial *Lancet* 369: 643–656

Brumme, Z. L., Winnie, W. Y., Keith, J., Robert, S. H., Julio, S. G., Michael, V. O. and Richard, H.P. (2003) Influence of polymorphisms within the CX3CR1 and MDR-1 genes on initial antiretroviral therapy response. *AIDS*. 17:201–208

Brumme, Z.L. and Harrigan,P.R. (2006) The impact of human genetic variation on HIV disease in the era of HAART. *AIDS reviews* 8:78-87

Butler I.F., Pandrea I., Marx P.A. and Apetrei C. (2007) HIV Genetic Diversity: Biological and Public Health Consequences. *Current HIV Research*. 5:23-45

Central Bureau of Statistics (CBS)[KENYA], Ministry of Health (MOH)[KENYA] and ORC Macro (2009). Kenya demographic and health survey 2008-2009. Calverton, MD: CBS,MOH and ORC Macro.

Cocchi, F., DeVico, A. L., Garzino-Demo, A., Cara, A., Gallo, R. C. & Lusso, P. (1996). The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection [see comments]. *Nature Medicine* 2: 1244-1247.

Coffin, J., Haase, A., Levy, J.A., Montagnier, L., Oroszlan, S. and Teich, N. (1986). Human immunodeficiency viruses. *Journal of virology* 64: 5230 - 5234

Clapham, P.R. and McKnight, A. (2002) Cell surface receptors, virus entry and tropism of primate lentiviruses. *Journal of General Virology*. 83: 1809-1829

Dean M., Carrington M., Winkler C., Huttley G.A., Smith M.W., Allikmets R., Goedert J.J., Buchbinder P., Vittinghoff E., Gomperts E., Donfield S., Vlahov D., Kaslow R, Saah A., Rinaldo C., Detels R and O'Brien J (1996). Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Science 273: 1856-1862.

Devries M.E., Cao H., Wang J ., Xu L ., Kelvin A.A., Ran L., Chau L.A., Madrenas J., Hegele R.A.and Kelvin D.J. (2003). Genomic Organization and Evolution of the CX3CR1/CCR8 Chemokine Receptor locus. *Journal of Biochemistry*. 278: 11985-11994

Donzella, G. A., Schols, D., Lin, S. W., Este, J. A., Nagashima, K. A., Maddon, P. J., Allaway, G. P., Sakmar, T. P., Henson, G., De Clercq, E. & Moore, J. P. (1998). AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nature Medicine* 4: 72-77

Doranz, B. J., Grovit-Ferbas, K., Sharron, M. P., Mao, S. H., Goetz, M. B., Daar, E. S., Doms, R. W. & O'Brien, W. A. (1997). A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. *Journal of Experimental Medicine* 186: 1395-1400.

Dragic, T. (2001) An overview of determinants of CCR5 and CXCR4 coreceptor function. *Journal of general virology* 82:1807-1814

Easterbrook P.J., Rostron T., Ives N., Troop M., Gazzard B.G and Rowland-Jones S.L. (1999). Chemokine Receptor Polymorphisms and Human Immunodeficiency Virus Disease Progression. *Journal of Infectious Diseases*. 180:1096-10105

Erin Q, Howard M and William P. (2004). The pharmacogenetics of antiretroviral therapy: AReview of studies to Date .....abacavir, Atazanavir, drug transporters and response to ART, metabolic effects of ART, Lipodystrophy, Chemokine reseptors and response to ART. *Clinical Infectious Diseases,* 39: 98-106

Faure S., Meyer L., Costagliola D., Vaneensberghe C., Genin E., Austran B., Delfraissy J., McDermott D.H., Murphy P.M., Debre P., Theŏdorou I. and Combadiere C. (2000). Rapid progression to AIDS in HIV+ Individuals with a structural variant of the Chemokine Receptor CX3CR1. *Science*. 287: 2274-2277

Faure S., Meyer L., Genin E., Pellet P., Debrè P and Theodorou C. (2003). Deleterious genetic influence of CX3CR1 genotypes on HIV 1 disease progression. *Journal of Acquired Immune Deficiency Syndrome.* 32: 335-337

Francesc V., Consuelo V., Pere D., Montserrat B., Enric P., David D., Hernando K., Joaquim P., Cristina G., Ma Anto` nia S., A ` ngels F., Elisabeth D., Mireia C., Milagros M., MD,k Cristo ´ bal R. and Simon M. (2005). Spanish HIV-1–Infected Long-Term Nonprogressors of More Than 15 Years Have an Increased Frequency of the CX3CR1 249I Variant Allele. *Journal of Acquired Immune Deficiency Syndrome*. 40: 527-531

François, R., Nathalie F., Catherine B., Jean S., Jean-Marie Z., Joël M., Goubau P., François S., Robert H and Jean-Claude S. (2002) Prevalence of HIV Co-Receptor Polymorphisms in HIV-Infected Patients and Uninfected Volunteers in Luxembourg. *HIV clinical trials* 3:195-201

Gabuzda, D. and Wang, J. (2000) Chemokine receptors and mechanisms of cell death in HIV neuropathogenesis. *Journal of NeuroVirology* 6:24-32

Garin, A., Tarrantino, N., Faure, S., Daoudi, M., Lecureuil, C., Bourdais, A., Debre, P., deterre, P. and Combadiere, C. (2003) Two novel fully functional isoforms of CX3CR1 are potent HIV coreceptors. *The journal of immunology* 171:5305-5312

Garret, R.H. and Grisham, C.M. (1999) *Biochemistry* 2nd edition. Saunders college publishing: Harcourt Brace, Orlando

Goodman, S.R. (2008) *Medical cell biology 3rd* edition. Academic press publishers: Waltham, Massachusetts

Hendel, Houria, Winkler, Cheryl, An, Ping, Roemer-Binns, Elisabeth, Nelson, George, Haumont, Philippe, O'Brien, Steve, Khalilli, Kamel, Zagury, Daniel, Rappaport, Jay, Zagury and Jean-François (2001). Validation of Genetic Case-Control Studies in AIDS and Application to the CX3CR1 Polymorphism [Epidemiology]. *Journal of Acquired Immune Deficiency Syndromes*. 26: 507-511

Henry V. D. and Alexander A. W. (2005) Mortality among Married Men in Rural Kenya and Malawi African Population Studies. *African Population Studies* 20: 165 – 177

Institute of Policy Analysis and Research (2004) HIV/AIDS Scourge in Nyanza Province: Poverty, Culture and Behaviour Change. *Policy Brief* 10: 11.

Khamadi S.A., Ochieng W., Lihana W.R., Kinyua J., Muriuki J., Mwangi J., Lwembe R., Kiptoo M., Osman S., Lagat N., Pelle R., Muigai a., Carter J., Oishi I., Ichimura H., Mwaniki D.L.,1 Okoth F.A.,Mpoke S., and Songok E.M. (2005) HIV Type 1 Subtypes in Circulation in Northern Kenya. *AIDS Research and Human Retroviruses*. 21: 810 - 814

Kwa, D., Boeser-Nunnink, B. and Schuitemaker, H. (2003) Lack of association between polymorphism in CX3CR1 and the clinical cause of HIV infection or virus phenotype evolution. *AIDS* 17: 981-985.

Levy, J.A. (2004) HIV pathogenesis: Knowledge gained after two decades of research. *Advances in dental research* 19: 10-16

Li C., Lu S.C., Hsieh P.S., Huang Y.H., Huang H.I., Ying T.H. and Shieh B. (2005) Distribution of human chemokine (C-X3-C) receptor 1 (CX3CR1) gene polymorphisms and haplotypes of the CC chemokine receptor 5 (CCR5) promoter in Chinese people, and the effects of CCR5 haplotypes on CCR5 expression. *International Journal of Immunogenetics* 32: 99-106

Livia M., Melissa N. and Vinod M. (2007) Spatial modeling of HIV prevalence in Kenya. Demographic and Health Research No.27. Calverton, MD: ORC Macro.

Lusso, P. (2006). HIV and the Chemokine System: 10 years later. *The EMBO Journal* 25:447 – 456

Mahalingam, S. and Karupiah, G. (1999) Chemokine and chemokine receptors in infectious diseases. *Immunolological Cell Biology* 77: 469-475

Mariani R., Wong S., Lubbertus C.,Wilkinson D.,Reinhart A., Larosa G., Nibbs R.,O'Brien T., Micheal N., Connor R., Macdonald M., Busch M., Koup R.A., and Landau N. (1999).

CCR2 – 641 Polymorphism Is Not Associated With Altered CCR5 Expression or Coreceptor Function. *Journal of Virology*. 73:2450-2459

Martinson J., Chapman N., Rees D.C., Liu Y and Clegg J.B. (1997). Global distribution of the CCR5 gene 32- base pair deletion. *Nature genetics*. 16: 100-103

Mcdermott D.H., Colla J.S., Kleeberger C.A., Plankey M., Rosenberg P.S., Smith E.D., Zimmerman P.A., Combadière C., Leitman S.F., Kaslow R.A., Goedert J.J. and Berger E.A. (2000) Genetic polymorphism in CX3CR1 and risk of HIV infection. *Science* 290: 2031-2035

McDermott D.H., Fong A.M., Yang Q., SechlerJ.M., CupplesA.L., Merrell M.N., Wilson W.F., Ralph B. D., O'DonnellC.J., Patel D.D. and Murphy P.M.(2003) Chemokine receptor mutant CX3CR1-M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans. *The Journal of Clinical Investigation*. 111:1241-1250.

McNicholl M.J, Smith D.K, Qari S.H, and Hodge T. (1997) Host Genes and HIV: The Role of the Chemokine Receptor Gene CCR5 and Its Allele (Δ 32 CCR5). *Journal of emerging infectious diseases* 3: 261 - 266

Ministry of Health sessional paper no. 4 1997. Aids in Kenya. Nairobi: MOH; 1997.

Ministry of Health, Republic of Kenya. 2005. AIDS in Kenya: Trends, Interventions and Impact. 7th Edition, 2005. Nairobi: National AIDS and STI Control Programme, Ministry of Health.

Ministry of Health, Republic of Kenya. Kenya AIDS Indicatory Survey 2007: National AIDS and STI Control Programme (NASCOP), Ministry of Health.

National AIDS Control Council (2010). United Nations General Assembly Special Session on HIV and AIDS (UNGASS-2010) country report - Kenya.

Nelson, K.E. and Williams, C.M. (2007). *Infectious disease epidiemiology: theory and practice.* 2nd edition. Jones and Bartlett publishers.

O'Brien S.J. and Moore J.P. (2003). The effect of genetic variation in chemokines and there receptors on HIV transmission and progression to AIDS. *Immunological Reviews* 177: 99-111

O'Brien T.R., McDermott D.H., Loannidis J.P.A., Carrington M., Murphy P.M., Havlir D.V. and Richman D.D. (2000). Effects of chemokine receptor gene polymorphisms on the response to potent antiretroviral therapy. *AIDS*.14:821-826

Puissant B., Roubinet F., Massip P.,Sandres-Saune K., Apoil P., Abbal M., Pasquier C.,Izopet J. and Blancher A. (2006) Analysis of CCR5, CCR2, CX3CR1, and SDF1 Polymorphisms in HIV-Positive Treated Patients: Impact on Response to HAART and on Peripheral T Lymphocyte Counts. *AIDS Research and Human Retroviruses* 22: 153-159

Qian, Y., Sun, H., Lin, K., Shi, L., Shi, L and Chu, J. (2008) Distribution of CCR5-_32, CCR2-64I, SDF1-3_A, CX3CR1-249I, and CX3CR1-280M in Chinese Populations *AIDS research and human retroviruses* 24:1391-1397

Schuitemaker H. and van Rij R. (2004) The role of polymorphisms in Chemokines and chemokine receptors in the clinical course of HIV-1 infection. *The journal of medicine*. 62:55-65

Singh, K.K., Hughes, M.D. and Chen, J. (2005) Genetic Polymorphisms in CX3CR1 Predict HIV-1 Disease Progression in Children Independently of CD4+ Lymphocyte Count and HIV-1 RNA Load. *Journal of infectious disease* 191:1971-1980

Smith M.W., Dean M., Carrington M., Winkler C., Gavin A.H., Deborah A., James J. G., O'Brien T.R., Lisa P., Richard K., Susan B., Eric V., Vlahov D., Hoots K and Margaret W. (1997). Contrasting Genetic Influence of CCR2 and CCR5 Variants on HIV-1 Infection and Disease Progression. *Science* 277: 959-965

Stanley, A. and Madhvan P. (1999). Current concepts in human Immunodeficiency virus infection and AIDS. *Clinical and diagnostic laboratory immunology*, 6: 295-305.

Steffen J., Julio A., Petra G., MaryJe S., Georg W., Kreutzberg., Alan Sher and Dan R. Littman (2000). Analysis of Fractalkine Receptor CX_3CR1 Function by Targeted Deletion and Green Fluorescent Protein Reporter Gene Insertion. *Molecular Cell Biology*. 20: 4106-4114

Su B., Jin L., Hu F., Xiao J., Luo J., Lu D., Zhang W., Chu J.,Du R., Geng Z., Qiu X., Xue J., Tan J., O'Brien S.J. and Chakraboty R. (1999). Distribution of Two HIV-1 Resistant Polymorphisms (SDF1- 3'A and CCR2-641) in East Asian and World Populations and Its Implication in AIDS Epidemiology. *Journal of Human Genetics*. 65:1047-1053

Su, B., Sun, G., Lu, D., Xiao, J., Hu, F., Chakraborty, R., Deka, R. and Jin, L. (2000). Distribution of three HIV-1 resistance-conferring polymorphisms (SDF1-3'A, CCR2-64I, and CCR5-Δ32) in global populations. *European Journal of Human Genetics*. 8: 975-979

Suresh P., Wanchu A., Sachdeva R.K. and Bhatnagar A. (2006). Gene Polymorphisms in CCR5, CCR2, CX3CR1, SDF-1 and RANTES in Exposed but Uninfected Partners of HIV-1 Infected Individuals in North India. *Journal of Clinical Immunology*. 26:476-482.

Toshio I. and Osamu Y. (2000). CX3CR1. Kan Research Institute, Science Center Building, 3 Kyoto Research Park, Kyoto, Chu-douji Kurita-Chou, 600-8815, Japan.

United Nations Programme on HIV diseases and AIDS (2008). 2008 Report on the global AIDS epidemic.

United State Agency for International Development (2007). Spartial modeling of HIV prevalence in Kenya. *Demographic and Health Surveys working papers* No.27

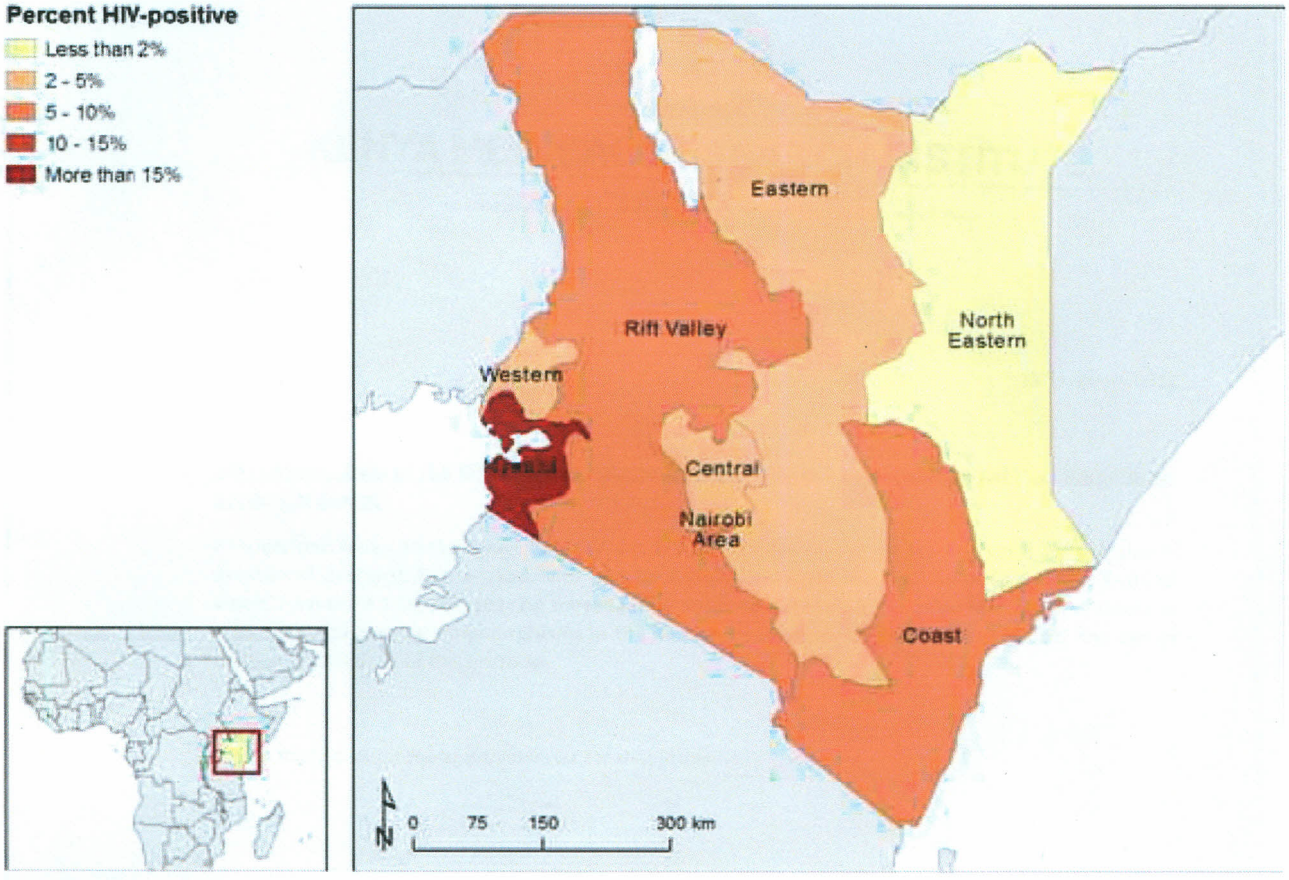
Wang, W., Chen, M., Chuang, C., Jeang, K. and Huang, L. (2000) Molecular biology of human immunodeficiency virus type 1. *Journal of Microbiological Immunology and infection* 33:131-140

Zlotnik A., Yoshie O. and Nomiyama H. (2006): The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biology* 7: 243

5. APPENDICES

APPENDIX 1

A MAP OF KENYA SHOWING HIV PREVALENCE PER PROVINCE.



Adapted from: USAID (2007), Demographic and Health Research.No.27

APPENDIX 2

A LETTER FROM DR. KHAMADI

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research P.O. Box 54628 - 00200, NAIROBI - Kenya
Tel: (254) (070) 2722541, 2713349; 0722-205901, 0733-400003; Fax: (254) (020) 2726115
E-mail: cvr@kemri.org

23rd January 2012

REF: Identification of the CX3CRI Gene Polymorphism T280M in a sampled Population of HIV infected persons in Kenya.

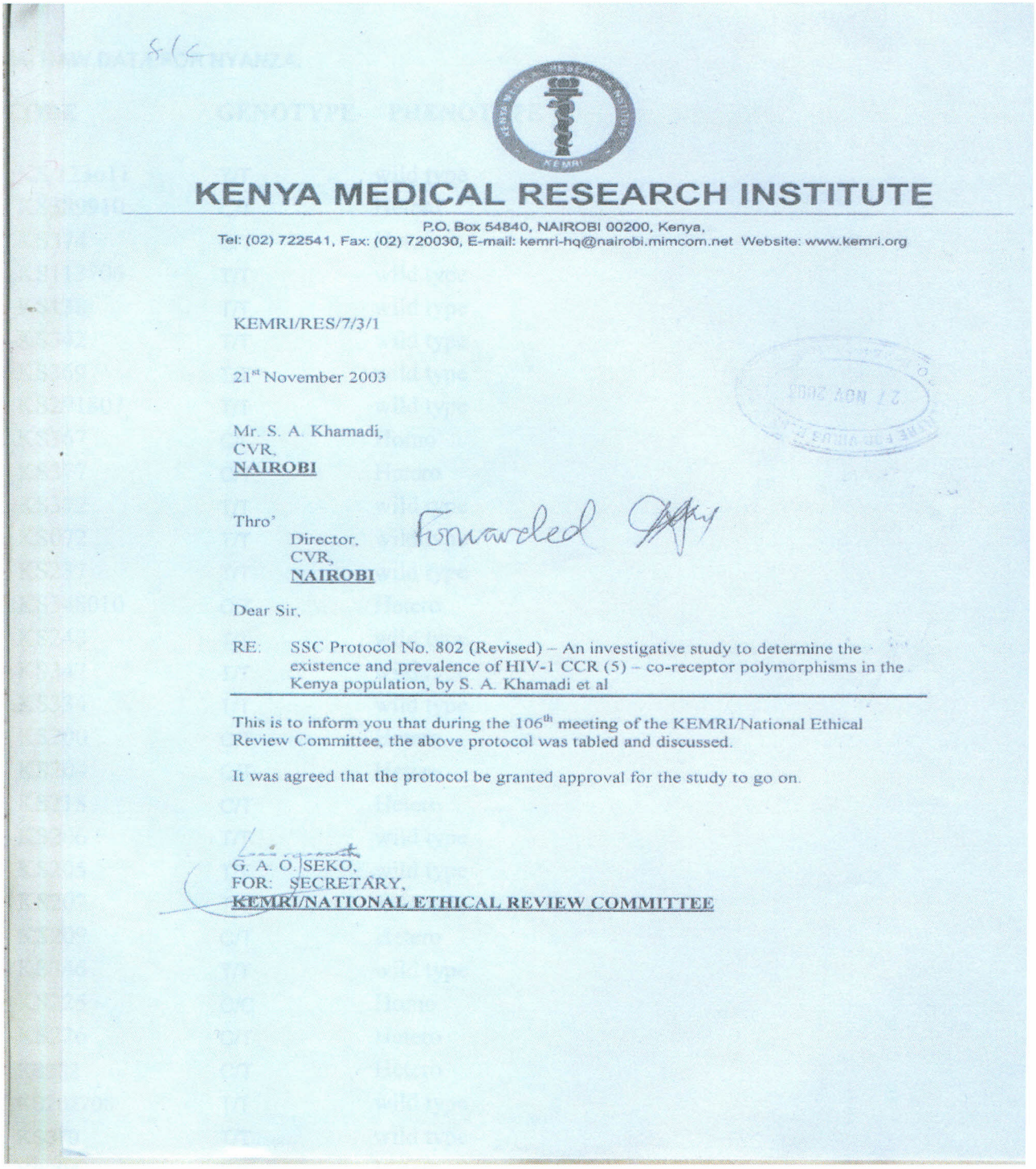
I supervised Felix Likhako Liyayi (I56/CE/12411/2004) for his Master of Science work for the above-mentioned protocol. We worked with him on samples collected from an approved KEMRI Protocol where I am the P.I i.e. **SSC 802: An Investigative Study to determine the existence and prevalence of HIV-1 CCR5 coreceptor polymorphisms in the Kenyan Population**. The work that Felix did was part of the study objectives of this protocol.

Please find attached the approval letter for that Study.

Samoel Khamadi, PhD.
Senior Research Officer, KEMRI.
Center for Virus Research.

APPENDIX 3

A LETER FROM ETHICAL COMMITTEE OF KEMRI



KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1

21st November 2003

Mr. S. A. Khamadi,
CVR,
NAIROBI

Thro'
Director,
CVR,
NAIROBI

Forwarded

Dear Sir,

RE: SSC Protocol No. 802 (Revised) – An investigative study to determine the existence and prevalence of HIV-1 CCR (5) – co-receptor polymorphisms in the Kenya population, by S. A. Khamadi et al

This is to inform you that during the 106th meeting of the KEMRI/National Ethical Review Committee, the above protocol was tabled and discussed.

It was agreed that the protocol be granted approval for the study to go on.

G. A. O. SEKO,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE

APPENDIX 2

RAW DATA FOR ALL THE 5 PROVINCES.

(A) RAW DATA FOR NYANZA.

CODE	GENOTYPE	PHENOTYPE
KS 123611	T/T	wild type
KS389910	C/T	Hetero
KS374	C/T	Hetero
KS113706	T/T	wild type
KS138	T/T	wild type
KS342	T/T	wild type
KS369	T/T	wild type
KS291807	T/T	wild type
KS367	C/C	Homo
KS377	C/T	Hetero
KS372	T/T	wild type
KS072	T/T	wild type
KS237	T/T	wild type
KS348010	C/T	Hetero
KS248	T/T	wild type
KS347	T/T	wild type
KS334	T/T	wild type
KS200	C/T	Hetero
KS204	C/T	Hetero
KS218	C/T	Hetero
KS206	T/T	wild type
KS205	T/T	wild type
KS207	T/T	wild type
KS209	C/T	Hetero
KS346	T/T	wild type
KS225	C/C	Homo
KS226	C/T	Hetero
KS382	C/T	Hetero
KS202705	T/T	wild type
KS379	T/T	wild type
KS394	C/T	Hetero
KS381	C/T	Hetero
KS866	T/T	wild type

KS038	T/T	wild type
KS001	T/T	wild type
KS3912	C/T	Hetero
KS339	C/T	Hetero
KS376	T/T	wild type
KS3912	T/T	wild type
KS202	T/T	wild type

(B) RAW DATA FOR CENTRAL

CODE	GENOTYPE	PHENOTYPE
002 163	C/T	Heterozygous
003 164	T/T	wild type
004 165	T/T	wild type
005 167	T/T	wild type
006 168	C/T	Heterozygous
007 169	T/T	wild type
016 178	T/T	wild type
009 171	T/T	wild type
010 172	T/T	wild type
011 173	C/T	Heterozygous
012 174	T/T	wild type
013 175	T/T	wild type
014 176	C/T	Heterozygous
015 177	C/T	Heterozygous
008 170	T/T	wild type
017 179	T/T	wild type
018 180	T/T	wild type
019 181	C/T	Heterozygous
020 182	T/T	wild type
021 183	T/T	wild type
022 184	T/T	wild type
023 185	C/T	Heterozygous
024 186	T/T	wild type
025 187	C/T	Heterozygous
026 188	T/T	wild type
027 189	T/T	wild type
028 190	T/T	wild type
029 191	T/T	wild type
030 192	C/T	Heterozygous
031 193	T/T	wild type
032 194	T/T	wild type
033 195	T/T	wild type

034 196	T/T	wild type
CC4180/06	C/T	Heterozygous
CC1082/06	T/T	wild type
7930	T/T	wild type
7946	T/T	wild type
8032	T/T	wild type
8179	T/T	wild type
7405	T/T	wild type

(C) RAW DATA FOR WESTERN

CODE	GENOTYPE	PHENOTYPE
BU 431	C/T	Heterozygous
BU 368	C/T	Heterozygous
BU 265	C/T	Heterozygous
BU 434	C/T	Heterozygous
BU 424	T/T	wild type
BU 421	T/T	wild type
BU 426	T/T	wild type
BU 388	T/T	wild type
BU 418	T/T	wild type
BU 390	C/T	Heterozygous
BU 391	C/T	Heterozygous
BU 427	T/T	wild type
BU 357	T/T	wild type
BU 363	T/T	wild type
BU 410	T/T	wild type
BU 392	T/T	wild type
BU 422	T/T	wild type
BU 364	T/T	wild type
BU 429	C/T	Heterozygous
BU 402	C/T	Heterozygous
BU 354	T/T	wild type
BU 401	T/T	wild type
BU 356	C/T	Heterozygous
BU 417	T/T	wild type
BU 430	C/T	Heterozygous
BU 406	T/T	wild type
BU 387	T/T	wild type
BU 374	T/T	wild type
BU 383	C/T	Heterozygous
BU341	T/T	wild type

BU 367	T/T	wild type
BU 369	T/T	wild type
BU 370	C/T	Heterozygous
BU 422	C/T	Heterozygous
BU 423	T/T	wild type
BU 355	C/T	Heterozygous
BU 400	C/T	Heterozygous
BU 418	T/T	wild type
BU 409	T/T	wild type
BU 411	T/T	wild type

(D) RAW DATA FOR NAIROBI PROVINCE

CODE	GENOTYPE	PHENOTYPE
STC 2470	T/T	wild type
STC 2469	T/T	wild type
STC 2459	T/T	wild type
STC 2457	T/T	wild type
STC 2452	C/T	Heterozygous
STC 2455	T/T	wild type
STC 2458	C/T	Heterozygous
STC 2453	C/C	Heterozygous
STC 2460	T/T	wild type
STC 2461	T/T	wild type
STC 2468	T/T	wild type
STC 2465	T/T	wild type
STC 2464	C/T	Heterozygous
STC 2467	T/T	wild type
STC 2451	C/C	Heterozygous
STC 2454	T/T	wild type
STC 2456	T/T	wild type
STC 2462	T/T	wild type
STC 2471	T/T	wild type
STC 2474	C/T	Heterozygous
STC 2478	T/T	wild type
STC 2476	T/T	wild type
STC 2488	C/C	Wild type
STC 2482	T/T	wild type
STC 3473	T/T	wild type
STC 2476	T/T	wild type
STC 2487	C/C	Homozygous
STC 2485	T/T	wild type

STC 2463	C/T	Heterozygous
STC 2466	C/T	Heterozygous
STC 2486	T/T	wild type
STC 2472	T/T	wild type
STC 2491	T/T	wild type
STC 2495	T/T	wild type
STC 2494	T/T	wild type
STC 2492	C/T	Heterozygous
STC 2483	T/T	wild type
STC 2484	C/T	Heterozygous
STC 2496	T/T	wild type
STC 2489	T/T	wild type

(E) RAW DATA FOR NORTH EASTERN PROVINCE

CODE	GENOTYPE	PHENOTYPE
TKMH 007	T/T	wild type
TLHC 029	T/T	wild type
TKMH 008	T/T	wild type
TKMH 006	C/T	Heterozygous
TLHC 031	T/T	wild type
TKMH 003PB	T/T	wild type
MYDH 39	T/T	wild type
TLHC 025	T/T	wild type
MYDH 006PB	T/T	wild type
MYDH 005	C/T	Heterozygous
MYDH 42	T/T	wild type
MYDH 43	T/T	wild type
TKMH 001	T/T	wild type
TLHC 026	T/T	wild type
TLHC 027	T/T	wild type
TKMH 004	T/T	wild type
TLHC 028	T/T	wild type
TLHC 006	T/T	wild type
TLHC 005	C/T	Heterozygous
TLHC 004	T/T	wild type
TLHC 003	C/T	Heterozygous
TLHC 002	C/T	Heterozygous
TLHC 001	T/T	wild type
LM 020	T/T	wild type
D1	T/T	wild type

ADH 005 PB	C/T	Heterozygous
ADH 009 PB	C/T	Heterozygous
TLHC 030	T/T	wild type
MYDH 038	T/T	wild type
MYDH 023	T/T	wild type
MYDH 022	T/T	wild type
TLHC 037	T/T	wild type
TLHC 040	T/T	wild type
MYDH 039	T/T	wild type
TKMH 020	C/T	Heterozygous
TKMH 014	T/T	wild type
TKMH 015	T/T	wild type
TKMH 019	T/T	wild type
TLHC 024	T/T	wild type
TLHC 037	T/T	wild type